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Tapetum structure and ontogeny in *Victoria* (Nymphaeaceae)

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Abstract
The tapetum is critical for successful pollen development. Innovations in tapetum ontogeny and in the composition and role of tapetal exudate are hypothesised to have been advantageous in early angiosperm evolution. Early-diverging angiosperm lineages exhibit considerable variation in tapetum development, indicating that the earliest angiosperms exhibited evolutionary lability in tapetum ontogeny. However, little or no data on tapetum development exist for many basal flowering plant taxa, including members of Nymphaeales. Here, tapetum ontogeny in the giant water lily *Victoria* is described along with the development of associated microspore characters. The tapetum in *Victoria* is secretory. Orbicules are present along both the inner tangential and radial plasmalemmae of the tapetal cells. The timing of orbicule production is synchronous with the timing of microchannel appearance within the exine, supporting hypotheses that exine microchannels function in transport and storage of tapetal exudate. Prior to tapetum degradation late in microspore development, tapetal cells extend into the locule and contact the developing pollen grains, which are held together in permanent tetrads. In a phylogenetic context, the presence of a secretory tapetum in *Victoria*, rather than an invasive type, indicates that the invasive tapetum likely arose at least twice in Nymphaeaceae. Thus, tapetum ontogeny may be even more labile in Nymphaeales than previously thought.

Keywords: *basal angiosperms, exine, microchannels, Nymphaeales,* Victoria, *tapetum*

The tapetum is a specialised tissue that lines the sporangium locule, forming a layer separating the sporogenous tissue from the sporangial wall. The tapetum plays a critical role in spore and microspore ontogeny (Pacini et al., 1985; Chapman, 1987), and abnormal tapetal development is associated with male sterility in seed plants (Canales et al., 2002; Yang et al., 2003; Colcombet et al., 2005; Zhang et al., 2006; Dun et al., 2011). A tapetal layer is found in all land plants; however, the developmental origin and specific function of the tapetum varies across plant groups (Chapman, 1987; Parkinson & Pacini, 1995). A primary function of the tapetum in all land plants is to provide nutrient material for the sporogenous tissue and developing spores (Maheshwari, 1950; Pacini et al., 1985; Chapman, 1987). Additionally, the tapetum is the source of exine precursors used in construction of the microspore wall, as well as callase, which depolymerises the callose wall surrounding microspore tetrads (Singh, 1978; Pacini et al., 1985). In angiosperms, the tapetum is also the source of sporophytic recognition proteins that are essential for self-incompatibility systems, as well as 'sticky' materials, such as pollenkitt or tryphine. Pollenkitt coats the pollen walls of many taxa, thereby facilitating insect pollination, and it can also function in recognition (Echlin, 1971; Dickinson & Lewis,

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1973; Heslop-Harrison et al., 1975; Howlett et al., 1975; Keijzer, 1987; Dickinson et al., 2000). The tapetum secretes these materials during the course of pollen ontogeny and, although there is considerable variation in timing, the tapetal cells typically degenerate late in pollen development (Maheshwari, 1950; Pacini et al., 1985).

Two primary types of tapeta are consistently recognised (Maheshwari 1950; Chapman, 1987; Furness & Rudall, 2001; Pacini, 2010). In the 'secretory' type, the layer(s) of tapetal cells are persistent for the greater part of pollen development, degrading *in situ* sometime during the free-microspore stage of anther development. In this type, tapetal cells secrete material into the anther locule but do not invade the locule themselves. In the 'invasive' or 'amoeboid' type, the tapetal protoplasts physically migrate into the anther locule and may, or may not, fuse to form an invasive plasmodium. The secretory type is the predominant type across all land plants and is undoubtedly the ancestral condition (Pacini et al., 1985; Furness & Rudall, 2001; Pacini, 2010).

The invasive tapetum is rare in gymnosperms (Pacini et al., 1985), but it has evolved independently several times in flowering plants, including at least four times in early-divergent angiosperm lineages (Furness & Rudall, 2001). Furthermore, several potential intermediate types of tapeta have been described in these lineages, including a 'cyclic-invasive' tapetum, in which individual tapetal cells invade the locule but then retreat back to the anther wall (Rowley et al., 1992; Furness & Rudall, 2001).

Innovations in tapetum development and changes in the role of tapetal exudate may have been advantageous to early angiosperms. Closer direct contact, which can be achieved via protrusion or invasion of the tapetum into the anther locule, has been hypothesised to increase microspore nourishment (Pacini & Franchi, 1982; Pacini, 1997). Moreover, molecules secreted by the angiosperm tapetum play a crucial role in insect pollination and sporophytic self-incompatibility systems and these, in turn, have been hypothesised to have facilitated early angiosperm diversification (e.g. Grant, 1949; Van der Pijl, 1960; Endress, 2001; Furness & Rudall, 2001; Gorelick, 2001). Understanding diversity in tapetum development in early-divergent lineages is key for evaluating these evolutionary hypotheses.

Nymphaeales, or water lilies, is an ancient angiosperm lineage comprising three families (Nymphaeaceae, Cabombaceae, Hydatellaceae) that evolved from either the basal-most or second-most basal node of the angiosperm phylogenetic tree (Löhne & Borsch, 2005; Saarela et al., 2007; APG, 2009). Nymphaeales includes taxa that exhibit secretory, invasive and cyclic invasive tapetum types (Rowley et al., 1992; Gabarayeva & El-Ghazaly, 1997; Gabarayeva et al., 2003; Taylor & Osborn, 2006; Taylor et al., 2008).

