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Heterochrony and its role in sex determination of cryptically dioecious *Consolea* (Cactaceae) staminate flowers

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Ovule development, megasporogenesis, and megagametogenesis were studied in six cryptically dioecious species of *Consolea*. All species showed uniform development typical for the Opuntioideae. Ovule development proceeds acropetally, but shows developmental asynchrony across floral morphs. At anthesis, female morph ovules are functional and available for fertilization, whereas staminate flower ovules are senescing and incapable of being fertilized. In occasional plants of some species, staminate flowers may reach anthesis with a few functional apical ovules capable of seed formation. Such plants are described as inconstant/leaky males. Ovule fertility differences across morphs are interpreted as resulting from heterochronic ovule development and senescence, although variation in embryo sac longevity cannot be ruled out. Significantly, ovule abortion follows a common pattern and timing in staminate flowers of both male morphs in all species. Thus, on the basis of this uniformity, a common origin for the cryptically dioecious breeding system in *Consolea* is hypothesized. Furthermore, staminate expression in *Consolea* appears to be controlled by a common, genetically determined heterochronic ovule developmental programme affecting the relative timing of ovule receptivity and flower opening. This is the first report of heterochrony as a mechanism of male sex determination. *Botanical Journal of the Linnean Society*, 2008, **156**, 305–326.

ADDITIONAL KEYWORDS: breeding system – effective pollination period – male sex determination – megagametogenesis – megasporogenesis – ovule development – senescence – unisexual flower.

INTRODUCTION

Dioecious species produce staminate (female sterile) and pistillate (male sterile) flowers on separate plants. In most cases, these pistillate and staminate flowers have an early bisexual stage in which both fertile whorls are initiated, and unisexuality is accomplished by abortion or stalling of the alternative sexual organ (Dellaporta & Calderon-Urrea, 1993; Wu & Cheung, 2000). Depending on the time of abortion there can be different morphological outcomes (Dellaporta & Calderon-Urrea, 1993; Di Stilio, Kramer & Baum, 2005). If, at flower inception, the alternative sex whorl is not initiated, the unisexual flowers will have no vestiges of the opposite sex organ, as in *Thalictrum dioicum* L. (Di Stilio *et al*., 2005). It has been hypothesized that such complete suppression may involve a two-step process, the first involving loss of function or abortion and the second involving loss of organ initiation via heterochrony (Mitchell & Diggle, 2005). If the abortion or stalling occurs later in flower development, the unisexual flowers will show an atrophied or vestigial sex whorl, as in *Asparagus officinalis* L. (Caporali *et al*., 1994). Finally, if abortion occurs very late in development, the staminate and pistillate flowers will retain the alternative, non-functional sex organ, indistinguishable in size and morphology from a functional sex organ. Flowers that are morphologically 'perfect', but functionally are either staminate or pistillate, characterize what is termed 'functional or cryptic dioecy' (Anderson & Symon, 1989; Mayer & Charlesworth, 1991), a breeding system difficult to recognize without close inspection.

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Abortion or stalling of the alternative sex whorl is associated with programmed cell death (PCD) (Wu & Cheung, 2000). In *Actinidia deliciosa* (A. Chev.) C. F. Liang & A. R. Ferguson (kiwifruit), a dioecious species, microspore degeneration in female flowers occurs by PCD (Coimbra, Torrão & Abreu, 2004). Male sterile flowers in both sunflower (Balk & Leaver, 2001) and rice (Ku *et al*., 2003) have premature PCD of the tapetum, resulting in microspore or pollen abortion. In a monoecious species, such as maize, PCD has been implicated in pistil abortion of female sterile flowers (Young, Giesler-Lee & Gallie, 2004). In *Vitis vinifera* L., a functionally dioecious species, Caporali *et al*. (2003) attributed sexual determination of staminate flowers to PCD in the ovule nucellus and integumentary layer. Although there is abundant literature concerning the role of PCD in male sterility, female sterility has not been studied as extensively.

Female sterility has also been identified as a mutation in otherwise normally hermaphroditic plants with perfect flowers, specifically in crop plants, such as tomato (*Lycopersicon esculentum* Mill.; Rick, 1946), potatoes (Arnason, 1943), lemon [*Citrus limon* (L.) Burm.f.; Wilms *et al*., 1983], and alfalfa (*Medicago sativa* L.; Rosellini *et al*., 2003), as well as in *Arabidopsis* (Reiser & Fischer, 1993). These and other mutants lead to the conclusion that normal megasporogenesis and megagametogenesis are correlated with normal ovule development (Reiser & Fischer, 1993).

Functionally staminate flowers of some cryptically dioecious species have a well-developed gynoecium, but are female sterile. Reasons for the nonfunctionality of the gynoecium can range from defects in the stigma to a lack of ovules. *Epigaea repens* L. (Wilson, 1893; Clay & Ellstrand, 1981) and *Chassalia corallioides* (Cordemoy) Verdc. (Pailler, Humeau & Figier, 1998) are examples of functionally staminate flowers with closed, unreceptive, and somewhat malformed stigmas. Functionally staminate flowers of *Labordia* Gudich. spp. and *Pernettya rigida* D.C. show a well-developed gynoecium that is completely empty (Motley & Carr, 1998; Anderson *et al*., 2000). When well-developed ovules are present in functionally staminate flowers, the reason for their nonfunctionality may be related to abnormal callose deposition, as in *Rauvolfia sellowii* Müll. Arg. (Koch, Bittrich & Kinoshita, 2002), or arrested megasporogenesis or megagametogenesis, as in *Asparagus officinalis* (Lazarte & Palser, 1979). Most studies [*Pimenta dioica* (L.) Merr., Chapman, 1964; *Citharexylum fruticosum* L., Tomlinson & Fawcett, 1972; *Deprea* Raf. spp., Sawyer & Anderson, 2000; *Maesa perlarius*, Utteridge & Saunders, 2001; Ma & Saunders, 2003] do not specify the reason for gynoecium failure in functionally staminate flowers, although their inability to set fruit is clearly stated.

Functionally staminate flowers of *Echinocereus coccineus* Engelm. do not set fruits despite the presence of a well-developed gynoecium (Hoffman, 1992). This genus, together with *Consolea* Lem., is the only reported case of cryptic dioecy in the Cactaceae. Only a few studies have investigated ovule development, megasporogenesis, and gametogenesis in this family. Most of the studies that do deal with either *Opuntia* Mill. spp. (Archibald, 1939; Tiagi, 1954; Maheshwari & Chopra, 1955) or other members of the family, such as *Cereus* Mill. spp. (Guignard, 1886), *Pereskia* Mill. spp. (Neumann, 1935), *Astrophytum* Lem. sp., *Thelocactus* Britton & Rose sp., and *Toumeya* Britton & Rose sp. (Engleman, 1960), *Mammillaria* Haw sp. (Tiagi, 1961), and *Consolea* spp. (Strittmatter, Negrón-Ortiz & Hickey, 2002; Negrón-Ortiz & Strittmatter, 2004).

The Caribbean endemic *Consolea* (Opuntioideae, Cactaceae) has nine species (Areces-Mallea, 2001), six accessible species of which were studied (see Appendix) and found to be subdioecious/cryptically dioecious (Strittmatter *et al*., 2002; Negrón-Ortiz & Strittmatter, 2004; Strittmatter, Negrón-Ortiz & Hickey, 2006). Developmentally, both staminate and pistillate flowers are initiated as perfect. Strittmatter *et al*. (2006) found that the breakdown of microsporogenesis in anthers of male sterile flowers occurs early, at the onset of meiosis, resulting in anthers bearing no pollen grains, and that the abortive process follows a common pattern in all investigated species. Male sterility in pistillate flowers appears to be directly related to tapetum anomalies, with other anther layers and tissues affected, and with normal patterns of PCD disrupted.

In all six of these *Consolea* species (see Appendix), staminate flowers have a well-developed gynoecium with aborted ovules at anthesis. In this study, ovule development, megasporogenesis, and megagametogenesis were investigated in pistillate (male sterile/female fertile) and staminate (male fertile/female sterile) flowers using light and laser confocal microscopy. The purpose of the study was to describe and compare ovule development, megasporogenesis, and megagametogenesis in female fertile and female sterile ovules of *Consolea*. Attention was focused on the female sterility process to determine when *Consolea* ovules become non-functional and which tissues are involved in the process. In addition, it was examined whether there are one or more pathways to female sterility. This study is, to our knowledge, one of the few (Strittmatter *et al*., 2002; Negrón-Ortiz & Strittmatter, 2004) to describe the critical stages of sex determination in staminate flowers of cryptically dioecious species in the Cactaceae.

MATERIAL AND METHODS

Pistillate and staminate flowers of *Consolea moniliformis*, *C. millspaughii*, *C. nashii*, *C. picardae*, *C. rubescens*, and *C. spinosissima*, at various developmental stages, were collected from natural populations (see Appendix). For light microscopy, whole flowers were placed in plastic vials containing formalin–acetic acid–alcohol (FAA) (D'Ambrogio de Argüeso, 1986), dehydrated in an ascending series of ethanol, and embedded in paraffin. Blocks were sectioned at 7–12 mm and stained with safranin and fast-green (D'Ambrogio de Argüeso, 1986). Sections were viewed using an Olympus BH2 light microscope and photographed using an Olympus AX-70 with a Photometrics digital camera. Images of half ovaries were taken with an Olympus SZX-12 stereoscope.

For pollen tube observation, styles, stigmas, and half of the ovary of open flowers were softened in 8 M NaOH for 48 h at room temperature, rinsed for a minimum of 1 h in distilled water, stained with aniline blue in 0.1 M K_3PO_4 (decolorized aniline blue) for a minimum of 4 h (Schou & Philipp, 1982), and then squashed and analysed for pollen tube growth using ultraviolet (UV) epifluorescence with a 4′,6-diamidino-2-phenylindole (DAPI) filter set (Ex 360/40, dichroic mirror 400LP, Em 460/50).

