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Pollen and anther development malfunction in distylous flowers of *Palicourea padifolia* (Rubiaceae)

Fallas del desarrollo de polen y anteras en flores diestílicas de *Palicourea padifolia* (Rubiaceae)

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Abstract

Background: Heterostyly is a genetic polymorphism in which flowers differ between individual plants of a species in heights at which stamens and style are reciprocally positioned. In these species, sexual selection theory predicts that different allocation patterns affect the functioning of polymorphism, enabling the evolutionary transition between heterostyly and dioecy.

Questions: Because heterostyly can transit into dioecy, does anther/pollen development differ between floral morphs (LS and SS) of *P. padifolia*? Is pollen/anther development malfunction associated with one morph more than the other?

Study species: *Palicourea padifolia* (Rubiaceae), a distylous plant.

Methods: Tiny floral buds to flowers at anthesis were collected, processed for microphotography, and examined to describe pollen developmental pathways in LS and SS flowers. In addition, we used the TUNEL test to detect programmed cell death.

Results: Stages of normal pollen development are fully described and illustrated in LS and SS flowers. Abnormalities due to tapetal degeneration were observed at various developmental stages; at later stages, SS flowers showed more abnormalities than LS flowers. The TUNEL test showed that degeneration was by programmed cell death.

Conclusions: Along with previous results of asymmetrical fecundity and pollen transfer of morphs in *P. padifolia*, our study of pollen development indicates that the SS morph was more prone to lose the male function, though male sterility is far from being complete, which it might be an initial step towards functional dioecy.

Key words: Heterostyly, male sterility, *Palicourea*, pollen development, Rubiaceae, tapetum layer.

Resumen

Antecedentes: Heteroestilia es un polimorfismo floral donde las flores se diferencian recíprocamente entre plantas de una especie en la posición de estambres y estilo. En estas especies, la teoría de selección sexual predice que patrones de asignación diferente afectan el funcionamiento del polimorfismo, permitiendo la transición evolutiva entre heteroestilia y dioecia.

Preguntas: Debido a que la heteroestilia puede transitar hacia la dioecia, ¿Difieren los morfos florales (LS y SS) de *P. padifolia* en el desarrollo de anteras y polen? ¿Las fallas del desarrollo de polen y anteras están asociadas con uno de los morfos más que con el otro?

Especie de estudio: *Palicourea padifolia* (Rubiaceae), una planta diestílica.

Métodos: Colectamos desde botones florales pequeños a flores en antesis, procesamos para microfotografía, y examinamos para describir el desarrollo del polen en flores LS y SS. Además, usamos la prueba de TUNEL para detectar muerte celular programada.

Resultados: Se ilustra y describe el desarrollo normal del polen en flores LS y SS. Observamos anormalidades por degeneración tapetal en varias etapas, pero en los más tardías hubo más anormalidades en SS que LS. La prueba de TUNEL mostró que la degeneración fue por muerte celular programada.

Conclusiones: En concordancia con resultados previos de fecundidad y transferencia de polen asimétrica entre morfos de *P. padifolia*, nuestro estudio de desarrollo de polen indica que el morfo SS está perdiendo la función masculina, aunque la esterilidad masculina dista de ser total, lo que podría ser el primer paso hacia la dioecia funcional.

Palabras clave: Desarrollo de polen, esterilidad masculina, heteroestilia, *Palicourea*, Rubiaceae, tapetum.

Heterostyly is a genetically controlled floral polymorphism characterized by the presence within populations of two (distyly) or three (tristyly) morphs that differ reciprocally in the position of the stigmas and anthers, functioning to promote cross-pollination (Barrett 2002, Ferrero 2014). The heterostylous syndrome often consists of three associated sets of traits enforcing disassortative mating between floral morphs: reciprocal herkogamy, self- and intramorph incompatibility, and an array of ancillary floral polymorphisms such as pollen grain size and number, stigmatic surface and shape, anther color, corolla length and the timing and amount of nectar reward (Ganders 1979, Barrett 1990, 1992, Lloyd & Webb 1992a, Dulberger & Ornduff 2000, Ornelas *et al*. 2004a, b). Although hundreds of studies on the morphology and function of heterostyly had been published (see reviews Barrett & Shore 2008, Weller 2009, Cohen 2010, Barrett 2019), most studies have documented morph-specific differences of the mature flowers (reviewed in Dulberger 1992, Barrett 2019), developmental pathways through the ontogeny of floral buds (*e.g*., Faivre 2000, Hernández & Ornelas 2007b, Cohen 2010), and in a number of pollen characters, such as size, number, shape and starch content (*e.g*., Dulberger 1975, 1992, Ganders 1979, Scribailo & Barrett 1991 a, b, Weller 2009). However, to our knowledge, only two published investigations have examined early developmental differences between or among floral morphs of heterostylous species (Domínguez *et al*. 1997, Bull-Hereñu *et al*. 2016).

Heterostyly has arisen independently in 28 angiosperm families (199 genera), presumably as an adaptation to similar selective pressures (Arroyo & Barrett 2000, Barrett *et al*. 2000, Barrett 2002, Naiki 2012). In Rubiaceae, there are more species with dimorphic heterostyly than in any other family of flowering plants (Darwin 1877, Vuilleumier 1967, Weller 2009, Ferrero *et al*. 2012), perhaps more than in all other families of flowering plants put together (*c*. 734 species in 109 genera; Anderson 1973, Naiki 2012). In *Palicourea* Aubl. (*c*. 200 species, Rubiaceae), most species are distylous (Vuilleumier 1967, Sobrevila *et al*. 1983, Taylor 1989), and most other genera in the tribe Psychotrieae are predominantly distylous, including *Psychotria* Subg. *Heteropsychotria* Steyerm., the closest relative of *Palicourea*, showing substantial variation in the expression of the polymorphism among species (Bawa & Opler 1975, Taylor 1993, Sá *et al*. 2016). Distyly seems to be the ancestral condition for *Palicourea*, but that has been replaced by self-fertile homostylous species on islands or isolated populations (Taylor 1993, 1997, Sobrevila *et al*. 1983, Consolaro *et al*. 2009). However, more recent molecular work and taxonomic treatments acknowledge the homoplasious nature of the floral characters used to define *Palicourea* (Bremer *et al*. 1995, Taylor 1996, 1997, Nepokroeff *et al*. 1999, Andersson & Rova 1999, Bremer & Eriksson 2009). Studies of development of pollen are surprisingly scarce for a large family such as Rubiaceae. Microscope images of mature pollen stage have been published for several genera mainly in systematic studies (*e.g*., Dessein *et al*. 2005) and three studies have comprehensively studied pollen development of Rubiaceae species (Hansson & El-Ghazaly 2000, El-Ghazaly *et al*. 2001, Li *et al*. 2010). However, studies of pollen development from early stages of flower development on distylous species are lacking.

