

DEVELOPMENTAL EVOLUTION OF THE PROGAMIC PHASE IN NYMPHAEALES

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DEDICATION

To my grandma:

Vera Lorraine Stevens Holmes (1921 – 2011)

Who inspires me to be a strong, independent, creative woman and who, above all, loved me.

And to my grandma:

Hazel Aileen Hagadorn Taylor (1906-1994)

Who let me water her cacti and climb her trees and whose (very thorny) rose bushes I encountered in one of my very first interactions with the botanical world.

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ABSTRACT

The period between pollination and fertilization, or the progamic phase, is a critical life history stage in seed plants and innovations in this life history stage are hypothesized to have played an important role in the diversification of flowering plants. Over the course of this dissertation research, I investigated progamic phase development in Nymphaeales (water lilies), an ancient angiosperm lineage that diverged from the basalmost or next most basal node of the angiosperm phylogenetic tree and that is represented in the oldest angiosperm fossil record. I used field experiments and microscopy to document pollination biology, breeding system, and reproductive developmental traits in two families of Nymphaeales: Cabombaceae (*Brasenia*, *Cabomba*) and Hydatellaceae (*Trithuria*). Nymphaeales exhibits considerable variation in reproductive traits and true carpel closure, wind-pollination, and a primarily selfing breeding system have arisen independently in the lineage. Pollen tube pathway length, timing of stigma receptivity, and pollen tube growth rates are conspicuous traits that have undergone considerable modification in concert with shifts in pollination biology and breeding system. Post-pollination developmental processes in Nymphaeales appear to experience selective pressures similar to those experienced by more derived angiosperms and to evolve in similar ways. Nymphaeales also exhibits traits, such as accelerated pollen tube growth, callosic pollen tube walls, and the formation of callose plugs, that are almost certainly plesiomorphic in angiosperms and may have facilitated modification of carpel structure and progamic phase ontogenies. The finding that pollen tube traits that underlie developmental flexibility were already in place before the divergence of Nymphaeales supports the hypothesis that innovations in male gametophyte development were instrumental in facilitating early angiosperm diversification.

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CHAPTER I: INTRODUCTION TO THE PROGAMIC PHASE AND NYMPHAEALES

INTRODUCTION

Angiosperms, or flowering plants, comprise the largest and most diverse group of plants on Earth. At over 260,000 species, this clade exhibits rich taxonomic and ecologic diversity that has dominated plant communities since the end of the Cretaceous (Lupia *et al.* 1999).

Angiosperms also comprise the youngest major plant clade, with angiosperm fossils first clearly present in the fossil record in the early Cretaceous (~140 mya; e.g. Friis *et al.* 2010), although molecular dates indicate an earlier crown group origin (e.g. 140-180 mya, Bell *et al.* 2005; 130 mya, Magallón and Castillo 2009; 182-257 mya, Smith *et al.* 2010).

The evolutionary success of angiosperms has been repeatedly attributed to innovations in reproductive biology. Angiosperms exhibit a suite of novel reproductive traits that include highly reduced male and female gametophytes, a closed carpel with an internalized pollen tube pathway, pollen tubes with callosic walls, and increased pollen tube growth rates, as well as mechanisms for pre-zygotic mate discrimination, such as pollen competition or self incompatibility (e.g. Stebbins 1970, 1974; Willson and Burley, 1983; Doyle and Donoghue 1986; Mulcahy and Mulcahy 1987; Williams 2008, 2009). The majority of these developmental events occur during the life history stage known as the progamic phase.

THE PROGAMIC PHASE

The progamic phase is the life history stage in seed plants that begins with pollination and ends with fertilization (Cresti *et al.* 1992). In angiosperms, progamic phase events take place within the confines of the closed carpel. A pollen grain lands on the stigma and germinates, with

the pollen tube emerging through the aperture of the pollen grain. The pollen tube grows through the style, typically through a secretion-filled canal or a region of specialized cells (the transmitting tract), to reach the ovarian cavity. Pollen tubes must then enter an ovule through the micropyle, traverse the nucellus, and deliver two sperm nuclei to the female gametophyte. One nucleus fuses with the female gamete (egg) to form the zygote and the other fuses with one or more polar nuclei to produce the endosperm. Achieving fertilization requires coordination among multiple ontogenies so that male and female gametes meet at the correct developmental stage (Cresti *et al.* 1992; Friedman 1999; Williams *et al.* 1999; de Graaf *et al.* 2001; Herrero 2003). Despite the necessity of tight coordination, lability in progamic phase ontogenies has allowed the evolution of diverse reproductive developmental schedules and flower structures. The duration of the progamic phase can vary from less than 30 min to over 12 months and the pollen tube pathway can range from less than 0.5 mm to over 500 mm in length (Maheshwari 1950; Williams 2008). Understanding the causes and consequences of this diversity is of considerable interest in studies of angiosperm evolution (Willson and Burley 1983; Mulcahy and Mulcahy 1987; Williams 2008).

It is known that both pre- and post-pollination processes affect fertilization biology. Pollen size and quality, determined prior to pollination, can influence pollen tube growth rates and lengths (Baker and Baker 1979; Williams and Rouse 1990), as can the size and genetic diversity of pollen loads that are delivered to stigmas during pollination (Németh and Smith-Huerta 2003; Mazer *et al.* 2010). After pollination, pollen tubes interact with each other in the style, under the influence of both female sporophytic tissues and the female gametophyte (de

Graaf *et al.* 2001). Environmental factors, such as temperature, also affect pollen tube growth rates (Hedhly *et al.* 2003).

While we have some understanding of the abiotic and biotic factors that influence progamic phase development, particularly pollen tube growth, our knowledge of what forces shape the evolution of the progamic phase in flowering plants is much more limited (but see Williams 2008). Comparative investigations of integrated progamic phase developmental processes set in a phylogenetic context are necessary to better understand when key traits arose and how labile each is in the context of the others. Characterizing developmental and structural traits of the progamic phase in early-diverging lineages of angiosperms is particularly important for understanding the role progamic phase traits may have played in angiosperm diversification.

EARLY-DIVERGENT ANGIOSPERM LINEAGES

There is increasing resolution in the angiosperm phylogenetic tree and it has become clear that several lineages diverged from the rest of angiosperms early in angiosperm history, from nodes basal to origin of the monocot plus eudicot clade that includes the vast majority of flowering plant species (e.g Qiu *et al.* 1999, 2006; Löhne and Borsch 2005; Saarela *et al.* 2007; APG III 2009). These include the Magnoliids (Magnoliales, Laurales, Canellales, Piperales) plus Chloranthales (*Ascarina*, *Chloranthus*, *Hedyosmum*, *Sarcandra*), Austrobaileyales (*Austrobaileya*, *Illicium*, *Kadsura*, *Schisandra*, and *Trimenia*), Nymphaeales, and Amborellales (*Amborella*; Figure 1.1; all figures referenced in this chapter are found in Appendix 1). In the following chapters, I will refer to these lineages as “early-divergent” and to the taxa that collectively comprise these lineages as “basal angiosperms.” This term is commonly used in the

literature to refer to these, or some subset of these taxa and references the basal nature of the nodes from which their respective lineage diverged. It is not meant to imply that these extant taxa are in any way necessarily ancestral or primitive. These lineages have had the same amount of time to diversify and evolve as all other angiosperms (see Crisp and Cook 2005).

Studies of these lineages, however, can provide valuable insight into early angiosperm history and angiosperm diversification, as traits that are shared across this basal grade have a strong likelihood of being ancestral in flowering plants. It is true that some shared traits are likely independently evolved in multiple lineages (Crisp and Cook 2005). For example, a transition to the aquatic habit in both water lilies and the basal eudicot, *Ceratophyllum* may have led to convergent evolution of traits associated with the aquatic lifestyle, such as absence of a vascular cambium, highly dissected leaves, and floating leaves with high photosynthetic rates (Crisp and Cook 2005). However, this only underscores the importance of comparative developmental studies of basal lineages. Understanding development can shed light on whether characters have evolved independently or whether they represent a plesiomorphic state. Studies of early-diverging lineages can also provide insight into the likely direction of evolutionary transitions and possible developmental constraints in early angiosperms (e.g. Friedman 2006).

NYMphaeALES AS A STUDY SYSTEM

The order Nymphaeales, or water lilies, has long been considered to be among the oldest independent lineages of angiosperms and molecular studies have consistently indicated that Nymphaeales diverged from the basal-most or next most basal node of the extant angiosperm phylogenetic tree (Figure 1.1; e.g. Walker 1974; Doyle and Donoghue 1986; Hamby and Zimmer

1992; Doyle 1998; Les *et al.* 1991; Qiu *et al.* 1999, 2006; Löhne and Borsch 2005; Saarela *et al.* 2007). Water lilies are also well represented in the oldest angiosperm fossil records (Friis *et al.* 2001, 2003, 2009, 2010; Wang and Dilcher 2006; Mohr *et al.* 2008; Taylor *et al.* 2008).

Nymphaeales traditionally encompasses two well-supported monophyletic families:

Cabombaceae (*Brasenia*, *Cabomba*) and Nymphaeaceae (*Victoria*, *Euryale*, *Nymphaea*, *Ondinea*, *Barclaya*, *Nuphar*; Les *et al.* 1999; Borsch *et al.* 2008) and recent molecular data have placed the family Hydatellaceae (*Trithuria*) as sister to traditional Nymphaeales (Saarela *et al.* 2007). The intergeneric relationships within Nymphaeales have been elucidated in recent years, including the separation of *Nymphaea* into five subgenera (Figure 1.1), although there is still conflict concerning the exact relationships among “core Nymphaeales” (*Nymphaea*, *Ondinea*, *Victoria*, *Euryale*). This conflict arises primarily due to probable paraphyly in *Nymphaea*. The clade comprising *Victoria* and *Euryale* is likely nested within *Nymphaea* (Figure 1.1) and monotypic *Ondinea* (*O. purpurea*) may be nested within the *Nymphaea* sub-genus *Anecypha* and synonymized with *Nymphaea* (*N. ondinea*; Borsch *et al.* 2007).