For confocal microscopy, longitudinal sections of the ovary, with attached ovules, were dissected from FAA-fixed flowers of *C. moniliformis* [Haiti and Fairchild Tropical Garden (FTG)] and *C. millspaughii* at different developmental stages. For pistillate flowers, the developmental stages used were from young open to past flowers. For staminate flowers, buds in various developmental stages up to anthesis and some senescent flowers were used. The longitudinal ovary slices were rinsed in water repeatedly and stained in aqueous Gills haematoxylin (Fisher #2 CS401-1D) for 25 min, destained in 2% acetic acid for 15 min, dehydrated in an ascending ethanol series to 100% ethanol, and then gradually replaced to 100% methyl salicylate (Stelly *et al*., 1984; Pereira, Lersten & Palmer, 1997). Alternatively, the ovary slices with ovules were stained with aniline blue in K_3PO_4 for 24 h and then dehydrated through 100% ethanol, or were first dehydrated to 100% ethanol and then stained with fast-green for 1 min. Gradual replacement with methyl salicylate was then performed, as described previously. The ovules were scraped from the placenta and mounted in methyl salicylate on excavated slides, and sealed with valap (1 vaseline : 1 lanolin : 1 paraffin). Slides were cold stored to avoid evaporation. The mounted ovules were observed in an Olympus FV500 Laser Scanning Confocal System with an argon ion laser (40 mW) at 85% energy output in order to increase tissue penetration. The excitation wavelength was 488 nm and the emission filter was 505 LP when using a normal fluorescein isothiocyanate (FITC) setting; in other instances, a customized 'enhanced' FITC setting of 405 nm and 488 nm laser excitation (DC 405BP/488BP) was used. Imaging was performed with a 505 LP emission filter. Image Pro-Plus 4.5 software was used to reconstruct embryo sacs (ESs) from confocal optical sections using maximum intensity. The confocal ES images are presented in grey scale and contrast inverted for clarity.

RESULTS

Ovule development

Mature ovules of *Consolea* are campylo-circinotropous (i.e. circinotropous with a curved nucellus; Fig. 1A), crassinucellate, and bitegmic. A spiral canal, the funicular canal, is formed within the funicle as a result of the circinotropous nature of the ovule (Fig. 1A). At maturity, this canal is lined with papillose epidermal cells that are directed towards the micropyle (Fig. 1B). Each of the two integuments are two cell layers thick, except at the micropylar end where the inner integument is swollen and multilayered (Fig. 1A, B). Megagametophyte development is of the *Polygonum* type, and the mature ES consists of an egg apparatus and two distinct polar nuclei; the antipodals are ephemeral. Ovule maturation is acropetal, evidenced mainly during meiosis, ovule abortion, and anthesis. At flower opening, pistillate flower ovules (Fig. 1C) at the base are mature and functional (Fig. 1F), whereas ovules at the apex are still immature (Fig. 1A); in some staminate flowers, ovules at the base of the ovary are shrivelled and consumed, whereas ovules at the top, although non-functional, still retain an apparently normal appearance (Fig. 1D, G). Other staminate flowers have all their ovules so consumed that the acropetal maturation wave is obscured (Fig. 1E, H).

The ovules in both pistillate and staminate flowers follow the same developmental pattern until the formation of the mature ES. The reference point established for the beginning of ovule abortion in staminate flowers was the presence of clear and unequivocal signs of abortion in basal ovules. *Consolea moniliformis* is presented as the standard species. In this study, a detailed description of the staminate flower ovule developmental sequence is presented and compared against anther developmental stages, in order to provide a time frame comparison for ovule abortion in the different species. The ovule developmental process is divided into five distinct morphological stages.

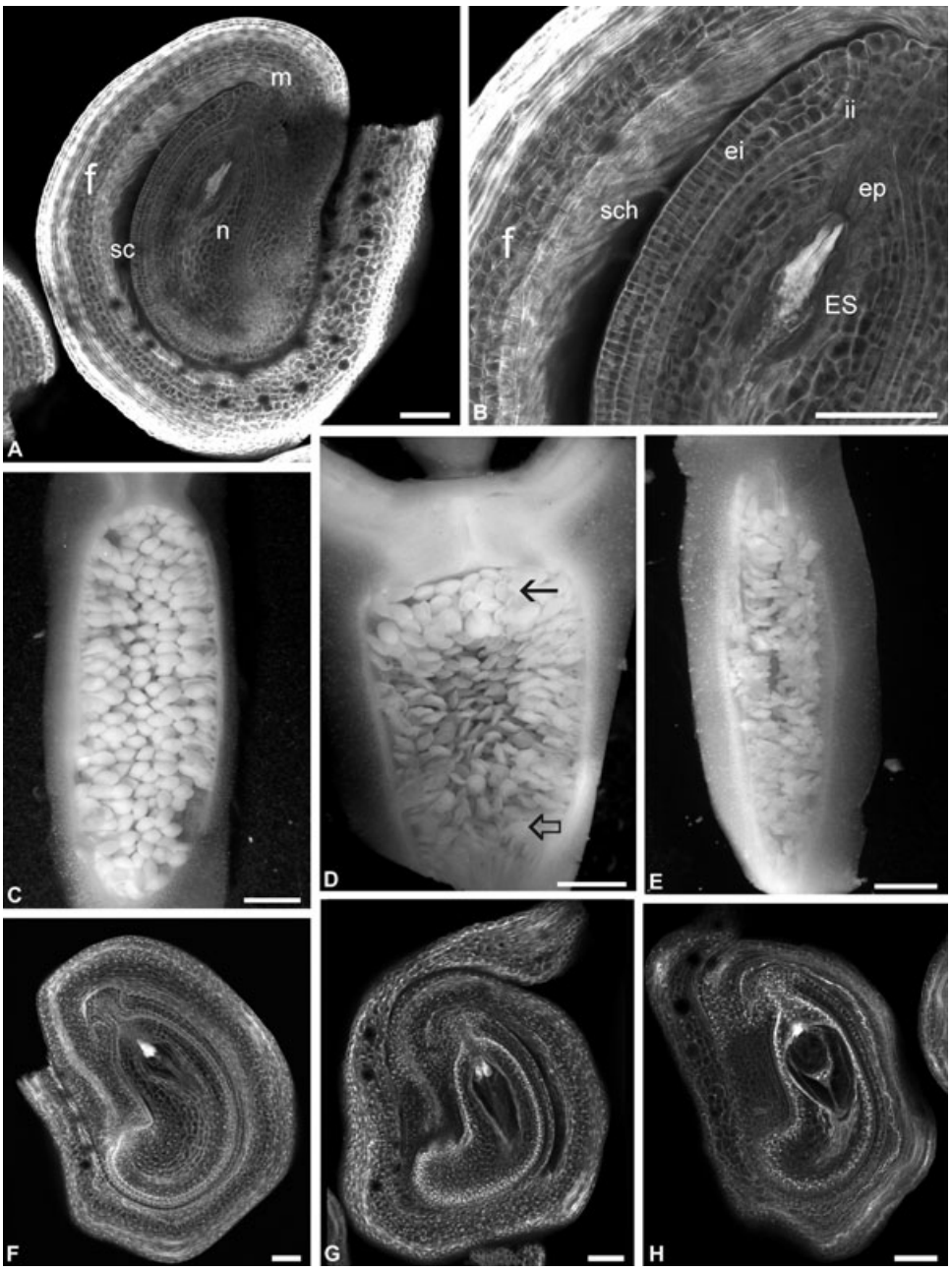

Figure 1. Confocal (A, B, F–H) and dissecting scope (C–E) micrographs of ovules and ovaries of open *Consolea moniliformis* flowers. A–C, F, Pistillate flower. A, Campylo-circinotropous ovule with curved nucella (n) and the micropyle (m) facing the spiral canal (sc) delimited by the encasing funicle (f). B, Detail of micropylar region of A, showing spiral canal hairs (sch), external integument (ei), internal integument (ii), epistase (ep), and embryo sac (ES). C–E, Longitudinal hand section of entire ovary. C, Healthy, functional ovules throughout the pistillate flower ovary. D, Acropetal maturation as evidenced by degenerated basal ovules (open arrow) and apparently healthy apical ovules (arrow) in staminate flower from leaky male. E, Degenerated ovules throughout the staminate flower ovary from male morph. F, Healthy mature functional ovule from C. G, Apical ovule from D showing signs of degeneration. H, Apical ovule from E showing extensive signs of degeneration. Scale bars: A, B, F–H, 100 μm; C–E, 2 mm.

Stage 1: ovule primordia

Ovules elongate away from the parietal placenta by funicular enlargement. By the time both integument primordia are present, the ovule is anatropous (Fig. 2A). The anthers at this stage are meristematic and poorly differentiated, with only the epidermis and one parietal layer surrounding the archesporium (Fig. 2B).

Stage 2: young circinotropous ovule

After the archespora differentiates, a periclinal division gives rise to an outer, small parietal cell and, at the chalazal end, to an inner, larger megaspore mother cell. The funicle totally encases the nucellus and integuments, forming the characteristic circinotropous ovule (Fig. 2C). The epidermal cells of the spiral canal are not yet elongated, and no spiral canal hairs are present (Fig. 2C). As ovule development progresses, both internal and external integuments elongate, and the internal integument proliferates at the tip defining the micropyle. At this stage, the megaspore mother cell is differentiated and elongate. Epidermal cells of the inner funicle surface have begun to elongate to form the incipient hairs that line the spiral canal. Anthers at this point have the characteristic four anther wall layers (epidermis, endothecium, middle layer, and tapetum) surrounding the microspore mother cells (Fig. 2D).

Stage 3: meiosis stage

This stage is somewhat more variable across individuals. In most cases, meiosis in the anthers and the basal ovules is coincident, so that, at the end of meiosis, megaspore and microspore tetrads are present in basal ovules and anthers, respectively. However, we have encountered cases in which anthers have just initiated meiosis (Fig. 2F), whereas some ovules already exhibit young ESs (Fig. 2I, J).

At meiosis, the ovules have a well-formed micropyle (Fig. 2E), and the nucellar epidermal cells have elongated to form an incipient epistase (Fig. 2E, H). The linear megaspore tetrad has a functional chalazal megaspore, and the remnants of the other three megaspores lie adjacent to the parietal cells (Fig. 2H). The spiral canal hairs have elongated, although they have not yet reached their mature size. Anthers show disintegration of the middle layer, leaving three layers around the tetrahedral microspore tetrads (Fig. 2G).

Stage 4: megagametophyte

During stages 4 and 5, acropetal maturation is much more obvious. Although ovules at the base of the ovary have already completed meiosis, ovules at the ovary apex have only reached the stage of the differentiated megaspore mother cell. Stage 4 begins after the chalazal megaspore gives rise to an eight-nucleated, seven-celled, *Polygonum*-type megagametophyte after three successive mitoses (Fig. 2J). Thus, this stage is defined as extending from the young, eight-nucleated, seven-celled ES (Figs 2I–J, 4G) to the mature, five-nucleated, four-celled megagametophyte (Figs 2N, 4A, H). As megagametophyte maturation takes place, several key developmental changes occur in the anther, including microspore release from the tetrads to pollen grain maturation (Figs 2L, M, 3B).

The nucellus curves slightly and, as a result, the micropyle and chalaza are no longer aligned (Fig. 2H). This curvature of the nucellus is what defines the ovule as campylotropous (Fig. 2I, K). In addition, the ES lies in close contact with the nucellar epidermis (epistase) as a result of parietal layer disintegration (Fig. 2N). The spiral canal hairs continue to elongate and take on a turgid appearance (Fig. 2I, K).

