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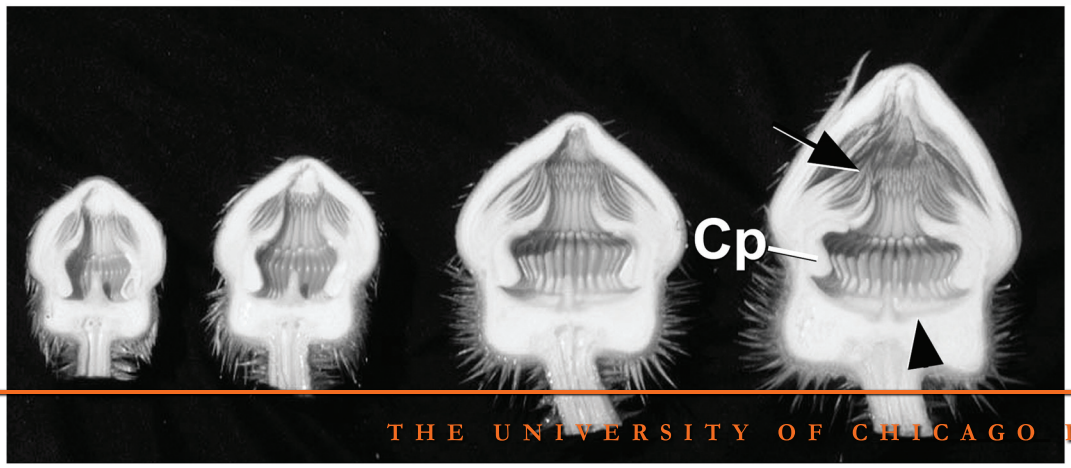

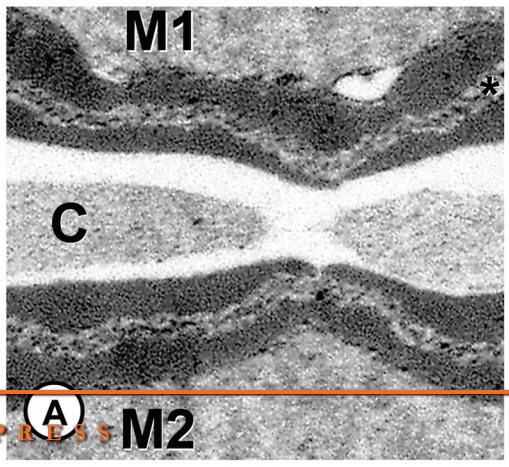

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On the Cover *Inset*, an emergent bud of the water lily *Victoria* (Nymphaeales) one day before flowering, with large platter-like leaves shown floating behind it. *Background*, montage showing sectioned floral buds at various stages of ontogeny (*lower left*) and images of developing pollen tetrads. Taylor et al. (pp. 1259–1276, in this issue) comprehensively characterize pollen development in *Victoria* for the first time, document the ultrastructure of the tectate-columellate exine, and demonstrate that permanent tetrads form in this genus through a never-before described process of exine protrusion and modification. Discovery of a novel pattern of compound pollen formation in this early-divergent angiosperm underscores the developmental lability in pollen wall formation and highlights the need for comparative ontogenetic analyses.

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POLLEN ONTOGENY IN *VICTORIA* (NYMPHAEALES)

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Premise of research. Water lilies (Nymphaeales) make up one of the oldest independent lineages of angiosperms. The giant water lily, *Victoria*, exhibits pollination and floral traits that are derived within Nymphaeales. Specialization in pollination and floral biology is often reflected in pollen traits, and in *Victoria*, this is evidenced by the production of permanent tetrads. Compound pollen has evolved many times across the angiosperm phylogeny, but compound pollen development has been investigated in only a few taxa, and the degree of developmental variation in microspore cohesion is unknown. This article comprehensively characterizes the pollen ontogenetic sequence in *Victoria* for the first time.

Methodology. Floral buds of *Victoria amazonica*, *Victoria cruziana*, and Longwood hybrid were field collected. Anthers at the sporogenous, microspore mother cell, tetrad, "free" microspore, and mature pollen grain stages were studied using combined LM/SEM/TEM.

Pivotal results. Microspore cohesion in *Victoria* differs from that exhibited by the few compound pollen-producing taxa that have been studied. In *Victoria*, the calymmate tetrads fuse via crosswall cohesion, but cytoplasmic connections are transient and do not serve as a template for wall bridge formation. Instead, the ektexines protrude at gaps in the callose wall, fuse, and are subsequently modified, resulting in continuous infratectal layers. In addition to revealing the pattern of permanent tetrad development, ontogenetic data demonstrate the presence of infratectal columellae, a character that has been debated in Nymphaeaceae. *Victoria* pollen grains also exhibit a rarely described membranous granular layer that forms beneath a well-defined endexine, which is composed of white-line-centered lamellae.

Conclusions. *Victoria* exhibits a never-before-described pattern of microspore cohesion during permanent tetrad formation. The new data underscore the developmental lability in pollen wall formation and the importance of ontogenetic data for characterizing ambiguous and enigmatic traits, particularly with regard to understanding the evolution of reproductive biology and phylogenetic relationships within early-divergent angiosperm lineages.

Keywords: compound pollen, Nymphaeaceae, Nymphaeales, pollen, pollen development, pollination, tetrads, *Victoria*.

Introduction

Victoria (Nymphaeaceae, Nymphaeales) is a South American water lily genus composed of two species, *Victoria amazonica* (Poep.) Sowerby and *Victoria cruziana* Orb. Both species are frequently grown in cultivation and can be crossed to yield hybrids (Schneider and Williamson 1993). The Longwood hybrid results from crosses with *V. cruziana* as the pollen parent and *V. amazonica* as the ovule parent, whereas the Adventure hybrid results from the reciprocal cross (Knotts 1999). *Victoria* is the largest of all water lilies, with floating leaves that can reach 1 m across and flowers that measure up to 50 cm in diameter (Schneider and Williamson 1993).

Nymphaeales is composed of three families (Cabombaceae, Hydatellaceae, Nymphaeaceae) and has long been considered to be among the oldest independent lineages of angiosperms (Takhtajan 1969; Walker 1974; Donoghue and Doyle 1989; Hamby and Zimmer 1992; Doyle 1998; Les et al. 1999). Water lilies are represented in the record of early angiosperm fossils (Friis et al. 2001, 2003, 2011; Taylor et al. 2008*a*), and phylogenetic studies have consistently indicated that Nymphaeales diverged from the basalmost or next-most-basal node of the extant angiosperm phylogenetic tree (Qiu et al. 1999, 2006; Löhne and Borsch 2005; Saarela et al. 2007). The intergeneric

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relationships within Nymphaeales have been elucidated in recent years; however, there is still conflict concerning the exact relationships among subfamily Nymphaeoideae (*Nymphaea*, *Ondinea*, *Victoria*, *Euryale*). Within this group, *Victoria* and the Asian genus *Euryale* consistently form a clade, which is currently thought to be nested within the genus *Nymphaea* (Borsch et al. 2007, 2008).

Among Nymphaeales and early angiosperms, *Victoria* is of particular evolutionary interest because the genus exhibits several characters that are likely derived within water lilies. Many of these characters, including large flowers, carpellary appendages, and thermogenesis, have been hypothesized to be correlated with pollination biology (Prance and Arias 1975; Lamprecht et al. 2002; Seymour and Matthews 2006; Borsch et al. 2008). In *Victoria*, pollination typically occurs via entrapment of large Dynastid beetles (*Cyclocephala*; Prance and Arias 1975; Seymour and Matthews 2006). Beetles are attracted to the sweet-smelling, thermogenic, first-night flowers, which open at dusk (Prance and Arias 1975; Seymour and Matthews 2006) and in which the beetles become trapped when the flower closes at dawn. Once in the flower, beetles feed on specialized carpellary appendages and deposit pollen (collected from other flowers visited the previous day) on the receptive stigmatic surface. On the second evening, the flower reopens, now with the anthers fully dehiscent, and the beetles push through pollen-covered stamens to escape (Prance and Arias 1975).

This specialized beetle pollination may have arisen very early in the evolution of Nymphaeaceae, and there is evidence that this ancient partnership represents one of the earliest examples of a mutualistic interaction between flowering plants and insects (Ervik and Knudsen 2003; Gandolfo et al. 2004; Thien et al. 2009). Alternatively, the transition to beetle pollination in *Victoria* may be more recent, depending on the dating of the divergence of the *Victoria-Euryale* clade (Borsch et al. 2008; Löhne et al. 2008). Like floral traits, pollen characters in *Victoria* are likely to reflect pollination biology, as they do in other water lilies (Osborn and Schneider 1988; Osborn et al. 1991; Taylor and Osborn 2006; Taylor et al. 2008*b*). Therefore, data on pollen development in *Victoria* may shed light on the trajectory of pollen evolution in water lilies and on the evolutionary history of beetle pollination in early angiosperms.

Studies of *V. cruziana* have revealed that mature pollen grains are held together in permanent tetrads (Erdtman 1954; Ueno 1962; Roland 1965, 1971; Khanna 1967). *Victoria* tetrads are calymmate, with a continuous exine surrounding each individual microspore (Roland 1965; Knox and McConchie 1986). Compound pollen is found in more than 50 angiosperm families and has undoubtedly originated independently many times in flowering plants (Walker and Doyle 1975; Walker 1976; Knox and McConchie 1986; Shukla et al. 1998; Copenhaver 2005). The mechanisms of wall cohesion have been inferred from studies of mature pollen grains from a variety of taxa (Skvarla and Larson 1963; Skvarla et al. 1975, 1976; Verhoeven and Venter 1994; reviewed in Knox and McConchie 1986). However, the developmental and architectural details have been described in only a few ontogenetic studies from a small number of taxa across all types of compound pollen (Dunbar 1973; Knox and Friederich 1974; Meyer and Yaroshevskaya 1976; Takahashi 1979; Takahashi and Sohma 1980, 1984; Knox and McConchie 1986; Waha 1987; Dahl and Rowley 1991; Fitzgerald et al. 1993, 1994; Tsou and Fu 2002, 2007). These studies have begun to elucidate the general patterns of microspore cohesion and have revealed that there is variation in the ontogeny of compound pollen formation. Investigating variation in the mechanisms of pollen wall cohesion in phylogenetically distant groups may provide key insight into the lability of the exine developmental program. *Victoria* is of considerable interest because it is nested within Nymphaeales, the earliest-diverging angiosperm lineage to evolve compound pollen.

The objective of this study was to comprehensively document pollen ontogeny in *Victoria* for the first time using combined LM, SEM, and TEM, with a special focus on development of the pollen grain wall within the calymmate tetrad. Tapetal characters were also investigated and will be described as they relate to pollen development, but tapetum ontogeny is discussed more in depth in Taylor et al. (2012). The developmental pattern of pollen wall formation in *Victoria* will be compared to that of other water lilies as well as to taxa that form permanent tetrads. Correlations to pollination biology will be discussed as well.