Among basal lineages, the Nymphaeales are particularly well suited for studying evolutionary transitions in pollination and fertilization biology because they exhibit much more variation in reproductive traits than other basal angiosperms. Nymphaeales is the earliest diverging lineage that includes extant species with hermaphroditic flowers and flower size ranges from 30-50 cm wide in *Victoria* (Figure 1.2A; Schneider and Williamson 1993) to 1-2 cm in diameter in Cabombaceae (Figure 1.2G–H; Williamson and Schneider 1993) to less than 2 mm in diameter in *Trithuria* (Figure 1.2I).

Nymphaeales also exhibit a range of pollination strategies. *Nymphaea* (Figure 1.2C), *Ondinea* (Figure 1.2D), *Barclaya* (Figure 1.2E), and *Nuphar* (Figure 1.2F) are pollinated by beetles, flies, bees, or some combination (Schneider and Moore 1977; Schneider 1983; Capperino and Schneider 1985; Schneider and Williamson 1993, 1994; Seymour and Matthews 2006; Thien *et al.* 2009), whereas *Victoria* (Figure 1.2A) is specialized for pollination via entrapment of Dynastid beetles (*Cyclocephala*; Prance and Arias 1975), *Cabomba* (Figure 1.2G) is fly pollinated (Schneider and Jeter 1982), and *Brasenia* (Figure 1.2H) is wind-pollinated (Osborn and Schneider 1988). *Trithuria* has been hypothesized to exhibit pollination by either wind or water (Rudall *et al.* 2007). Evolution of these various pollination strategies has had consequences for reproductive morphology and phenology in Nymphaeales and may have consequences for progamic phase development.

There is also variation in sexual system within Nymphaeales, particularly in the genus *Trithuria*. Of the twelve species of *Trithuria*, four have bisexual reproductive structures, four are dioecious, and four are cosexual (monoecious), with male and female reproductive structures on the same plant (Yadav and Janarthanam 1995; Sokoloff *et al.* 2008). Among early-divergent angiosperm lineages, this variation in sexual system is unique to *Trithuria*. Most other basal genera, including all of Nymphaeaceae and Cabombaceae, as well as *Austrobaileya*, *Illicium*, and *Trimenia* (Austrobaileyales) exhibit only bisexual flowers, whereas other genera, including *Amborella*, *Hedyosmum*, *Ascarina*, and those in the family Myristicaceae (Magnoliales), exhibit only unisexual flowers and are dioecious (e.g. Endress 2001, 2010). Diversity in sexual system in *Trithuria* may have consequences for breeding system, reproductive morphology, and pollen tube development.

Nymphaeales also exhibits diversity in carpel morphology. Both Cabombaceae and Hydatellaceae are apocarpous, with carpels free, whereas Nymphaeaceae are syncarpous, with multiple carpels fused together (Williamson and Schneider 1993; Schneider and Williamson 1993). Furthermore, carpels in Cabombaceae and Hydatellaceae are sealed only by secretion, whereas carpels in Nymphaeaceae are partially fused at maturity (Endress 2001, 2005; Rudall *et al.* 2007). Cabombaceae have long stylar necks and few ovules per carpel (*Brasenia* = 2, *Cabomba* = 3), while Nymphaeaceae lack styles and have deep ovaries filled with numerous ovules (Schneider and Williamson 1993). Hydatellaceae lack styles, having instead long stigmatic hairs, and have ovaries containing one ovule (Rudall *et al.* 2007). Carpel structure provides the context for developmental events that occur during the progamic phase and, therefore, variation in carpel form potentially underlies modification of these developmental events.

GOALS OF THE DISSERTATION RESEARCH

The goals of this research were (1) to document post-pollination biology and the progamic phase developmental program in Nymphaeales and (2) to determine the consequences of ecological transitions, such as shifts in pollination syndrome, habitat, or breeding system, on reproductive morphology and progamic phase processes. This dissertation work focuses on five water lily species comprising two families: Cabombaceae (*Brasenia*, *Cabomba*) and Hydatellaceae (*Trithuria*). I chose to focus on Cabombaceae and Hydatellaceae because they exhibit some of the greatest structural and ecological divergence in water lilies, yet next to nothing was previously known about progamic phase development in these families. All

previous investigations of the progamic phase in water lilies were in Nymphaeaceae (*Nymphaea capensis*, Orban and Bouharmont 1995; *Nuphar polysepala*, Friedman and Williams 2003).

In Chapter II, I describe a comparative investigation of progamic phase development in the two genera of Cabombaceae and discuss evolutionary transitions in this family in light of their divergent pollination syndromes (wind vs. fly). In Chapter III, I discuss a study of reproductive development, pollination syndrome, and breeding system in *Trithuria submersa*. I expanded on this work in a comparative study, described in Chapter IV, of progamic phase development in three *Trithuria* species, *T. submersa*, *T. austinensis*, and *T. australis*. This chapter explores the evolution in pollen tube growth and progamic phase development in the context of breeding system divergence in *Trithuria*. Finally, in Chapter V, I highlight several conclusions regarding progamic phase evolution in water lilies that can be drawn from the results of this dissertation work, in combination with results from previous studies of Nymphaeales reproductive biology (e.g. Friedman and Williams 2003; Williams *et al.* 2010).

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APPENDIX 1

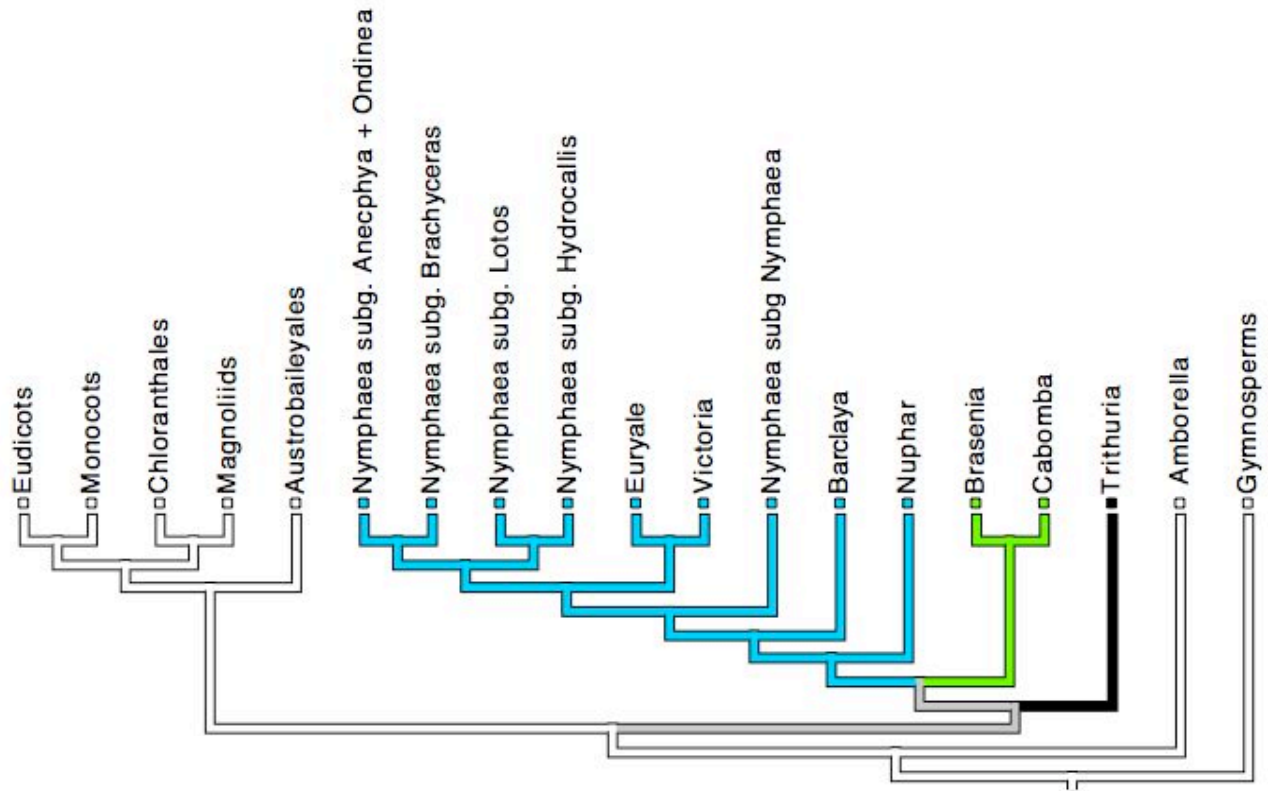


Figure 1.1. Phylogenetic relationships among genera of Nymphaeales and other selected angiosperm lineages (modified from APG III 2009; Borsch *et al.* 2008; Saarela *et al.* 2007). Note hypothesized paraphyly of the genus *Nymphaea*, which is divided into 5 subgenera. Blue branches = Nymphaeaceae, green = Cabombaceae, black = Hydatellaceae, gray = water lily stem lineage.