Megagametophyte maturation can be witnessed through morphological changes in the synergids. Shortly after cytokinesis in the ES, the synergids lack a chalazal vacuole and have no defined filar apparatus (Figs 2J, 4G). As the ES elongates and matures, both the characteristic synergid chalazal vacuole and filar apparatus become evident (Figs 2N, 4H). After the antipodals have disappeared, the synergids acquire their mature hooked morphology with a well-defined filar apparatus, chalazal vacuole, and nuclei positioned closer to the micropylar end (Fig. 3D). Other cells within the megagametophyte also experience changes as the megagametophyte matures. The central cell enlarges and becomes filled with starch grains (Fig. 2N, O), and the antipodals disintegrate (Fig. 4A). The nucellar cells lie in close contact with the megagametophyte as the latter matures and enlarges (Figs 2H, J, K, 4A, G). To accommodate the growth of the ES, nucellar cells surrounding the ES degenerate; however, the nucellus continues to tightly surround the ES (Fig. 4A, H). The elongation and darker staining of the chalazal nucellar cells directly behind the megagametophyte indicate hypostase formation, which occurs as the ovule continues to enlarge. Evidence from whole cleared ovules corroborates the observations with serial sectioning. Ovules from flower buds with anthers between meiosis and the free microspore stage have complete, although immature, ESs and no signs of abortion are evident (Fig. 4G). The nucellar cells tightly surround the young ES and the spiral canal hairs are incompletely elongated.

Even after the ovules have reached the megagametophyte stage, the anthers continue their development. The nuclei of the newly released microspores migrate from a central position (Fig. 2L) towards the cell wall and the first mitotic division takes place (Fig. 2M). The young pollen grain thus consists of a vegetative or pollen tube cell and a generative cell (Fig. 2M). During these changes, the anther wall is composed of epidermis, endothecium, and tapetum (Fig. 2M).

Throughout stage 4, i.e. mature ovule with mature ES, ovule development is identical in pistillate and staminate flowers; their developmental stages are

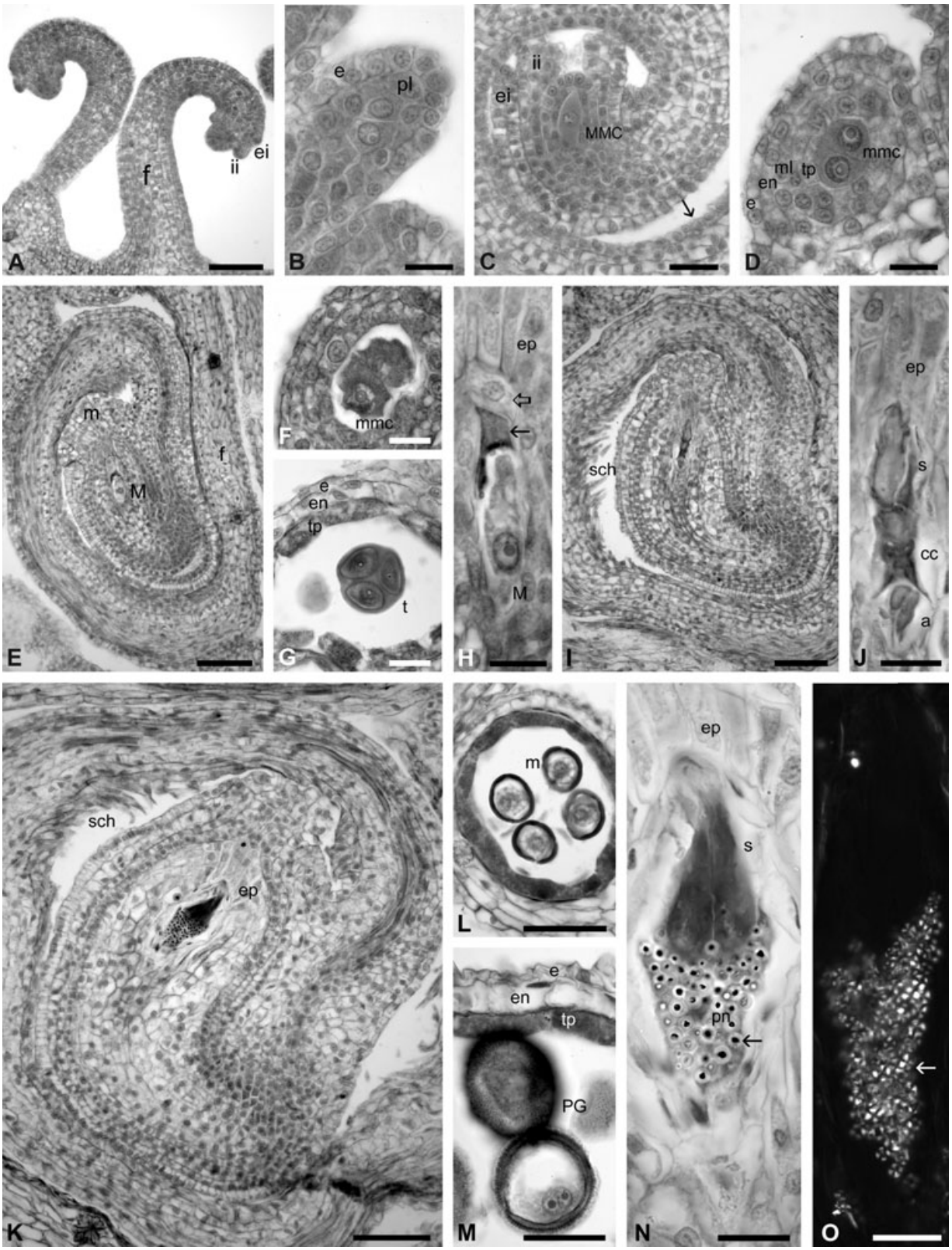
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Figure 2. Bright field micrographs of ovules and comparable anther developmental stages of *Consolea moniliformis* staminate flower bud, stages 1–4. A, B, Stage 1. A, Ovule primordia with funicles (f) bending in opposite directions; external (ei) and internal (ii) integument primordia can be seen. B, Anther primordium with only epidermis (e) and parietal layer (pl) surrounding the archesporic tissue. C, D, Stage 2. C, Young circinotropous ovule with megaspore mother cell (MMC), developing spiral canal hairs (arrow), and external and internal integuments. D, Microspore mother cells (mmc) surrounded by four anther layers: the epidermis (e), endothecium (en), middle layer (ml), and tapetum (tp). E–H, Stage 3. E, Young ovule with micropyle (m) and functional megaspore (M). F, Microspore mother cells at prophase I. G, Microspore tetrads (t) surrounded by three anther layers: e, en, and tp. H, Detail of megaspore tetrad from E showing a functional chalazal megaspore (M), remnants of aborted megaspores (arrow), parietal layer (open arrow), and epistase (ep). I, J, Stage 3–4. I, Campylo-circinotropous ovule with young megagametophyte and well-developed spiral canal hairs (sch). J, Detail of young megagametophyte from I composed of synergids (s), central cell (cc), and antipodals (a). K–O, Stage 4. K, Mature ovule with well-developed spiral canal hairs and embryo sac. L, Free microspores (m). M, Immature, bicellular pollen grains (PG). N, Detail of embryo sac from K, with well-developed synergids (s) and central cell filled with starch grains (arrow) that obscure the polar nuclei (pn). O, Polarized light micrograph of N showing starch grains (arrow). Scale bars: A, E, H, K, L, 100 μm; B, D, F, G, I, J, N, O, 25 μm; C, M, 50 μm.

synchronized until meiosis. After that point, pistillate flowers lack a stamen-associated reference frame, and it is not possible to determine whether ovules in pistillate and staminate flowers are developmentally synchronized. In addition, from stage 4 onwards, ovules in pistillate and staminate flowers follow different paths (Table 1). These floral morphs are described separately, beginning with ovule degeneration in staminate flowers prior to anthesis.

Stage 5: abortion

By the end of stage 4, ovules at the ovary base exhibit early signs of abortion. The ES cavity has slightly enlarged and a small space between the ES and the nucellus becomes evident (Fig. 3A); moreover, some of the spiral canal hairs have collapsed (Fig. 3A, C). However, the ES shows no signs of degeneration at this stage (Fig. 3D). These changes coincide with a still tetrasporangiate anther whose wall is now composed of only two layers: the endothecium with fibrous thickenings and the epidermis (Fig. 3B). Within the pollen grains, the generative cell has migrated into the starch grain-filled vegetative cell cytoplasm (Fig. 3B).

Shortly after this, unequivocal signs of abortion are evident in the ovules at the ovary base. The ES cavity has enlarged considerably as a result of degenerating nucellar cells, and therefore is no longer tightly embracing the ES (Fig. 3E). The synergids and egg cell of the distended ES exhibit less dense cytoplasm than in previous stages (Fig. 3H, 4I). Specifically, the chalazal vacuoles in the synergids have enlarged considerably (Fig. 3H, 4I). In addition, the hairs of the spiral canal have collapsed and their crushed walls stain darkly (Fig. 3E).

The anther is still tetrasporangiate, although the intersporangial septum begins to disintegrate at this stage (Fig. 3F). Pollen grains are three-celled; the generative cell has divided to form two sperm cells (Fig. 3G). When the intersporangial septum has disintegrated and the anther is not yet dehiscent (Fig. 3J; probably caused by mechanical rupture during sectioning), pollen grains are mature, and the cytoplasm of the vegetative cell is filled with starch grains that obscure the nuclei of the vegetative and sperm cells (Fig. 3K).

From this stage until anthesis (from here to flower opening), the ovules progressively degenerate and lose cellular mass. The nucellar cells surrounding the ES continue to degenerate, the ES cavity enlarges further (Fig. 3I), and the ES becomes even more distended (Figs 3I, 4J, K). The central cell has numerous, large starch grains and the polar nuclei remain unfused (Fig. 4J, K). The funicle also loses cell mass, giving the ovule an irregular, wrinkled appearance (Fig. 3I). In addition, some of the testa cells around the micropylar end and at the chalazal end develop secondary wall thickenings and dark contents.

At flower opening, all the ovules in the ovary are aborted, with those at the base almost totally consumed (Fig. 3L), but those at the ovary apex conserving a more 'normal' outward appearance (Fig. 1D, G). In some flowers, all the ovules appear to be totally consumed (Fig. 1E, H). Abundant pollen grains lay on the stigmatic surface and produce pollen tubes (Fig. 5A) that can be traced within the style's transmission tissue to the ovary apex. With decolorized aniline blue, ovules from open staminate flowers show intense fluorescence localized in the micropylar end of the ES (Fig. 5B, D), and sometimes at the chalazal region (Fig. 5B), and a widened ES cavity is evident (Fig. 5D). The acropetal abortion process can also be witnessed with this technique. Ovules from the ovary base show a more intense fluorescence as well as a more evident widened ES cavity (Fig. 5D), whereas, in some cases, ovules at the ovary apex display very little or no fluorescence.