Palicourea padifolia (Roem. and Schult.) C. M. Taylor and Lorence deviates from perfect reciprocity as originally described for heterostylous species. Anthers of the long-styled flowers (hereafter LS morph) are positioned well above the stigma heights in the short-styled flowers (hereafter SS morph), whereas stigma-anther separation in the LS morph is less pronounced in comparison with the SS morph in most studied populations (Hernández & Ornelas 2007a). This results in a lower reciprocity between lower-level sex organs and a difference in the degree of herkogamy between the floral morphs (Hernández & Ornelas 2003), which promotes more effective pollen between SS anthers and LS stigma than in the opposite direction (Ornelas *et al*. 2004a). In addition, morph differences in ancillary floral traits and resource allocation to reproduction have been documented in *P. padifolia*. Flowers of SS individuals have significantly longer corollas, larger stigmatic surfaces, and fewer pollen grains than those of LS individuals. By contrast, LS flowers secrete more nectar, develop more pollen grains, and produce more floral buds per inflorescence than SS flowers (Contreras & Ornelas 1999, Ornelas *et al*. 2004a, b, González *et al*. 2005). SS individuals produce almost twice as many fruits as LS individuals after outcross hand-pollinations (Contreras & Ornelas 1999, Ornelas *et al*. 2004a, b). However, morph differences in fruit production vary among years under natural conditions (González *et al*. 2005). Furthermore, the disproportionate allocation to gynoecium with increasing corolla mass in SS morphs (Hernández & Ornelas 2007b) supports the idea that morph differences in allocation are not a mere result of developmental or architectural constraints. Pollen performance and/or developmental differences in resource allocation to male and female function between morphs might account for these differences (*e.g*., Bull-Hereñu *et al*. 2016). There are also morph differences in pollen viability; the proportion of pollen grains that deeply stained for starch among LS flowers was twice as high as the proportion among SS flowers (A. Hernández, J. F. Ornelas, F. Ortega-Escalona & G. Ángeles, unpublished data). It is possible that the higher content of starch in LS pollen play a role in terms of more resources for reaching the ovules through

shorter SS pistils. If so, longer styles would intensify interference by allowing small differences in pollen tube growth rate to be expressed. Thus, the documented dissimilarities between morphs in *P. padifolia* should lead to differences in their abilities to gain reproductive success through female versus male function. Because *P. padifolia* is morphologically distylous with complete intra-morph incompatibility, the vector-mediated asymmetrical pollen transfer between morphs, which is not consistent with Darwin's hypothesis of disassortative pollination, and allocation differences between floral morphs are likely the conditions for gender specialization in distylous species and less likely selfing avoidance because intra-morph-incompatibility would greatly avoid self-fertilization (see also Zhou *et al*. 2015, Jiang *et al*. 2018).

Since Darwin (1877), heterostylous species have been portrayed as the best-documented cases to study transitions of heterostyly to dioecy (Wyatt 1983). Sexual selection theory predicts different allocation patterns in distylous flowers (reviewed in Casper 1992), in which gender specialization favors maleness in the floral morph with the higher pollen donation efficiency (typically the SS flowers), and femaleness in the opposite receiving more pollen (LS flowers). Pollinator-driven asymmetrical pollen transfer between the floral morphs might create the initial ecological conditions for the transition to dioecy from distyly (Darwin 1877). An asymmetrical reproductive success between floral morphs may be produced by morph-biased shortage of pollen, asymmetrical pollinator service, and/or high proportion of sterile pollen produced by LS or SS individuals (male sterility). The higher pollen production by LS flowers, its successful delivery on stigmas of SS flowers by hummingbirds, and higher deposition of illegitimate pollen on their stigmas (Ornelas *et al*. 2004a, Hernández & Ornelas 2007a), favor the male function in LS morph and might represent an initial early step of gender specialization in *P. padifolia* in a transition from heterostyly to the evolution of separate sexes. Here, our study addresses the hypothesis that heterostyly in *P. padifolia* can be an intermediate stage in the origin of dioecy, exploring the idea that an initial step could occur if pollen/anther development malfunction becomes associated with one morph more than the other. Specifically, we used light microscopy and scanning electron microscopy to assess whether one of the floral morphs of *P. padifolia* is more prone to lose the male function through developmental stages of anthers and pollen grains. We hypothesize that pollen development in *Palicourea padifolia* deviates from the normal ontogenetic stages observed in other Rubiaceae, in which floral morphs would differ in manifestations of the male function. According to our previous particular results of fecundity and pollen transfer of morphs in *P. padifolia* favoring maleness in the LS morph and femaleness in the SS morph, we expect that SS flowers would be more prone to lose the male function (male sterility) because the female function in this morph is bigger. Thus, the transition to dioecy in this species would be with the SS morph acting as female function and the LS morph as male function.

Material and methods

Species. Palicourea padifolia, a long-lived, 2-7 m tall understory shrub, is morphologically distylous with complete intra-morph incompatibility (Ree 1997, Contreras & Ornelas 1999). It occurs in middle-elevation cloud forests from eastern Mexico to Panama (Taylor 1989, Gutiérrez-Rodríguez *et al*. 2011). One-day yellow flowers have epipetalous, tubular corollas in which stamens are attached to the internal surface of the corolla tube and are visible up to the internal ring of trichomes that encloses the nectar chamber (Contreras & Ornelas 1999). Pollination is primarily by hummingbirds; they transfer pollen more effectively from LS to SS flowers than in the opposite direction (Ornelas *et al*. 2004a), leading to asymmetrical pollen flow in most studied populations (Hernández & Ornelas 2007a).

Plant material. Plants used in this study were sampled from a population in a cloud forest remnant, at the Parque Ecológico Francisco Xavier Clavijero near Xalapa, Veracruz, Mexico (19º 30´ N, 96º 57´ W; at 1,225 m above sea level). In this population (> 500 reproductive individuals), the LS and SS morphs are equally present (Hernández & Ornelas 2007a). Field observations indicate that floral buds take 30-70 days to develop from < 6 mm to anthesis (Hernández & Ornelas 2007a). After initiation, buds develop to a point at which growth is arrested; at this stage they are consistently about 20 % of their final size to anthesis. Buds remain at this arrested stage for about 25 days. Once buds begin to grow again, a 15-d period of rapid growth begins and then buds open within a predictable range of days (Hernández & Ornelas 2007a). A positive relationship between bud length and number of pollen grains per anther, and significantly different between floral morphs, was observed by Hernández & Ornelas (2007a), but morph differences in pollen number were not detected at smaller floral buds. Therefore, the early stages of pollen development and hence the most significant developmental events, proceed very rapidly (J. Márquez-Guzmán, pers. comm.). Descriptions of pollen developmental stages were made on samples collected from LS and SS plants in May of 2006, 2007, 2008 and 2009 (> 100 plants and > 600 buds). Samples were collected from different plants each year because plants do not flower each year (González *et al*. 2005). Although material was collected in different years for preliminary work, the same general pattern was observed in different years.