Methods

Twenty-two floral buds (11 from *Victoria amazonica*, 6 from *Victoria cruziana*, and 5 from Longwood hybrid) representing a diverse range of developmental stages were collected from the private ponds of Ben and Kit Knotts in Cocoa Beach, Florida (fig. 1*A*–1*D*). Anthers were dissected from the buds in the field, fixed in 3% glutaraldehyde (in 0.2 mol/L phosphate buffer, pH 7.4) for 24 h, and then buffer washed four times. Specimens were postfixed in buffered 1% osmium tetroxide for 3 h and buffer washed four times.

Anthers for LM and TEM were dehydrated in a graded series of ethanol and acetone rinses and gradually infiltrated and embedded in Spurr epoxy resin. Embedded anthers were sectioned on an ultramicrotome with a diamond knife. Thick sections (850 nm) were stained with Richardson's stain (azure II and methylene blue) and examined/imaged with bright-field and differential interference contrast illumination on an Olympus BHS compound light microscope. Thin sections (90 nm) were collected on 1 × 2-mm slot grids and dried onto Formvar support films. Grids were stained with 1% uranyl acetate and lead citrate and then examined/imaged using a JEOL JEM-100SX transmission electron microscope at 80 kV.

Anthers for SEM were dehydrated in a graded ethanol series, critical-point dried, and mounted onto aluminum stubs with colloidal graphite. To view morphological features of the anther wall and the locular contents, some anthers (while in 70% ethanol) were either transversely fractured with a double-edged razor blade at room temperature or frozen in liquid nitrogen and then fractured. Additional anthers were transversely fractured after critical-point drying. Dried anthers were also macerated using a syringe needle and mounted onto aluminum stubs with double-sided adhesive tape. All stubs were sputter-coated with gold/palladium and examined/imaged using a JEOL JSM-6100 scanning electron microscope at 5 kV.

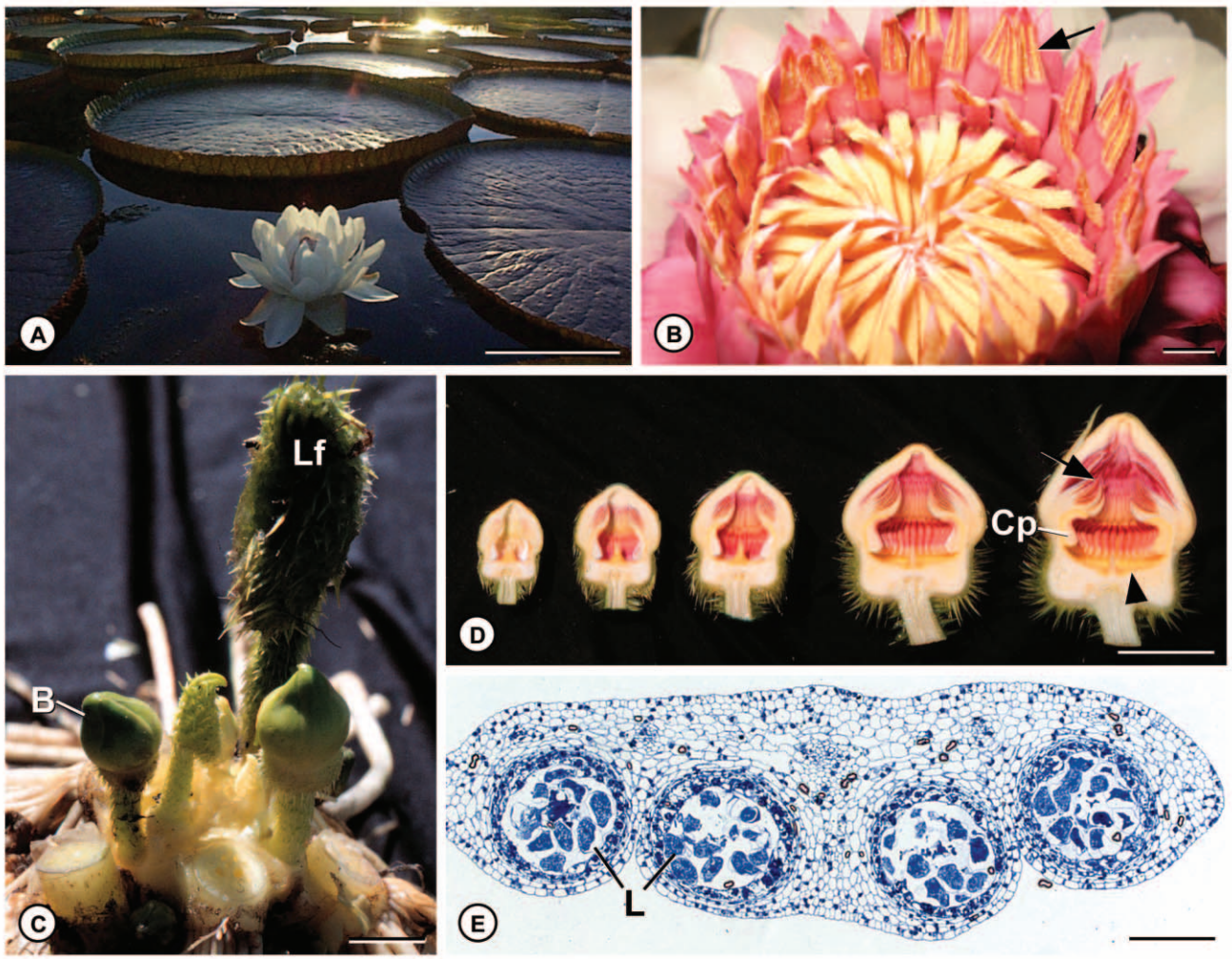

Fig. 1 Habit and flower morphology—*Victoria*. *A*, Whole *Victoria* plant with large, floating platter-like leaves and first-night flower. *B*, Detail of a second-night flower. The outer two to three whorls of the laminar stamens are reflexed, whereas the inner whorls are bent over the central stigmatic cup. The anther portion of the stamen (arrow) is dehiscent, and pollen is visible, falling out of the anthers onto the abaxial surfaces of the stamens below. *C*, Detail of floral buds (B). Buds develop in the leaf axils while submerged; here the mature leaves were removed at the base of petioles. Two immature leaves (Lf) are also present. *D*, Longitudinal sections of a developmental series of floral buds. Whorls of laminar stamens (arrow) surround an inner whorl of carpellate appendages (Cp), which arch over the central stigmatic cup (arrowhead). *E*, Transverse section through the anther region of a laminar stamen. Four locules (L) are located on the adaxial surface. Scale bars = 20 cm (*A*), 10 mm (*B*), 5 cm (*C*, *D*), 100 μm (*E*).

Results

We did not observe substantial variation in pollen development among *Victoria amazonica*, *Victoria cruziana*, and Longwood hybrid, so data from these taxa are combined to describe the general pattern of ontogeny in *Victoria*. Although the data below are presented in discrete ontogenetic stages, it is important to note that developmental events occur on a continuum.

Stamen Morphology and Ontogenetic Timing

Stamens in *Victoria* are laminar and tetrasporangiate. Although the laminar stamens lack distinct filament and anther regions, the elongated region of the microsporophyll in which the microsporangia are positioned is referred to here as the "anther" (fig. 1*B*, 1*E*).

Pollen development is asynchronous within buds, such that different anthers from the same bud contain microspores in different developmental stages. For example, in one bud, 14% of sectioned anthers contained late tetrads, 43% contained early "free" microspores, and 43% contained late free microspores. In another bud, 11% of sectioned anthers contained sporogenous tissue, 56% contained microspore mother cells, 22% contained tetrads, and 11% contained early free microspores. However, pollen development is typically synchronous within individual anthers (fig. 1*E*). Anthers open and release pollen grains on the second evening of the 2-d floral cycle, prior to flower opening (fig. 1*B*).

Sporogenous Tissue and Microspore Mother Cells

Sporogenous tissue is found in anthers within the smallest buds. Individual sporogenous cells are polygonal and slightly larger than surrounding cells. In late sporogenous cells, the large nucleus and nucleoli are distinct (fig. 2*A*).

Sporogenous cells differentiate into microspore mother cells, each of which exhibits a single large nucleus, relatively few small vacuoles, and abundant starch grains (fig. 2*B*–2*D*). Early in development, microspore mother cells are appressed against each other and the tapetum, filling the entire anther locule (fig. 2*B*, 2*C*). Cytomictic channels are present between adjacent microspore mother cells. As the microspore mother cell stage progresses, cells develop an electron-dense microspore mother cell coat (fig. 2*E*) and separate from one another. A layer of callose develops between the plasmalemma and microspore mother cell coat (fig. 2*D*–2*F*), with the callose thickening to 1.2 μm by the end of the microspore mother cell stage.

Tetrads

Microspore mother cells undergo meiotic divisions to each form a tetrad of microspores, and the entire tetrad is enveloped by callose and a persistent microspore mother cell coat (fig. 3*A*–3*C*). The vast majority of tetrads are tetrahedral, with some rhomboidal tetrads present as well (fig. 3*A*–3*C*), indicating that cytokinesis is simultaneous. In Longwood hybrid, polyads containing more than eight microspores within each callose envelope are also produced (fig. 3*A*).

Early tetrad stage. Following cytokinesis, callose septae develop between the individual microspores (fig. 3*B*, 3*C*), such that microspores are separated from one another. Cytoplasmic connections between adjacent microspores are occasionally observed (fig. 3*C*, 3*D*). These narrow connections appear to be transient structures and are observed in locules in which only microspore mother cells undergoing meiosis and very early tetrads are present. In the earliest tetrads, the callose directly abuts the plasmalemma of each individual microspore (fig. 3*E*). Shortly after, the primexine develops between the callose envelope and the plasmalemma (fig. 3*F*). Cytoplasmic connections were not observed in tetrads with primexine formation. Despite the collection of numerous floral buds, as well as the sectioning of a large number of anthers within these buds, including many with microspore tetrads, very few tetrads were observed that exhibited primexine formation.

Late tetrad stage. The late tetrad stage is marked by the presence of a defined tectum, infratectum, and foot layer (fig. 4*A*–4*C*). The tectum is homogenous and is of similar thickness in the proximal and distal walls. In contrast, the foot layer is much thinner in the distal wall than in the proximal wall, averaging only 26 nm in thickness compared to 102 nm, respectively (table 1). The thickness of the infratectal space is similar throughout the developing wall, and well-defined infratectal columellae are present. The columellae occur at regular intervals and are, on average, 25 nm wide at this stage (fig. 4*D*, 4*E*, 4*G*; table 1).

During the tetrad stage, localized regions without callose are present between the adjacent microspores (fig. 4*A*). No cytoplasmic connections were observed in the late tetrad stage. At the locations without callose, the corresponding tectal layers of adjacent microspores are relatively thin, and the plasmalemma of at least one of the microspores typically bulges outward slightly (fig. 4*A*). In later tetrads, the plasmalemma and the walls of adjacent microspores protrude through the intermicrospore space such that the adjacent exines are in contact with each other and form a "wall bridge" between the microspores (fig. 4*B*). These contact points are hereafter termed "points of primary cohesion."