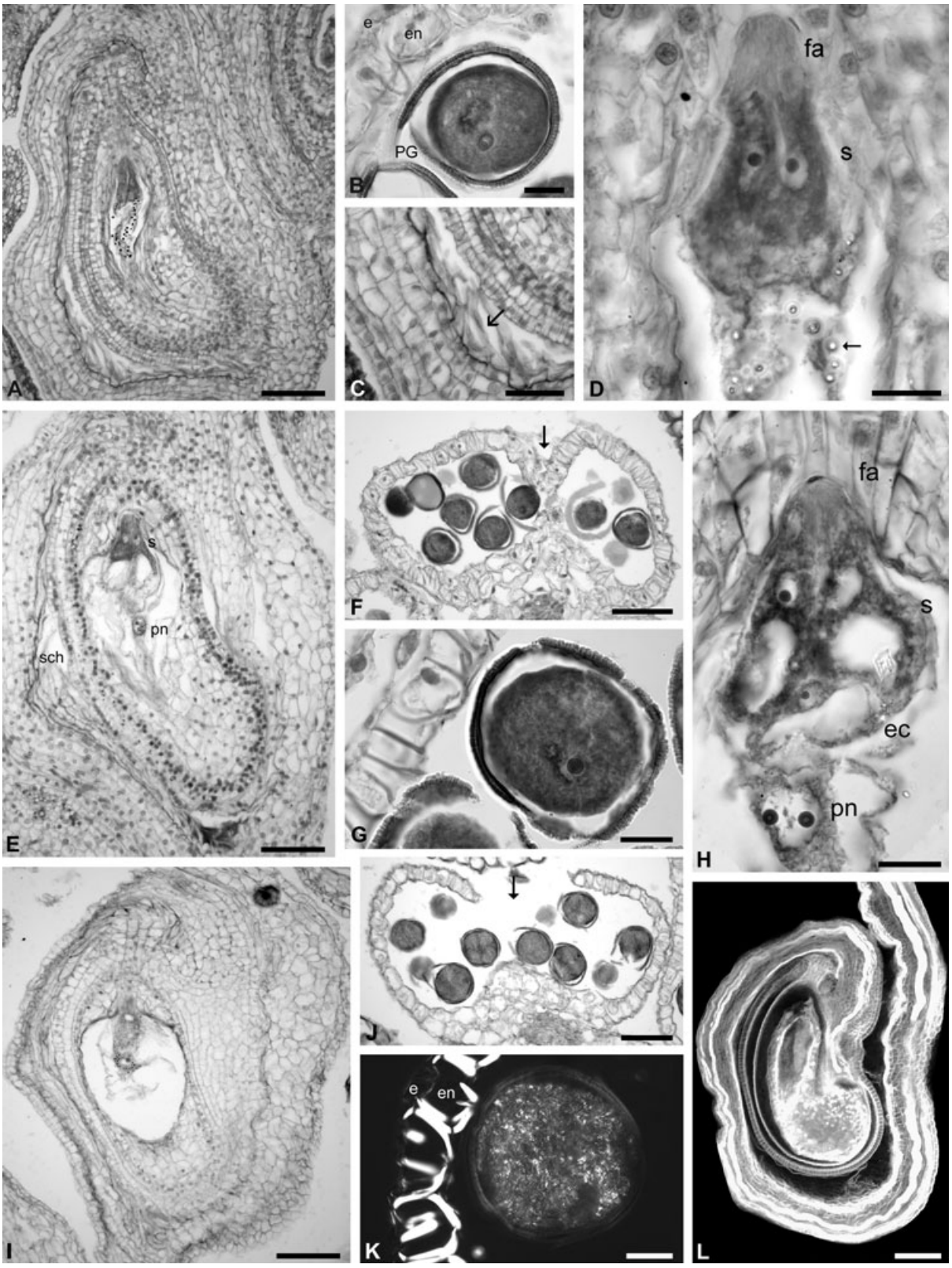
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Figure 3. Bright field micrographs of ovule and comparable anther developmental stages of *Consolea moniliformis* staminate flower bud, stages 4–5. A–D, End of stage 4. A, Ovule showing the early abortive signs of crushed spiral canal hairs (C, arrow) and slightly widened embryo sac (ES) cavity. B, Bicellular pollen grain (PG) with generative cell embedded in the vegetative cell cytoplasm (e, epidermis; en, endothecium). D, Detail of synergids (s) from ES at same stage as A, showing a well-developed filar apparatus (fa), nuclei located towards the micropyle, and relatively small chalazal vacuoles; starch grains (arrow) are abundant in the central cell. E–L, Stage 5. E, Degenerating ovule with increasingly widened ES cavity, completely crushed spiral canal hairs (sch), and distended ES with synergids (s) and polar nuclei (pn) visible. F, Bilocular anther theca showing partially dissolved intersporangium septum (arrow). G, Detail of pollen grain with vegetative or tube cell nucleus visible. H, Detail of ES from same stage as ovule in E, with greatly distended and vacuolated synergids (s), egg cell (ec), and unfused polar nuclei (pn). I, Consumed ovule with shrivelled funicle, and greatly enlarged ES cavity; the polar nuclei are the only remnants of the ES. J, Unilocular (dehiscent as a result of sectioning artefact) anther with intersporangial septum disintegrated (arrow). K, Polarized light micrograph showing a mature pollen grain; the starch contents obscure the vegetative and sperm cell nuclei; anther wall composed of thin epidermis (e) and endothecium (en) with fibrous thickenings. L, Confocal micrograph of a completely consumed ovule as it appears at flower opening. Scale bars: A, E, F, I, J, L, 100 μm; B, D, G, H, K, 25 μm; C, 50 μm.

The same abortive signs previously described, based on paraffin sections, were observed in whole cleared ovules, i.e. collapsed spiral canal hairs, enlarged ES cavity (Fig. 1G, H), and a morphologically distinct ES (Fig. 4I). Although the overall appearance is not one of an already aborted ovule, both the nucellus and funicle appear hyaline and less tightly packed (Fig. 1G, H) when compared with ovules from pistillate flowers at flower opening (Fig. 1F). Changes in the ES morphology are also evident. The synergids are distended, their chalazal vacuole is more prominent, and their cytoplasm is less dense, as evidenced by a mild fluorescence (Fig. 4I). The unfused polar nuclei in the central cell are, in most cases, positioned further away from the egg cell (Fig. 4I), and the central cell cytoplasm contains numerous large starch grains. As the flower progresses towards anthesis, ovule degeneration becomes more accentuated: the ES cavity is greatly widened (Fig. 4J), the spiral canal hairs are hardly visible (Fig. 6C), and the distended ES has synergids with a chalazal vacuole that occupies a larger volume than the cytoplasm (Fig. 4J). In addition, the crushed cells of the nucellus and inner integument sometimes fluoresce intensely (Fig. 4J, K) and the funicle cellular mass shrinks considerably, leaving behind little more than the vascular bundle (Fig. 3L). The polar nuclei remain close to each other, but never fuse (Fig. 4J, K), and, in some cases, they are the last recognizable remnant of the ES after the egg apparatus has become disorganized (Fig. 4K). Some cells of the testa acquire secondary wall thickenings as well as darkly stained contents. As stated previously, the degree of ovule degeneration at anthesis varies along the length of the ovary (as an acropetal abortion wave), amongst flowers in the same individual, between individuals (males), and, as discussed later, between males and leaky males (Fig. 1D, E).

Ovules from pistillate flowers

Because pistillate flowers initiate anther abortion very early during microspore mother cell meiosis, successive ovule developmental stages in pistillate flowers lack a stamen-associated reference frame. During stage 3, ovules complete meiosis and megaspore tetrads are visible, whereas anthers have microspore mother cells that are still in early prophase I. Pistillate flower ovule development parallels that which was described for early stages of staminate flowers up to ES formation. Rather than degenerate, however, pistillate flower ovules reach anthesis with a mature ES, and there are no signs of abnormality in either the megagametophyte or surrounding sporophytic tissues (Fig. 4A). Acropetal maturation is evidenced here, as some ovules at the ovary apex have immature ESs that still retain the antipodals.

At flower opening, pollen grains on the stigma surface produce pollen tubes that descend through the transmitting tissue of the style and reach the ovary apex. After reaching the ovary locule, the pollen tubes exit the transmitting tissue and proceed down to the papillose placenta surface. To reach the micropyle and ultimately the ES, the pollen tubes travel through the spiral canal and interact with the spiral canal hairs (Figs 1D, 5C, E). Ovules at this stage exhibit no fluorescence, and some present modified testa cells with dark contents (Fig. 5C, E). The pollen tube penetrates through the micropyle, the epistase, and finally reaches one of the ES synergids (L. I. Strittmatter, R.J. Hickey & V. Negrón-Ortiz, unpubl. data).

Whole cleared ovules show no signs of degeneration either in the sporophytic or gametophytic tissue at anthesis. The nucellar cells embrace the ES tightly and the turgid and healthy spiral canal hairs show a slight fluorescence (Figs 1A, B, 6A). The ES has elongated synergids with dense cytoplasm that, in most cases, obscures their nuclei (Figs 1B, 4A). The filar apparatus