For both floral morphs, fresh floral buds at different developmental stages of *P. padifolia*, from very tiny floral buds (< 3 mm) to fully developed floral buds (14-16 mm), were collected from living plants at the Parque Ecológico Francisco Xavier Clavijero for light microscopy (LM) and scanning electron microscopy (SEM). The fresh buds were immediately fixed in FAA (10 % formaldehyde, 5 % glacial acetic acid, 50 % ethanol, and 35 % distilled water) in the field. In addition to buds, flowers at anthesis were collected from both floral morphs and fixed as described until use in microscopy analysis. When back in the laboratory, floral buds of each morph were measured with a caliper (precision 0.1) and sorted into seven size classes based on bud length (< 3.0, 3.1-5.0, 5.1-7.0, 7.1-9.0, 9.1-11, 11.1-13.0, > 13.1 mm). This classification was made to guide the finding of developmental stages in the androecium, proceeding very rapidly toward the free microspore stage during the first two size classes. Then, the seven size classes were associated with five developmental stages confirmed by microscopic examination (< 3.0-mm buds: sporogenous tissue stage, microspore mother cell stage, tetrad stage; 3-4 mm buds: free microspore stage; > 5 mm buds: mature pollen stage).

Microscopy. For LM and SEM, the anthers were dehydrated in a graded ethanol series (30, 50, 70, 85, 96 and 100 %) and stored in 70 % ethanol. For the histological analysis the dehydrated anthers were gradually infiltrated and embedded in LR-W (London resin white) by mixing absolute alcohol and LR-W (3:1, 2:2, 1:3), each change kept at 4 ºC for 1 h and a change to LR-W at 4 ºC for 12 h. The embedded specimens were transferred to gelatin capsules and kept at 56 ºC for 12 h. For observing pollen development, embedded specimens were sectioned with a RMC MT-990 (Boeckeler Instruments Inc., Tucson, Arizona, USA) motorized precision microtome at 1.5-2 µm. Longitudinal and cross-sections were stained with 1 % toluidine blue O and imaged with an Olympus Provis AX-70 Evolution MP 6MPX (Olympus America Inc., Center Valley, Pennsylvania, USA) microscope.

For SEM, anthers of buds and flowers at anthesis (15 randomly-chosen samples per floral morph) were further dehydrated in absolute ethyl alcohol and critical-point dried with CO_2, glued with silver paste to SEM stubs, coated with a layer of gold (JEOL fc-1100 ionizer), and imaged with a JEOL JSM-6360LV (Peabody, Massachusetts, USA) scanning electron microscope at 80 kV.

Lastly, several anthers from flowers at anthesis were squashed and pollen was acetolyzed according to Erdtman (1966) and imaged with an Olympus Provis AX-70 microscope.

Out of the plants sampled in 2008 (n = 27, 14 LS and 13 SS), we randomly chose 5 floral buds per developmental stage from each plant (n = 25 per plant; 350 LS floral buds, 325 SS floral buds) to determine the relative frequency of abnormalities in each morph. We used a Chi-square test to investigate morph differences in abnormalities. Samples were scored as abnormal only when other described abnormalities besides tapetal degeneration were observed in a given developmental stage.

TUNEL assay. To detect the mechanism for degeneration during anther development in *P. padifolia* SS flowers, we used the TUNEL assay (TUNEL, AP, ROCHE Mannheim, Germany; Cat. no. 1684 809), which is a hallmark of programmed cell death (PCD), following Gavrieli *et al.* (1992), Coimbra *et al.* (2004), Flores-Rentería *et al.* (2013) and Márquez-Guzmán *et al.* (2016). Sections were viewed using an Olympus FV1000 confocal microscope (Olympus Optical, Tokyo, Japan).

Results

Normal development. Normal anther and pollen development are fully described and illustrated in LS and SS flowers. Although the development of the pollen is a continuous process, results are presented next for five key ontogenetic stages, from sporogenous tissue to microspore mother cell (MMC), tetrad, free microspore, and mature pollen stages. Abnormalities are described following the description of normal anther and pollen development.

Sporogenous tissue stage. *Palicourea padifolia* has tetrasporangiate anthers (Figure 1A), with raphides distributed in the connective tissue. The differentiation of the sporogenous tissue initiates in buds < 3 mm in length. At the time the sporogenous tissue is differentiating, the tissue forming the anther wall is divided periclinally and anticlinally (Figure 1B). The anther wall typically consists of epidermis, endothecium, one or two middle layers and tapetum. The tapetum is one layered and tapetal cells are uninucleate. Pollen grains are dispersed at the tricellular stage.

Microspore mother cell stage (MMC). Microspore mother cells (MMCs) are found in buds < 3 mm long. Sporogenous tissue differentiates into MMCs, which are large and contain a sizable nucleus. In this stage five layers are differentiated in the anther wall: epidermis, endothecium, two middle layers and the tapetum (Figure 1C). Large vacuoles are present in tapetal cells. As development proceeds, the callose layer is getting thicker until dyad formation (Figure 1D).

Tetrad stage. Tetrads are found in buds < 3 mm long. Once the MMCs are surrounded by callose, meiosis I occurs (Figure 1E) and the meiosis II division occurs immediately after forming tetragonal tetrads of microspores, which are still surrounded by the callose layer. In this developmental stage, the tapetal cells grow radially and contain large vacuoles occupying most of the cellular space and displacing the cytoplasm and nucleus to the periphery (Figure 1F).

Free microspore stage. Free microspores are found predominantly in buds between 3 and 7 mm long, with the majority of these buds ranging between 3 and 4 mm long. At the end of the tetrad stage, the callose layer disassociates, and the microspores become separated from one another, initiating the free microspore stage. In the locule some microspores are spherical, all presenting cytoplasmic content (Figure 2A). At this time of development, three layers, from the outside to the inside, constitute the anther wall: epidermis, endothecium and tapetum. The uninucleate, tapetal cells increase in size and the vacuoles become larger and cytoplasm becomes restricted to the margins of the vacuole (Figure 2B). Then, pollen grains keep growing, accumulating reserves and become on the surface surrounded by the exine layer (Figure 2C).