At the points of primary cohesion, the tectal layers often appear to be disrupted (fig. 4*B*, 4*D*) and adjacent tectal layers are not continuous with each other (fig. 4*D*). As ontogeny proceeds, the adjacent tectal layers fuse to form a clearly continuous layer at the periphery of the primary cohesion point (fig. 4*C*, 4*E*). The tectum is typically absent from the center of these wall bridges, such that the infratectal layers of the adjacent microspores are continuous (fig. 4*C*). Residual sporopollenin, presumably of tectal origin, is sometimes apparent in the center of the wall bridge. In some cases, the tectum and the infratectum both become continuous between adjacent microspores and the center of the bridge is composed of homogenous sporopollenin (fig. 4*E*). In every case, a distinct foot layer underlies the wall bridge, and in no case was a connecting strand of cytoplasm observed within the wall bridge (fig. 4*A*–4*E*). One to several contact points were observed between each adjacent microspore in a single section (fig. 4*C*, 4*F*).

The endexine is initiated in the late tetrad stage, after formation of the points of primary cohesion. The endexine is deposited as thin, white-line-centered lamellae in both the proximal and distal walls, including beneath the aperture and the wall bridges (fig. 4*G*, 4*H*). The endexine is never present within wall bridges (fig. 4*E*).

The homogenous tectum is absent in the apertural region, but the foot layer and endexine form the apertural membrane (fig. 4*H*). On the apertural membrane, small globular elements of sporopollenin are present above the foot layer (fig. 4*H*).

Free Microspores

Early free microspore stage. The callose envelope around the tetrad degrades, marking the beginning of the free microspore stage. However, microspores are held together at the points of primary cohesion and do not separate from one another (fig. 5*A*, 5*B*). We still refer to microspores that are not surrounded by callose as free microspores. In early free microspores, remnants of the callose septae are sometimes present in the pockets between the points of primary microspore cohesion and at the center of the tetrad where all four microspores meet (fig. 5*C*). As the early free microspore stage progresses, this callose fully degrades (fig. 5*A*, 5*D*).

Between the tetrad and early free microspore stages, the tectum and the foot layer each thicken in the proximal wall to an average of 226 and 264 nm, respectively (fig. 5*D*; table 1). However, the thickness of both the tectum and the foot layer can vary significantly along the length of each microspore (fig. 5*C*, 5*D*). The thickening of the proximal tectal layers causes the adjacent tectal layers to contact each other and fuse such that they form one continuous common tectal layer (fig. 5*D*). This additional cohesion of adjacent microspore walls is referred to here as "secondary cohesion." In these regions, only the tectal layers fuse, whereas the infratectal and foot layers of each adjacent microspore remain distinct (fig. 5*D*). The

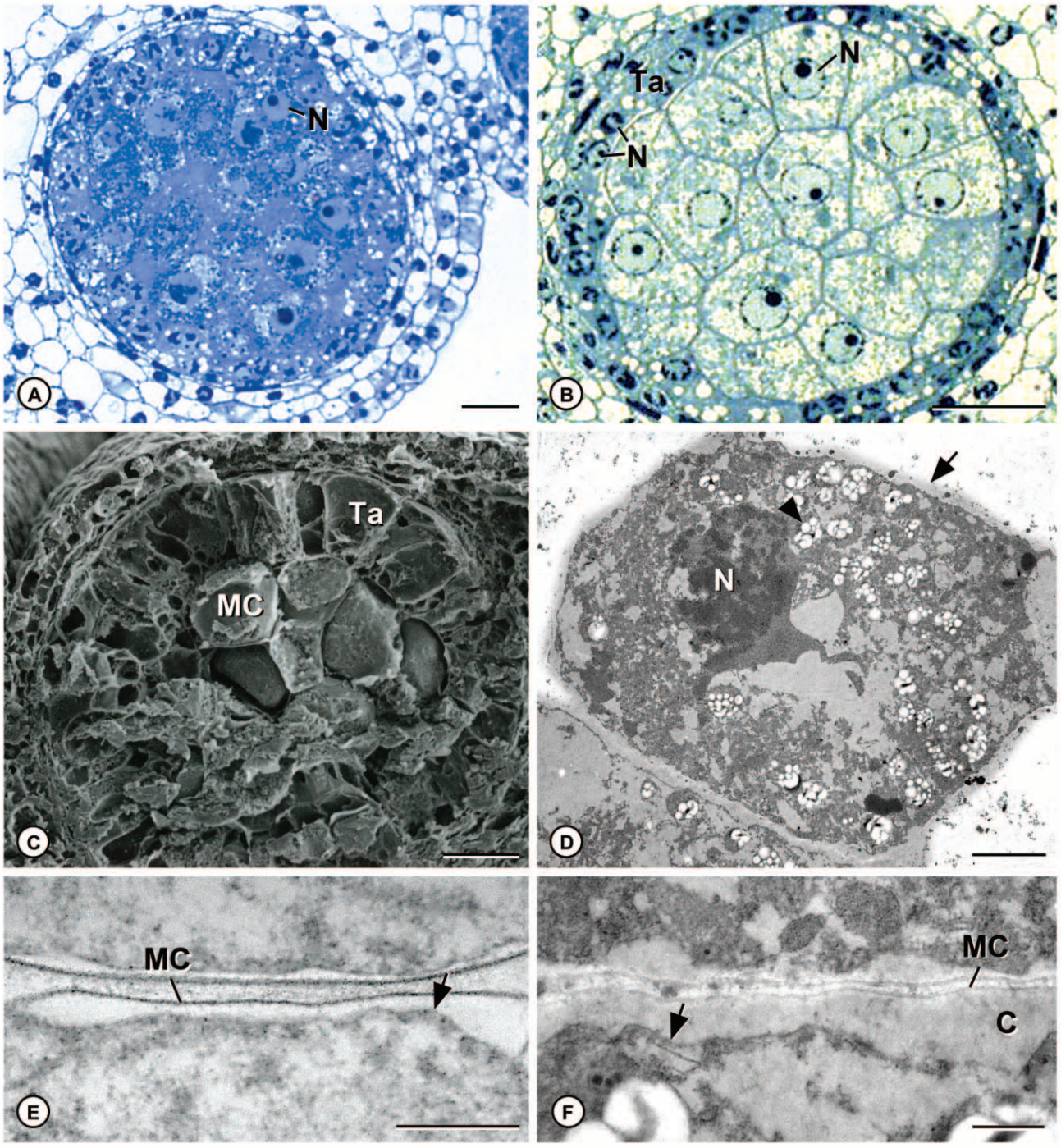

Fig. 2 Sporogenous tissue and microspore mother cell stage—*Victoria*. *A*, Transverse section of anther locule with sporogenous tissue. The nuclei (N) and nucleoli are apparent. *B*, Transverse section of anther locule with differentiated microspore mother cells and tapetum (Ta). Microspore mother cells have a single large nucleus (N), whereas tapetal cells are often binucleate. *C*, Anther locule with differentiated microspore mother cells (MC) and tapetum (Ta). *D*, Microspore mother cell with a thin layer of callose surrounding the cell (arrow), a single large nucleus (N), and abundant starch granules (arrowhead). The microspore mother cells have separated such that the cell is adjoining only one other cell. *E*, Detail of plasmalemma of two adjacent microspore mother cells. The plasmalemma (arrow) is separated slightly from the microspore mother cell coat (MC). *F*, Detail of two adjacent microspore mother cells with callose (C) deposited between the plasmalemma (arrow) and the microspore mother cell coat (MC) of the lower cell. The callose layer is unevenly thickened. Scale bars = 25 μm (*A*–C), 5 μm (*D*), 500 nm (*E*, *F*).

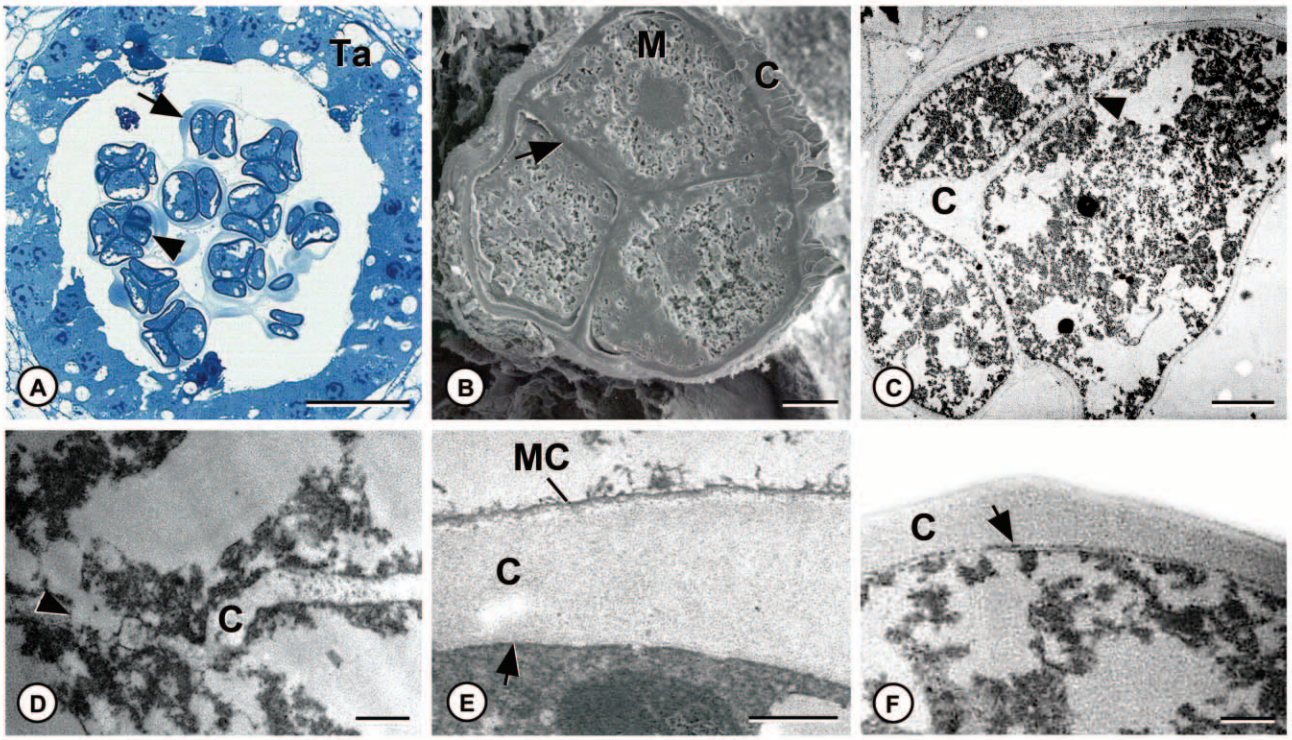

Fig. 3 Early tetrad stage—*Victoria*. *A*, Transverse section of anther locule with tetrads surrounded by callose (arrow) and secretory tapetum (Ta). Tetrads are tetrahedral or rhomboidal, with at least one polyad (arrowhead) present. *B*, Section through a tetrad showing three microspores (M) in section. Callose (C) surrounds the exterior of the tetrad, and callose septae (arrow) extend between the individual microspores. *C*, Section through a tetrad showing three microspores in section divided by callose septae (C). A single cytoplasmic connection (arrowhead) is present between two of the microspores. *D*, Detail of cytoplasmic connection (arrowhead) between two microspores shortly after meiosis. A thin callose septum (C) separates the microspores. *E*, Detail of distal surface of a microspore shortly after meiosis. A thick callose (C) layer separates the plasmalemma (arrow) and the persistent microspore mother cell coat (MC). *F*, Detail of distal wall of an early tetrad showing early primexine formation (arrow) between a thick layer of callose (C) and the microspore plasmalemma. Scale bars = 50 μm (*A*), 10 μm (*B*), 5 μm (*C*), 500 nm (*D–F*).

adjacent tectal layers in all regions of the wall do not fuse simultaneously, so gaps in the common tectum are apparent throughout the fused microspore walls (fig. 5*B*, 5*D*). During the early free microspore stage, electron-dense microchannels are first apparent in the developing exine. These extremely narrow channels dissect the tectum and foot layers in all regions of the microspore wall (fig. 5*D*).