Figure 1.2. Representative staminate flowers of water lily genera. A–F: Nymphaeaceae: (A) *Victoria cruziana* with anthers (arrow) dehiscing over central gynoecium. (B) *Euryale ferox*. (C) *Nymphaea odorata* with dehiscing anthers (arrow) surrounding and bending over central syncarpous gynoecium. (D) *Ondinea purpurea* with dehiscing anthers (arrow) reflexed away from central stigmas. (E) *Barclaya motleyi*. (F) *Nuphar lutea* with dehiscing anthers (arrow) reflexed from the central syncarpous gynoecium (arrowhead). G–H: Cabombaceae: (G) *Cabomba caroliniana* with six dehiscent anthers (arrow) and three carpels (arrowhead) aggregated in the floral center. (H) *Brasenia schreberi* with many dehiscing anthers (arrow) supported by long, slender filaments above centrally aggregated carpels (arrowhead) (I) Hydatellaceae: *Trithuria austinensis* male plant with three dehiscing anthers (arrow) held above the water surface on long filaments. Photo credit (B, D, E): EL Schneider.

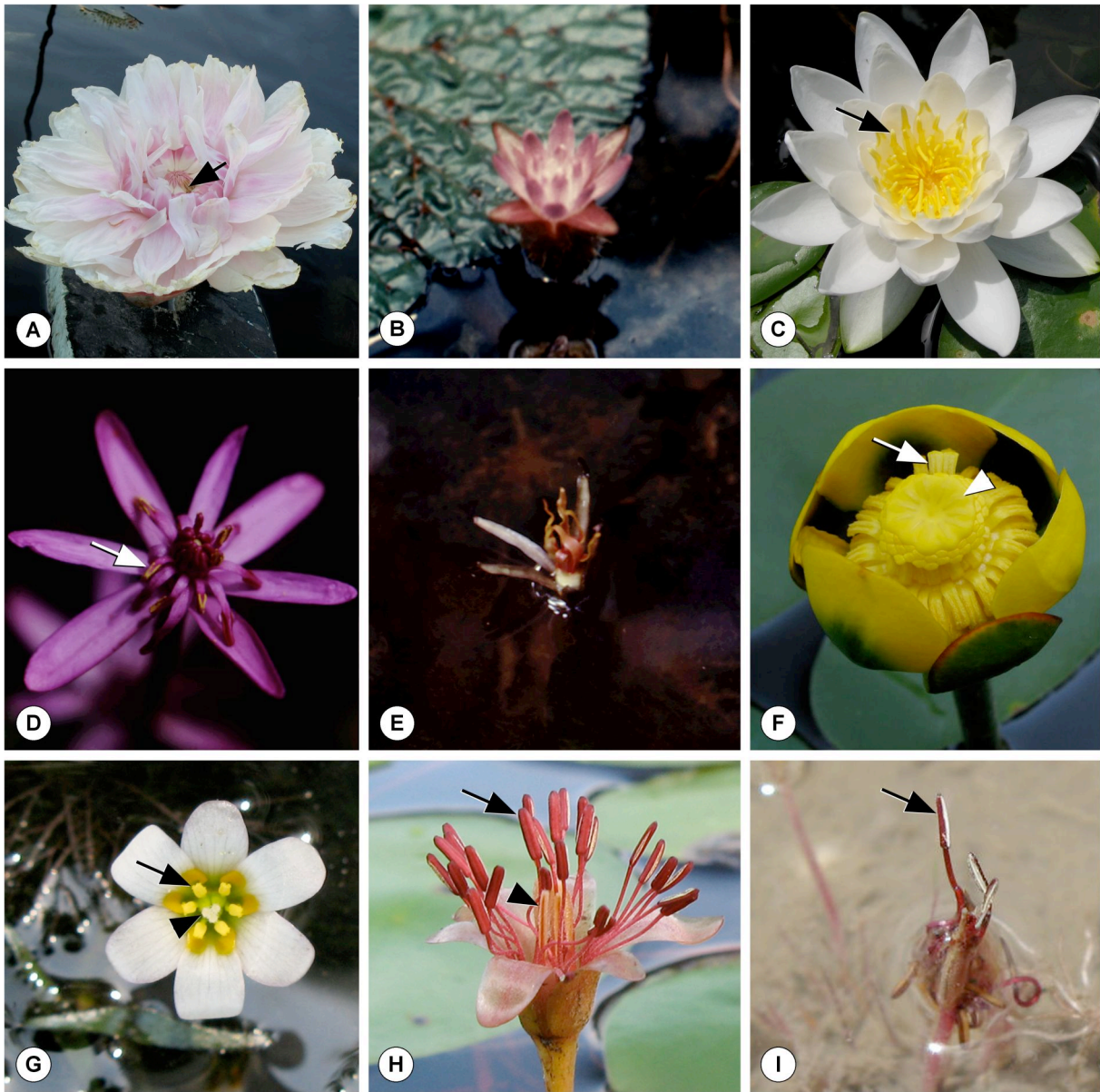


Figure 1.2

CHAPTER II: CONSEQUENCES OF POLLINATION SYNDROME EVOLUTION FOR POST-POLLINATION BIOLOGY IN AN ANCIENT ANGIOSPERM FAMILY

This chapter is a slightly modified version of an original research article published in the June 2009 issue of the *International Journal of Plant Sciences*.

Taylor M.L. and J.H. Williams. 2009. Consequences of pollination syndrome evolution for postpollination biology in an ancient angiosperm family. *International Journal of Plant Sciences* 170: 584–598.

In the following chapter, the words “we” and “our” refer to my co-author and me. My contributions to this paper include (1) co-formation of the original hypothesis (2) modification of experimental pollination techniques and completion of all field experiments (3) completion of all microscopical analyses (4) construction of figures, and (5) most of the writing.

ABSTRACT

Evolutionary shifts from insect to wind pollination involve a host of modifications to floral structure and phenology, but little is known about how floral modifications that facilitate pollination might affect the fertilization process. Within the water lily family Cabombaceae, there is evidence that wind pollination arose recently in *Brasenia*, whereas the sister genus *Cabomba* became specialized for fly pollination. Both species have an apomorphic stylar extension, which in *Brasenia* became greatly elongated to produce a much larger stigmatic surface. Consequently, pollen tubes in *Brasenia* must travel much farther to reach ovules, and because mean pollen tube growth rates are similar (750–950 $\mu\text{m/h}$), fertilization occurs approximately four hours later in *Brasenia* than in *Cabomba*. In both genera, pollen tubes grow between cells of the sub-stigmatic ground tissue and then within an open, secretion-filled stylar canal and ovarian cavity. In *Brasenia*, early pollen tube development is slower than in *Cabomba*, which may be a result of displacement of flower opening to an earlier, cooler time of day. Our results show that modifications to carpel ontogeny and structure associated with the transition to wind pollination had consequences for pollen tube development and fertilization.

INTRODUCTION

The progamic phase is the life history stage in seed plants that begins with pollination and ends with fertilization. In angiosperms this process takes place within the confines of the closed carpel (stigma, transmitting tract, ovarian cavity, and ovule) and requires coordination among multiple ontogenies so that male and female gametes meet at the correct developmental stage (Friedman 1999; Williams *et al.* 1999; Herrero 2003). The progamic phase is characterized

by tremendous developmental and structural diversity (Maheshwari 1950). For example, in angiosperms, the duration of the progamic phase can vary from less than 30 min to over 12 months and the pollen tube pathway can range from less than 0.5 mm to over 500 mm in length (Williams 2008). Understanding the forces that shape such diversity has long been of interest (Willson and Burley 1983; Mulcahy and Mulcahy 1987; Williams 2008).

It is well known that fertilization biology is affected by both pre- and post-pollination processes. For example, pollen size and quality (determined by the paternal parent) can influence pollen tube growth rates and lengths (Baker and Baker 1979; Williams and Rouse 1990). After pollination, development of pollen is contingent on properties of the carpel, which forms the pollen tube pathway within which pollen tubes interact, and signaling by the female gametophyte (de Graaf *et al.* 2001). Competition and selection can occur during pollen germination, pollen tube growth, and fertilization, causing both maternally-derived and paternally-derived progamic phase traits to evolve.

Conversely, progamic phase traits might become modified indirectly when selection acts primarily on floral traits. We know that pollinator identity can often be predicted on the basis of floral biology (Stebbins 1970; Faegri and van der Pijl 1979; Waser 1983; Proctor *et al.* 1996; Peeters and Totland 1999; Weller *et al.* 2006) and shifts in pollination syndrome are often associated with dramatic changes in floral morphology and flowering schedule (Fulton and Hodges 1999; Schemske and Bradshaw 1999; Castellanos *et al.* 2003; Friedman and Barrett 2008). Because the progamic phase takes place within the flower, changes in floral size or phenology might affect coordination of the fertilization process. Shifts in the size of flowers are strongly correlated with shifts between insect and wind pollination (Friedman and Barrett 2008)

and, if the size of the carpel scales with that of the flower, then the length of the pollen tube pathway will be affected. Changes in pollen tube pathway length will, in turn, affect the duration of the progamic phase, unless pollen tube growth rate evolves to compensate. Changes in pollination syndrome can also affect the timing and duration of pollen dispersal, which must, in turn, have consequences for the timing of stigma receptivity. Changes in the onset or duration of stigma receptivity may then have downstream effects on pollen tube growth or ovule longevity. There is a wealth of comparative studies on the evolution of floral and pollination biology (e.g. Lloyd and Barrett 1996), but relatively few on comparative progamic phase biology (e.g. Williams and Rouse 1990; Williams 2008), and none that we know of that connect the two. In this study, we hypothesized that the floral traits involved in pollination syndrome divergence also caused developmental changes in the progamic phase. We sought to determine the nature of such changes by measuring rates, timing, and durations of progamic phase ontogenies in *Brasenia schreberi* JF Gmelin and *Cabomba caroliniana* Gray, within the water lily family Cabombaceae. *Brasenia* is wind pollinated (Osborn and Schneider 1988), while *Cabomba* is pollinated by small flies (Diptera; Schneider and Jeter 1982). Their floral morphologies and phenologies differ in many attributes related to their divergent pollination syndromes (Schneider and Jeter 1982; Osborn and Schneider 1988).