Victoria (Nymphaeaceae) is endemic to South America and comprises two species, *Victoria amazonica* Sowerby and *V. cruziana* Orbign. These two species are common in cultivation and can produce hybrids. The 'Longwood' hybrid is the result of a cross between *V. cruziana* as the pollen parent and *V. amazonica* as the ovule parent. *Victoria* is well known for its massive floating leaves, reaching up to 3 m in diameter, and large flowers that can reach up to 50 cm across (Schneider & Williamson, 1993). Within Nymphaeaceae, *Victoria* traditionally forms a clade with the Asian genus *Euryale*, with this *Victoria–Euryale* clade being sister to *Nymphaea.* However, recent phylogenetic analyses indicate that (a) the *Victoria–Euryale* clade is instead nested within *Nymphaea* and (b) there is still some lack of resolution in the relationships among genera forming the core Nymphaeaceae (*Victoria*, *Euryale*, *Nymphaea* and *Ondinea*; Borsch et al., 2007, 2008; Löhne et al., 2008). Several of the reproductive traits exhibited by *Victoria*, including enlarged flowers and specialised beetle pollination, are thought to be derived within Nymphaeales (Endress, 2010).

Due in part to the importance of the tapetum in pollen development and the variation that has been discovered in early-divergent angiosperm lineages, there have been an increasing number of studies that have documented tapetum development in these plants (e.g. Rowley et al., 1992; Gabarayeva & Rowley, 1994; Gabarayeva & El-Ghazaly, 1997; Tobe et al., 2000; Furness & Rudall, 2001; Taylor & Osborn, 2006; Taylor et al., 2008; Zhou & Fu, 2008). However, there are many basal taxa, such as *Victoria*, for which tapetum ontogeny has not been described in detail and which may provide a clearer picture of tapetum evolution in angiosperms.

One might expect *Victoria* to exhibit an invasive tapetum due to its highly specialised pollination biology and its close relationship with *Nymphaea*, a genus that includes at least two species that exhibit a cyclic invasive tapetum (*N. colorata* Peter and *N.*

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through a 'Longwood' hybrid anther locule containing microspore (M) tetrads encased in callose (*arrow*). Many of the tapetal cells (T) have pulled away from one another radially, where the cells face the locule (*arrowheads*). **F.** TEM, detail of the tapetum of a 'Longwood' hybrid anther as in (**E**), showing that the four tapetal cells (T1–T4) have begun to separate along their radial faces (*arrowhead*). Scale bars – 100 μm (A), 50 μm (E), 25 μm (B, C), 10 μm (D), 2 μm (F).