The infratectum increases only slightly in thickness in the proximal wall early in the free microspore stage (table 1), whereas the columellae nearly double in width to 38 nm in this stage (fig. 5*D*, 5*E*; table 1). In early free microspores, the ringlike aperture of each microspore can be clearly observed (fig. 5*B*). On average, the endexine is thicker in the early free microspore stage than in the late tetrad stage, particularly in the distal wall and the apertural membrane (fig. 5*E*; table 1).

Late free microspore stage. In late free microspores, the tectal layers of adjacent microspores are nearly entirely fused, although the occasional gap in the common tectum is still present (fig. 6*A–6C*, 6*G*). The points of primary cohesion remain visible as places where the infratectal or foot layers meet (fig. 6*D*). Between the early and late free microspore stages, the tectum and foot layer each approximately double in thickness in both the distal and proximal regions. On average, the tectum of each individual microspore is slightly thinner in the distal wall (327 nm) when compared to the proximal wall (432 nm; table 1). Moreover, the distal tectum is considerably thinner than the common tectal layer that is formed from the fusion of adjacent proximal tectal layers (864 nm; table 1). The tectal layer, particularly in the distal wall, is transected by numerous microchannels that exhibit a regular, radial orientation (fig. 6*E*).

The foot layer remains an order of magnitude thinner in the distal wall than in the proximal wall (43 nm vs. 482 nm; table 1). In contrast, the infratectal space becomes thinner in late free microspores than in early free microspores, particularly in the proximal wall (table 1). The infratectal columellae continue to thicken in the late free microspore stage, the columellae sometimes take on a spherical appearance, likely due to the constraint of the narrowing size of the infratectal space. Together, the columellae fill much of the infratectal space (fig. 6*E*). In the aperture, the sporopollenin elements atop the foot layer have enlarged (fig. 6*F*).

By the late free microspore stage, the endexine has increased in thickness in the distal wall (fig. 6*F*), but it has become

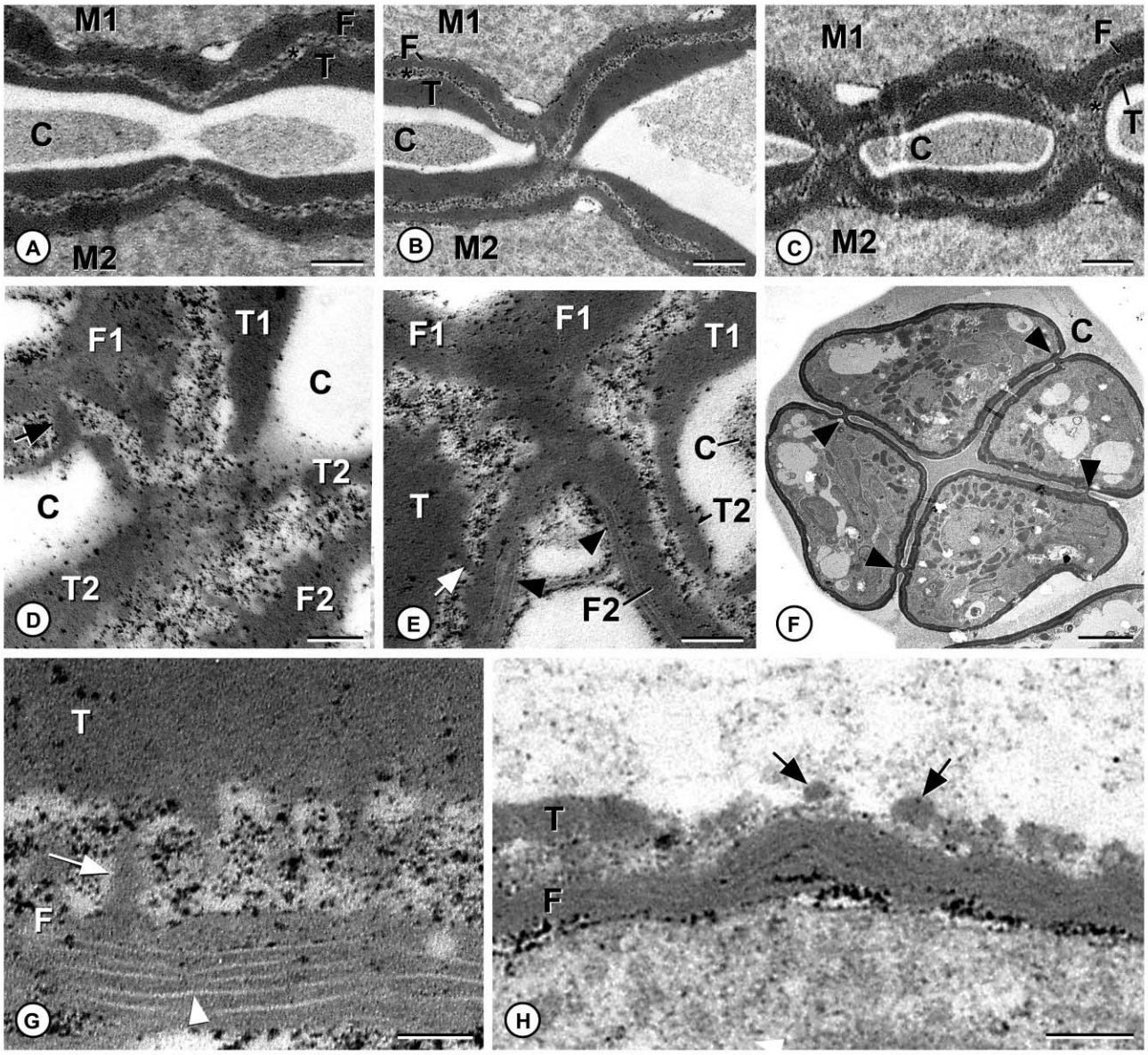

Fig. 4 Late tetrad stage—*Victoria*. *A*, Detail of proximal walls of two adjacent microspores (M1, M2). The tectum (T), foot layer (F), and infratectum (asterisk) are present. The callose septum (C) between the microspores is absent from a small region that corresponds to areas of the adjacent exines that exhibit a thin tectum. *B*, Detail of proximal wall of two adjacent microspores (M1, M2). The callose (C) between the microspores has receded further, and the two adjacent tecta (T) are in contact at a point of primary cohesion. The adjacent infratectal (asterisk) and foot layers (F) remain distinct from each other. *C*, Detail of two adjacent microspores (M1, M2) with two points of primary cohesion. The tectal layers (T) at the points of cohesion have disappeared, and the tectal and infratectal (asterisk) layers of the two adjacent microspores are now continuous. The foot layers (F) remain distinct. Remnants of the callose septum (C) are present between the points of cohesion. *D*, Detail of primary point of cohesion illustrated in *B*. The adjacent tectal layers (T1, T2) are touching but are disrupted at the point of contact and do not form a continuous layer at the periphery of the wall bridge. The infratectal layers and foot layers (F1, F2) remain distinct. The callose (C) between the microspores is disrupted. There is no evidence of a cytoplasmic connection or channel that connects the two adjacent microspores and serves as a substructure for the wall bridge. Infratectal columellae (arrow) are evident near the point of primary cohesion. *E*, Detail of primary point of cohesion similar to *C*. In this tetrad, a band of homogenous sporopollenin is present within the wall bridge, and the infratectal layers and the columellae (arrow) remain distinct. The adjacent tectal layers (T1, T2) have fused into a continuous tectal layer (T) at the periphery of the point of primary cohesion. This continuous tectal layer is thick on the left side and thin on the right side. The disrupted callose septum (C) is visible to the right of the point of cohesion. The foot layers (F1, F2) underlie the wall bridge, and a lamellate endexine (arrowheads) underlies the foot layer. *F*, Entire tetrad surrounded by callose (C). In this plane of section, one point of primary cohesion (arrowheads) is present between each adjacent microspore. *G*, Detail of nonapertural distal wall showing a thick tectum (T), infratectal columellae (arrow), a relatively thin foot layer (F), and several endexine lamellae (arrowhead). *H*, Detail of microspore aperture. The tectum (T) is largely absent from the aperture, but globular sporopollenin elements (arrows) are present on top of the foot layer (F), and these span the apertural membrane. Scale bars = 500 nm (*A*–*C*), 100 nm (*D*, *E*), 5 μm (*F*), 50 nm (*G*), 250 nm (*H*).

Table 1

Mean Thickness (nm) of Distal and Proximal Pollen Wall Layers at Sequential Developmental Stages

	Distal wall				Proximal wall			
Wall layer	Late tetrad	Early free microspore	Late free microspore	Mature grain	Late tetrad	Early free microspore	Late free microspore	Mature grain
Tectum	139	155	327	877	177	226	864[a] (432)	988[a] (494)
Infratectum	107	103	95	73	112	156	72	83
Foot	26	22	43	292	102	264	482	405
Endexine	71	80	108	123	44	190	111	95
MGL	...	...	152	249	...	...	177	185
Intine	...	...	...	1625	...	...	...	1123

Note. Mean $n = 5$ microspores/stage. Ellipses indicate that the layer is not present in a measurable amount at that stage. MGL = membranous granular layer.

[a] The proximal tectum in the late free microspore and mature grain stages represents the common tectum of two adjacent microspores. The tectum of each microspore, assuming equal thickness for both, is shown in parentheses.

compressed in the proximal wall (fig. 6*D*, 6*E*; table 1). During this stage, globular elements develop beneath the endexine, between the developing exine and the plasmalemma (fig. 6*E*, 6*F*). These elements are not part of the lamellate endexine, as they do not form around white-line lamellae and appear only after the lamellate endexine is fully formed. However, these elements more closely resemble the material of the endexine in electron density than that of the intine, which is not yet apparent. The layer of globular elements corresponds to the membranous granular layer (MGL) described by El-Ghazaly and Huysmans (2001). Near the end of the late free microspore stage, the intine begins to develop beneath the globular elements. Before the intine is initiated, however, exine cohesion of adjacent microspores is nearly complete (fig. 6*G*).

During the late free microspore stage, the tapetal cells separate from each other along their radial walls and elongate so that they protrude more deeply into the anther locule (fig. 6*A*) than in previous stages. Orbicules are also present between tapetal cells and within the anther locule (fig. 6*H*).

Mature Pollen Grains

Mature pollen grains are held together in permanent tetrads (fig. 7*A*–7*C*) that average 78.0 μm in diameter. The majority of tetrads are tetrahedral (fig. 7*A*, 7*C*), with some rhomboidal tetrads also present (fig. 7*A*, 7*B*). Occasional polyads are produced by Longwood hybrid (fig. 7*A*, 7*D*). The nonapertural surface of pollen grains is psilate, but the apertural membrane is ornamented with sculptural elements that average 0.59 μm in diameter (fig. 7*C*–7*E*).