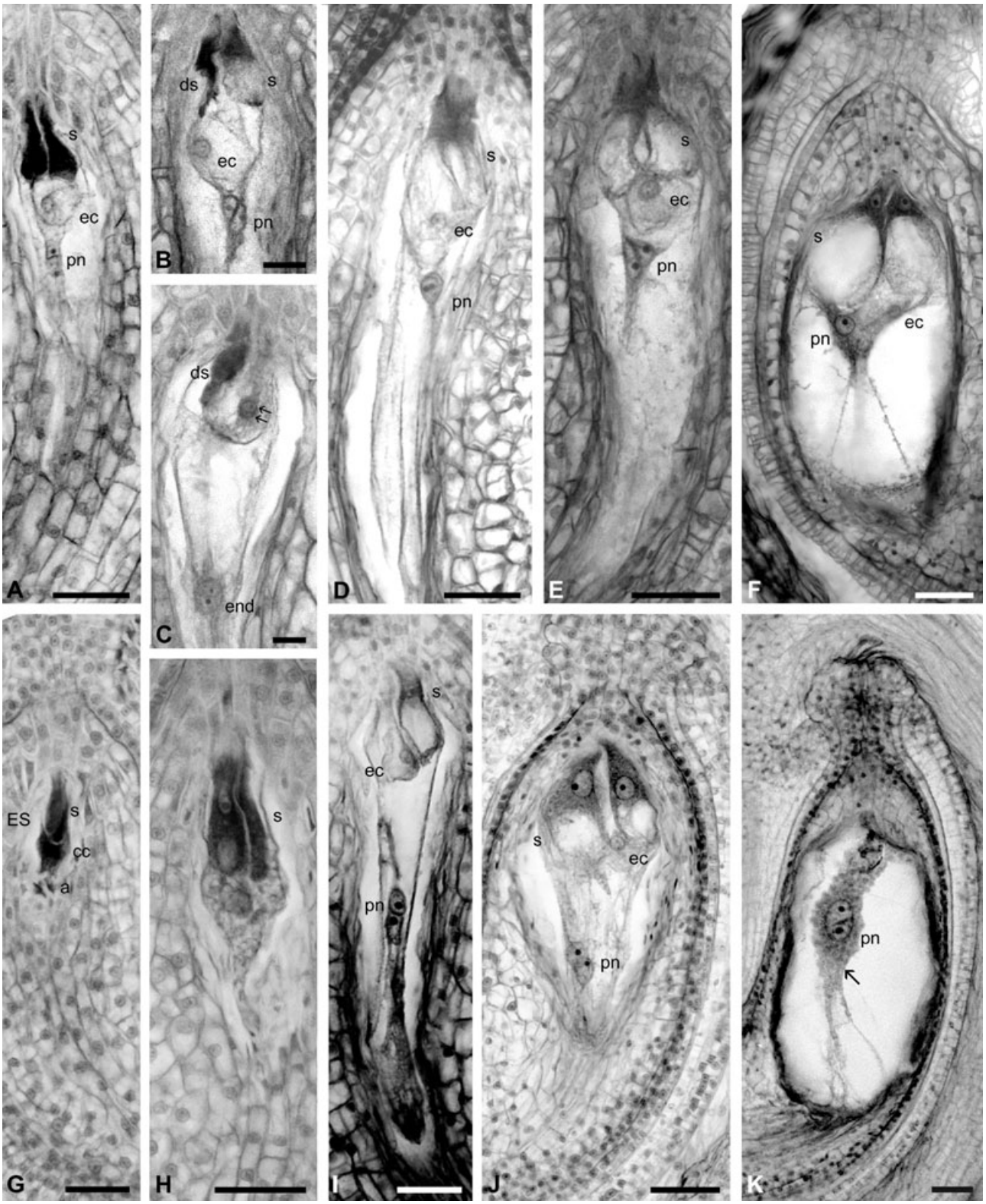
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Figure 4. Embryo sacs (ESs) of *Consolea moniliformis* reconstructed from confocal optical section stacks. A–F, Open pistillate flowers with and without pollen tubes. A, Young ES showing synergids (s) with very small chalazal vacuoles and dense cytoplasm; the egg cell (ec), partially obscured by the synergids, is located close to the unfused polar nuclei (pn). B, Recently fertilized ES with one degenerating synergid (ds) that received the pollen tube and one intact synergid (s) with a large chalazal vacuole; egg cell/zygote (ec) and unfused polar nuclei (pn) also visible. C, Advanced fertilization stage, showing the receptive degenerating synergid (ds) attached to the young zygote prekaryogamy (arrows) and an endosperm nucleus (end). D, Distended ES from a senescing ovule deprived of pollen, showing synergids (s) with large chalazal vacuoles and unfused polar nuclei (pn). E, More advanced degenerating/senescing stage from a nonfertilized ovule in a fruit. F, Almost completely disintegrated ES from a senescent unfertilized ovule. G–K, Staminate flower buds. G, Very young, immature, seven-celled ES with synergids (s), central cell (cc), and antipodals (a). H, Mature ES showing well-developed synergids (s) with filar apparatus and small chalazal vacuoles, and central cell with starch grains. I, Degenerating ES with distended synergids (s) and polar nuclei (pn) situated distant from the egg cell. J, Very distended ES in widened ES cavity. K, Completely degenerated ES showing remnants of unfused polar nuclei (pn) and starch grains (arrow). Scale bars: A, D–H, J, K, 50 μm; B, C, I, 25 μm.

Table 1. Types of ovule present in different floral morphs during development

Flower morph	Pollen grains present on receptive stigma	Flower stage			
		Flower bud-stage 3	Flower bud-stage 4	Open-anthesis	Past-senescent
Pistillate	No (FTG)	Healthy unfertilized ovules	→	Healthy unfertilized ovules	Degenerating unfertilized ovules
Pistillate	Yes (wild population)	Healthy unfertilized ovules	→ Healthy unfertilized ovules → Fertilized functional ovules	Healthy unfertilized ovules Fertilized functional ovules	Few degenerating unfertilized ovules Numerous fertilized functional ovules
Staminate	Yes (wild population)	Healthy unfertilized ovules	Degenerating unfertilized ovules	→	→
Staminate from leaky male	Yes (wild population)	Healthy unfertilized ovules	→ Numerous degenerating unfertilized ovules → Some apical healthy unfertilized ovules	Numerous degenerating unfertilized ovules Some apical healthy unfertilized ovules Few apical fertilized functional ovules	Numerous degenerating unfertilized ovules Few apical fertilized functional ovules

FTG, Fairchild Tropical Garden, cultivated female plants – no pollen grains available.

is well developed and can be seen contrasting with the synergid's cytoplasm because of a lack of fluorescence (Fig. 1D). The chalazal vacuole is relatively small (Fig. 4A). The central cell contains numerous starch grains, and the polar nuclei lie close to the egg apparatus, and to one another, although they are not fused (Fig. 4A). At the ovary apex, the ovules have a young and immature ES, with long and narrow synergids (Fig. 1A) and sometimes antipodals. At the ovary base, the ovules have a mature ES composed only of the egg apparatus and unfused polar nuclei (Figs 4A, 6A) constituting the female germ unit (minimum number

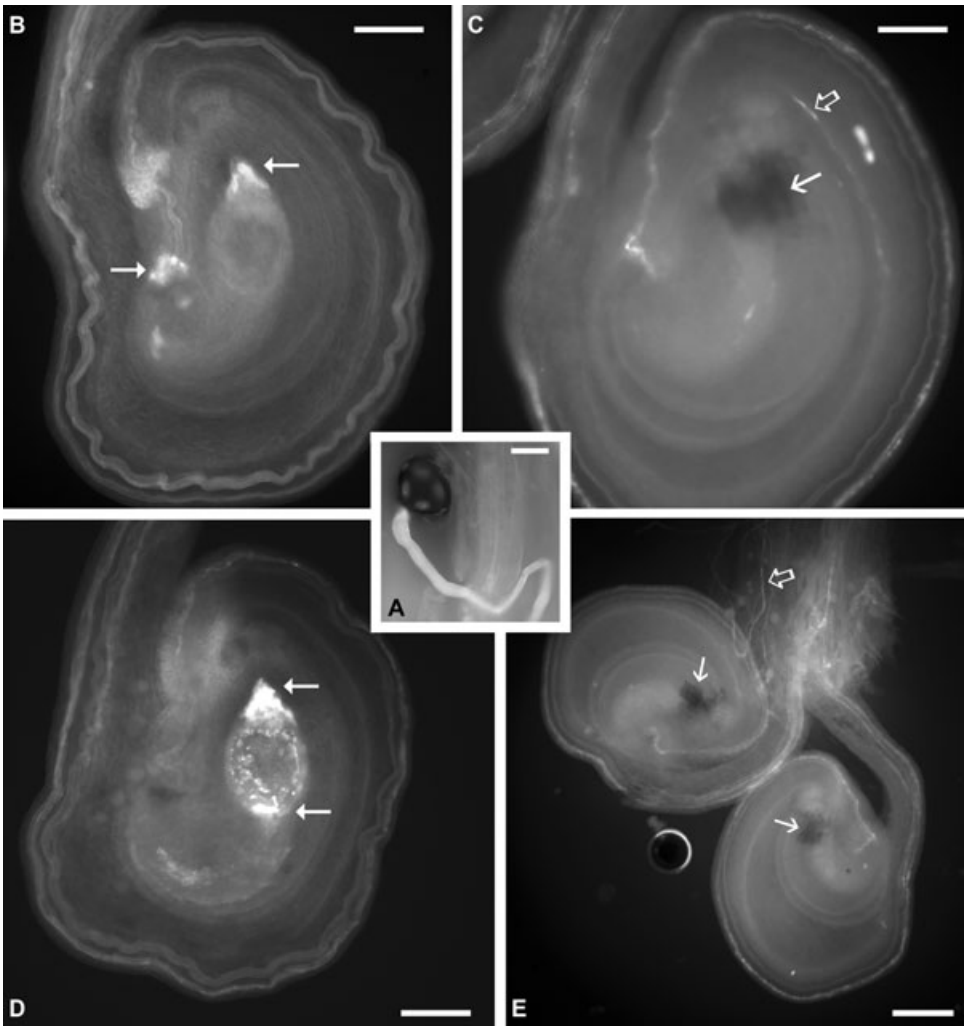

Figure 5. Epifluorescence micrographs from open staminate (A, B, D) and pistillate (C, E) *Consolea moniliformis* flowers stained with decolorized aniline blue. A, Pollen grain with pollen tube in stigma of staminate flower. B, Degenerating apical ovule with fluorescent callose at the micropylar end of the embryo sac (ES) and at the chalazal region of the ovule (arrows). C, Healthy, fertilized or almost fertilized ovule with pollen tube in the spiral canal (open arrow) and darkened testa cells (arrow). D, More extensive degeneration of basal ovule with cellular mass loss and more extensive fluorescence in the ES area (arrows). E, Apical ovules with pollen tubes descending through the placenta and funicles (open arrow), darkened testa cells (arrow), and no signs of fluorescence. Scale bars: A, 50 μm; B–E, 100 μm.

of cells necessary for fertilization). When mature, both synergids have dense cytoplasm and small chalazal vacuoles (Fig. 6A). In ovules from *C. moniliformis* FTG plants (hereafter FTG plants), some outer integument cells close to the micropyle develop secondary wall thickenings and dense cellular contents. These cells seem to be part of the developing testa and cast a shadow over the ES, obscuring somewhat synergid fluorescence.

These healthy unfertilized ovules follow two different pathways depending on the presence of pollen tubes. If pollen tubes reach the ovules, they become fertilized functional ovules; if no pollen tubes reach them, they become degenerating unfertilized ovules (Table 1). Fertilized functional ovules show elongating external funicular epidermal cells and no signs of degeneration in either the ES or tissues of the ovule (Fig. 6D).

Unfertilized ovules ultimately begin to degenerate. Early degeneration signs include collapsed spiral canal hairs and a somewhat widened ES cavity (Figs 4D, 6B, E). Compared with the healthy unfertilized ovules, the synergids are distended and their chalazal vacuole is more prominent (Fig. 4D, E). In addition, the synergid cytoplasm is less dense, as evidenced by a mild fluorescence, and, in most cases,

the polar nuclei in the central cell are positioned further away from the egg cell. As degeneration progresses, the ES cavity enlarges further (Fig. 4F), and the spiral canal hairs are completely collapsed and darkly stained (Fig. 6F). In addition, the nucellar cells surrounding the ES cavity are highly fluorescent (Fig. 6F). In ovules from FTG plants, more cells of the incipient testa develop secondary wall thickenings and dense contents. These cells produce a shadow that obscures the fluorescence of the synergids. This interference intensifies as the ovules degenerate; thus, degenerating unfertilized ovules from these past flowers without access to pollen grains have a more extensive area of modified integument. Unfertilized ovules from FTG plants and *C. millspaughii* female plants never show fused polar nuclei, a degenerated synergid, or signs of seed development as in fertilized functional ovules.