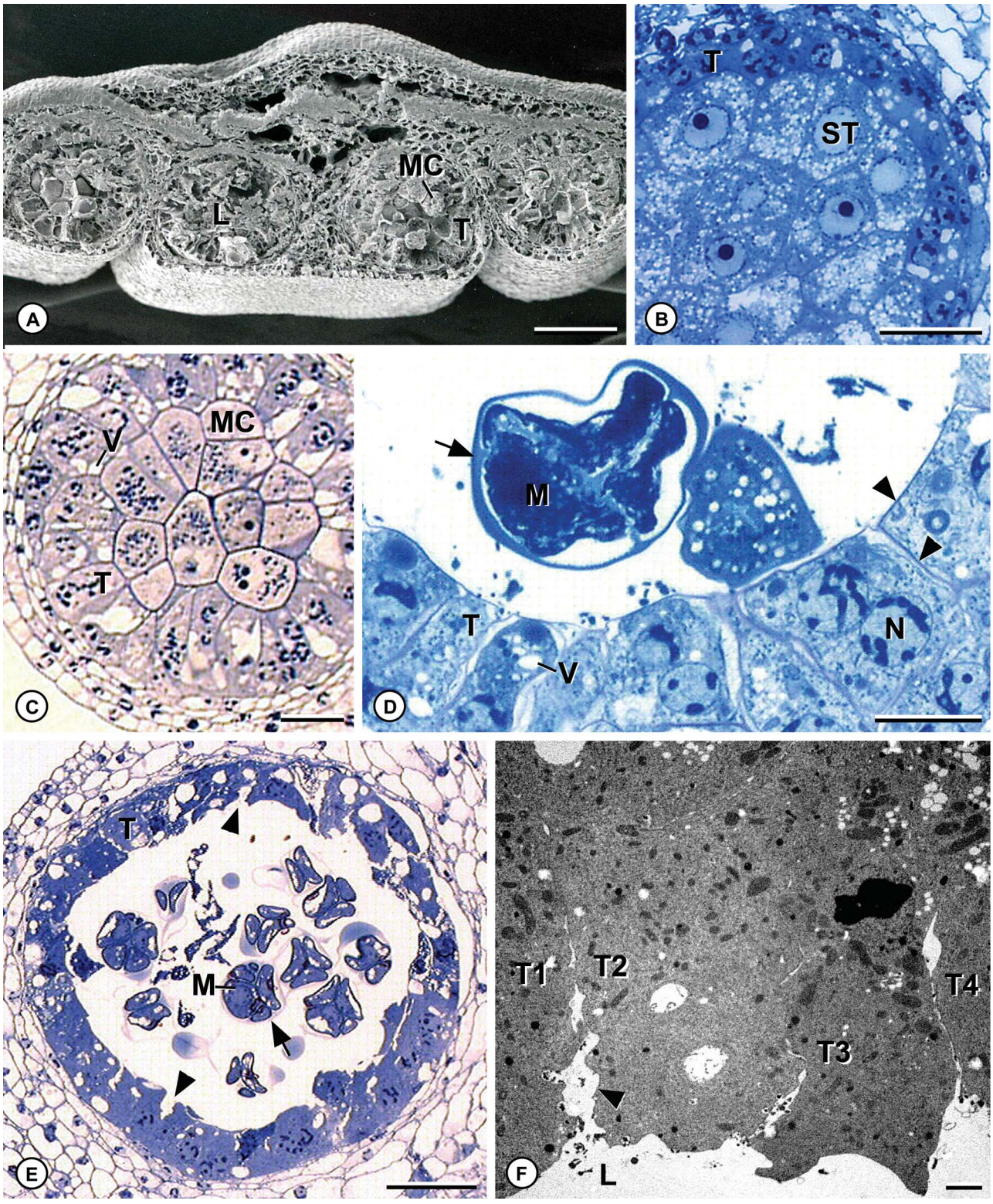

Figure 1. Tapetum development in *Victoria*. **A.** SEM, transverse section through a *V. cruziana* stamen during the early microspore mother cell stage. The tapetum (T) and microspore mother cells (MC), which fill the entire locule (L), are visible in all four anther locules. **B.** LM, section through a locule of a *V. amazonica* anther during the late sporogenous tissue stage showing densely packed sporogenous tissue (ST) and already-differentiated tapetal cells (T), which have not yet elongated. **C.** LM, section through a *V. cruziana* anther locule containing early microspore mother cells (MC). The tapetal cells (T) have elongated into the locule and have few small vacuoles (V). **D.** LM, section through a *V. cruziana* anther locule during the early tetrad stage. Microspore (M) tetrads are enveloped in callose (*arrow*), and the tapetal cells (T) are binucleate (N) and contain a small number of vacuoles (V). The tapetal cells are tightly appressed against one another radially, with very little intercellular space between them, and the margins (*arrowheads*) are continuous in shape and non-invaginated. **E.** LM, section

mexicana Zucc.; Rowley et al., 1992; Gabarayeva & Rowley 1994; Gabarayeva & El-Ghazaly 1997). Furness and Rudall (2001) list the tapetum type of *Victoria* as secretory, based on a short report for *V. regia* Lindl. (= *V. amazonica*) by Wunderlich (1954). Khanna (1967*a*) also reports a secretory tapetum. However, there are neither published descriptions nor published micrographs of tapetum structure or ontogeny in *Victoria*, other than one brief account of the 'behaviour' of tapetal nuclei accompanied by line drawings (Khanna, 1967*b*).

The goal of this study was to characterise the complete pattern of tapetum ontogeny in *Victoria* for the first time, as well as the development of selected microspore characters closely associated with tapetal biology. Our results bear on the question of tapetum diversity in early-divergent angiosperms and add clarity to the understanding of evolutionary relationships among core Nymphaeaceae.

Material and methods

Plant material was collected from private ponds in Cocoa Beach, Florida, USA, owned by Kit and Ben Knotts. Twenty-two floral buds (11 from *Victoria amazonica*, six from *V. cruziana*, and five from the 'Longwood' hybrid) representing the full range of developmental stages were collected. The buds were dissected in the field and the anthers were fixed in 3% glutaraldehyde (buffered in 0.2 mol/L phosphate buffer, pH 7.4) for 24 h and then buffer-washed. The anthers were post-fixed in buffered 1% osmium tetroxide for 3 h and buffer-washed.

For light microscopy (LM) and transmission electron microscopy (TEM), anthers were dehydrated in a series of ethanol/acetone rinses and gradually infiltrated/embedded in Spurr epoxy resin. Embedded anthers were sectioned on an ultramicrotome with a diamond knife. Thick sections for LM (850 nm) were stained with Azure II and Methylene Blue (Richardson's Stain) and imaged on an Olympus BHS compound light microscope with bright-field and differential interference contrast illumination. Thin sections for TEM (90 nm) were collected on copper slot grids (1×2 mm in size), dried onto formvar-support films, and stained with uranyl acetate (1%) and lead citrate (Taylor & Osborn, 2006; Taylor et al., 2008). The anthers were then imaged at 80 kV using a JEOL JEM-100SX transmission electron microscope.

For scanning electron microscopy (SEM), anthers were dehydrated in an ethanol series, critical point dried and mounted onto aluminium stubs using colloidal graphite. Some anthers were fractured with a double-edged razor blade using several techniques: (a) while still in 70% ethanol at room temperature, (b) following being frozen in liquid nitrogen and (c) after critical point drying. The aluminium stubs with the mounted specimens were sputter-coated with gold-palladium and imaged at 5 kV using a JEOL JSM-6100 scanning electron microscope.

Results

Stamina in *Victoria* are laminar with four elongated microsporangia positioned on the adaxial surface; in transverse section, the microsporangia are round (Figure 1A). Although the laminar stamina have no distinct filament and anther regions, we refer to the region of the microsporophyll, in which the microsporangia are positioned as the 'anther'.

We did not observe substantial variation in tapetal ontogeny among *Victoria amazonica*, *V. cruziana* and the 'Longwood' hybrid, so data from these taxa are combined to describe the general pattern of tapetum development in *Victoria*.