The distal tectum in mature grains is 877 nm thick on average, whereas the proximal tectum of individual microspores are only half as thick; however, each is fused to form a common tectal layer that is 988 nm thick (table 1). The distal foot layer has thickened significantly since the late free microspore stage to reach a thickness of 292 nm, whereas the proximal foot layer has not thickened further (table 1). The infratectum is very narrow, and the mature infratectal columellae, which are 109 nm wide on average, clearly span across the entire infratectal space (fig. 7*F*, 7*G*; table 1). The wall bridges—which consist of the infratectal layer and sometimes the foot layer but never the endexine—remain distinguishable and mark the initial points of primary cohesion between adjacent microspores (fig. 7*B*).

The endexine is compressed in the proximal wall and to a lesser degree in the nonapertural distal wall, so that the individual lamellae are visible in only a few places (fig. 7*F*, 7*G*). The membranous granular layer remains apparent between the compressed endexine and the now fully developed intine. At the mature grain stage, the MGL is composed of globular elements that are much more electron dense than the intine and that strongly resemble the endexine in composition (fig. 7*F*). The mature intine is thick and bilayered (fig. 7*F*; table 1).

Discussion

Ontogenetic Timing

Pollen development in *Victoria* was asynchronous among the anthers within individual buds, with some buds containing all developmental stages ranging from sporogenous tissue to late free microspores. This was unexpected as all anthers dehisce over the course of a single night (Prance and Arias 1975). Flowers of *Lilium longiflorum* exhibit a similar pattern of asynchrony in pollen development that disappears before anthesis (Jansen et al. 1995). Among water lilies, both *Brasenia* and *Cabomba* have nearly synchronous pollen development within flowers (Taylor and Osborn 2006; Taylor et al. 2008*b*), but the relative timing of pollen development in other members of Nymphaeaceae is unknown.

A summary of key pollen wall ontogenetic characters is illustrated in figure 8*A*–8*F*. Primexine formation occurs in the early tetrad stage (fig. 8*B*). The small number of tetrads exhibiting primexine formation in our material, compared to the large number of microspores found that exhibited both earlier and later developmental stages, indicates that the primexine forms rapidly in *Victoria*. *Brasenia* and *Cabomba* exhibit a similar ontogenetic pattern, in which the entire tetrad stage, and particularly early exine development, occurs rapidly (Taylor and Osborn 2006; Taylor et al. 2008*b*).

The tectum, foot layer, and infratectal columellae are initiated in the middle tetrad stage, and these layers all exhibit

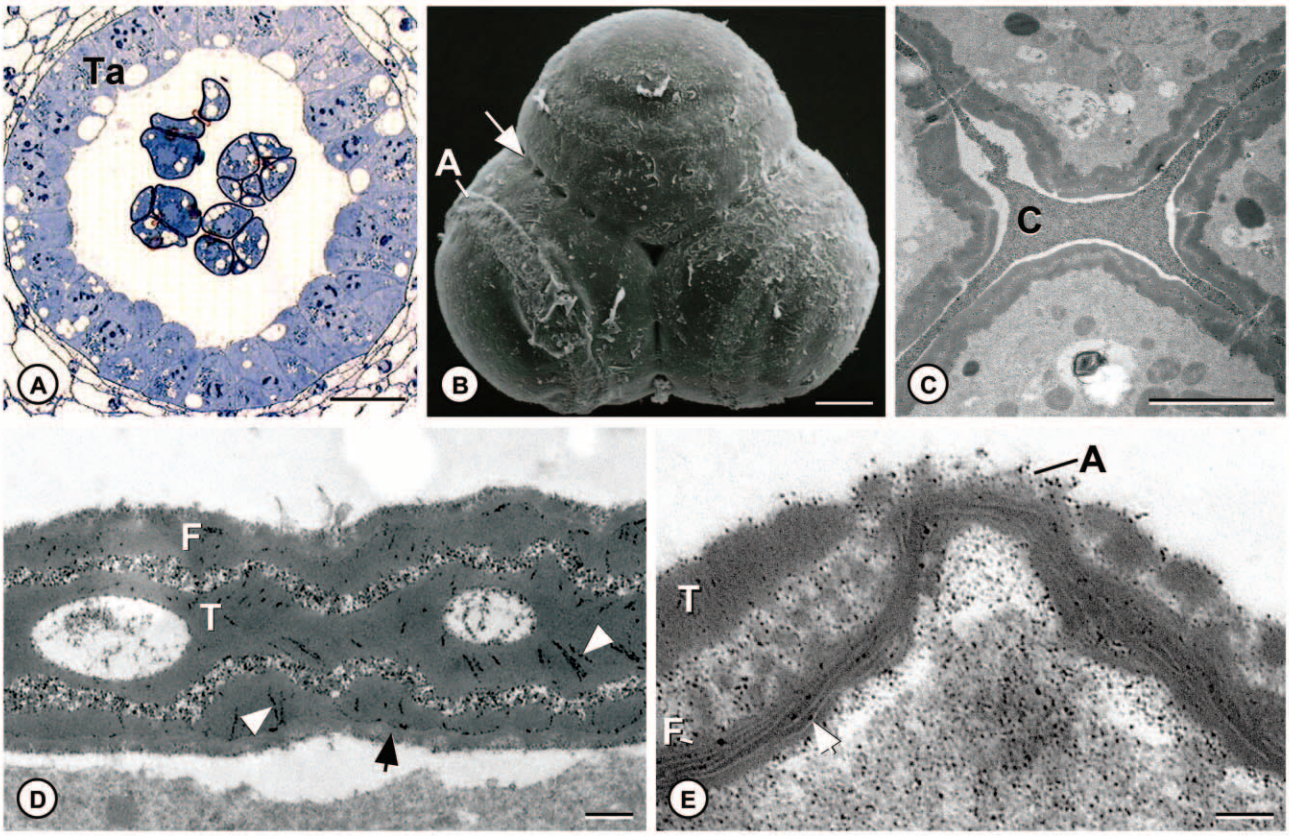

Fig. 5 Early free microspore stage—*Victoria*. *A*, Section through anther locule with early free microspores. Both tetrads and polyads are present. The callosic envelope surrounding the tetrads has dissociated, as have the callose septae between the microspores, but the tectum is not fully fused. The tapetum (Ta) is present as a palisade layer lining the anther locule. *B*, Entire tetrad of early free microspores. The points of primary cohesion between the microspores are visible (arrow). The ringlike apertures (A) of each microspore appear as rough bands. *C*, Detail of center of tetrad in section showing residual callose (C) still present between the adjacent microspores. *D*, Detail of proximal walls of two adjacent microspores. The tectal layers have fused in continuous stretches to form a single, fused tectum (T). The tectum and foot layers (F) are dissected with thin microchannels (arrowheads). The endexine is initiated, with lamellae faintly visible (arrow) beneath the foot layer. *E*, Detail of distal wall and apertural region of a single microspore. This section is slightly oblique, resulting in a granular appearance of the infratectal columellae. The tectum (T) is absent from the aperture (A), but the foot layer (F) and endexine lamellae (arrow) are present. Scale bars = 50 μm (*A*), 10 μm (*B*), 5 μm (*C*), 500 nm (*D*), 100 nm (*E*).

significant sporopollenin deposition by the late tetrad stage (fig. 8*C*, 8*D*). This is also true of pollen in other water lilies, including *Brasenia* (Taylor and Osborn 2006), *Cabomba* (Gabarayeva et al. 2003; Taylor et al. 2008*b*), and *Nymphaea* (Gabarayeva and Rowley 1994; Gabarayeva and El-Ghazaly 1997). In *Victoria*, the endexine also begins to develop in the late tetrad stage (fig. 8*D*), which is in contrast to other water lilies, in which the endexine is not initiated until the early free microspore stage (Gabarayeva and El-Ghazaly 1997; Gabarayeva et al. 2003; Taylor and Osborn 2006; Taylor et al. 2008*b*). After callose degradation in *Victoria*, all structural components of the exine undergo further increase in size. In the late free microspore stage, globular elements that make up the enigmatic membranous granular layer develop beneath the endexine (fig. 8*E*). Initiation of the intine occurs very late in the free microspore stage (fig. 8*F*).

Pollen Wall Characters and Ultrastructural Architecture

All nonapertural regions of the pollen wall exhibit a tectum, infratectum, foot layer, endexine, membranous granular layer, and intine. The tectum is absent from the aperture, but this region is ornamented with relatively large sporopollenin elements deposited on the foot layer.

Tectum. The individual tectum of each microspore is thinner along the proximal surface than along the distal surface, a phenomenon also observed by Roland (1965). Because the individual microspores fuse to form a calymmate tetrad, the presence of the tectum of the adjacent microspore limits the degree of thickening that can occur on the proximal side. However, after cohesion of the two proximal tecta, the common tectum is as thick (and often thicker) as the distal tectum on the individual microspores.