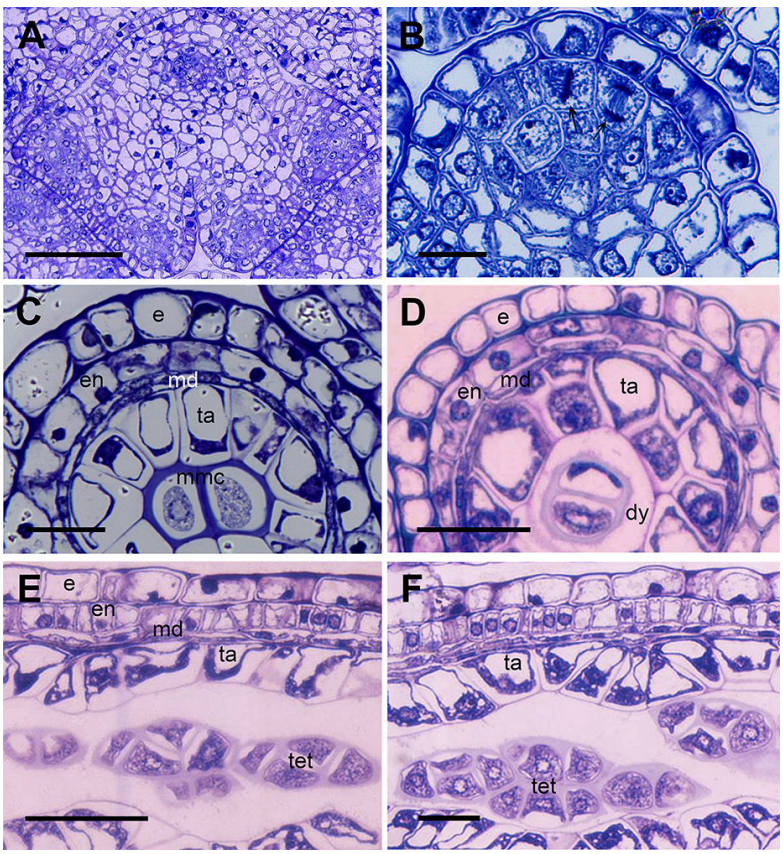

Figure 1. Anther development and microsporogenesis of *Palicourea padifolia* flowers. (A) *Sporogenous tissue stage*. Cross-section of an entire LS anther showing four locules, the anther loculi containing sporogenous tissue. Note that the sporogenous cells have relatively large nuclei and that a distinguishable tapetum has not yet differentiated. Scale bar = 36 µm. (B) Detail of SS anther development showing the microsporangium. Note the mitosis divisions (arrows), which give rise to the inner layers of the anther wall and sporogenous cells. Scale bar = 20 µm. (C) *Microspore mother cell stage* (MMC). Microsporangium of LS anther with MMCs towards the center and the anther wall differentiated into five layers: epidermis, endothecium, two middle layers and tapetum. Scale bar = 20 µm. (D) Cross-section of a microsporangium showing dyad formation (LS) surrounded by a callose layer. Note that the uninucleate tapetal cells contain a large vacuole that displaces the nucleus and cytoplasm to the periphery, and are tightly adjoined to each other in contact with the middle layers of the anther wall. Scale bar = 25 µm. (E) *Tetrad stage*. Longitudinal section of a single locus showing tetrahedral tetrads (SS) with underling callose layer surrounding the entire tetrad. Anther wall formed by the epidermis, endothecium, obliterate middle layers and tapetum. Scale bar = 16 µm. (F) Detail of the tetragonal tetrad formation (LS). Note that a thick callose layer surrounds the tetrad. Scale bar = 18 µm. dy = dyads, e = epidermis, en = endothecium, md = middle layer, mmc = microspore mother cells, ta = tapetum, tet = tetragonal tetrads.

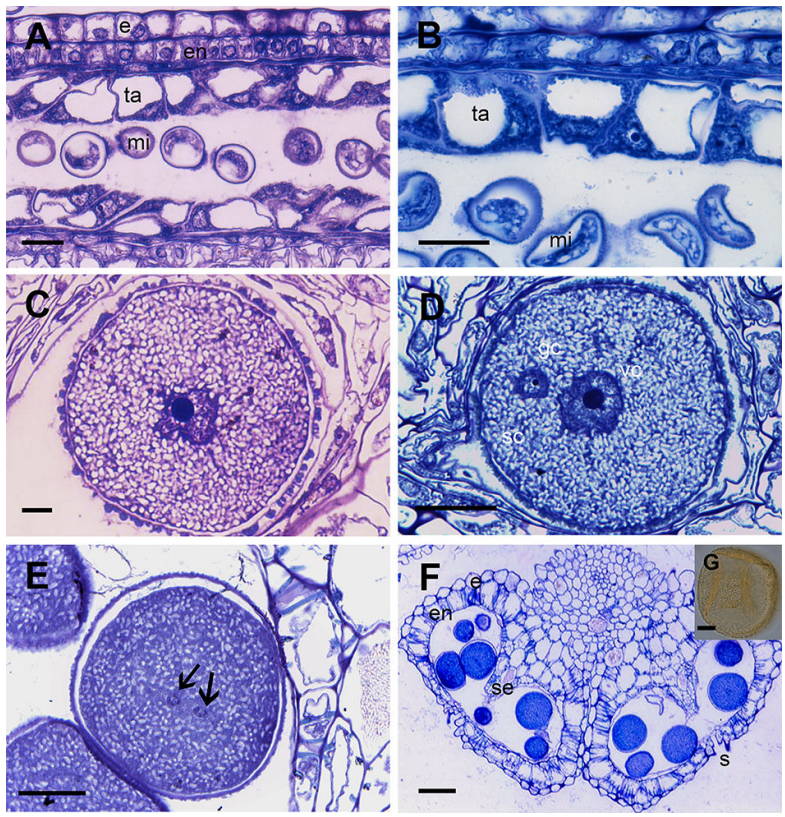

Figure 2. Anther development, microsporogenesis and microgametogenesis of *Palicourea padifolia* flowers. (A) *Free microspore stage*. Longitudinal section of a single locus showing free microspores at the early free microspore stage (LS). Note that the tapetal cells remain closely abut and contain large vacuoles. Scale bar = 19 µm. (B) Uninuclear tapetum (LS) with large vacuole and dense cytoplasm. Scale bar = 10 µm. (C) *Mature pollen stage*. Haploid pollen grain (SS) with dense cytoplasm. Note exine deposits on the surface. Scale bar = 10 µm. (D) Bicellular pollen grain (SS) with vegetative cell and generative cell. Tapetum fully collapsed. Scale bar = 20 µm. (E) Mature pollen grain (LS), tricellular (arrows). Scale bar = 20 µm. (F) Cross-section of an anther showing mature pollen stage (LS). Note the thickenings of the endothecial cells and that pollen grains are ready to exit since the septum that separates the microsporangium and the stomium are broken. Scale bar = 100 µm. (G) Acetolyzed pollen grain (LS). The pollen grain is inaperturate with scabrate-verrucose ornamentation. Scale bar= 10 µm. gc = generative cell, vc = vegetative cell, e = epidermis, en = endothecium, md = middle layers, mi = microspore, mpg= mature pollen grain, ta = tapetum, se = septum, s = stomium, sc = cells sperm.

Mature pollen stage. Mature pollen stage is exclusively found in buds > 5 mm long and in mature flowers at anthesis. Pollen grains pass through a mitotic division to form bicellular pollen grains: a vegetative cell and a smaller generative cell (Figure 2D). Then the generative cell undergoes a second division to form two sperm cells (Figure 2E). At this stage the mature anther wall consists of epidermis and endothecium cells whose walls have thickenings. Septa separating the sporangia break, allowing the release of pollen grains (Figure 2F). Finally, the pollen grains when leaving the anther are formed by the wall of the pollen grain (exine and intine) and three cells: a vegetative and inside of their cytoplasm two spermatic cells. Mature pollen grains are inaperturate with scabrate-verrucate surface ornamentation (*e.g.*, Luo *et al*. 2009, Figure 2G).