Sporogenous tissue stage

The tapetum in *Victoria* is first apparent as a differentiated tissue lining the microsporangium in the late sporogenous tissue stage. At this stage, cells of the sporogenous tissue are still tightly appressed and

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underlying middle layers (ML). **B.** TEM, detail of three tapetal cells (T1–T3) in *V. amazonica*. Note that the inner tangential margin of the tapetum (T2) is in direct contact with the exine (E) of a developing microspore (M) and that the radial plasmalemmae of the tapetal cells (*black arrowheads*) are separated by an intercellular space. Pro-orbicules (*arrows*) and sometimes orbicules are present within invaginations of the tapetal cells' plasmalemmae, both in the intercellular spaces between tapetal cells and between the tapetum and the microspore. The microspore exine is dissected with narrow microchannels (*white arrowhead*). **C.** TEM, section through a binucleate (N) tapetal cell of 'Longwood' hybrid that is protruding into the locular space. The plasmalemma is irregular in outline, and orbicules are concentrated around the tapetal cell within the locular space (*arrows*). **D.** TEM, detail of two tapetal cells (T1–T2) in 'Longwood' hybrid. The plasmalemmae along the radial faces (*arrowheads*) are irregular and slightly invaginated in comparison to the outer tangential surface (*arrow*), where the tapetal cells directly abut cells of the middle layer (ML). Note also the abundant endoplasmic reticulum, lipid bodies (LB) and relatively large vacuoles (V) within the cells. **E.** TEM, detail of the inner tangential plasmalemma facing the anther locule of a 'Longwood' hybrid tapetal cell. Pro-orbicules (*arrow*) are present within pockets formed by invaginations in the plasmalemma (*arrowhead*). **F.** TEM, detail of the radial plasmalemmae of two adjacent tapetal cells (T1–T2) of 'Longwood' hybrid. Orbicules (*arrow*) are present in the intercellular space. **G.** TEM, section through the tapetum of 'Longwood' hybrid. Tapetal cells (T) are almost fully separated radially, but have not separated from the anther middle layers (ML). Scale bars – 25 μm (A, G), 5 μm (C), 2 μm (D), 1 μm (B), 500 nm (E, F).

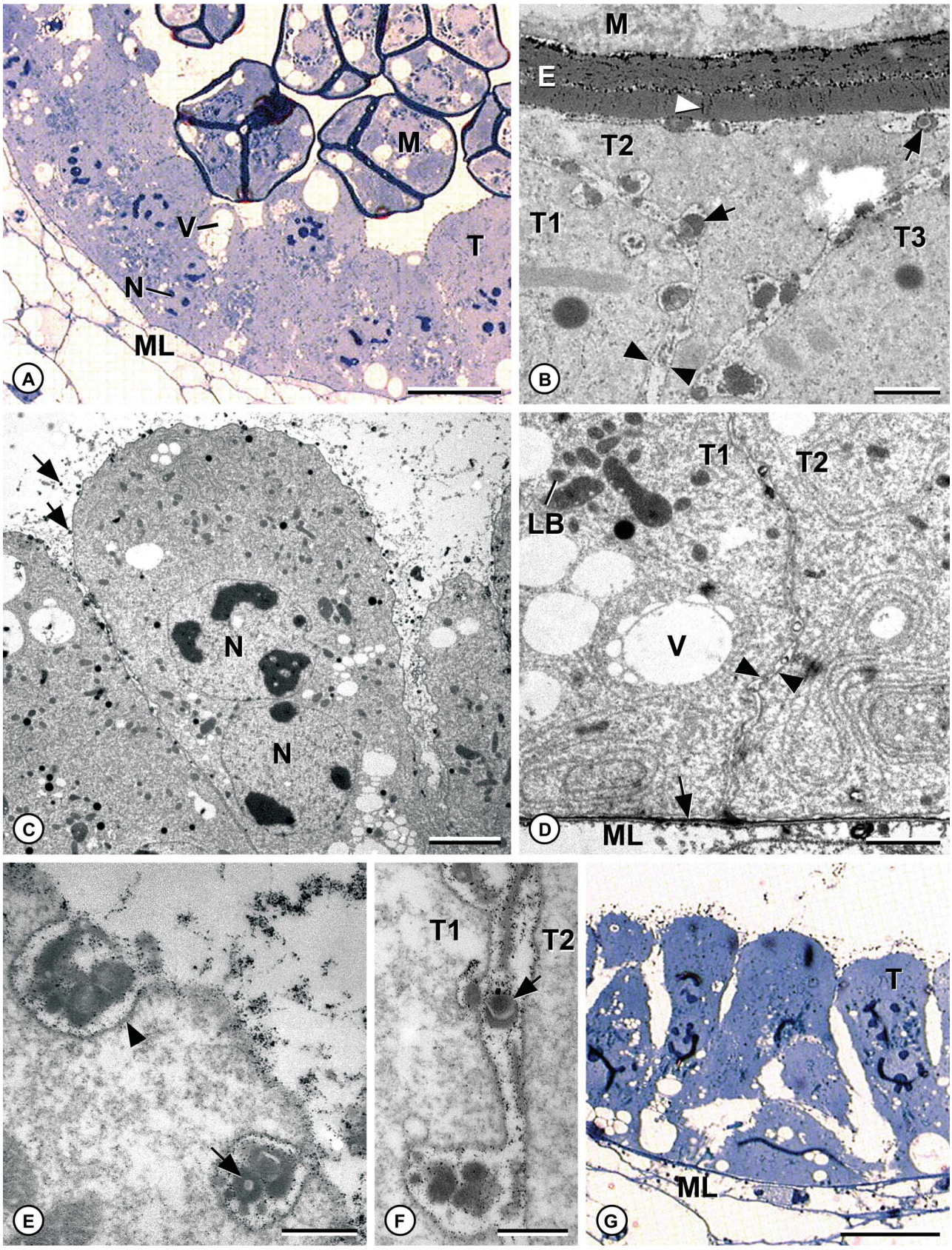

Figure 2. Tapetum development during the 'free' microspore stage in *Victoria*. **A.** LM, section through a 'Longwood' hybrid anther with overtopping and protruding tapetal cells (T). The microspore (M) tetrads at the periphery of the locule are in direct contact with the tapetal cells, whereas the central tetrads are not. Note also the large nuclei (N) and greater number of vacuoles (V) within the tapetal cells and the

have not developed a microspore mother cell coat (Figure 1B). The cells that comprise the tapetum are rectangular, with the long axis of each cell oriented parallel to the middle layers (Figure 1B).