Cabombaceae is an important family in which to study evolutionary transitions in pollination and fertilization biology because of its position within Nymphaeales s.l. (Nymphaeaceae, Cabombaceae, Hydatellaceae), a lineage that is represented in the oldest angiosperm fossil records (Friis *et al.* 2001; Friis *et al.* 2003; DW Taylor *et al.* 2008), and that diverges from the basal-most or next most basal node of the extant angiosperm phylogenetic tree

(e.g Qiu *et al.* 1999, 2006; Löhne and Borsch 2005; Saarela *et al.* 2007). Cabombaceae possesses a suite of reproductive traits that are thought to be plesiomorphic in angiosperms. Their bisexual flowers are apocarpous, with open, ascidiate carpels and few ovules (Endress 2001). They have monosulcate pollen (Osborn *et al.* 1991) and likely a four-celled female gametophyte with diploid endosperm (Galati 1985; Williams and Friedman 2002; Rudall *et al.* 2008). Their pollination syndromes have been well studied (Schneider and Jeter 1982; Osborn and Schneider 1988), but little is known about their pollination to fertilization biology. Thus, the divergence in pollination syndrome between *Brasenia* and *Cabomba* is of general interest in that it represents an evolutionary transition in floral biology between two genera that lack many of the usual floral features characteristic of derived lineages of monocots and eudicots.

The specific goal of this study was to characterize progamic phase ontogenies and mature traits (from stigma receptivity to egg receptivity) in *Brasenia* and *Cabomba* and to identify differences that might be associated with their divergent pollination syndromes. We also review what is known of floral biology as it relates to the progamic phase in Nymphaeaceae and other outgroups to better understand apomorphies of Cabombaceae.

METHODS

FIELD SITES

Experimental pollinations of *Brasenia schreberi* J.F. Gmel were conducted in June and July of 2006 and 2007 at Monterey Lake, Putnam County, TN, USA (36°06' N, 85°14' W).

Experimental pollinations of *Cabomba caroliniana* Gray were conducted in July 2006 and 2007

at Raccoon Creek, Jackson County, AL, USA (34°46' N, 85°50' W). Voucher specimens from both populations are deposited in the University of Tennessee Herbarium (TENN). Because temperature is known to affect rates of development, daily mean and 7:00 am (Central Daylight time) temperature were obtained from the Monterey, TN and Scottsboro, AL Weather Stations for the duration of the field study (National Climactic Data Center, online at <<http://www.ncdc.noaa.gov/oa/ncdc.html>>). In addition, the hourly temperature and relative humidity during May and June 2007 were measured at Monterey Lake (<10 m from plants) with a HOBO Pro Series Weatherproof Data Logger (Forestry Suppliers, INC., Jackson, MS).

Pollen exclusion treatments

Brasenia emergent buds were caged on the day before pollination treatment with cages modified from Osborn and Schneider (1988). One-quart Styrofoam cups with bottoms removed and three “windows” cut out of the sides were attached to 20 cm² bases constructed from one-quarter-inch insulating Styrofoam. The center of each base was removed to allow cages to float over flowers, held in place with floating leaves. To exclude windborne pollen, cages were covered with Spunbond Polypropylene non-woven fabric with a maximum gap diameter of 70 µm.

Cabomba first-day flowers were caged before anther dehiscence. *Cabomba* cages were constructed by cutting away the top two-thirds of 1-qt. Styrofoam cups. Cup bottoms were removed and replaced with bridal veil (maximum gap diameter = 200 µm) to exclude insects. Cages were placed over flowers and attached to peduncles with nylon fishing line. Caging

treatments had no apparent effect on the timing of floral submergence, emergence, opening, or closing in either *Brasenia* or *Cabomba*.

Stigma receptivity and pollen viability

The duration of stigma receptivity was determined by measuring pollen germination success following experimental pollinations. Dehiscent anthers were collected across the lake from pollen recipients and kept in a desiccation chamber on ice to conserve pollen viability. To mimic natural wind pollination in *Brasenia*, anthers were held approximately 3 cm above first-day flowers and tapped twice to release pollen. To simulate fly pollination in *Cabomba*, dehiscent anthers were gently brushed across the stigmas of first-day flowers. *Brasenia* flowers were pollinated at 7:30 am, 8:30 am, 9:30 am, 10:30 am, and 11:30 am, with the first pollination time corresponding to the time when anthers typically first dehisced. Pollen germination rate was calculated as the percentage of pollen germinated on two stigmas/flower collected at least one hour after pollination (hap). *Cabomba* flowers were pollinated at 11:00 am, 12:00 pm, 12:30 pm, 1:00 pm, 2:00 pm, and 3:00 pm. Again, the first pollination time corresponded to the onset of anther dehiscence. Pollen germination rate was calculated as above from one stigma/flower collected at least one hap. For both species, 10 second-day flowers were experimentally pollinated to assess stigma receptivity beyond the first day.

Stigmas were placed onto glass slides immediately after collection. A drop of stain solution (1mg/1mL aniline blue [AB] plus 1mg/mL of sodium azide in 0.33 M K₃PO₄ and 10 mL glycerol; Marshall and Diggle 2001) was added. Slides were covered, stored in a humid chamber for 24 h, and examined under fluorescent light with an Olympus (Lake Success, New York,

USA) BX60 compound microscope. Pollen was scored as germinated if a clearly defined pollen tube, at least one-half the length of the pollen grain, was seen emerging from the pollen aperture. The number and location of germinated and ungerminated grains was recorded.

To determine if differences in pollen germination percentage over time might be due to differences in pollen viability over time rather than to stigma quality, *in vitro* germination success of *Brasenia* pollen was determined. Pollen was tapped onto depression slides containing 10 % sucrose solutions at 8:00 am, 9:00 am, 10:00 am, 11:00 am, 12:00 pm, 8:00 pm, and 8:00 am the following day. Slides were incubated for 24 h before viewing as above. Germination percentage was calculated from 100 grains/slide with four replicates/treatment.

Pollen germination

Once the timing of stigma receptivity was determined, additional flowers were caged and experimentally pollinated at the optimal time to determine typical time to pollen germination. *Brasenia* stigmas were collected and fixed at 15, 30, 45, 60, 75, and 90 min after pollination (map), and *Cabomba* stigmas were fixed at 15, 30, 45, 60, 120, 180, and 240 map. Stigmas were stained, stored, and observed as above. As water content of dispersed pollen can affect germination speed (Franchi *et al.* 2002), we measured both fresh and dry weight of pollen from dehiscing anthers. Dry weight was calculated after drying fresh pollen at 50° C for 72 h. Water content was expressed as a percent of fresh weight.

To determine whether pollen load size differed by location on the stigma (top, middle, bottom thirds of stigma) or whether germination success was affected by pollen load size or

location on the stigma, one- and two-way ANOVAs, respectively, were run on SAS 9.13 (SAS Institute Inc, Cary, NC, USA), with plant as the experimental unit.

Pollen tube growth and ovule development

To investigate pollen tube growth, first-day flowers of *Brasenia* and *Cabomba* were pollinated as discussed earlier. *Brasenia* flowers were collected at 2, 4, 6, 8, 10, 16, 24, 28, 34, 48, 72, and 96 hap, and *Cabomba* flowers were collected at 1, 2, 4, 6, 8, 10, 22, 24, 28, 32, and 48 hap.

Whole carpels were removed from flowers and chemically fixed for at least 24 h in 3:1 95 % ethanol: acetic acid, FAA (40 % formaldehyde, glacial acetic acid, and 95 % ethanol), or Karnovsky's fixative (50 % gluteraldehyde and 16 % paraformaldehyde in 0.2 M Phosphate buffer [pH 7.4]). Specimens fixed in 3:1 and FAA were then rinsed in 70 % EtOH, while those fixed with Karnovsky's fixative were buffer-washed and dehydrated in a graded ethanol series. All specimens were stored in 70 % EtOH. To document pollen tube lengths, as well as overall pollen tube and carpel morphology, specimens were hand sectioned, placed on glass slides, stained with aniline blue for 4-8 h, and viewed under fluorescent light with a Zeiss Axioplan II compound microscope (Carl Zeiss, Oberkochen, Germany). Imaging of carpel anatomy and pollen tubes and analysis of sizes of structures was performed using Zeiss Axiocam camera and Axiophot 4.0 micrograph analysis software. Maximum pollen tube pathway length was measured from the stigmatic apex to the apical micropyle along the pollen tube pathway. The distance the pollen tube had reached into the carpel and the actual pollen tube length (pollen grain to tube tip) were measured. Ovules were scored as entered if a pollen tube was observed within the

micropyle. For observation of pollen tube nuclei, aniline blue-stained material was rinsed in distilled water, and stained with 4',6-diamidino-2-phenylindole (DAPI) for four to five hours. For histological analysis, carpels were dehydrated to 95 % EtOH, and then infiltrated and embedded in JB-4 polymer (Polysciences, Inc., Warrington, PA, USA) following standard protocols. Specimens were serial-sectioned with a Sorvall Dupont JB-4 microtome (Newtown, Connecticut, USA), using glass knives. Serial sections (5 μ M) were mounted on glass slides and stained with 0.1 % toluidine blue O (TBO) for general histology and imaged with a Zeiss Axioplan 2 compound microscope.

Pollen tube growth rates were calculated at each timepoint from the leading pollen tube in each carpel as length from pollen grain to tube tip divided by time since pollination minus 15 minutes to account for pollen germination (see “Results”; mean n /timepoint = 6 and 11 for *Brasenia* and *Cabomba*, respectively). We also estimated average growth rate within a time interval as the difference between average pollen tube lengths at any two time points divided by the time interval.