Abnormalities. Based on the pattern and timing of tapetal development and disappearance *P. padifolia* showed a parietal tapetum (also known as secretory or glandular; Pacini 1997). Abnormalities in pollen development were observed at several ontogenetic stages. Pollen development with tapetal degeneration occurred during the MMC stage (microsporogenesis), which is more evident during the free microspore stage (microgametogenesis). Low-frequency abortion and abnormal pollen development were observed in the MMC stage, but increased in the young pollen grains immediately after meiosis. Microsporangia with aborted pollen grains or that were completely empty were also observed at later stages.

Microspore mother cell stage. The most frequent abnormalities during this stage of development in both morphs were related to the tapetum and/or mother cells of the microspores. In the latter is often observed inside the cytoplasm collapsed and absence of nucleus. This cell degeneration becomes more evident when comparing these cells with other normal cells in the same locule (Figure 3A). It is also frequently observed tapetal cells appearing empty and with very thick walls between them, indicating their atrophic condition. MMCs surrounded by a thick wall of callose, contain a collapsed cytoplasm and an apparent absence of nucleus (Figure 3B).

Free microspore stage. At this stage the greatest diversity of abnormalities is observed during the stage of meiosis. Locules are often observed with free microspores, totally abnormal with or without traces of cytoplasm and irregular shapes away from the spherical shape of normal microspores (see Figure 2A). In addition, the tapetum either does not exist (Figure 3C) or presents a completely atrophic structure as the free microspores (Figure 3D).

Mature pollen stage. Hypertrophied structures are presented at this stage in the anther locule, reminiscent of the pollen grains by irregular deposition exine forming its wall and the presence of a granular cytoplasmic deposited externally to a large central vacuole. The tapetum is abnormally persistent at this stage with its cell integrity and the collapsed protoplast occupies the central area of the tapetum cell (Figure 3E, F). No cases where there is a normal tapetum but abnormal pollen grains were observed. Finally, abnormalities occurred at the stage of anther dehiscence, in which one or more of the sporangia are sterile due to never having formed normal pollen grains (Figure 3G).

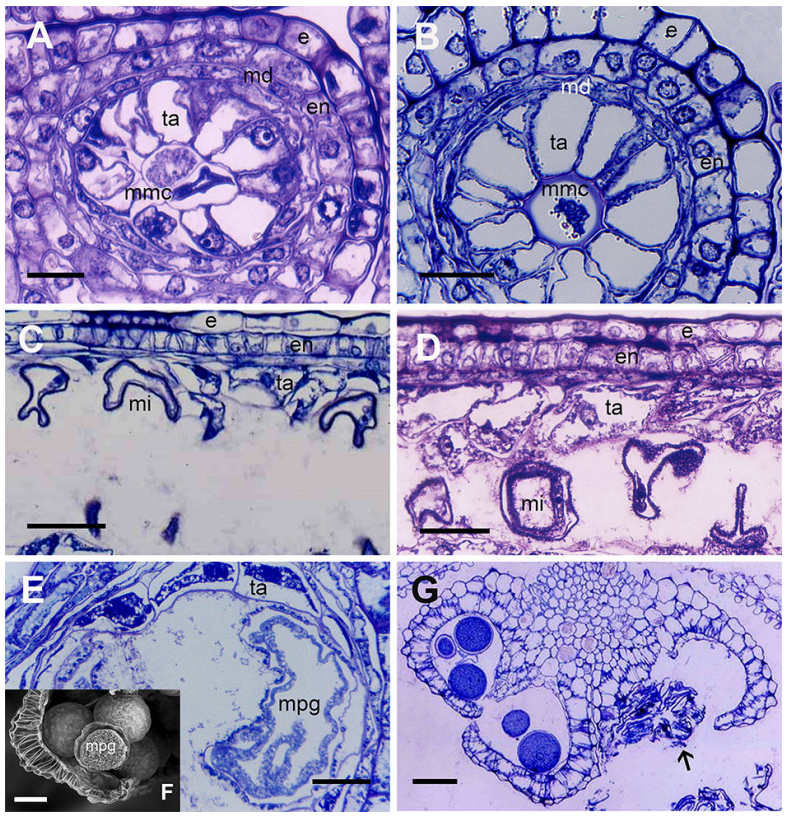

Figure 3. Abnormalities of *Palicourea padifolia* flowers. (A) *Microspore mother cell stage*. Cross-section of a microsporangium (SS). One of the MMCs is degenerating. Scale bar = 10 µm. (B) Cross-section of a microsporangium (SS). MMCs collapsed, covered by callose deposition. Tapetum hypertrophied. Scale bar = 20 µm. (C) *Free microspore stage*. Longitudinal-section of a microsporangium (SS). Free microspores folded lacking cytoplasm. Tapetum becomes thinner with little cytoplasmic content. Scale bar = 22 µm. (D) Longitudinal-section of a microsporangium (LS). Free microspores are abnormal, folded. Tapetal cells with large vacuoles are invading the anther locule. Scale bar = 30 µm. (E) *Mature pollen stage*. Detail of two folded pollen grains (SS), with irregular exine deposits on the surface. Scale bar = 20 µm. (F) Mature pollen stage (SS) with irregular exine deposits (inset, SEM). Scale bar = 50 µm. (G) Mature tetrasporangiate LS anther with collapsed pollen grains in two microsporangia. Scale bar = 100 µm. e = epidermis, en = endothecium, md = middle layer, mi = microspore, mmc = microspore mother cell, mpg = mature pollen grains, ta = tapetum.

Abnormalities in pollen development differed by floral morph (χ^2 = 5.11, df = 1, p = 0.023), with a higher proportion of samples with abnormalities in the SS across developmental stages (LS = 5/350 floral buds, 1.4 %, SS = 14/325 floral buds, 4 %). Tapetal degeneration is a normal phenomenon, and in the examined samples abnormalities occurred more often in the microspore mother cell and free microspore stages. Abnormalities were more often observed in the mature pollen stage (63 % of all samples with abnormalities), and at this stage the number of examined samples with abnormalities were significantly higher in the SS morph (LS = 5/70 floral buds, 7.1 %, SS = 13/65 floral buds, 22 %; χ^2 = 5.77, df = 1, p = 0.016). Assuming that abnormalities occurring at an early stage of anther development are carried through to the mature pollen stage (if the phenomenon is genetically based), the morph difference was upheld (LS = 17/70 floral buds, 24.3 %, SS = 29/65 floral buds, 44.6 %; χ^2 = 6.21, df = 1, p = 0.012). Because these percentages were derived from a randomly selected sample of flowers in 2008, we are confident that they reflect the true pattern of abnormal pollen development in *P. padifolia*.