Microspore mother cell stage

Following differentiation of the sporogenous tissue, the newly formed microspore mother cells expand in size. As the individual microspore mother cells begin to separate, with each developing a microspore mother cell coat, the tapetal cells elongate perpendicular to the middle layers. This elongation results in a thick tapetum tissue that is in direct contact with the peripheral microspore mother cells. The young microspore mother cells fill the entire anther locule (Figure 1A, C). As this ontogenetic stage progresses, the microspore mother cells develop a thin callose wall and separate from each other and from the tapetal cells, creating more intralocular space. During the microspore mother cell stage, tapetal cells are binucleate and typically have few, small vacuoles (Figure 1C).

Tetrad stage

The tapetal cells remain connected to the anther middle layers as the microspore mother cells undergo meiosis and form tetrads, each of which is held together by a thickened layer of callose (Figure 1D, E). In the early tetrad stage, the binucleate tapetal cells are tightly appressed to each other radially, and the cells form a distinctive palisade (Figure 1D). Vacuoles are more numerous than in the microspore mother cells stage but are typically still small (Figure 1D, E). During the late tetrad stage, tapetal cells begin to separate from each other along their radial faces (Figure 1E, F). The plasmalemma of each tapetal cell also becomes slightly invaginated, both along the cell margin facing the locule and radially between the tapetal cells (Figure 1F).

'Free' microspore stage

Mature pollen grains of *Victoria* are held together in permanent tetrads. Thus, the end of the tetrad stage and the beginning of the 'free' microspore stage is marked by the dissolution of callose, but not the separation of microspores (Figure 2A).

During the early 'free' microspore stage, the tapetal cells elongate, overtop each other, and begin to separate further from one another such that there is intercellular space between them (Figure 2A–C). The plasmalemma along the inner tangential margin (facing the locule) and the radial margins of the tapetal cells is quite irregular in shape (Figure 2A–C). However, the plasmalemma does not become invaginated where the tapetal cells contact the anther middle layers (Figure 2D). At this stage, the tapetal cells extend more deeply into the locule than in previous stages and directly contact the developing microspore tetrads (Figure 2A, B). The degree of contact varies greatly; some microspore tetrads, particularly those near the periphery of the anther locule, are in contact with tapetal cells on at least two sides, whereas others have little or no direct contact with tapetal cells (Figure 2A). The tetrads do not fill the locule, so there is abundant locular space, and not all microspores are pressed against the tapetum.

During progression of the 'free' microspore stage, tapetal cells become increasingly vacuolated, with vacuoles increasing in both size and number (Figure 2A, C). Tapetal cells also contain abundant endoplasmic reticulum and lipid bodies (Figure 2B–D). At this stage, the developing orbicules, or pro-orbicules, are abundant within pockets of the plasmalemma (Figure 2B, E, F). Numerous fully-developed orbicules that exhibit an electron-dense outer layer and an electron-translucent core are present within some of the invaginations, as well as within other regions of intercellular space (Figure 2B, E, F). Orbicules in *Victoria* range in size from 0.19 μm to 0.35 μm and are abundant in both the anther locule and between tapetal cells (Figure 2C–F). Near the end of this ontogenetic stage, the tapetal cells are quite separated from one another along their radial faces, but the cells do not separate from the anther middle layer to invasively migrate into locule (Figure 2D, G).

During the 'free' microspore stage, narrow channels become apparent in the tectum and foot layer of the developing pollen wall. These 'microchannels' are detectable as electron-dense lines dissecting the exine perpendicular to the microspore plasmalemma (Figure 2B). The microchannels are regularly organised and occur in both the distal and proximal exine walls. As the 'free' microspore stage progresses and the exine thickens, the microchannels become less organised in orientation (Figure 3E).

At the end of the 'free' microspore stage, tapetal cells recede from the anther locule and begin to undergo programmed cell death (Figure 3A, B). During apoptosis, the contents of the tapetal cells are increasingly secreted into the locule (Figure 3C) and the tapetal cells become highly vacuolated (Figure 3B, D). Tapetal cell death occurs only when the intine has been initiated and the distal walls of the individual microspores are almost entirely fused within the developing permanent tetrads (Figure 3B).