RESULTS

Floral cycle and anther dehiscence

Brasenia and *Cabomba* are strongly protogynous; both typically have a two-day floral cycle with the female phase on the first day and male phase on the second (for further discussion, see Osborn and Schneider 1988; Schneider and Jeter 1982). In *Brasenia*, floral buds emerged between 5:00 and 6:00 am, with second-day flowers emerging slightly earlier than first-day flowers, and opened at approximately 7:00 am. Carpels of first-day (female phase) flowers are

reflexed outward, exposing their long stigmatic crests (Figure 2.1A; all figures referenced in this chapter are found in Appendix 2). Anthers are closed and on short filaments below stigmas (Figure 2.1A).

Brasenia first-day flowers closed by 1:00 pm and became submerged in the late afternoon. Flowers reemerged the next morning, opening with anthers elevated on elongated filaments well above the central aggregation of carpel tips (Figure 2.1B). Anther dehiscence began at approximately 7:30 am. Anthers within a flower dehiscence simultaneously and generally emptied within minutes of opening. Within the population, all flowers began to release pollen within 1 h of each other and almost all anthers were empty within 2 h after the first flowers opened, thus pollen dispersal was generally over by about 9:30 am. Flowers closed and submerged as in first-day flowers.

Cabomba floral buds emerged between 10:00 am and 11:00 am and opened approximately 30 min later. Second-day flowers emerged and opened first, followed by first-day flowers. The carpels of first-day flowers remain aggregated so that stigmas are much closer together than in *Brasenia* (compare Figures 2.1A, C), however, anthers remain closed and basally positioned (Figure 2.1C). *Cabomba* first-day flowers remained open until approximately 5:00 pm, then closed and submerged.

As with *Brasenia* stamens, filaments elongated overnight so that anthers were held above stigmas when second-day flowers opened (Figure 2.1D). All anthers within a flower dehiscence simultaneously and all second-day flowers within the population began to release pollen at approximately the same time, concurrent with the opening of first-day flowers. Pollen remained

in the open anthers throughout the day. Second-day flowers were generally completely closed by 5:00 pm. In both *Brasenia* and *Cabomba*, seeds develop beneath the water surface.

Stigma receptivity and pollen germination

Pollen is mature when second-day flowers open and was bicellular on stigmas in both *Brasenia* and *Cabomba*. The water content of pollen grains was 72 ± 7.2 % just after flower opening in *Brasenia* and 81 ± 3.4 % 1-2 h after flower opening in *Cabomba*. During the approximate period of pollen dispersal in *Brasenia* (7 am - 9 am) the mean relative humidity was 91.7 ± 12.9 % (Monterey Lake site; May 23, 2007 to June 30, 2007).

In *Brasenia*, 74 % of pollen germinated successfully on stigmas at 7:30 am, when pollen is typically released (Figure 2.2A). Pollen germination percentage declined slightly at 8:30 and 9:30 am, and was significantly lower at 10:30 and 11:30 am (Figure 2.2A). Because pollen used in pollinations became progressively older, we tested *in vitro* pollen germination percentage and found it to be relatively constant over the entire period that hand-pollinations were performed (although lower than *in vivo* germination, germination percentage did not drop until more than 12 h after anther dehiscence (Figure 2.2A). Therefore, the decline in *in vivo* pollen germination percentage seen at 3 and 4 hap was not due to declining pollen viability or vigor, but to decreased stigmatic receptivity.

Anatomical observations also indicate declining female support for pollen germination in *Brasenia*. In pollinations of stigmas that were 3 or more hours old, many pollen grains exhibited an open sulcus from which pollen cytoplasm had been expelled (Figure 2.3A), instead of emitting a pollen tube (cf. Figures 2.3A, B). No burst grains were observed in 7:30 am

pollinations and very few were seen in 8:30 am or 9:30 am pollinations. Thus, *Brasenia* stigmas were most receptive when pollen was first being released and the receptive period ended a little over 2 h later, at least 2 h before flowers closed.

In *Cabomba*, first-day flowers open about 10:30 am and 61 % of pollen germinated in 11:00 am pollinations (Figure 2.2B). In contrast to *Brasenia*, germination success was high for the entire time the flower was open (Figure 2.2B). In addition, stigmas from second-day flowers still supported low levels of pollen germination, although pollen did not adhere as well.

Pollen germinated before 15 map in both species, and *Cabomba* was already near its maximum germination percentage at this time (Figure 2.4B), whereas the maximum was not approached until 60 map in *Brasenia* (Figure 2.4A). *Brasenia* has a stigmatic crest that was on average 4.3 ± 0.7 mm in length ($n = 26$), which results in a large stigmatic surface area of approximately 5 mm^2 (Figure 2.5A). Thus, position on the stigma might have a greater effect on pollen germination success in *Brasenia*. In *Cabomba*, the stigma is restricted to the very tip of the carpel (Figure 2.5B) and averages only 0.4 mm in length and 0.75 mm^2 in surface area (Figure 2.5B).

In our hand pollinations, designed to mimic wind pollination, a one-way ANOVA indicated pollen load varied by stigmatic region ($n = 87$ flowers, $p \leq 0.0001$). Significantly more pollen was received on the upper one-third than on the middle, and significantly more pollen was received on the middle than on the bottom one-third of the stigmatic crest (Post-Hoc Tukey's Studentized Range Test). Neither stigmatic region nor pollen load size had a significant effect on pollen germination percentage ($p = 0.441$ and 0.840 respectively; $n = 29$ plants). Under natural

pollination, the average pollen load per stigma was three pollen grains, with a maximum load of ten grains ($n = 16$).

Brasenia pollen tube path and development

Pollen grains in *Brasenia* were captured by unicellular papillae or secretions present on the stigmatic crest (Figures 2.3A–C). Pollen tubes emerged and grew either along the surface of the papillae or through the secretions toward the ground tissue of the stylar neck (Figures 2.3B–C). Whether originating at the carpel tip or the lateral margins of the stigmatic crest, pollen tubes penetrated the cuticle to enter the ground tissue of the carpel and grew towards the stylar canal, between large obliquely elongated cells (Figures 2.5A, 2.6A–B). These cells comprise the outer layer of the substigmatic region (Figure 2.6C) and clearly act as transmitting tissue (compare ground tissue that does not underlie stigmatic tissue on right side of Figure 2.6C). Pollen tubes exited the ground tissue through a single conspicuous layer of secretory epidermal cells (Endress 2005) to enter the open stylar canal (Figure 2.6A, C). The stylar canal extends through the length of the carpel, from the ovarian cavity to near the carpel tip, where the mouth opens from a slightly ventral position (Figure 2.5A; Endress 2005). Pollen tubes grew within secretions in the canal and only occasionally contacted the canal wall (Figures 2.6C–D).

Since the *Brasenia* stigmatic crest is oriented parallel to the stylar canal, the distance that pollen tubes must grow to reach ovules varies. At 1 hap, all pollen tubes had entered the stylar canal but not yet reached the ovarian cavity, and the tips of leading pollen tubes were 1000 - 3000 μm below the mouth of the stylar canal (Figure 2.7A). At 2 hap, most leading pollen tubes had entered the ovarian cavity, and at 4 hap, some leading pollen tubes had reached the apical

ovule (Figure 2.7A). Pollen tubes were first observed entering the apical micropyle at 6 hap (Figures 2.6E, 2.7A) and at 8 and 10 hap all apical ovules had been entered ($n = 8$, Figure 2.7A). With one exception, in both species the apical ovule was entered first and in both species only one pollen tube entered each micropyle.

Aniline blue staining indicated pollen tube walls were strongly callosic at all times (Figures 2.3B-C, 2.6A). Callose plugs apparently formed by greater callose deposition along one edge of the tube wall to form a bulge extending into the pollen tube lumen that eventually seals it off along all sides (Figure 2.6A, D-E). Callose plugs generally did not form until 2 hap, and some pollen tubes reached the ovarian cavity without having formed a plug. After 4 hap, when most leading pollen tubes were entering the ovarian cavity, callose plugs were common, especially near the pollen grain (Figure 2.6A), and pollen tubes contained many callose plugs thereafter (Figure 2.6D).

Cabomba pollen tube path and development

In *Cabomba*, pollen grains were captured by unicellular papillae and secretions of the small stigmatic region surrounding the mouth of the stylar canal (Figures 2.5B, 2.8A, C-E). Some pollen germinated directly adjacent to the carpel mouth and tubes grew directly into the secretion-filled stylar canal. However, most pollen tubes followed a pattern identical to *Brasenia*, in which pollen tubes penetrated the cuticle (Figures 2.8C-E) and then entered substigmatic ground tissue, growing between obliquely-oriented cells to enter the stylar canal below its mouth (Figures 2.8A-C, F). As in *Brasenia*, strongly callose-walled pollen tubes grew within secretions to the ovarian cavity (Figure 2.8F), and few or no callose plugs were observed at 1 or 2 hap

(Figure 2.8F). At 4 hap and later, numerous callose plugs were present (not shown); however, apical ovules had already been penetrated at this time (Figure 2.7B). In second-day flowers, cell walls at the base of the stigmatic region were strongly callosic, indicating pollen tubes could no longer penetrate styles (Figure 2.8H).

In *Cabomba*, leading pollen tubes first reached the area near the apical micropyle at 1 hap and first entered the apical ovule at 2 hap, although there was a great deal of variation in how far tubes had grown at that time (Figure 2.7B). In some pollen tubes, two sperm nuclei were observed at 2 hap, whereas in others the generative cell was in anaphase or telophase of mitosis II at 2 hap (Figure 2.8G). In some cases, ovule entry still had not occurred by 10 hap (Figure 2.7B), yet the leading pollen tube was seen near the apical micropyle. In several carpels, the leading pollen tube had passed the apical micropyle and was approaching a more basal micropyle.