The same signs of degeneration were observed in *C. millspaughii* ovules from past/senescent pistillate flowers derived from a population composed only of female plants. When compared with *C. moniliformis* ovules, the only observable difference was the reduced number of modified testa cells in the integument in *C. millspaughii.* Thus, degenerating unfertilized ovules are found in past flowers that have no pollen grains or pollen tubes and also, but in low numbers, amongst incipient seeds. These are ovules that, for some reason, have not been fertilized, and that age and degenerate until eventually they become re-absorbed. Their degeneration seems to proceed somewhat more slowly than in flowers with no pollen grains or pollen tubes available, i.e. FTG plants. Whereas most of the incipient seeds have enlarged considerably, the degenerating unfertilized ovules show only early signs of degeneration, and, in some instances, slightly elongated external funicle cells.

Ovules from 'leaky' males

In the *C. moniliformis* population in Haiti, some plants bearing morphologically staminate flowers also bore occasional fruits. When analysed in the field, all flowers from such plants had functional anthers with abundant pollen grains. However, when the ovaries were dissected, not all of the ovules appeared aborted. Closer examination showed that, in most cases, these superficially 'healthy' ovules showed extensive signs of abortion (Fig. 1G), but were not as consumed as in the case of normal male flowers (Fig. 1H). Ovules from the basal and middle regions of the ovary showed typical abortion signs, including an enlarged ES cavity, distended ES, aborted nucellar cells, and collapsed spiral canal hairs. However, because both maturation and abortion are acropetal, ovules at the ovary apex were sometimes still functional at the start of anthesis. Indeed, these few functional apical ovules are capable of being fertilized and, if so, the ovary develops into a fruit. Such fruits have fewer seeds than a female fruit. Most flowers, however, reach anthesis with all the ovules already degenerating and no fruits are formed. Similar leaky males were found in wild populations of *C. corallicola* (Negrón-Ortiz & Strittmatter, 2004), *C. nashii* and *C. picardae* (L. I. Strittmatter, pers. observ.), and *C. spinosissima* (Strittmatter *et al*., 2002).

In general, leaky male ovules from flower buds at different developmental stages are identical to those found in males, although most flowers show ovules with a lesser degree of degeneration. In both open and past flowers, ovules ranged from those fully aborted to those with very few to no signs (Fig. 6D) of degeneration, and to some that were fertilized (Table 1). The fertilized ovules were indistinguishable from fertilized ovules in pistillate flowers, and synergid degeneration patterns related to fertilization were identical.

These plants have been termed 'leaky males'. They are not hermaphrodites because only occasionally do their staminate flowers produce fruit, and these fruits have only very few seeds clumped at the ovary apex.

Anomalies

Ovules lacking an ES were found in all flower types examined (Fig. 6H). In these ovules, the ES cavity was filled with nucellar cells resembling the hypostase (Fig. 6H, arrow). In some open staminate flowers, apparently healthy ovules from the ovary apex were in fact ovules lacking an ES (Fig. 6H). Such ovules seemed to retain their integrity longer when compared with normal ESs containing staminate ovules from the same ovary apex (Fig. 6I).

In other cases, degenerated ovules had both synergids completely disintegrated, forming a fluorescent mass at the micropylar end of the ES, instead of a distended ES with greatly enlarged synergids, characteristic of staminate flowers at this stage.

DISCUSSION

Ovule abortion

Female sterility is absolute in staminate flowers of dioecious angiosperm species. If ovules are present, they are either developmentally defective or, for various reasons, degenerate. Studies of ovule degeneration in staminate flowers of dioecious *Aparagus officinalis*, *Vitis vinifera* ssp. *silvestris*, *C. spinosissima*, and *C. corallicola*, using histological methods, are the only reports in which the degeneration of various ovular tissues has been described (Lazarte & Palser, 1979; Strittmatter *et al*., 2002; Caporali *et al*., 2003; Negrón-Ortiz & Strittmatter, 2004). Ovules

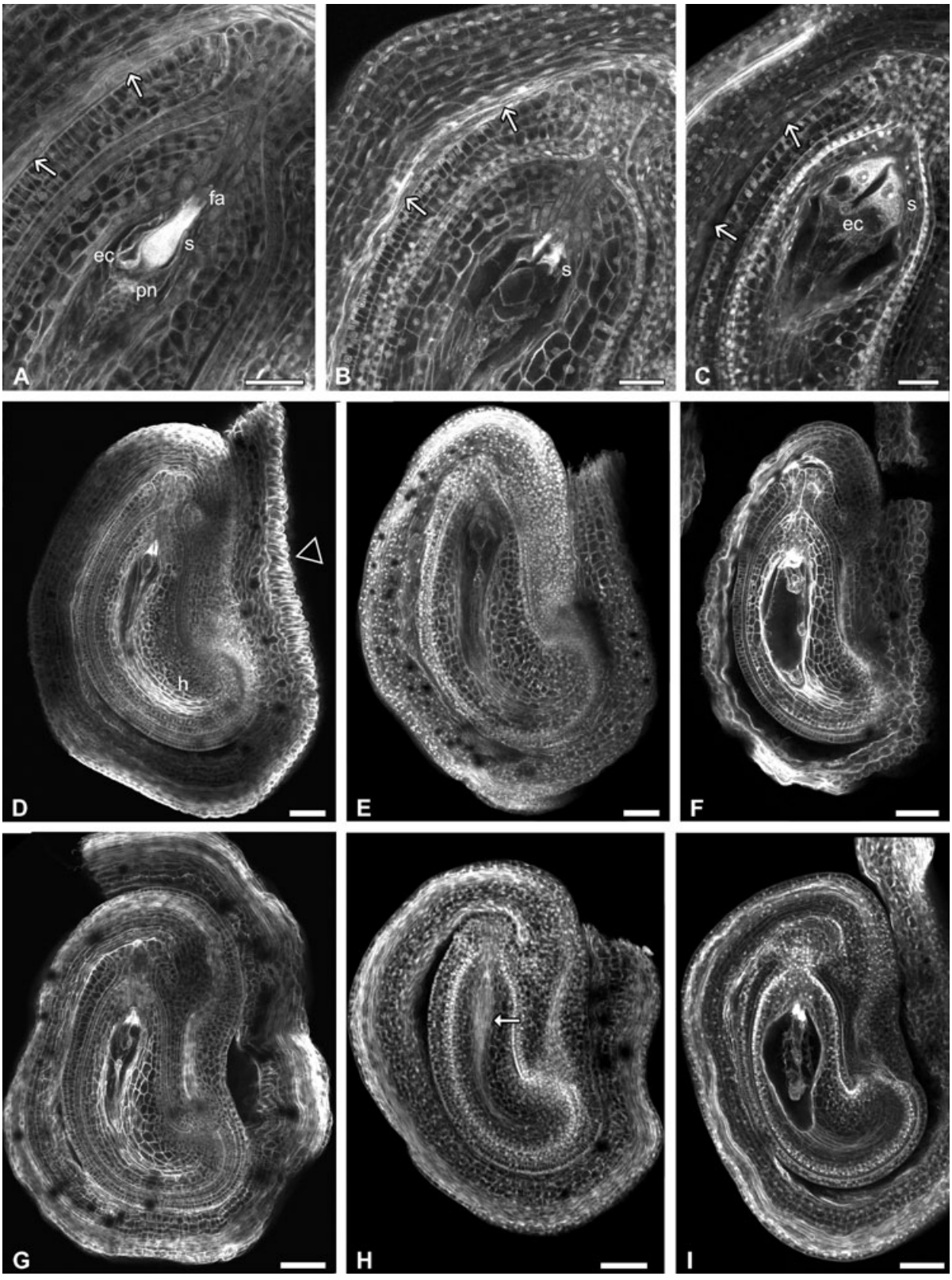
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Figure 6. Confocal micrographs of *Consolea moniliformis* ovules. A–C, Detail of ovule micropylar region. A, Mature, functional ovule from open pistillate flower, showing mildly fluorescent spiral canal hairs (arrows), a highly fluorescent synergid (s) and its filar apparatus (fa), an egg cell on side view (ec), and polar nuclei (pn) barely visible because of starch grains. B, Unfertilized, senescent ovule from past pistillate flower, with crushed spiral canal hairs (arrows), widened embryo sac (ES) cavity, and collapsed synergids (s). C, Degenerated ovule from staminate flower bud from male morph, with faint spiral canal hairs (arrow), a greatly widened ES cavity, distended synergids (s), and a highly fluorescent internal layer of internal integument. D–F, Ovules from past pistillate flowers. D, Fertilized, external epidermal hairs elongating (arrowhead), nucellar cells tightly surrounding the ES, and well-developed hypostase (h). E, Senescent ovule from a plant (FTG) deprived of pollen; the ES cavity is enlarged and the ES is distended and dimly fluorescent. F, Senescent unfertilized ovule showing extensive signs of degeneration, such as consumed funicle, crushed and highly fluorescent nucellar cells delimiting the widened ES cavity, and a nearly consumed ES. G, Degenerating apical ovule from past staminate flower from leaky male; few degenerating signs, such as crushed spiral canal hairs and slightly widened ES cavity, can be seen. H, I, Apical ovules from open staminate flower from male. H, Anomalous ovule showing almost no degeneration signs and no ES; hypostase-like cells (arrow) fill what would have been the ES cavity. I, Degenerated and partially consumed apical ovule showing enlarged ES cavity, shrivelled funicle, and crushed spiral canal hairs. Scale bars: A–C, 50 μm; D–I, 100 μm.

from staminate flowers of both *A. officinalis* and *V. vinifera* ssp. *silvestris* show degeneration of nucellar and outer integument cells (Lazarte & Palser, 1979; Caporali *et al*., 2003), whereas ovules from staminate flowers of *C. spinosissima* and *C. corallicola* show signs of degeneration in the nucellus and ES (Strittmatter *et al*., 2002; Negrón-Ortiz & Strittmatter, 2004). What is not clear is whether nucellus degeneration is a consequence or cause of ovule abortion.