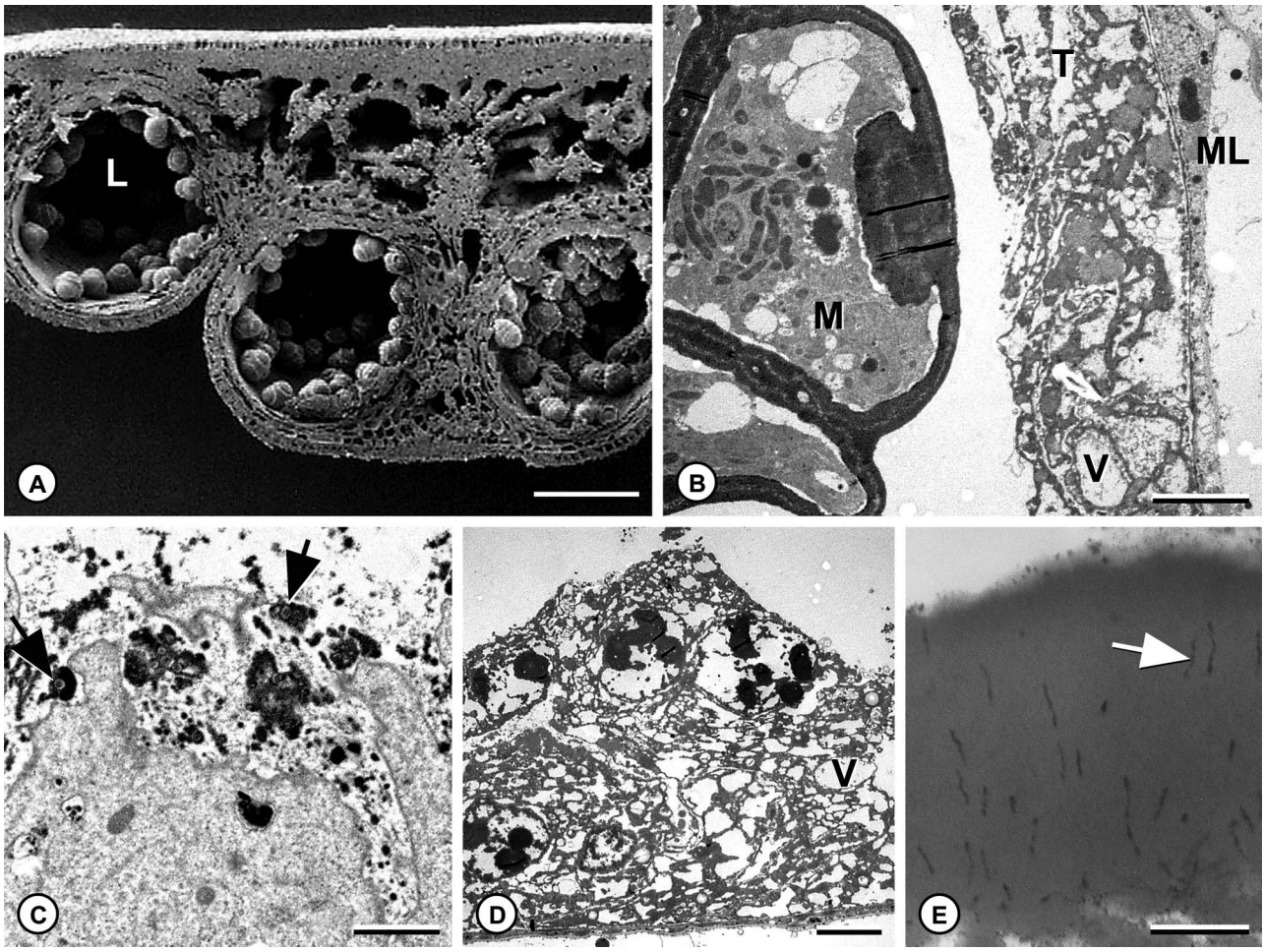

Figure 3. Tapetum development in the late 'free' microspore stage in *Victoria*. **A.** SEM, transverse section through a stamen of 'Longwood' hybrid showing three microsporangia. The tapetum is almost fully degraded and the permanent microspore tetrads are distributed throughout the locules (L). **B.** TEM, detail of a tapetal cell (T) in 'Longwood' hybrid with an adjacent microspore (M) tetrad. The tapetal cells contain many vacuoles (V) and have undergone significant degradation, but the cells remain attached to the anther middle layer (ML). **C.** TEM, detail of a 'Longwood' hybrid tapetal cell. The plasmalemma is highly irregular and the cell is secreting its contents, including numerous orbicules (*arrows*), into the anther locule. **D.** TEM, detail of a 'Longwood' hybrid tapetal cell later in degradation than the cell shown in (**C**). Note that the cell is highly vacuolated (V). **E.** TEM, detail of the microspore exine showing the thick tectum in transverse section. Note that the tectum is dissected with electron-dense microchannels (*arrow*). Scale bars – 250 μm (A), 5 μm (B, D), 2 μm (C), 100 nm (E).

Discussion

This study is the first to describe and micrographically illustrate tapetal ontogeny in *Victoria*. Several developmental characters are considered with regard to what is known from other members of Nymphaeales and discussed in the context of current phylogenetic hypotheses for water lilies and other basal angiosperms.

Ontogenetic timing

The tapetum first becomes differentiated during the sporogenous tissue stage. Tapetal cells are binucleate and remain appressed to each other until the late tetrad stage, when they begin to separate radially. During the 'free' microspore stage, the tapetal cells become increasingly vacuolated and elongated such that they extend further into the anther locule. The tapetal cells never become detached from the wall of the anther. Abundant orbicules are present along the inner tangential and radial surfaces of the tapetal cells and in the anther locule. The tapetum is persistent throughout microspore wall development, with the tapetal cells undergoing apoptosis *in situ* after exine ontogeny is nearly complete and intine development is initiated.