The ovule and female gametophyte

Ovules in *Brasenia* and in *Cabomba* are anatropous, bitegmic, and crassinucellar (Galati 1985; Williamson and Schneider 1993). Carpels in *Brasenia* typically contained two ovules, whereas in *Cabomba*, they almost all contained three (Figure 2.5B). In ovules observed at the onset of stigma receptivity, the female gametophyte in both species was cellularized to form a four-celled/four-nucleate gametophyte containing an egg cell, two intact synergids, and a central cell with a single nucleus positioned near the micropylar pole in *Cabomba*, but closer to the chalazal pole in *Brasenia* ($n = 5/\text{genus}$; collected at 10:30 am and 7:30 am, respectively).

In *Brasenia* the apical ovule micropyle was $6621 \pm 868 \mu\text{m}$ ($n = 33$) and the basal ovule was $7573 \pm 697 \mu\text{m}$ ($n = 11$) from mouth of the stylar canal (Figure 2.7A). The distance from the entrance of the micropyle to the nucellus was $106.5 \pm 14.0 \mu\text{m}$ and the one to two cell layers of nucellus were $31.2 \pm 7.1 \mu\text{m}$ thick ($n = 10$). The equivalent lengths in *Cabomba* were $2039 \pm 882 \mu\text{m}$ ($n = 52$) to apical ovule, $2869 \pm 1004 \mu\text{m}$ ($n = 46$) to basal-most ovule, $100.4 \pm 3.6 \mu\text{m}$ through the micropyle, and $24.8 \pm 4.2 \mu\text{m}$ through nucellus ($n = 11$).

Pollen tube growth rate

The overall maximum sustained growth rate, measured at the time ovules were first entered was $742 \mu\text{m/h}$ in *Brasenia* ($n = 4$) and $963 \mu\text{m/h}$ in *Cabomba* ($n = 12$; Figure 2.9). In *Brasenia*, pollen tube growth rate was slowest during early growth, averaging $309 \mu\text{m/h}$ over the first 45 minutes of growth, then increased to $991 \mu\text{m/h}$ from 1 to 4 hap and finally slowed to $623 \mu\text{m/h}$ from 4 to 6 hap (Figure 2.9). In contrast, in *Cabomba* pollen tube growth rate was $1935 \mu\text{m/h}$ over the first 45 minutes and then slowed to $476 \mu\text{m/h}$ from one to two hap (Figure 2.9). In *Brasenia*, growth rates ranged from $96 - 1705 \mu\text{m/h}$, whereas in *Cabomba* they varied from $281 - 2724 \mu\text{m/h}$.

DISCUSSION

The progamic phase begins with deposition of pollen on a receptive stigma and ends with fusion of the male and female gametes (Cresti *et al.* 1992). The timing of stigma receptivity relative to differentiation of the egg and central cell nucleus of the female gametophyte

determine the overall duration of the progamic phase. A number of aspects of pollen development must co-evolve with pollen tube pathway characters to maintain synchrony during this phase. Below we first discuss the great number of shared characters present in *Brasenia* and *Cabomba*, and in their sister family Nymphaeaceae, that relate to progamic phase timing. Then we address the question of how complex developmental interactions were affected by transformations in floral morphology and phenology associated with their divergent pollination syndromes.

Ancestral character states of the progamic phase in Cabombaceae

Duration of the progamic phase. Both *Brasenia* and *Cabomba* have very short progamic phases. Female gametophytes were anatomically near maturity at the time of stigma receptivity in both species and pollen tubes reached the micropyle within 2 h in *Cabomba* and within 6 h in *Brasenia*. This pattern is similar to other Nymphaeales which have nearly coincident stigma receptivity and female gametophyte maturity and a correspondingly short period of time between pollination and pollen tube entry of the ovule, such as in *Nuphar polysepala* (Williams 2008), *Nymphaea nouchali* (Orban and Bourharmont 1998) and *Nymphaea odorata* (Conard 1905; Williams 2009). Ovule entry occurs between 1 and 6 hap in *Nymphaea capensis* (Orban and Bourharmont 1995) and by 8 hap in *N. polysepala* (Williams 2008). The traits that determine the short duration of the progamic phase in Cabombaceae can be inferred to have evolved sometime before the divergence of Nymphaeaceae and Cabombaceae (Figure 2.10). In most other basal angiosperms, pollen tubes reach the ovules after a slightly longer period of 14-72 hap (Williams 2008; 2009).

Male gametophyte development. Pollen germination in Cabombaceae occurs within 15 min and is among the fastest known in angiosperms (see Nepi *et al.* 2001). Pollen germination in *Nuphar* and *Nymphaea* is also rapid (Figure 2.10; Orban and Bouharmont 1995; Friedman and Williams 2003; Williams *et al.* 2010).

Cabombaceae pollen is monosulcate, with a single large aperture that extends along the entire length of the pollen grain. Monosulcate pollen is generally thought to germinate more slowly than other pollen types because pollen with multiple apertures allows more opportunity for direct contact between apertures and the stigma to facilitate rapid hydration (Heslop-Harrison 1979a, 1979b; Furness and Rudall 2004). However, pollen grains of Cabombaceae have large and long apertures that increase the area of stigma contact. Large apertures can have a cost of increasing water loss (Heslop-Harrison 1979a, 1979b), but pollination in this study took place in an environment with high relative humidity, which likely reduces this risk.

Perhaps the most important trait underlying rapid germination is the high water content (>70 %) of Cabombaceae pollen grains at dispersal. Pollen that has low water content at dispersal undergoes physiological dormancy, allowing it to better withstand the stresses of the environment. Upon pollination, it must become rehydrated, delaying germination (Nepi *et al.* 2001; Franchi *et al.* 2002). Such “partially dehydrated” pollen is typical of gymnosperms and many angiosperms (Franchi *et al.* 2002). Pollen with a high water content at dispersal, such as in Cabombaceae, maintains an active metabolic state during dispersal and does not have to become hydrated in order to germinate (Nepi *et al.* 2001; Franchi *et al.* 2002). However, partially hydrated (PH) grains are less likely to have mechanisms that control water loss and are more vulnerable to desiccation once in the external environment (Pacini and Franchi 1999; Nepi *et al.*

2001). Both *Brasenia* and *Cabomba* exhibit pollen characters typical of PH grains (see Franchi *et al.* 2002), including a thick exine and intine to slow water loss and a short period of viability (< 24 h). They also lack furrows, which are characteristic of pollen that must undergo large changes in volume during dehydration and rehydration.

In Cabombaceae, pollen was bicellular at pollination, as previously seen in *Cabomba* (Padmanabhan and Ramji 1966; Batygina and Shamrov 1983; Galati 1985) and *Brasenia* (Taylor and Osborn 2006) and this trait is considered plesiomorphic in angiosperms (Brewbaker 1967). Tricellular pollen has been reported in some Nymphaeaceae *Euryale ferox*, *Victoria cruziana* and *Nymphaea stellata* (Khanna 1964, 1967), as well as *Nuphar lutea* (Batygina and Shamrov 1983), but bicellular pollen was seen in *Barclaya longifolia* (Batygina and Shamrov 1983) *Nuphar polysepala* (Friedman and Williams 2003) and *Nymphaea odorata* (Williams unpublished data). Transitions to tricellular pollen are generally thought promote faster pollen germination and/or tube growth (Mulcahy and Mulcahy 1983), but the data from our study suggest that hydrated, bicellular pollen can germinate and grow tubes quite rapidly. Pollen tubes in *Cabomba* and *Brasenia* grow at average rates of over 700 $\mu\text{m/h}$, similar to rates in *Nymphaea* and *Nuphar*, which range from approximately 600 to 800 $\mu\text{m/h}$ (Williams 2008). Pollen tube growth rates in Nymphaeales are much more rapid than in other basal angiosperms (Williams 2008) and approach those more typical of derived angiosperms (Maheshwari 1950).

As is typical in angiosperms, pollen tubes in Cabombaceae have prominent callosic walls, especially early in pollen tube development (Figure 2.3B; 2.8A). In both species, callose plugs begin to develop between one and two hap. In *Cabomba*, this is just after mitosis II, which is the general pattern in angiosperms (Mulcahy and Mulcahy 1983). *Brasenia* and *Cabomba* produce

numerous callose plugs, though plugs are not common until 4 hap. Similarly, *Nymphaea* and *Nuphar* both produce many callose plugs (Williams 2008).

Pollen tube pathway. Both *Brasenia* and *Cabomba* have open, ascidiate carpels that are sealed by secretion, a trait thought to be plesiomorphic in angiosperms (Tucker and Douglas, 1996; Doyle and Endress 2000; Endress 2001, 2005). The presence of a distinct extension of the carpel to form a hollow style is apomorphic in Cabombaceae (Figure 2.10). The long stylar canal is the major portion of the pollen tube pathway of *Brasenia* and *Cabomba*.

Both *Cabomba* and *Brasenia* have stigmatic tissue surrounding the open mouth of the stylar canal, and one might expect pollen tubes to follow a path of least resistance entirely within secretions. This would resemble *Amborella*, in which pollen tubes grow laterally along the sub-stigmatic surface until they reach the open mouth of the stylar canal (Williams 2009). However, in both *Cabomba* and *Brasenia* pollen tubes clearly penetrate the cuticle of the sub-stigmatic surface and then grow between tightly-packed ground tissue cells to reach the stylar canal. Once pollen tubes enter the stylar canal, they grow freely within secretions (instead of along the inner epidermal surfaces) to the top of the ovarian cavity and then to the micropyle.