Partial female sterility can also be found in hermaphroditic plants. This can occur either naturally or by induced mutations, and abortion only takes place in a certain percentage of the ovules. More attention has been devoted to partial female sterility of economically important crops, because of the negative economic impact of ovule degeneration on seed and fruit production. In these plants, when ovule abortion occurs, a wide range of ovule and ES anomalies are displayed (Bradbury, 1929; Harrold, 1935; Arnason, 1943; Rick, 1946; Hartman & Howlett, 1954; Wilms *et al*., 1983; Alburquerque, Burgos & Egea, 2002; Rosellini *et al*., 2003). Amongst hybrids, female sterility is commonly the result of arrested ES development, either at meiosis or during mitotic divisions of the megaspore (Greenleaf, 1941; Carapetian & Rupert, 1989; Liu, Xu & Zhang, 2004). Partial female sterility in economically important crop species ranges from failure to form an ES, arrested ES development, and consequently immature ESs at anthesis, to degenerated ESs at anthesis (Bradbury, 1929; Harrold, 1935; Arnason, 1943; Rick, 1946; Hartman & Howlett, 1954; Wilms *et al*., 1983; Alburquerque *et al*., 2002; Rosellini *et al*., 2003). These gametophyte abnormalities are often accompanied by a certain degree of male sterility (Arnason, 1943; Rick, 1946; Liu *et al*., 2004). As expected, lethal ovule mutants also display a wide range of ovule or ES anomalies, including incomplete integument elongation (Robinson-Beers, Pruitt & Gasser, 1992; Lang, Ray & Ray, 1994), arrested ES development (Christensen, Subramanian & Drews, 1998), failure to fuse the polar nuclei (Christensen *et al*., 1998), or failure to undergo fertilization (Pereira, Ilarslan & Palmer, 1997).

In *Consolea,* the ovules of all morphs are essentially identical. Their development is morphologically comparable, and all form a functional ES near or before anthesis. In all morphs, ovule maturation within individual ovaries proceeds acropetally. For example, in pistillate flowers at anthesis, this acropetal maturation is expressed as immature apical ovules grading into mature, receptive basal ovules. This developmental and spatial continuum of ovule development and receptivity may allow for more effective fertilization in ovaries with numerous ovules by extending pollination for the ovary as a whole. Because ovules with immature ESs do not attract pollen tubes (De Martinis & Mariani, 1999; Higashiyama, 2002), fertilization of some ovules can be delayed, and therefore the effective pollen receptivity is extended. However, in the absence of pollen, maturation continues and results in an acropetal senescence sequence in unpollinated pistillate flowers. These acropetal maturation and senescence sequences seen in pistillate flowers also explain the ovule abortion patterns observed in staminate flowers. When ovule abortion/degeneration occurs, identical morphological changes are observed in all morphs. According to our data, differences in ovule fertility amongst morphs are simply a matter of timing. It is hypothesized that abortion of normally developing ovules in *Consolea* staminate flowers, including males and leaky males, is either the result of heterochrony in ovule development and senescence programmes or in ES longevity/resting stage (Fig. 7).

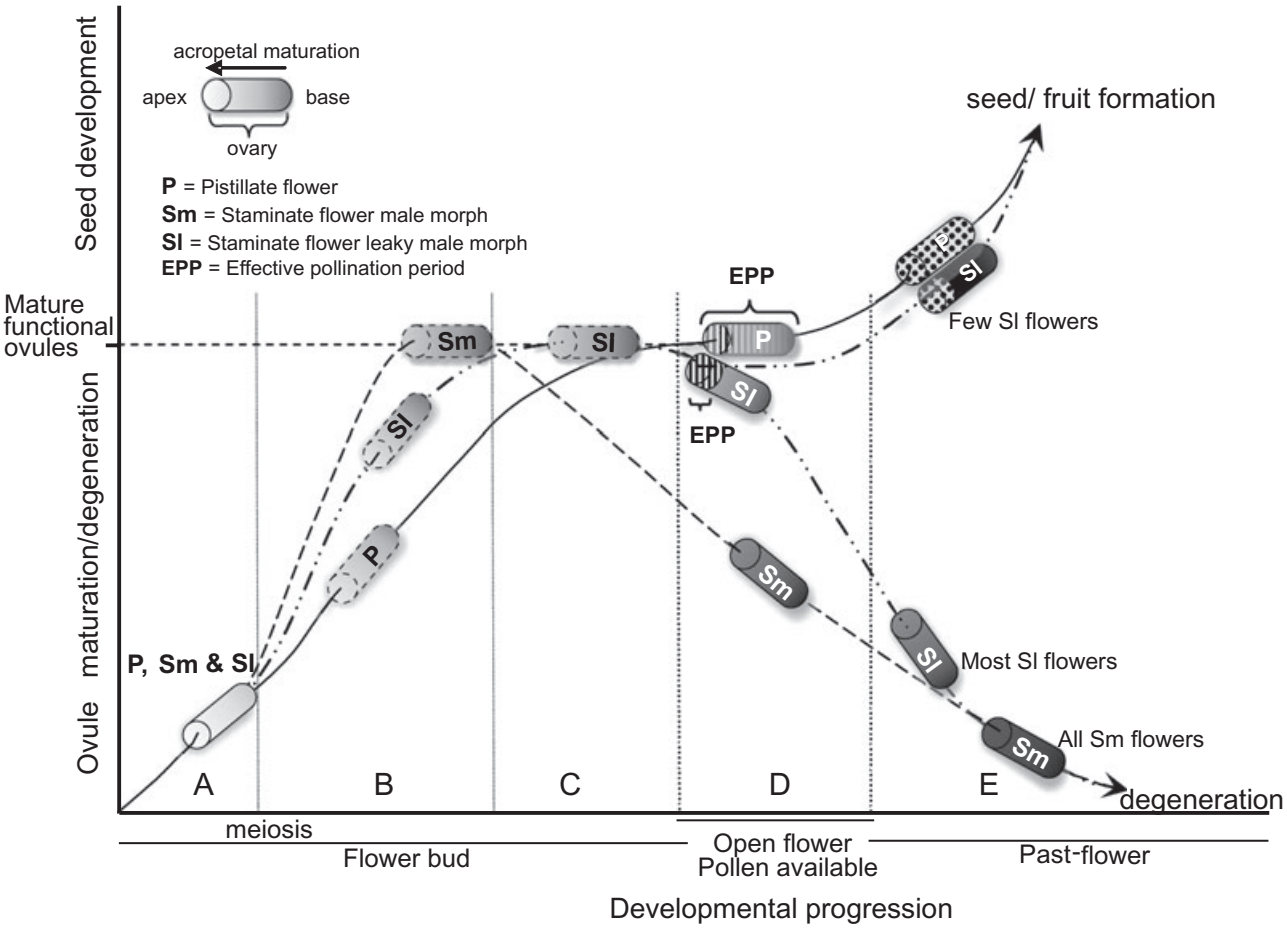

Figure 7. Graphic representation of ovule development, from inception to degeneration or seed formation, in the three flower morphs of *Consolea* spp. Each cylinder depicts an ovary orientated with its apex to the left (younger developmentally, lighter) and its base to the right (older developmentally, darker); vertical stripes represent regions with ovules capable of being fertilized; dotted patterns identify fertilized ovules or seeds. Although synchronized at early stages (A), they become developmentally desynchronized (B, C) as they approach flower opening. Ovules of both Sm (B) and Sl (C) reach maturity (mature functional ovule stage) before flower opening and start to degenerate before pollen becomes available. At flower opening (D), the ovules of the three morphs are at different developmental stages: P have mature functional ovules capable of fertilization, Sm have ovules that are degenerating, and Sl occasionally have apical ovules that are still functional and capable of fertilization, whereas the rest of the ovules are degenerating. As a result, EPP differs amongst the three morphs: P have the longest EPP because of a staggered ovule maturation, some Sl have a very short EPP available for the functional apical ovules, and Sm and most Sl completely lack an EPP because their ovules have degenerated. P form fruits with numerous seeds (E), whereas the few Sl that set fruit have a reduced number of seeds (E). All past Sm abscise and no fruits or seeds are produced. Cylinders with broken outlines indicate that their developmental position relative to one another is estimated.

Ovule longevity and the effective pollination period (EPP)

In general, ES formation is tightly correlated with the development of the surrounding sporophytic ovular tissues (Grossniklaus & Schneitz, 1998), which supply nutrients to the developing female gametophyte. When the ES is fully differentiated, its development stops and it enters a resting stage that lasts until pollination, or the arrival of the pollen tube to the micropyle (Willemse & van Went, 1984). However, this 'resting stage' might represent a more dynamic period during which small physiological changes occur, as observed in *Nicotiana tabacum* L. (Tian & Russell, 1997). For example, changes in the quantity and distribution of Ca^{2+} have been recorded in the synergids and other cells of the ES during ES maturation and at pollen tube arrival (Tian & Russell, 1997). If pollen tubes are delayed, however, these ovules reach a point at which they can no longer be fertilized (Tian & Russell, 1997). Thus, ovule and ES

longevity control an EPP, determined by the ovule and female gametophyte longevity minus the necessary time for the pollen tube to fertilize the megagametophyte (Stösser & Anvari, 1982). The duration of EPP is highly variable amongst species, although, in commercial fruit trees, it is rather short and acts as a limiting factor for fruit set (Stösser & Anvari, 1982).

Because of the importance of fruit set in commercial species, such as cherry, apricot, peach, apple, pear, and almond, several authors have concentrated their efforts on determining the factors affecting ovule longevity (Eaton & Jamont, 1964; Marro & Lalatta, 1978; Stösser & Anvari, 1982; Beppu, Suehara & Kataoka, 2001; Alburquerque *et al*., 2002). In certain cultivars in which fruit production is very low, it has been found that some ovules at anthesis have an ES that is either immature or degenerate (Hartman & Howlett, 1954; Marro, 1976), or the cultivar has a shortened ES longevity that translates into a shortened EPP (Eaton, 1962; Stösser & Anvari, 1982). In the latter case, the window for effective fertilization is too short, and most ovules senesce before pollen tubes can reach them (Eaton, 1962; Stösser & Anvari, 1982). This plasticity in EPP seems to be genetically determined, as different species, including cultivars, exhibit different EPPs (Eaton, 1962; Marro, 1976; Marro & Lalatta, 1978; Stösser & Anvari, 1982; Sanzol & Herrero, 2001). However, within a cultivar, environmental conditions, such as temperature (Beppu *et al*., 2001) and/or hormones, can affect EPP (Sawhney & Dabbs, 1978; Okamoto & Omori, 1991). Hormones and, specifically, gibberellin (GA), 2,4-dichlorophenoxyacetic acid, benzyladenine, and kinetin, have been shown to accelerate ovule degeneration, and consequently to affect EPP (Stösser & Anvari, 1982; Beppu *et al*., 2001). However, in different plant species, the application of GA_3 has diverse effects (Sawhney & Dabbs, 1978; Okamoto & Omori, 1991). Beppu *et al*. (2001) found that *Prunus avium* L. flowers, developed at high temperatures, exhibited high endogenous levels of GA. The authors suggested that higher levels of GA may induce the early degeneration of ES at anthesis, and thus provide the link between the effect of environmental conditions and hormones on ES longevity. Similarly, in *Pyrus* L. spp., the application at bloom of a polyamine, putrescine, delayed ovule senescence and extended EPP in 'Comice' pear (Crisosto *et al*., 1992). Polyamines have been known to delay senescence in some systems, simulating the effects of cytokinins, but have also been implicated in the metabolic pathway of ethylene, inhibiting its production (Crisosto *et al*., 1992). However, putrescine's role in delaying ES senescence in 'Comice' pear was not tied to the inhibition of ethylene production (Crisosto *et al*., 1992).