TUNEL assay. Signals of DNA fragmentation were observed in anthers of SS morphs, which showed more abnormalities during pollen development; only high-quality images of the SS floral morph are presented (Figures 4 and 5). The assay was not conducted during the free microspores stage because the microspores were collapsed and nuclei in the anthers were degraded and they could not be visualized even using DAPI. DNA fragmentation signals are shown in green as a result of the fluorescein incorporation at the 3´-OH in DNA breaks. Positive control tissues (MMCs) were treated with DNase, after which nuclei were seen with an intense green signal. In the case of the negative control, the DNA transferase reaction did not include TdT and therefore no localized fluorescence was expected and only a general green background was observed (Figure 4). Positive and negative controls are indicated in the figure.

TUNEL-positive signals (DNA fragmentation) were detected at the MMC stage, specifically in the epidermis, endothecium and tapetum. However, TUNEL-positive signals were undetected in the nuclei of the MMCs, indicating that DNA fragmentation had not occurred at these stages (Figure 5A-D). DNA fragmentation was also observed at the pollen grain stage, in the nuclei of the pollen grains and the tapetal cells. However, degeneration in the tapetal cells is normal to observe at this developmental stage (Figure 5E-H).

Discussion

This study is the first on pollen development in *Palicourea padifolia* and the first to report the entire developmental sequence in both SS and LS flowers of a distylous species in the genus using LM and SEM. In general, pollen development in *P. padifolia* followed the normal ontogenetic stages previously described in two species of Rubiaceae, namely on *Mitriostigma axillare* (Hansson & El-Ghazaly 2000) and *Rondeletia odorata* (El-Ghazaly *et al.* 2001). Li *et al.* (2010) reported that the occurrence of normal tapetum PCD following meiosis in the SS morph of *Mussaenda pubescens* (Rubiaceae) fits well with normal cases of tapetum development following meiosis, while degradation of tapetum in the LS morph was relatively earlier to normal in the SS morph. The tapetum pre-degradation was interpreted as the developmental stage that probably accounts for male sterility in the LS morph. Several key pollen developmental characters of *P. padifolia* are discussed next in light of what is known from studies of other heterostylous species, particularly the abnormalities of SS flowers in the context of male sterility. Because the plant material was collected in a single population and differences between morphs were not absolute (*i.e.*, number of individuals with abnormalities rather than just the number of flowers per floral morph), note that the study was not designed to detect that variation. However, we acknowledge

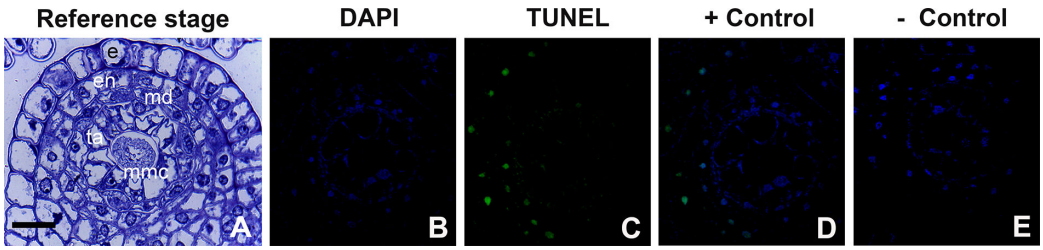

Figure 4. TUNEL assay to detect DNA fragmentation in SS flowers of *Palicourea padifolia*. (A) Cross-section of the anther at stage of MMC stained with toluidine blue. (B) DAPI staining showing the nuclei with blue fluorescence. (C) TUNEL assay: green fluorescence indicates nuclei that are positive for DNA fragmentation. (D) *Positive control.* Overlapping images exposing nuclei with DNA fragmentation. (E) *Negative control*. Nuclei with DNA fragmentation are not visualized (green fluorescence) but nuclei with DAPI are visualized with blue fluorescence in the overlapping images. e = epidermis, en = endothecium, md = middle layers, mmc = microspore mother cell.

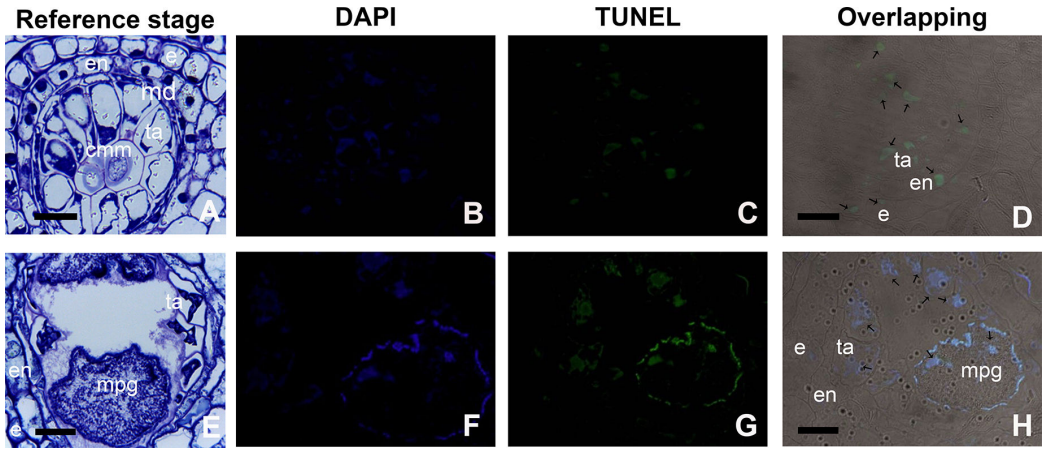

Figure 5. DNA fragmentation detection by TUNEL assays in SS flower anthers of *Palicourea padifolia*. (A-D) Cross-section of anthers at the MMC stage. (A) Cross-section of the anther at stage of MMC stained with toluidine blue. The anther wall is composed of epidermis, endothecium, middle layers and tapetum; MMCs at the center. (B) DAPI staining evidencing the nuclei with blue fluorescence. (C) TUNEL assay: nuclei with green fluorescence (positive signal for DNA fragmentation). (D) Overlapping DAPI and TUNEL images. Nuclei with DNA fragmentation observed in the epidermis, endothecium, and tapetum. Scale bar: D = 10 µm. (E-H) Cross-section of anthers in mature pollen stage. (E) Detail of a pollen grain. The anther wall is composed of epidermis, endothecium, and tapetum stained with toluidine blue. (F) DAPI staining evidencing blue fluorescence in the nuclei of pollen grains and tapetum. Note fluorescence of the exine. (G) TUNEL assay: green fluorescence in the nuclei of pollen grains and tapetum. (H) Overlapping DAPI and TUNEL images. Nuclei of the tapetum and pollen grains (arrows) are fragmented. Scale bar: H = 70 µm. e = epidermis, en = endothecium, md = middle layers, mmc = microspore mother cell, mpg = mature pollen grains, ta = tapetum.

that the linkage between morph type and male sterility is not complete, and could vary among individuals and years and across populations if the effect is a genotypic one, *i.e.*, genotypic variation in loss of male function (Rosas & Domínguez 2009, Zhou *et al*. 2012, 2017, Kappel *et al*. 2017, Shore *et al*. 2019).