In Nymphaeaceae, carpels of *Nymphaea* and *Nuphar* are also ascidiate, but they undergo post-genital fusion at the periphery, just below the stigmatic crests (Endress 2001) and pollen tubes must grow through these developmentally fused tissues (Orban and Bourharmont 1995). Thus, in both Cabombaceae and Nymphaeaceae, pollen tubes pass through a short region of ground tissue early in development and then grow through secretions for the majority of the pollen tube pathway. Some of the fastest pollen tube growth rates measured were during the period that included growth through ground tissue.

Summary. *Brasenia* and *Cabomba* inherited a very short progamic phase, already present in the common ancestor of the family (Figure 2.10), but determined by a unique combination of plesiomorphic and apomorphic characters. Rapid pollen germination and rapid pollen tube growth rates can be inferred to have arisen early in Nymphaeales (Figure 2.10), whereas pollen tube growth through solid ground tissue may or may not have had independent origins in Cabombaceae and Nymphaeaceae (Williams 2009). Cabombaceae retains angiosperm plesiomorphies such as open carpels and bicellular pollen, but has an apomorphic stylar neck, whereas Nymphaeaceae lack the stylar neck, but some taxa originate partially closed carpels and possibly tricellular pollen. The variability of the many pollen and carpel traits contribute to speeding the fertilization process and reflect a general developmental flexibility of reproductive traits among early lineages of angiosperms.

Ancestral pollination syndrome of Cabombaceae

Brasenia schreberi exhibits many traits that are specialized for wind pollination. At dehiscence, anthers are elevated and tepals are strongly reflexed, nectar is absent, and flowers produce smaller and many more pollen grains than does *Cabomba* (Osborn and Schneider, 1988). Because insects, especially flies and bees, may occasionally visit *Brasenia* flowers, comparative studies of pollination syndromes often consider *Brasenia* to have a mixed wind/insect pollination syndrome (e.g Thien 2000; Hu *et al.* 2008; Thien *et al.* 2009). However, at Monterey Lake insects primarily visited second-day, male-phase flowers to feed on pollen and this occurred in late morning (MLT personal observation), after the decline of stigma receptivity (this study). Moreover, *Brasenia* pollen is unornamented and lacks pollenkit, so it is less able to

adhere to insect bodies in the first place (Osborn and Schneider 1988). Therefore, like Osborn and Schneider (1988), we consider *Brasenia* to be primarily wind-pollinated.

In contrast, *Cabomba caroliniana* and all other extant members of Nymphaeaceae are pollinated by beetles, flies, bees, or some combination (Schneider and Moore, 1977; Schneider 1983; Schneider and Williamson 1993; Capperino and Schneider, 1985; Seymore and Matthews, 2006; Thien *et al.* 2009). Nymphaeaceous fossil flowers from the early Cretaceous (115-125 mya; Friis *et al.* 2001) and late Cretaceous (90 mya; Gandolfo *et al.* 2004) have also been interpreted to be entomophilous (Friis *et al.* 2001). *Cabomba* itself appears to be specialized for fly pollination. Petals are not strongly reflexed, flowers are light in color, anthers and stigmas are exposed, petals have exposed nectaries, and pollen is ornamented and covered with copious pollenkitt (see Schneider and Jeter 1982; Osborn *et al.* 1991; ML Taylor *et al.* 2008).

We consider it likely that the ancestor of extant Cabombaceae exhibited a generalist pollination syndrome and extant *Brasenia* and *Cabomba* each represent derived specializations for wind and flies, respectively (Osborn and Schneider 1988). Several other extant basal genera, including *Amborella* (Thien *et al.* 2003), *Trimenia* (Bernhardt *et al.* 2003), and *Saururus* (Thien *et al.* 2000), display a combined wind/insect pollination syndrome, whereas many other basal groups exhibit flowers with a generalist insect pollination strategy (Thien *et al.* 2000, 2009).

Cabomba pollen exhibits specializations such as sculptural rods on the surface of the grain and microchannels that form in the pollen wall. These are hypothesized to function in the distribution and storage of the copious pollenkitt it produces (Osborn *et al.* 1991; ML Taylor *et al.* 2008). In contrast, pollen in *Brasenia* and Nymphaeaceae do not have these features (Osborn *et al.* 1991; Taylor and Osborn 2006; ML Taylor *et al.* 2008). Furthermore, fossil Cabombaceae

pollen from the Tertiary (*Brasenia purpurea* Michx = *B. schreberi*) and Quaternary (*B. schreberi*) is unornamented and is identical to modern *Brasenia* pollen (Jessen *et al.* 1959; Lloyd and Kershaw 1997). Thus, parsimony suggests that specializations of *Cabomba* pollen evolved from either a generalist insect or wind/insect pollinated ancestor.

The common ancestor of extant Cabombaceae is unlikely to have been exclusively wind pollinated. *Brasenia* exhibits both a perianth and UV reflectance in its flowers (Osborn and Schneider 1988), traits that function in insect attraction and are unnecessary for wind pollination. Furthermore, the long style associated with wind pollination in *Brasenia* is likely a derived feature, as short styles have been inferred to be the ancestral state in Nymphaeales and for all angiosperms (Williams 2008). Therefore, it seems likely that *Brasenia* became specialized for wind pollination sometime after its divergence from *Cabomba*.

Transitions from insect to wind pollination are not uncommon in angiosperms and have occurred at least 65 times, while the converse seems to be much less frequent (Stebbins 1970; Linder 1998; Culley *et al.* 2002). Wind-pollinated flowers have generally evolved from dioecious ancestors that had small (< 1 cm wide), unshowy flowers, with a generalist insect pollination syndrome (Friedman and Barrett 2008). However, because of its occurrence in an early-diverging angiosperm lineage, the evolutionary transition to wind pollination in *Brasenia* has some unique qualities relative to such transitions among derived lineages. The flowers of the common ancestor of *Brasenia* and *Cabomba* were likely generalist pollinated, but they were likely similar in size to those in extant Cabombaceae (> 1 cm wide), were almost certainly bisexual, and were probably showy, with a colored and UV reflective perianth.

Interestingly, most other early-divergent angiosperms, especially those that are wind-pollinated, have small flowers. The relatively larger flower size of *Brasenia* reflects its evolutionary developmental history: wind pollination became more efficient when a larger stigmatic surface evolved through elongation of an ancestrally short stylar neck. Elongation of the neck involved enlargement of the whole carpel (compare carpels in Figure 2.5), and flower size increased because other floral parts had to also enlarge in order to enclose the larger carpels during repeated submergences of the mature flower.

The consequences of a shift in pollination syndrome on progamic phase biology

The evolutionary transition to wind pollination in *Brasenia* affected floral structure as well as phenology. In *Brasenia*, flowers open 3 h earlier than in *Cabomba*, and thus stigma receptivity and pollen dispersal occur during the cooler, more humid hours of the early morning. Pollen is almost completely dispersed by wind very soon after anther dehiscence and stigma receptivity is correspondingly short (Figure 2.11). Earlier flowering probably reduces desiccation of wind-dispersed pollen and might also provide an escape from pollinivorous insects. In *Cabomba*, pollen germination and pollen tube growth take place during the warmest part of the day, when insect pollinators are more active. Pollen is presented to flies throughout the entire time the pistillate flower is open and stigmas maintain a similar period of receptivity (Figure 2.11). No other Nymphaeales exhibit as brief a period of pollen dispersal and stigma receptivity as seen in *Brasenia*, suggesting this pattern evolved as a consequence of the origin of wind pollination.

Brasenia has a stylar neck that is more than four times as long as that of *Cabomba*, and much of its epidermal surface is papillate and functions as stigmatic tissue with nearly seven times the surface area as in *Cabomba*. *Brasenia* flowers fit a pattern often seen in wind pollinated species relative to animal-pollinated species in which larger stigmatic surfaces and shorter periods of stigma receptivity arise to increase pollen reception (Whitehead 1969).

In the case of *Brasenia* the expanded stigma, developed by elongation of the stylar neck, had the secondary effect of increasing pollen tube pathway length. In both species, pollen tubes first grow a short distance through substigmatic ground tissue and then grow within secretions of the stylar canal and ovary to the micropyle. In *Brasenia* pollen tubes must grow an additional distance of 0.5-6.5 mms entirely because of their longer stylar canal. However pollen tube growth rates within secretions of the stylar canal were similar in both species (Figure 2.9). Thus, the longer progamic phase of *Brasenia*, defined by a shift to an earlier time of pollination and retention of a similar fertilization time (~ 1 pm in both species; Figure 2.9), is due in large part to failure of pollen tube growth rate to have tracked the evolution of stylar canal length.

Brasenia pollen did germinate slightly slower and overall average pollen tube growth rate was slightly slower (742 $\mu\text{m}/\text{h}$ compared to 963 $\mu\text{m}/\text{h}$ in *Cabomba*). Slower early development of pollen in wind- versus animal-pollinated groups has been seen as a female strategy for increasing the quantity and quality of pollen on stigmas when pollen is limiting (Willson and Burley 1983). Our data are consistent with this scenario, but such a pattern might also arise as a consequence of differences in the intensity of pollen competition. In both *Brasenia* and *Cabomba*, one pollen tube approaches and enters each micropyle, so pollen competition is strongest before the ovary is reached. We found very low and dispersed pollen loads in *Brasenia*

under both natural and artificial pollination, and although we did not measure pollen loads on the small, capitate stigma of *Cabomba*, insect pollination generally results in increased pollen loads (Faegri and van der Pijl 1979; Willson 1983). Thus, both stigma structure and pollination syndrome favor stronger pollen competition within the stylar canals of *Cabomba* than in *Brasenia*. Because the common ancestor of Cabombaceae also likely had small, insect-pollinated stigmas, *Cabomba* probably has a long history of strong pollen competition and would be expected to have evolved a shorter progamic phase, consistent with our findings. In either case, selection for rapid fertilization seems to have acted strongly on early development in Nymphaeales in general, and in *Cabomba* pollen tubes “get out of the gate” rapidly as a result of both exceptionally fast pollen germination and to very fast early pollen tube growth (Figure 2.9). Pollen tubes often slow down in the ovary in other angiosperms (Herrero 2003), and this was the case in *Cabomba*, as well. However, slower late growth rates in *Cabomba* might be caused by the occurrence of mitosis II (Brewbaker and Majumder 1961), which, in leading pollen tubes, takes place in the ovary within 2 hap.