The tectum is dissected by narrow microchannels that ini-

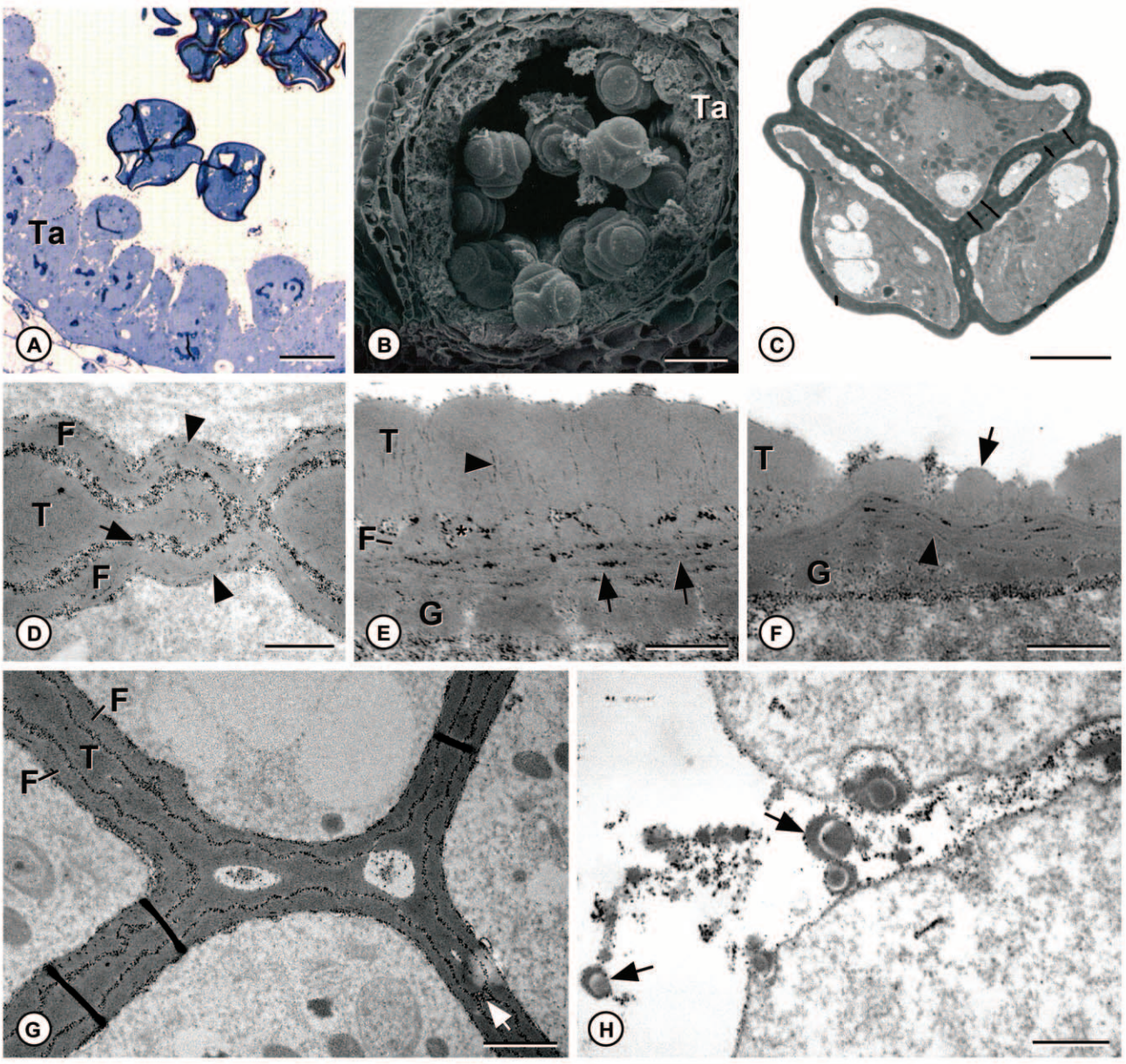

Fig. 6 Late free microspore stage—*Victoria*. *A*, Portion of anther locule with permanent tetrads. The tapetal cells (Ta) overtop each other and extend into the locule. *B*, Whole tetrads present in anther locule, lined by tapetum (Ta). *C*, Section of entire tetrad with proximal tectal layers nearly completely fused. *D*, Detail of two adjacent proximal walls. Simple fusion of the tectum (T) is nearly complete, and one point of primary cohesion, where the infratectal layers meet, is apparent. Infratectal columellae (arrow) are rodlike and span the infratectal space. Endexine lamellae (arrowheads) are visible beneath the foot layer (F). *E*, Detail of distal microspore wall exhibiting a thick tectum (T) with radially oriented microchannels (arrowhead), relatively thin foot layer (F), and robust infratectal columellae (asterisk). Endexine lamellae (arrows) are present, and homogenous sporopollenin granules (G) that will make up the membranous granular layer are beginning to develop beneath the endexine lamellae. *F*, Detail of aperture. The tectum (T) is absent, but large spherical sporopollenin elements (arrow) are present atop the foot layer. Endexine lamellae (arrowhead) and granules of the membranous granular layer (G) are present under the aperture. *G*, Detail of center of a tetrad. Two gaps are still present in the common tectum (T). The foot layer (F) and infratectal layer of the individual microspores remain mostly distinct; however, one point of primary cohesion, where the infratectal layers meet, is visible (arrow). *H*, Detail of adjacent tapetal cells. Orbicules (arrows) are present in the intercellular space between the radial membranes of the cells, as well as in the anther locule. Scale bars = 25 μm (*A*), 50 μm (*B*), 10 μm (*C*), 500 nm (*D–F*, *H*), 2 μm (*G*).

tially become detectable during the early free microspore stage. These first appear as electron-dense elements running perpendicular to the microspore plasmalemma, and as ontogeny proceeds and the exine thickens, the microchannels become less regular in orientation. The microchannels are apparent throughout the tectum of mature pollen grains, as well as within the foot layer. The developmental timing of microchannels within the exine is synchronous with the timing of orbicule production by the secretory tapetum, supporting hypotheses that the microchannels function in the transport and storage of tapetal exudate (Taylor et al. 2012).

Infratectum. There have been conflicting interpretations of exine ultrastructure in *Victoria*, primarily regarding the structure of the infratectum. *Victoria* pollen has been described as having a granular infratectum (Ueno 1962), an infratectum with short, indistinct columellae that give the appearance of a granular layer (Roland 1965), and a homogenous exine with a few internal cavities (Walker 1976). In phylogenetic analyses, the infratectum of *Victoria*, or more generally Nymphaeoideae, is typically coded as either granular or granular-intermediate (Doyle and Endress 2000; Doyle 2005; Borsch et al. 2008). However, this coding is often reported as provisional or based on disputed characterizations of published data for *Nymphaea*.

The structure of the infratectum in *Victoria* is difficult to interpret, and in certain instances, infratectal elements do appear granular in nature. Without having studied numerous floral buds and all developmental stages, it would be difficult to state unequivocally that the infratectal elements are indeed rod-shaped columellae and not spheroidal elements. However, columellae are observed spanning the infratectum at every developmental stage and are particularly clear in the tetrad stage. Our ontogenetic data show that the infratectal space becomes compressed during the free microspore stage, due to thickening of the tectum and the foot layers. This compression of the infratectal space causes the columellae to increase in width throughout the free microspore stage, sometimes filling nearly all the infratectal space and taking on a granular appearance. Furthermore, many of the granular-type elements that are observed are clearly in oblique section or cross section. This is particularly true near the aperture and the points of primary cohesion, where significant wall deformation occurs. Therefore, we agree with Roland's (1965) assessment that the exine in *Victoria* is of the tectate-columellate state and should not be considered granular. Gabarayeva and Rowley (1994) describe a similar infratectal structure in *Nymphaea colorata*.

Some authors have considered the infratectum of *Victoria* to be structurally different enough to be potentially scored as a third, intermediate state (Doyle 2005; Borsch et al. 2008). The idea of an intermediate state between columellate and granular infratecta types was proposed by Doyle and Endress (2000) to describe the infratecta of Cabombaceae and *Barclaya*, although these authors coded Nymphaeoideae as granular at that time. Whereas we understand the rationale for coding the infratectal type as intermediate at the time, we do not think that there is sufficient reason to continue describing the infratectum of *Victoria* as intermediate rather than columellate, given the new ontogenetic data. Moreover, we emphasize that if the infratectum in *Victoria* is considered intermediate, then it is a modification of an ancestral columellate infratectum, rather than an intermediate state between an ancestral granular type and a derived columellate type (Doyle 2005).

Historically, all genera of Nymphaeales have been reported to have granular infratecta (Ueno and Kitaguchi 1961; Ueno 1962; Walker 1974, 1976), which was hypothesized to be plesiomorphic in angiosperms (Walker 1976; Donoghue and Doyle 1989) and a shared trait between Nymphaeales and gymnospermous anthophytes (Osborn et al. 1991, 1993; Osborn and Taylor 1995; Osborn 2000). However, further study of Nymphaealean pollen, including *Brasenia* (Osborn et al. 1991; Taylor and Osborn 2006), *Cabomba* (Osborn et al. 1991; Gabarayeva et al. 2003; Taylor et al. 2008*b*), and *Trithuria* (Hydatellaceae; Remizowa et al. 2008; M. L. Taylor and J. M. Osborn, unpublished data), have revealed that these water lily taxa clearly have columellate infratecta. The infratectum in *Nymphaea* has been described as columellate as well (Rowley et al. 1992; Gabarayeva and Rowley 1994; Gabarayeva and El-Ghazaly 1997; Gabarayeva et al. 2001). As water lilies and other early-divergent angiosperms lineages predominately exhibit columellate pollen, granular pollen is considered to be derived within extant flowering plants (Doyle and Endress 2000; Borsch et al. 2008).

Endexine and membranous granular layer. The endexine in *Victoria* is composed of white-line lamellae that are apparent in the tetrad and free microspore stages. In mature grains, the endexine becomes developmentally compressed so that the individual lamellae are not always apparent. Roland (1965) described the endexine in *Victoria* as granular, but this description was almost certainly of the sporopollenous granules that make up the membranous granular layer, which is clearly visible in Roland's micrograph, rather than the compressed endexine lamellae, which are not (Roland 1965, fig. 10).

The MGL is an enigmatic layer that develops in a range of taxa during the late free microspore stage, after endexine lamellae deposition and before intine initiation (El-Ghazaly and Huysmans 2001). This layer is characterized by small sporopollenin granules that often coalesce into a more homogenous layer as development proceeds (El-Ghazaly and Huysmans 2001). This type of wall layer has been described in taxa across the angiosperm phylogeny, including *Betula* (Dunbar and Rowley 1984; El-Ghazaly and Huysmans 2001), *Catharanthus* (El-Ghazaly 1990), *Cucurbita* (Ciampolini et al. 1993), *Helleborus* (Echlin and Godwin 1969), *Ludwigia* (Skvarla et al. 1975), *Nelumbo* (Kreunen and Osborn 1999), and *Zea* (Skvarla and Larson 1966). Due to the location and timing of onset of development of this layer, it has often been described as part the endexine or, occasionally, the intine. However, unlike the intine, the MGL resists acetolysis and has a different mode of deposition and chemical composition from endexine lamellae (El-Ghazaly and Huysmans 2001).

Within early-divergent lineages, an MGL has been described in *Nymphaea* (Gabarayeva and El-Ghazaly 1997; El-Ghazaly and Huysmans 2001), but it is not present in *Brasenia* (Taylor and Osborn 2006), *Cabomba* (Gabarayeva et al. 2003; Taylor et al. 2008*b*), or *Trithuria* (Remizowa et al. 2008; M. L. Taylor and J. M. Osborn, unpublished data). *Amborella* also lacks an MGL (Hesse 2001). However, *Austrobaileya* exhibits a layer composed of sporopollenin "endexine granules" beneath the endexine lamellae (Zavada 1984), and a "spongy" inner