CONSOLEA'S EPP

In *Consolea*, staminate flowers from male (Sm) and leaky male (Sl) morphs, and pistillate flowers (P), show a synchronized ovule development until they reach meiosis (stage 3), as depicted in Figure 7. After this stage, pistillate flowers lack an anther reference time frame, and therefore exact time correlations amongst flower morphs are difficult for stages between meiosis and flower opening. When flowers open, a time frame for comparative ovule development amongst morphs is regained. At this time, basal ovules from pistillate flowers are mature and receptive, whereas ovules of staminate flowers from males and leaky males are generally aborted and consumed (Fig. 7). Occasionally, however, some apical ovules of staminate flowers from leaky males are still functional, and can be fertilized to form seeds (Fig. 7). As explained earlier, EPP is the ovule and ES longevity minus the time that it takes for the pollen tube to reach the ES. Thus, EPP for *Consolea* pistillate flowers is a time period embedded within flower opening, when pollen grains and pollen tubes become available and the ovules are capable of fertilization. It was observed that basal ovules from pistillate flowers are mature and functional at flower opening, and, as acropetal maturation proceeds, other ovules become functional and EPP is extended. By contrast, all ovules from the male morph completely lack an EPP, because the ovules are already degenerate when pollen becomes available (Fig. 7). In the case of leaky males, some ovaries reach flower opening with a few apical ovules capable of being fertilized, but with the remaining ovules already initiating degeneration; thus EPP for the staminate flowers of leaky males is extremely short. Most leaky male flowers, however, lack an EPP as all of their ovules are degenerated at flower opening. The presence of a short EPP for some staminate flowers of leaky males seems to be the result of a slower senescence process in leaky male flowers when compared with the staminate flowers of males (Fig. 7). Between meiosis and flower opening (stages 4–5), ovule and ES maturation become desynchronized across the three flower morphs.

Moreover, ES longevity seems to be tightly related to ovule longevity in *Consolea*. In the absence of an ES, cues for ovule degeneration appear to be initially absent. This is evidenced by the conserved integrity of ovules with no ES, which escape initial degeneration and persist longer than ovules with an ES (Fig. 6H, I). Ovules missing an ES have also been described in tomato (Rick, 1946) and avocado (Tomer & Gottreich, 1978); however, no data with respect to ovule longevity were provided. In addition to this ES control over ovule senescence, external controls can also be exerted. Unfertilized ovules in developing fruits of *Consolea*

pistillate flowers show a slower degeneration pattern, possibly as a result of hormonal changes, such as those associated with seed and fruit formation. Although no particular hormone has been shown to delay ovule senescence, applied exogenous substances, such as paclobutrazol (blocks GA production; Beppu *et al*., 2001; Beppu, Aida & Kataoka, 2005) and putrescine (polyamine capable of inhibiting ethylene production; Crisosto *et al*., 1992), can do so. In addition, ovary development on pollination has been related to auxin production by fertilized ovules (Gustafson, 1939). This suggests that alterations in hormonal balance within the ovary may play a secondary role in delaying senescence of the unfertilized ovules.

Leaky males

The presence of leaky males in both dioecious and cryptically dioecious species is not uncommon. *Deprea paneroi* C. Benítez de Rojas & M. Martínez, a cryptically dioecious Solanaceae endemic to Venezuela, also has a few 'hermaphrodite' plants, i.e. males that exceptionally produce fruits (Sawyer & Anderson, 2000). *Polyscias pancheri* Harms (Araliaceae), endemic to New Caledonia and also functionally dioecious, includes a few males that bear a small number of morphologically perfect flowers (Schlessman, Lowry & Lloyd, 1990). Even in dioecious species, such as *Hymenanthera chathamica* Kirk. (Violaceae), in which staminate flowers have a reduced and atrophied gynoecium, some male plants occasionally develop ovules; these, however, abort just before anthesis (Beuzenberg, 1961). *Dombeya ciliata* Cordem., *D. pilosa* Cordem., and *D. delilei* Planch. (Sterculiaceae) are endemic to La Réunion Island, and are cryptically dioecious, although some of the males produce a small number of fruits, and are thus classified as having leaky dioecy (Humeau, Pailler & Thompson, 1999). Finally, in one of the more extensively studied dioecious species, *Asparagus officinalis*, the staminate flower gynoecium exhibits variable maximum development (Lazarte & Palser, 1979). Males that are homogametic (yy) have small and rudimentary pistils, whereas heterogametic males (xy) can develop pistils almost identical to pistillate flowers, and can sometimes produce 10–12 fruits per plant (Lazarte & Palser, 1979). Thus, the presence of an occasional fruit on otherwise male morphs, leaky males, is not surprising.

Consolea leaky males can be distinguished in the field from normal males because the latter lack fruits. Leaky males produce abundant pollen and bear fewer fruits than females. As previously described, staminate flowers from leaky males seem to have a slower abortion wave that occasionally results in some functional apical ovules that are able to be fertilized and form seeds (Fig. 7). The presence of leaky males in *Consolea* is evidence of a more relaxed sterility control than is seen in the female morph. This relaxation might be a function of later acting ovule suppression. Because ovules are not only formed but reach a functional stage, they have a greater chance of escaping abortion than do anthers that abort early and fully (Strittmatter *et al*., 2006). Further studies are necessary to assess whether the proportion of functional ovules at flower opening exhibited by leaky males is affected by environmental conditions.

Heterochrony

Heterochrony is defined as the evolutionary change in either the developmental rate or timing of an already established genetic programme (Li & Johnston, 2000). Heterochrony has been implicated in morphological floral evolution, not only differentiating species but also differentiating floral morphs and mating systems within species. Specifically, cleistogamous flowers seem to have evolved from chasmogamous flowers by a reduced developmental rate and extended growth (Li & Johnston, 2000). Similarly, Georgiady & Lord (2002) proposed heterochrony as the mechanism involved in the evolution of the inbred flower form of *Lycopersicon pimpinellifolium* (L.) P. Miller. In dioecious species in which flowers are unisexual from inception, heterochrony has been postulated as a possible process to transition from unisexual flowers with abortive organs to unisexual flowers without abortive organ remnants (Mitchell & Diggle, 2005). In this context, it is interesting to note that *C. corallicola* secondary staminate flowers born on the areoles of unfertilized primary flower pericarps sometimes lack the gynoecium from inception (Negrón-Ortiz & Strittmatter, 2004), thus indicating a trend towards complete morphological dioecy, probably through heterochrony.

Our data suggest that males and leaky males in *Consolea* have either an intrinsically shorter ES longevity, or a faster ovular development and thus earlier temporal beginning to ES longevity (Fig. 7). We observed what appears to be extreme EPP variation amongst morphs, so extreme that staminate flowers of the male morph completely lack an EPP. This variation in EPP is possibly the result of a heterochronic mutation affecting the rate of ovule development or ES longevity amongst flower morphs (Fig. 7). It is hypothesized that male sex determination in *Consolea* is achieved simply by heterochronic ovule and ES developmental programmes amongst flower morphs. Although the variation in ES longevity cannot completely be discarded, evidence for an accelerated ovule developmental programme is provided by the slight but distinct asynchrony of ovules at the meiosis stage. Specifically, some ovaries from staminate flowers had

ovules with young ESs, whereas the anthers were still at the early meiosis stage (Fig. 3F, I, J). These ovules not only had a young megagametophyte, but also well-developed sporophytic tissues, such as spiral canal hairs and nucella [cf. Fig. 3E (young ovule with megaspore tetrad), 3I (ovule with young ES), and 3K (ovule with mature ES)]. Such ovules are further along their developmental programme than ovules of other staminate flowers, indicating that the most probable cause of early senescence of staminate flower ovules is a faster rate of development rather than a shorter ES longevity. Thus, we favour ovule heterochrony as the mechanism involved in the determination of the staminate flower morph in *Consolea*. Heterochrony therefore appears to play a role in the evolution of unisexuality from bisexual flowers, not only by suppressing organ formation after the organ has become non-functional (Mitchell & Diggle, 2005), but also by rendering the organ non-functional. This latter role of heterochrony represents a novel mechanism of sex determination. Supportive evidence is provided by the presence of vestigial carpels with metabolically active cells in staminate flowers of cucumber (Yang *et al*., 2000). The carpel in this case is not aborted; rather, it is merely small and undifferentiated, retaining primordial status through anthesis. Yang *et al*. (2000) proposed that the phytohormonal regulation of sex expression in cucumber staminate flowers acts to retard the developmental rate of the carpel primordia. Similarly, endogenous hormone levels have been shown to affect EPP by accelerating ovule and ES development/degeneration in cultivated cherries (Stösser & Anvari, 1982; Beppu *et al*., 2001; Beppu *et al*., 2005). Thus, a possible regulation of the accelerated ovule developmental programme seen in *Consolea* staminate flowers may be explained by fluctuations in endogenous hormone levels. These fluctuations could, in turn, affect the temporal variation in ovule receptivity/viability (EPP), and therefore control male sex determination.

In spite of the presence of leaky males, the ovule abortive pattern in *Consolea* is conserved within and amongst the studied species. Thus, it is proposed that male sexual expression is a genetically determined heterochronic developmental phenomenon, and the only lability seen is in the relative temporal relationship between ovule receptivity and flower opening. The extremely conserved ovule abortion pattern, together with the uniform abortion pattern in anthers, suggests that the common ancestor for *Consolea* was cryptically dioecious.

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APPENDIX

SPECIMENS INVESTIGATED

Pickled material deposited at the Bruce Fink Laboratory MU, Oxford, OH, USA

Species	Location	Date collected
Consolea millspaughii (Britton) A. Berger	Bahamas, Long Island: Females – Mortimers, across the street from the Anglican Church Males – north of Stella Maris	December 2000
Consolea nashii (Britton) A. Berger	Bahamas: Great Inagua, south of Matthew Town	December 2000
Consolea picardae (Urb.) Areces	Dominican Republic: South-east, road to Bayahibe, 2 km from the deviation to Higüey	August 2000
Consolea moniliformis (L.) A. Berger	Dominican Republic: Lago Enriquillo	August 2000
Consolea rubescens (Salm-Dyck) Lem. (collected by Dr Negrón-Ortiz)	Puerto Rico: Cabo Rojo	December 2001
Consolea spinosissima (Mill.) Lem.	Jamaica: Hellshire Hills	December 1999