Tapetum type

Early-divergent angiosperms exhibit diversity in tapetum type including several intermediate types,

indicating that the earliest angiosperms exhibited evolutionary lability in tapetum ontogeny (Furness & Rudall, 2001). Diversity in tapetum type is also found within Nymphaeales. *Brasenia schreberi* J.F.Gmel. (Taylor & Osborn, 2006), *Cabomba aquatica* Aubl. (Gabarayeva et al., 2003) and *Nuphar pumila* DC. (Zhou & Fu, 2008) exhibit a secretory tapetum, which has also been reported in *Nymphaea alba* L. (as *Castalia alba* (L.) Wood; Schnarf, 1931). In contrast, tapetal cells of *Cabomba caroliniana* A. Gray physically detach from the anther wall and invasively migrate into the locule, but the cells retain some characteristics of a secretory tapetum (Taylor et al., 2008). Both *Nymphaea colorata* and *Nymphaea mexicana* exhibit a 'cyclic' invasive tapetum, in which tapetal cells physically migrate into the locular space but then retreat back to the anther wall multiples times during ontogeny (Rowley et al., 1992; Gabarayeva & El-Ghazaly, 1997).

This investigation has documented that the tapetum in *Victoria* is secretory, thereby confirming previous reports of a secretory tapetum in the genus. Yet, while tapetal cells do not detach from the anther wall, they do separate from one another and extend into the anther locule and directly contact the developing microspores, often in multiple places. The degree of radial separation of the tapetal cells and their extension into the anther locule varies among angiosperms, including water lilies. For example, radial separation occurs in *Cabomba caroliniana* before the tapetal cells detach from the middle layers and invade the anther locule (Taylor et al., 2008). However, radial separation of tapetal cells was not observed in *Brasenia* (Taylor & Osborn, 2006) and images of the tapetum in *Nuphar* by Zhou and Fu (2008, figures 6–9) show some radial separation, but no overtopping and very little extension into the anther locule. It should be noted that it is challenging to characterise and compare the degree of tapetal cell protrusion among other basal angiosperm taxa, because this character is difficult to measure and is typically not reported in studies of tapetum ontogeny. This underscores the importance of describing tapeta beyond the typical classification schemes (i.e. 'secretory', 'amoeboid') in ways that more fully characterises the range of complexity exhibited by different taxa.

Orbicules

Orbicules, or Ubisch bodies, are small bodies characterised by an electron-dense outer layer that is comprised of sporopollenin and a more electron-translucent core that is comprised of lipids and polysaccharides (Huysmans et al., 1998; Furness & Rudall, 2001). In *Victoria*, lipidic pro-orbicules are first apparent within invaginations of the plasmalemma. These invaginations are present on the radial faces between tapetal cells, as well as along the inner tangential surfaces of the tapetal cells lining the anther locule. Clearly-defined orbicules with an electron-dense outer layer and an electron-translucent inner core are also apparent within these invaginations, as well as in the intercellular space between tapetal cells and in the anther locule.

Orbicules are almost exclusively associated with the secretory tapetum type (Furness & Rudall, 2001). However, orbicules have been reported in several taxa that exhibit invasive tapeta including the water lilies *Nymphaea colorata* (Rowley et al., 1992), *N. mexicana* (Gabarayeva & El-Ghazaly, 1997) and *Cabomba caroliniana* (Taylor et al., 2008) as well as *Gentiana acaulis* L. (Gentianaceae; Lombardo & Carraro, 1976) and *Modiolastrum malvifolium* (Griseb.) K.Schum. (Malvaceae; Galati et al., 2007).

Nearly all water lily genera for which tapetal ontogeny has been described to date, produce orbicules, including those with secretory tapeta having cells that degrade *in situ* (i.e. *Brasenia*, Taylor & Osborn, 2006; and *Victori*a, this study) and those with invasive tapeta. Orbicules are not reported in *Cabomba aquatica*, which has a secretory tapetum (Gabarayeva et al., 2003).

The definitive function of orbicules is undetermined, but they have been hypothesised to contribute to microspore wall development (Maheshwari, 1950), to aid in pollen dispersal (Heslop-Harrison, 1968; Heslop-Harrison & Dickinson, 1969; Keijzer, 1987), to be a bi-product of tapetal metabolism (Heslop-Harrison, 1968; Dickinson & Bell, 1972) and/or to aid in the degradation of tapetal cells (Rowley & Erdtman, 1967; see Huysmans et al., 1998, for further discussion).

Regarding the pollen dispersal hypothesis, Heslop-Harrison (1968) and Heslop-Harrison and Dickinson (1969) have suggested that orbicules aid in dispersal by forming a non-wettable surface on the inside of the anther locule. Given this, Suarez-Cervera et al. (1995) further hypothesised that orbicules may be associated with wind pollination. No such correlation, however, has been observed (Huysmans et al., 1998) and the water lily taxa that produce orbicules exhibit a range of pollination syndromes including wind pollination in *Brasenia* (Osborn & Schneider, 1988; Osborn et al., 1991; Taylor & Williams, 2009), fly pollination in *Cabomba* (Schneider & Jeter, 1982; Osborn et al., 1991) and beetle pollination in *Victoria* (Prance & Arias, 1975). Therefore, within Nymphaeales, orbicule production is not associated with a particular tapetum type or pollen vector.

Exine microchannels

Both the tectum and foot layer of the mature pollen wall are dissected with narrow microchannels. These exine elements have not been previously described in *Victoria*. Microchannels first become apparent during the early 'free' microspore stage as electron-dense elements running perpendicular to the microspore plasmalemma. As the exine thickens, the microchannels become less regularly organised.

In *Victoria*, the exine microchannels become apparent at the same time that the tapetum is secreting material into the anther locule. Such microchannels in other taxa have been shown to facilitate the transport of sporophytic tapetal material to the cytoplasm of the developing microspore (Rowley et al., 1987, 2003; Abadie et al., 1988; Carretero & Rodriguez-Garcia, 1995). Microchannels have also been hypothesised to store pollenkitt within the pollen wall of *Cabomba* as an adaptation to enhance fly pollination (Osborn et al., 1991; Taylor et al., 2008). In *Victoria*, the synchronous ontogenetic timing of microchannel appearance and tapetal degradation indicates that the exine microchannels could have either or both of these functions.