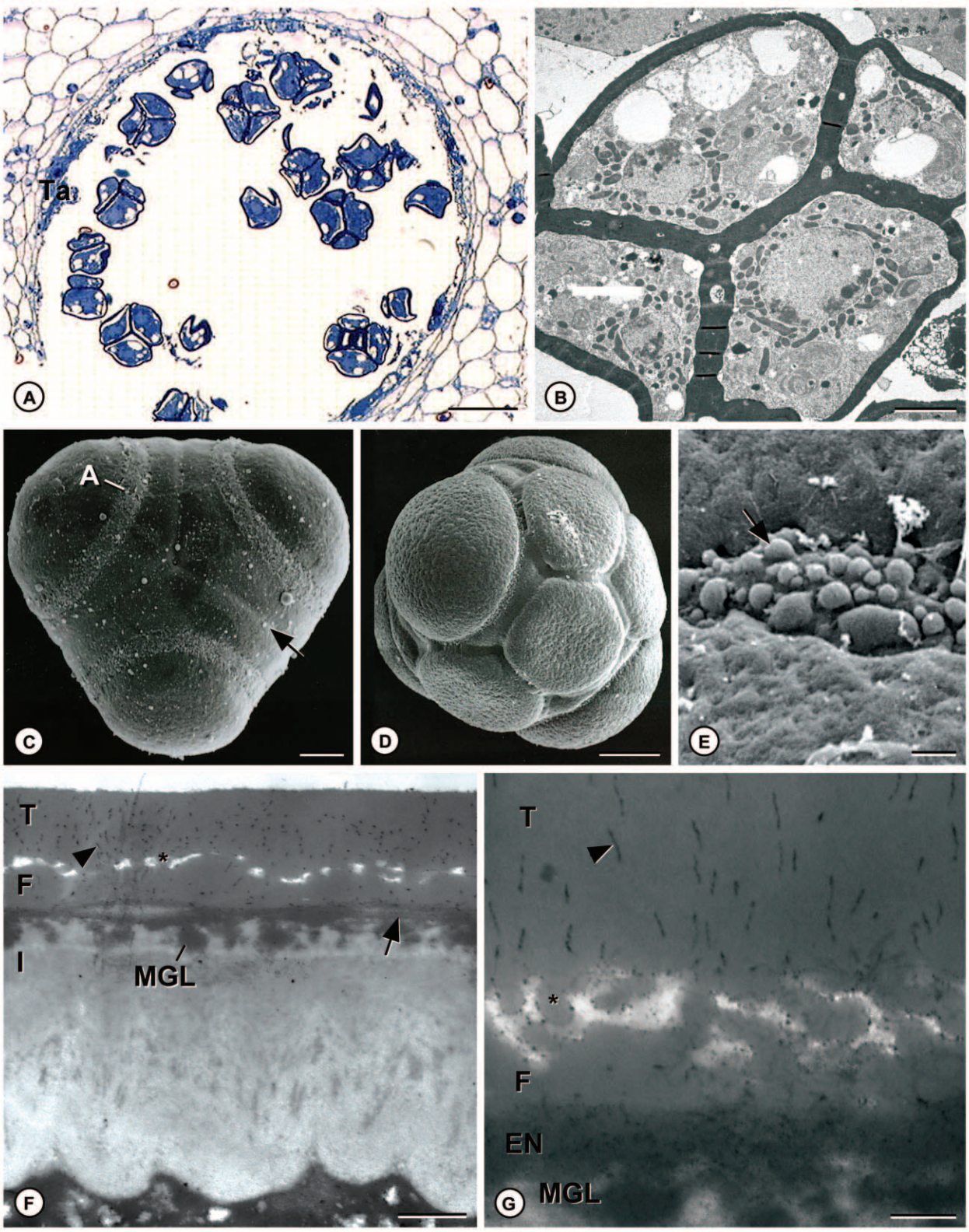

Fig. 7 Mature grain stage—*Victoria*. *A*, Anther locule with degraded tapetum (Ta) and mature tetrads. *B*, Detail of a mature tetrad in section. The proximal tectal layers have nearly fully fused. *C*, Whole tetrad showing ringlike apertures (A) of each of the three microspores in view and zone of fusion between adjacent microspores (arrow). Orbicules are visible on the surface of the tetrad. *D*, Polyad with eight microspores in view. *E*, Detail of surface of the aperture. The apertural membrane is ornamented with spherical sporopollenin bodies (arrow). *F*, Transverse

endexine has been reported in Schisandraceae (Sampson 2000*a*).

Mature angiosperm pollen typically exhibits a much thinner endexine with fewer lamellae than gymnosperm pollen (Osborn et al. 1993; Osborn and Taylor 1994, 1995, 2010; Osborn 2000; Doores et al. 2007). Osborn (2000) hypothesized that this is due to the evolutionary loss of the inner lamellae and substructural white-line units. If so, then the sporopollenin granules that make up the MGL may represent late-formed sporopollenin, originally of endexinous origin, that is unable to aggregate onto the white-line units that have been lost (Osborn 2000). This hypothesis is supported by the fact that the elements that make up the MGL are similar to the endexine in their ability to resist acetolysis (El-Ghazaly and Huysmans 2001). Whether the MGL is regarded as a truly distinct layer, its broader characterization in a phylogenetic context may yield data regarding both the evolutionary significance of this layer as well as lability in endexine development.

Development of the Permanent Tetrad

Compound pollen, defined as an association of two or more grains united in such a way that the unit has properties of its own (Knox and McConchie 1986), is produced by more than 580 species in more than 50 angiosperm families across the angiosperm phylogeny (Knox and McConchie 1986; Copenhaver 2005). Compound pollen units, including dyads, triads, tetrads, polyads, and pollinia, are classified into two basic types: (1) the calymmate type, in which a tectum surrounds the whole tetrad without interruption, forming a continuous layer, and (2) the acalymmate type, in which the tectum is interrupted between individual grains (Van Campo and Guinet 1961; Roland, 1965; Knox and McConchie 1986).

Two primary mechanisms of microspore cohesion are recognized: (1) simple cohesion, in which the ektexine surfaces of adjacent microspores become intermeshed and fuse, and (2) crosswall cohesion, in which connections, or "wall bridges," form between adjacent grains (Skvarla et al. 1975, 1976; Knox and McConchie 1986). Both types of cohesion can result in either calymmate and/or acalymmate pollen units, and there is no apparent correlation between the mechanism of cohesion and the type of pollen unit produced.

Originally defined as consisting of an ektexine outer layer and an endexine core (Skvarla et al. 1975), wall bridges composed of a variety of wall layers have been described (Kenrick and Knox 1979; Takahashi 1979; Knox and McConchie 1986; Fitzgerald et al. 1993; Verhoeven and Venter 1994; Sampson 2000*b*, 2007; Furness 2012).

The mechanism of fusion has typically been determined by observing the ultrastructure of the mature wall. Only a few published studies have directly documented microspore cohesion through ontogenetic study (Takahashi 1979; Takahashi and Sohma 1980, 1984; Knox and McConchie 1986; Dahl and Rowley 1991; Fitzgerald et al. 1993, 1994).

Pollen of *Victoria* is dispersed in calymmate tetrads. Calymmate tetrads in *Victoria cruziana* have been previously described by Ueno (1962) and Roland (1965, 1971). Ueno (1962) and Khanna (1967) reported that pollen tetrads often separated into single grains at the time of dispersal. However, we found no evidence of pollen monads within anthers in our study, and given the complete cohesion of adjacent microspore walls, it is doubtful that monads are common. Furthermore, no monads were observed in extensive observations of dispersed pollen on the stigmatic cup following hand pollinations (M. L. Taylor, unpublished data).

The new ontogenetic data clearly indicate that the mechanism of fusion in *Victoria* is through crosswall cohesion. Wall bridges form in the late tetrad stage at points of primary cohesion along the proximal walls. This is followed by secondary, simple cohesion of the remaining proximal tectal layers during the free microspore stage. The steps of tetrad fusion along the proximal walls in *Victoria* are summarized in figure 8*G*–8*M*.

Cytoplasmic connections, also called cytoplasmic strands or cytoplasmic channels, are connections that form between adjacent microspores following meiosis, presumably due to either incomplete cytokinesis or dissolution of adjacent cell walls at the site of the connection (Knox and McConchie 1986). To date, cytoplasmic connections have been observed only in taxa that produce permanent tetrads (Knox and McConchie 1986) and are thought to serve as sites for transport of tapetal nutrients or hormones between grains of the tetrad and to ensure developmental synchrony (Heslop-Harrison 1968; Fitzgerald et al. 1993; Pacini and Hesse 2002). It has also been hypothesized that cytoplasmic channels may serve as a template for primexine and exine deposition, providing the substructure for wall bridge formation (Takahashi and Sohma 1980; Knox and McConchie 1986).

Tsou and Fu (2002, 2007) suggest that this is always the case, despite the fact that these authors did not observe cytoplasmic channels. In fact, exine accumulation on the cytoplasmic connections has been developmentally documented in only a few taxa, notably, *Pyrola japonica* (Takahashi and Sohma 1980) and *Calluna* (Dahl and Rowley 1991), both in Ericaceae. Furthermore, cytoplasmic connections were not observed in *Chimaphila japonica* (Takahashi 1979; Takahashi and Sohma 1980) and are transient and do not serve as a template for primexine deposition or exine accumulation in *Acacia paradoxa*, despite wall development around the rest of the microspore (Fitzgerald et al. 1993). For the vast majority of taxa that produce compound pollen via crosswall cohesion, the ontogenetic data regarding wall bridge formation are absent or incomplete.

In *Victoria*, there is no evidence that cytoplasmic connec-

section of mature distal pollen wall. The tectum (T), infratectal columellae (asterisk), and foot (F) layers are distinct. Microchannels (arrowhead) are visible dissecting the tectum and foot layer. The endexine lamellae are compressed, such that the individual lamellae are apparent in only a few locations (arrow). The membranous granular layer (MGL) is apparent beneath the endexine, and the thick, bilayered intine (I) is present. *G*, Detail of mature exine showing the thick tectum (T) dissected with microchannels (arrowhead), infratectal columellae (asterisk), relatively thin foot layer (F), compressed endexine (EN), and MGL. Scale bars = 50 μm (*A*), 5 μm (*B*), 10 μm (*C*, *D*), 1 μm (*E*), 500 nm (*F*), 200 nm (*G*).