Sporogenous male sterility. The phenotypic expression of male sterility often involves pollen abortion; however, male sterility ranges from indehiscent anthers that contain viable pollen to the absence of male reproductive organs in flowers (Kaul 1988). Embryological studies have detected that failure in pollen development during or after the tetrad stage is often associated with tapetal degeneration (Raghavan 1997), and tapetal degeneration during pollen development is a well-documented cause of male sterility (Kaul 1988). Two investigations have examined pollen developmental differences between or among morphs in heterostylous species. In tristylous Pontederiaceae, the three anther levels within flowers enter meiosis at different times, and differences in pollen mother cell numbers, as well as size, are present prior to meiosis (Richards & Barrett 1984, 1992). Abnormal pollen development was documented during meiosis in distylous *Erythroxylum havanense* (Erythroxylaceae), and the failure to develop a fertile androecium among SS flowers seemed a consequence of abnormalities observed at the sporogenous tissue, the tapetum layer, and the microspore (Domínguez *et al*. 1997).

In *P. padifolia*, we observed that the tapetal cells begun to degenerate shortly after microspore release from the tetrad, which is normal pollen development. Presumably, this cell death is programmed and occurs after microspores are released from the tetrad for proper microspore development and further differentiation into mature functional pollen grains (reviewed in Wu & Cheung 2000). However, we also observed manifestations of male sterility with more abnormalities at the MMC, tetrad, and young pollen grain stages. Abnormal microsporogenesis in *P. padifolia* expressed early in development at the MMC stage, indicates that abnormalities are manifestations of male sterility due to tapetal degeneration. Post-meiotic abortion also produced morph differences in *P. padifolia*; anthers with all microsporangia empty or with microsporangia empty and with aborted pollen grains (the only result that maters to fitness) were more often observed in samples of SS flowers. We believe that the shifts in timing of tapetum PCD cause the phenomenon of DNA degeneration in developing pollen. Our results indicate that the early degeneration of the tapetum, or indications of abnormality such as hypertrophication, causes MMC, microspore, or pollen grain degeneration because of a lack of nourishment or correct signaling during development, and that pollen sterility is likely caused by a defective tapetum.

Our developmental study showed that signals of pollen sterility were less often observed among samples of LS anthers, which suggests a higher investment of resources in the production of pollen; reallocation of those resources to produce more and/or better seeds in SS plants. Given the invariant number of ovules per flower in *P. padifolia*, compensation effects could be expected in seed mass or total seed production (Cuevas-García *et al.* 2005). Hernández & Ornelas (2007a) showed morph differences in resource allocation in *P. padifolia*, in which LS flowers invested twice as much biomass to the male function than SS flowers in relation to the female function. However, data on reproductive compensation and detailed descriptions of female gametophyte development are needed to discern whether populations with females and hermaphrodites (gynodioecy) are an intermediate condition by which dioecy evolves from hermaphroditism (see also Vázquez-Santana *et al.* 1996, Li *et al.* 2010).

Our pollen development data and results of the TUNEL assay suggest a higher incidence of abnormalities (collapsed sporangia and pollen abortion) and DNA fragmentation (in the nuclei of the pollen grains) at the pollen grain stage of SS flowers. Abnormalities were observed in both morphs at various developmental stages due to the mis-timing of tapetal degeneration. However, more abnormalities occurred in the pollen grain stage of SS flowers, indicating SS partially male sterility. The TUNEL assay implies early senescence due to malnutrition from a defective tapetum, in which mutations might affect the timing of the already present PCD in the tapetum, and that the mis-timing of tapetal degeneration would cause pollen cell death by lack of proper nutrition or signal timing from the tapetum. Furthermore, the developmental pathways of SS flowers of *P. padifolia* resemble those in gynodioecious species leading to male sterility with cytoplasmic male sterility, closely linked to tapetal degeneration (Kaul 1988, Conley *et al.* 1994, Cuevas-García *et al.* 2005, Li *et al.* 2010). Regardless of these similarities, the higher incidence of male sterility among SS flowers would provide the mechanism for the transition to functional dioecy from distyly (reciprocal herkogamy). Further comparative pollen developmental studies that examine pollen developmental patterns of homostylous relatives (or species with distylous and homostylous populations) should provide new insights to the understanding of male sterility in the functioning of heterostyly.

Transition from distyly to dioecy. Heterostyly is one of the hypothesized steps in a pathway from homostyly to heterostyly to sexual dimorphism (dioecy) in Rubiaceae (Pailler & Thompson 1997, Pailler *et al.* 1998a) and in other families (*e.g.*, Erythroxylaceae; Pailler *et al.* 1998b, Avila-Sakar & Domínguez 2000, Rosas & Domínguez 2009), and male sterility might be the mechanism for the initial spread of gender specialization leading to the transition of heterostyly into dioecy (Muenchow & Grebus 1989, Shultz 1994, Domínguez *et al.* 1997). However, there is evidence that under some ecological scenarios, heterostyly evolves into other derived reproductive systems (Ganders 1979, Bawa & Beach 1981, Lloyd & Webb 1992b, Castro *et al.* 2004, Sakai & Wright 2008, Consolaro *et al.* 2011). For example, with inbreeding advantage (*i.e.*, pollinator shortage, population bottlenecks, and/or colonizing events), natural selection would favor recombination within the distyly supergene and the consequent evolution of homostyly (Barrett *et al.* 1989, Barrett 1990, Carlson *et al.* 2008, Barrett *et al.* 2009, de Vos *et al.* 2012, 2014). In contrast, on the basis of sexual selection and sex allocation theory, floral morphs may exhibit gender specialization and gain differential reproductive success through male versus female function (reviewed in Casper 1992) and, eventually, heterostyly or reciprocal herkogamy evolves into dioecy (Muenchow & Grebus 1989, Barrett *et al.* 2000, Barrett 2002, Rosas & Domínguez 2009, Li *et al.* 2010).