We did not observe pollenkitt on the surface of *Victoria* pollen grains and there have been no published reports of this tapetal secretion in *Victoria*; however, it is still possible that *Victoria* pollen grains exhibit pollenkitt. All of the *Victoria* anther and pollen material in this study was chemically fixed. Our investigations of *Cabomba caroliniana* demonstrate that whereas mature pollen grains exhibit copious amounts of pollenkitt, both on the exine surface and within its layers, this pollenkitt was only observed in grains that had not been chemically fixed in alcohol-based media (Osborn et al., 1991; Taylor et al., 2008). *Victoria* is pollinated by dynastid beetles (*Cyclocephala*; Prance & Arias, 1975; Schneider et al., 2003; Seymour & Matthews, 2006) and, based on visual observations, Prance and Arias (1975) described *Victoria* pollen as dry and adhering to beetle bodies with a sticky floral secretion produced by the central cavity of the flower (the stigmatic cup). In other members of Nymphaeaceae, similar stigmatic secretions have been shown to wash pollen grains off insects (Schneider, 1982, 1983; Capperino & Schneider, 1985) and serve as the location of pollen germination (Williams et al., 2010). In *Victoria*, it is possible that this secretion functions to adhere pollen to beetles, but at this time we cannot unequivocally discount the possible occurrence and role of pollenkitt.

Phylogenetic implications

Recent phylogenetic studies of Nymphaeaceae have indicated that traditional genus circumscriptions need to be re-assessed in this family and that there is a lack of resolution regarding the relationships among core Nymphaeaceae (Borsch et al., 2007, 2008; Löhne et al., 2008). Combined sequence data indicate that *Victoria* is sister to *Euryale* and that this clade may be nested within *Nymphaea*, diverging from the Nymphaeales stem lineage after the origin of subgenus *Nymphaea*, which contains *N. mexicana*, and before the origin of the clade comprising the rest of *Nymphaea*, including *N. colorata* (Borsch et al., 2007, 2008).

Due to this hypothesised relationship between *Victoria* and *Nymphaea* and the presence of a cyclic invasive tapetum in *N. mexicana* and *N. colorata*, we hypothesised that *Victoria* might exhibit an invasive tapetum. Instead, the presence of a secretory tapetum in *Victoria* indicates that the cyclic invasive tapetum was either lost in *Victoria* or, more likely, arose at least twice in *Nymphaea*. An invasive tapetum has been shown to have evolved independently in Nymphaeaceae, Annonaceae, Monimiaceae and Winteraceae (Furness & Rudall, 2001). The probable occurrence of three independent origins of the invasive tapetum in Nymphaeales, two in *Nymphaea* and one in *Cabomba caroliniana* (Taylor et al., 2008), indicates that lability in tapetum type is even greater in basal angiosperms than previously hypothesised. Greater resolution of the relationships within core Nymphaeaceae, as well as further investigations of tapetum type in a range of species will increase our understanding of tapetum evolution in basal angiosperms.

Nymphaeales exhibit several potentially intermediate tapetum types that, placed in a phylogenetic context, may help resolve the evolutionary steps in the transition from a secretory type to more derived invasive tapetum types. The cyclic invasive tapetum in *Nymphaea* is hypothesised to be intermediate between secretory and plasmodial tapetum types (Tiwari & Gunning, 1986; Furness & Rudall, 2001). In the plasmodial type, tapetal cell protoplasts invade the locule and then fuse to form a periplasmodium that replaces the locular fluid (Pacini et al., 1985; Furness & Rudall, 2001). The tapetum in *Cabomba caroliniana*, in which secretory tapetal cells invade the anther locule in a non-cyclical manner, is also hypothesised to be an intermediate type (Taylor et al., 2008). In the present study, we hypothesise that the tapetum of *Victoria*, in which tapetal cells extend into the anther locule and contact the developing microspores in *Victoria* may be more derived than the tapeta of *Brasenia* or *Nuphar*, but

less derived than the tapeta exhibited by *Nymphaea* and *Cabomba*. Due to their great diversity in tapetal ontogeny, water lilies are a key group, in which to focus further investigations.

Conclusion

Victoria is an important genus in which to investigate reproductive characters in early-diverging angiosperm lineages. *Victoria* is hypothesised to be nested within core Nymphaeales and exhibits several traits that are thought to be derived in basal angiosperms. Therefore, characterising reproductive development in *Victoria* is critical for understanding the extent of variation in early-diverging angiosperm lineages, yet much regarding the pattern of reproductive development in *Victoria* is still unknown.

This study characterised tapetum ontogeny and the development of associated exine characters for the first time. The presence of a secretory tapetum indicates that specialised beetle pollination in *Victoria* is not associated with an invasive tapetum. Moreover, the concurrent development of exine microchannels with tapetal secretory activity lends support to the hypothesis that microchannels functionally play a role in the transport and/or storage of tapetal exudate.

A secretory tapetum in *Victoria* indicates that there is more lability in tapetum ontogeny in basal angiosperms than previously thought, and the finding that tapetal cells separate along their radial faces and elongate such that they extend more deeply into the anther locule, unlike tapetal cells of *Brasenia* and *Nuphar*, highlights the variation in tapetum ontogeny that is present within early-divergent angiosperm lineages. This also underscores the necessity of describing the complete ontogenetic pattern and using care when assigning types.

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