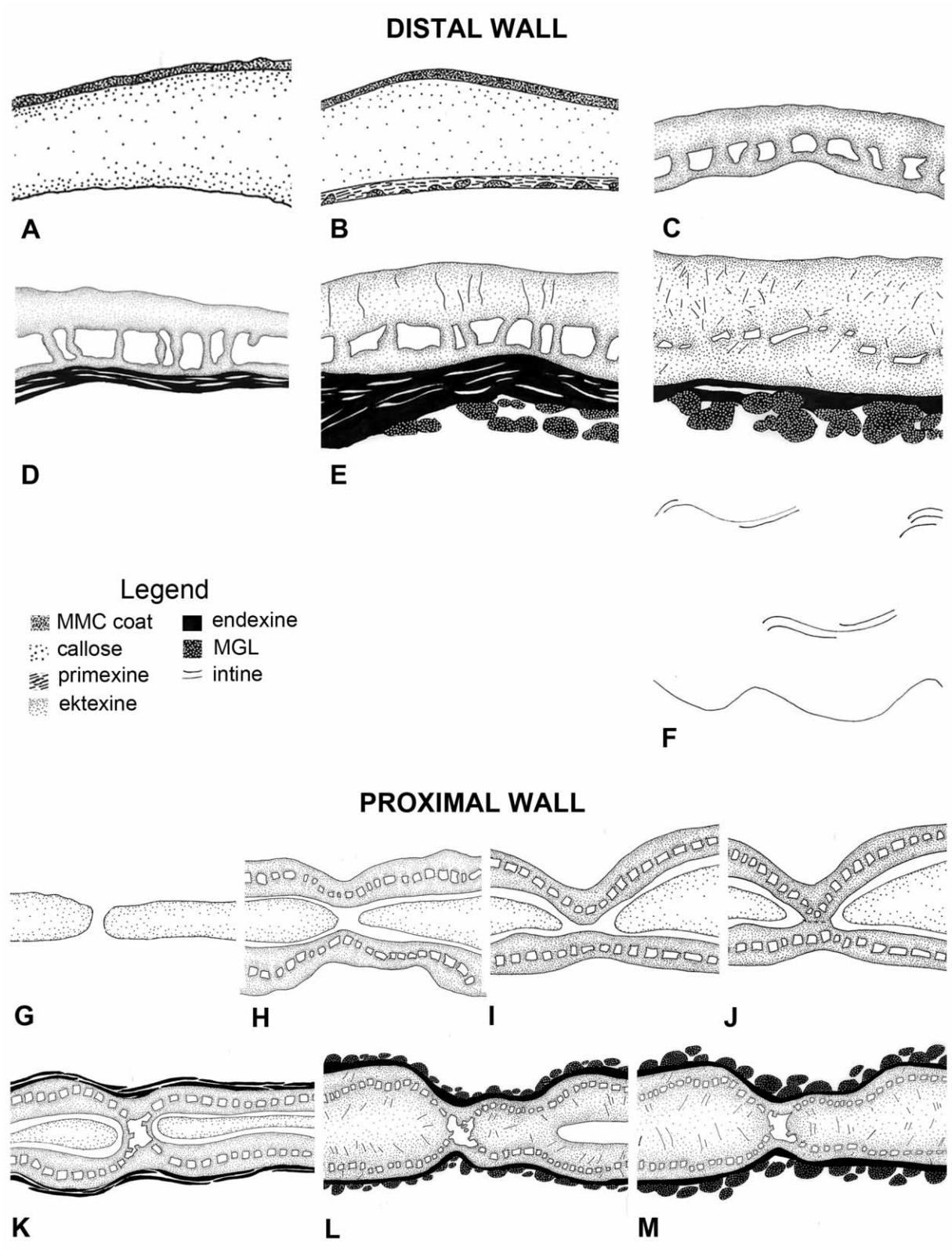

Fig. 8 Summary of major ontogenetic events during pollen wall development in *Victoria*. Distal wall: *A*, microspore mother cell stage; *B–D*, tetrad stage; *E*, free microspore stage; *F*, mature grain stage. Proximal wall: *G–K*, tetrad stage; *L*, free microspore stage; *M*, mature grain stage. Characters illustrated include microspore mother cell (mmc) coat (small, dense stipples); callose (large, light stipples); primexine (horizontal dashes); ektexine (dense stipples); endexine (solid black); membranous granular layer (mgl; black with white stipples); and intine (discontinuous wavy lines).

tions serve as a template for developing wall bridges. In this study, cytoplasmic connections were observed in only very early tetrads of *Victoria* (fig. 8*G*). By the middle tetrad stage, cytoplasmic channels were absent, but gaps in the callose septae were present (fig. 8*H*). It is possible that transient cytoplasmic connections prevent continuous callose deposition during the early tetrad stage, thereby determining the location of callose gaps and, subsequently, the wall bridges. Gaps in the callose septae between adjacent microspores have not been reported in any other water lily in which pollen development has been studied, all of which are typically dispersed as monads (Gabarayeva and El-Ghazaly 1997; Gabarayeva 2003; Taylor and Osborn 2006; Taylor et al. 2008*b*).

Absence of, or irregularities in, callose formation has been found to lead to permanent tetrad formation in several taxa, including *Pandanus odoratissimus* (Periasamy and Amalathas 1991), *Juncus bufonius* (Meyer and Yaroshevskaya 1976), and sterile anthers of *Sorghum bicolor* (Warmke and Overman 1972). Similarly, permanent tetrads develop in *quartet 3* mutants of *Arabidopsis*, due to a defect in the degradation of the microspore mother cell wall. This causes the microspores to be held together after callose degradation and results in exine cohesion (Rhee et al. 2003). Temporal changes in callose formation and/or degradation play a role in compound pollen formation in all of these taxa; however, the developmental steps leading to tetrad formation, for which published ultrastructural data are limited, appear to vary widely. Moreover, none of these taxa seem to form wall bridges in the manner that *Victoria* does.

In *Victoria*, the ektexine underlying the callose gaps was observed to protrude into the intermicrospore space (fig. 8*I*), and contact between adjacent tectal layers was observed. In these tetrads, there was disruption of adjacent tectal layers at the contact point. The adjacent tectal layers clearly did not form the continuous layer at the periphery of the point of cohesion that was observed in later tetrads and in free microspores (fig. 8*J*). These early developmental events were observed only in tetrads in which the endexine was not yet initiated, an event that occurs during the late tetrad stage in *Victoria*. This is evidence that callose gaps, ektexine projections, and partial contact of tectal layers were true ontogenetic events during the development of wall bridges and not artifacts of sectioning. The endexine was never incorporated into wall bridges, which would be expected if the pollen wall was developing on an underlying cytoplasmic substructure that connected the adjacent microspores. Rather, endexine lamellae were observed beneath the wall bridges, oriented parallel to the plasmalemma in each individual microspore (fig. 8*K*). During the free microspore stage, the gaps between the points of primary cohesion become filled in as a result of simple cohesion of the adjacent tectal layers (fig. 8*L*, 8*M*).

Wall bridges in *Victoria* have a different ultrastructure from those found in other early-divergent angiosperm lineages for which wall bridges have been described. These include *Lactoris* (Piperales), in which the adjacent walls are composed of only the foot layer and are joined internally via spherical elements extending between them (Sampson 1995), and *Takhtajania* and *Pseudowintera* (Winteraceae), in which wall bridges are plugged by endexine (Sampson 2000*b*, 2007). Instead, the ultrastructure of mature wall bridges in *Victoria* more closely resemble those in *C. japonica*, which have a core composed of columellae and tectal layers (Ericaceae; Takahashi 1979).

Compound pollen has originated independently many times in angiosperms, and despite limited developmental data, considerable variation has been described, not only in mature pollen wall ultrastructure but also in the pattern of compound pollen formation (Takahashi 1979; Takahashi and Sohma 1980; Knox and McConchie 1986; Fitzgerald et al. 1993; this study). This indicates that the pollen developmental program is labile and that there are multiple evolutionary pathways to the production of compound pollen. Significantly more ontogenetic data are needed in order to determine the variation in developmental mechanisms that have led to the formation of compound dispersal units across angiosperms and whether pollen cohesion mechanisms exhibit a phylogenetic signal.

Function of Permanent Tetrads and Pollination Biology

In addition to Nymphaeales, there have been multiple origins of compound pollen in early-divergent angiosperm lineages, including Canellales, Laurales, Piperales, and Magnoliales (Waha 1987; Sampson 1995, 2000*a*, 2007; Doyle 2005; Tsou and Fu 2007; Doyle and Le Thomas 2012), and several types of compound pollen are present in the angiosperm fossil record from the Lower Cretaceous (Doyle et al. 1990). Within Nymphaeales, in addition to *Victoria*, permanent tetrads have been reported in *Nymphaea tetragona* (Ueno 1962) and *Trithuria inconspicua* (=*Hydatella inconspicua*; Furness and Rudall 1999; Remizowa et al. 2008). The development of permanent tetrads has not been studied in either of these taxa; however, in the case of *T. inconspicua*, the tetrads are collapsed and potentially sterile (Furness and Rudall 1999; Remizowa et al. 2008). The production of permanent tetrads in these taxa indicates at least two, and likely three, origins of compound pollen in Nymphaeales.

The repeated evolution of compound pollen among early-divergent angiosperm lineages indicates that it provided a fitness benefit. Compound pollen is thought to be advantageous in insect pollination systems because it increases the number of pollen grains transferred with each pollination event, thereby improving the probability of successful fertilization of ovules, especially in taxa with a large number of ovules (Shukla et al. 1998). The earliest angiosperms are hypothesized to have exhibited generalist pollination (Bernhardt and Thien 1987; Thien et al. 2009), and compound pollen may have provided a fitness benefit in relatively unspecialized early pollination systems.

Permanent tetrads in *Victoria* are part of a suite of reproductive characters, including large flowers, floral thermogenesis, and starchy carpel appendages, whose evolution accompanied the transition to pollination via beetle entrapment, and we hypothesize that permanent tetrads increase pollination efficiency in *Victoria*. Each *Victoria* ovary contains several hundred ovules, so stigmatic pollen loads must be large to achieve a high percentage of seed set. Furthermore, the stigma in *Victoria* is receptive for only one night (Prance and Arias 1975), so the window for pollen transfer is relatively short. Permanent tetrads ensure that four functional pollen grains are transferred per dispersal event. It is possible that, in addition to increasing the number of pollen grains transferred, tetrads also adhere

more readily to beetles than would monads, due to their larger size and increased surface area. At 78.0 μm in diameter, permanent tetrads of *Victoria* are among the largest pollen dispersal units in water lilies, comparable with the fly-pollinated grains of *Cabomba* that measure 61 μm × 81 μm on average, and are twice as large as pollen grains of *Nymphaea odorata* (Williams et al. 2010). The surface of pollen grains in *Victoria* is smooth, so the tetrahedral shape of the tetrad may also increase the ability of pollen to adhere to the insect body. *Victoria* pollen has been described as dry and adhering to beetles via sticky floral secretions (Prance and Arias 1975). However, the possibility of pollenkitt in *Victoria* cannot be discounted, and the extensive network of microchannels within the exine may serve as a reservoir for pollenkitt or other tapetal secretions (Taylor et al. 2012).

Specialized beetle pollination is derived in Nymphaeaceae, but there is evidence that this syndrome evolved in Nymphaeaceae as early as 100 million years ago (Ervik and Knudsen 2003; Gandolfo et al. 2004; Thien et al. 2009) and predated the evolution of large flowers. The fossil genus *Microvictoria* from the earliest Upper Cretaceous has minute flowers that are structurally similar to those of *Victoria* and are hypothesized to have exhibited similar pollination via beetle entrapment (Gandolfo et al. 2004). Unfortunately, pollen grains of *Microvictoria* are unknown (Gandolfo et al. 2004). The paleotropical species *Nymphaea lotus* and members of *Nymphaea* subg. *Hydrocallis*, including *Nymphaea rudgeana*, exhibit pollination via beetle entrapment (Cramer et al. 1975; Ervik and Knudsen 2003; Löhne et al. 2008). However, permanent tetrads are not found in these beetle-pollinated *Nymphaea* species. *Nymphaea tetragona*, in which permanent tetrads have been described (Ueno 1962), is reported to be pollinated by flies (Hill 1988). Therefore, we hypothesize that compound grains are associated with the exceptionally large flowers of *Victoria* rather than beetle pollination itself. Comparative investigations of floral and pollen morphology and pollination ecology among members of Nymphaeoideae may provide insight into the potential selective advantages and the functional consequences of compound pollen.

Conclusion

Pollen development has been comprehensively characterized in *Victoria* for the first time. New ontogenetic data for *Victoria* pollen have provided clarity about important pollen characters that were previously ambiguous in the genus and/or for which there were conflicting reports in the literature. Most significantly, these include confirmation of a columellate infratectum, a lamellate endexine, and the presence of a membranous granular layer in *Victoria*. *Victoria* has also been shown to exhibit a never-before-described pattern of microspore cohesion in the formation of permanent tetrads, in which wall bridges develop from protrusions of the ektexine that occur at callose gaps. Despite the fact that considerable variation in the pattern of microspore cohesion has been described, there have been few attempts to determine the evolutionary significance of this variation or the functional consequences of compound pollen. Our findings in *Victoria* underscore the need for comparative studies using complete ontogenetic sequences, consistent terminology, and accurate characterizations of the literature.

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