Congeners of distylous species in Rubiaceae, dispersed among 21 tribes in the family according to Barrett & Richards (1990), are often homostylous species, suggesting that heterostyly has evolved repeatedly in the Rubiaceae (Castro *et al.* 2004, Sá *et al.* 2016). As first pointed out by Darwin (1877), sexual dimorphism could evolve from heterostyly as a result of gender specialization, with the LS and SS floral morphs no longer making equal contributions to offspring as paternal and maternal parents. Several authors have proposed that dioecy evolves from heterostyly when pollen transfer between floral morphs is highly asymmetrical (Ornduff 1966, Beach & Bawa 1980). The classical Darwinian explanation for the transition to dioecy from distyly proposes that the pollinator-driven asymmetrical pollen transfer between floral morphs, resulting from low reciprocity among sexual organs (*e.g.*, Feinsinger & Busby 1987, Lau & Bosque 2003, Ornelas *et al.* 2004a, Stone & Thomson 1994, Hernández & Ornelas 2007a, García-Robledo 2008) and lower accessibility of low-level sexual organs and pollinator behavior (Liu *et al.* 2016, Yuan *et al.* 2017), would favor maleness in the morph with higher pollen donation efficiency (usually SS), and femaleness in the morph receiving more legitimate pollen (usually LS) (Darwin 1877, Ornduff 1966, Lloyd 1979, Beach & Bawa 1980). Asymmetries in pollen flow might be an important first step towards gender specialization. Here we showed that failures in pollen transfer, which deviates *P. padifolia* from the expected disassortative pollination in distylous plants (Darwin 1877, Lloyd & Webb 1992b, Barrett 2002, 2019, Jiang *et al.* 2018), are potentially accompanied by major male sterility in SS flowers.

Asymmetrical pollen transfer has been observed in several distylous *Palicourea* species, with higher percentages of compatible pollination in the LS-morph than in the SS-morph in *P. fendleri* (Lau & Bosque 2003) and *P. tetragona* (Martén-Rodríguez *et al*. 2013), while other species (*P. lasiorrachis*, Feinsinger & Busby 1987; *P. demissa,* Valois-Cuesta *et al*. 2011a, b, 2012) including *P. padifolia* (Ornelas *et al*. 2004a, Hernández & Ornelas 2007b) have the opposite pattern, *i.e.,* more compatible pollination in the SS-morph than in the LS-morph. In *P. padifolia*, Hernández & Ornelas (2007a) found that pollen receipt was not affected by reciprocal herkogamy, typically associated with the efficiency of this floral polymorphism in promoting disassortative pollination, and legitimate cross-pollination was reduced significantly in LS plants with increased stigma-anther separation. Accordingly, increased herkogamy in SS flowers might favor gender specialization, SS flowers being more effective as receptors of legitimate pollen (femaleness in SS flowers) and LS flowers as pollen donors (maleness in LS flowers). In *Palicourea fendleri*, Lau & Bosque (2003) found that *Amazilia tobaci* hummingbirds were more efficient depositing legitimate pollen on stigmas of SS flowers than on those of LS flowers. Using stuffed hummingbirds as pollen vectors, Ornelas *et al*. (2004a) found that long-billed hummingbird species transferred significantly more pollen from LS towards SS flowers than in the opposite direction in *P. padifolia*. Although asymmetrical pollen transfer between floral morphs seemed to favor SS plants of *P. padifolia*, the observed variation in pollen removal and pollen receipt was not explained by the variation in hummingbirds' bill morphology, nor did they find evidence that hummingbird bill morphology solely explained the differences in fruit production between LS and SS-morph individuals (Ornelas *et al*. 2004b). If we consider that the bill size of a hummingbird is the determining factor in the direction of pollen flow between morphs in hummingbird-pollinated *Palicourea* species, Valois-Cuesta *et al*. (2011b) proposed that long-billed hummingbird species play a major role in pollen transfer between reproductive organs at low levels (from anthers of LS flowers towards stigmas of SS flowers), whereas short-billed hummingbird species would be more efficient in the transfer of pollen grains among reproductive organs positioned at higher levels (from anthers of SS flowers towards stigmas of LS flowers). Accordingly, the most frequent long-billed hummingbird species deposited 2 times more legitimate pollen grains on SS emasculated flowers than LS emasculated flowers in *Palicourea demissa*. In contrast, the less frequent short-billed hummingbird species deposited 2.3 times more legitimate pollen on LS than SS emasculated (Valois-Cuesta *et al*. 2012). Accordingly, the pollinator-driven selective regime would favor a process of sexual divergence between the floral morphs and eventually the evolution of separated sexes. This is unlikely the sole explanation for gender specialization because reciprocal herkogamy, stigmatic pollen loads and the pollinator communities visiting flowers of *P. padifolia* populations vary across time and space (Hernández & Ornelas 2007a, Hernández-Ramírez 2018). Instead, long- and short-billed hummingbird species may complement each other in the legitimate pollination of *P. padifolia* across populations. For instance, Deschepper *et al*. (2018) surveyed pollinator communities visiting flowers of the distylous *Primula veris* (Primulaceae) in which the positioning of the anthers and stigmas differ between flowers of forest and grassland populations. They measured anther-stigma separation in the two habitats, assessed pollen uptake on the head and proboscis of each of the pollinator species observed, and compared stigmatic pollen deposition and subsequent seed set between SS and LS flowers of *P. veris*. The forest and grassland *P. veris* populations contained distinct pollinator communities, in which long- and short-tongued insects complemented each other in the legitimate pollination of this distylous species and that differences in floral morphology do not impact on reproductive success. Lastly, other ecological conditions inducing reproductive costs (*i.e*., nectar robbers, herbivores; see also Ornelas *et al*. 2004b, Chautá *et al*. 2017) might contribute as selective mechanisms responsible for gender specialization and the evolutionary transition of heterostyly. However, little is known about the early stages of this transition, including the genetic and developmental basis for the evolution of unisexuality such as sterility mutations differentially expressed between the floral morphs (*e.g*., Domínguez *et al*. 1997, Pailler *et al*. 1998a, del Carlo & Buzato 2006, Rosas & Domínguez 2009, Li *et al*. 2010).

In previous work with *P. padifolia*, it has been suggested that the reproductive differences between floral morphs are due to asymmetrical pollinator service favoring SS individuals (Ornelas *et al*. 2004a, Hernández & Ornelas 2007a). When the possibility of asymmetrical pollen flow was ruled out by means of experimental pollination (hand-pollination and pollination by stuffed hummingbirds), the fecundity differences between floral morphs held (Ornelas *et al*. 2004a), suggesting that asymmetrical pollinator service is not the sole explanation for the observed differences in fecundity where SS plants develop almost twice the number of fruits developed by LS plants. Here our results suggest that abnormalities of pollen development in *P. padifolia* are biased to partially male sterility in SS morph. Although male sterility is far from being complete, the SS-biased male sterility would provide the initial conditions for the transition of heterostyly and the selective pressures promoting gender specialization in *P. padifolia*. Further study of the anther transcriptome and digital gene expression profiling is necessary to identify candidate genes

contributing to anther and pollen development, particularly those that regulate tapetum and pollen development (*e.g.*, Yue *et al*. 2017). In addition, development of female structures must be investigated, especially how the incompatibility system works in each morph. A relaxation of the incompatibility system in the SS morph might be an explanation for femaleness more than maleness in this morph. Comparisons of anther and pollen transcriptomes through development would undoubtedly reveal the gene-expression differences along the pathway from homostyly to heterostyly to gender specialization and sexual dimorphism (dioecy) in Rubiaceae (Yue *et al*. 2017, Barrett 2019).

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