Environmental effects also add to the differences in developmental rates. The pollen population effect (Brewbaker and Majumder 1961; Cruzan 1986) predicts faster germination of *Cabomba* pollen because of its larger and more concentrated pollen loads. Our hand pollinations, designed to mimic natural pollination, resulted in consistently larger pollen loads in *Cabomba*. Faster pollen tube growth in *Cabomba* might also be due to warmer temperatures over its growth period (see Hedhly *et al.* 2003), as a result of starting the progamic phase later in the day.

CONCLUSION

Cabombaceae and Nymphaeaceae have an extremely short progamic phase relative to other groups of early-divergent angiosperms, especially woody perennials. Shifts to very short progamic phases are also seen in other aquatic early-divergent lineages and are primarily caused by speeding of pollen development after pollination (Williams 2009). In Nymphaeales, the very short progamic phase was achieved by shifts to faster pollen germination, faster pollen tube growth rates, and a novel pattern of pollen tube growth through solid ground tissue.

The common ancestor of Cabombaceae possessed a carpel with a short stylar neck and capitate stigma. Under the assumption that *Brasenia* represents a derived case of wind pollination, its style became elongated to produce a larger stigma, and the duration of stigma receptivity and pollen dispersal were shortened and shifted earlier in the day. Fertilization occurs at the same time of day in both species; therefore, as the period of pollen tube growth became lengthened, pollen germination and early tube growth became displaced to a cooler time of day. Environmental and developmental effects might explain slower pollen tube growth in *Brasenia*, but differences in the historical intensity of pollen competition between these wind- and insect-pollinated taxa might also have been important.

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APPENDIX 2

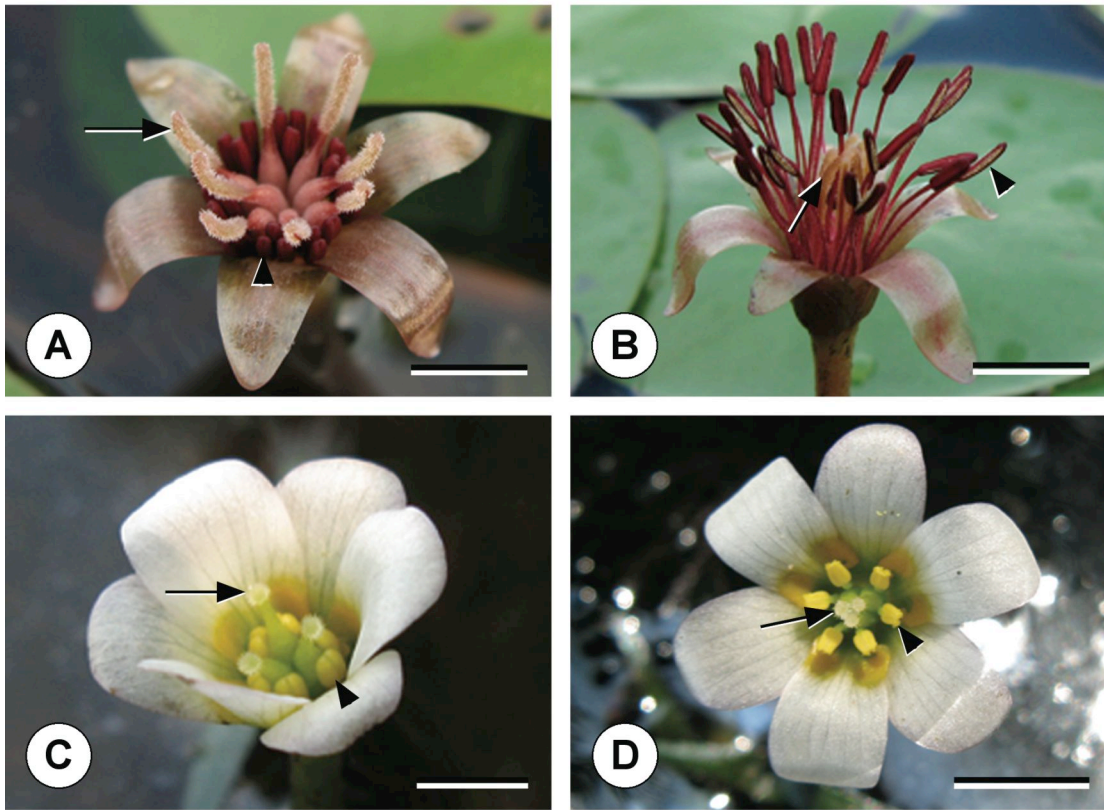


Figure 2.1. Floral cycle of *Brasenia* and *Cabomba*. Stigmatic crests indicated with arrows and anthers with arrowheads. (A) *Brasenia* first-day flower showing reflexed tepals and stigmatic crests. Closed anthers are positioned beneath stigmas because filaments are undeveloped. (B) *Brasenia* second-day flower showing elongated filaments supporting dehiscent anthers and centrally aggregated cluster of stigmas. (C) *Cabomba* first-day flower showing slightly reflexed tepals, exposed stigmatic surfaces, and short stamens. (D) *Cabomba* second-day flower showing more strongly reflexed tepals, dehiscent anthers borne upon elongated filaments and centrally aggregated stigmas. Scale bars = 5.0 mm.

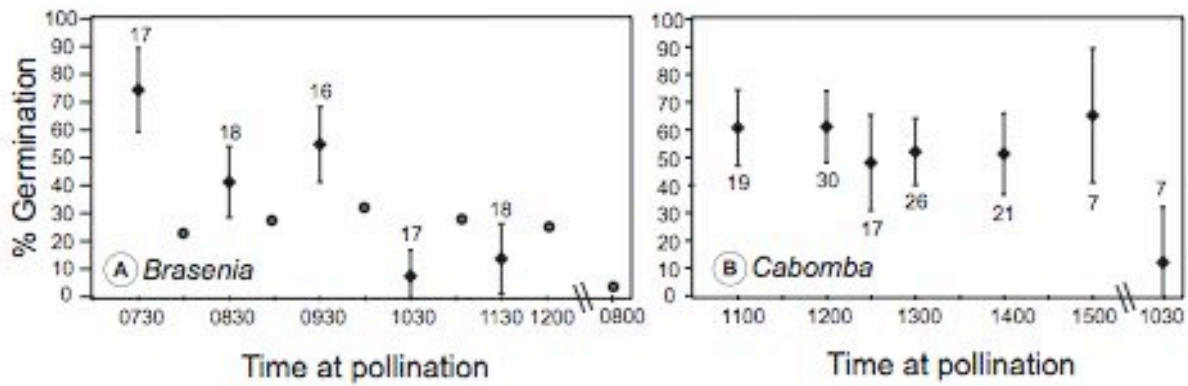


Figure 2.2. Duration of stigma receptivity in (A) *Brasenia* and (B) *Cabomba*, as determined by pollen germination percentage at different times after flower opening (pollen fixed 1 h after pollination). Bars are 95 % confidence intervals and numbers refer to the number of maternal plants sampled. *In vitro* pollen germination success of *Brasenia* pollen is represented by circles without bars in A.

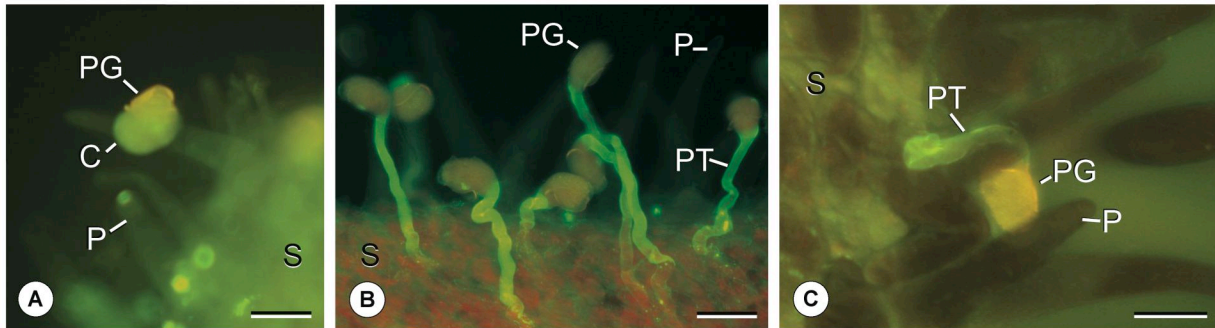


Figure 2.3. Pollen germination in *Brasenia*. (A) Burst pollen grain 2 h after 10:30 am pollination. (B) Pollen grains with well-developed pollen tubes 2 h after 7:30 am pollination. Note strong fluorescence of callose walls of pollen tubes growing within stigmatic secretions, which is still visible after they penetrate stigmatic ground tissue. (C) Germinated pollen grain on stigmatic papilla showing the pollen tube growing along papilla surface (1 h after pollination). Scale bars = 50 μ m. Abbreviations: C = pollen cytoplasm, P = stigmatic papillae, PG = pollen grain, PT = pollen tube, S = stigmatic surface. Stain: aniline blue.

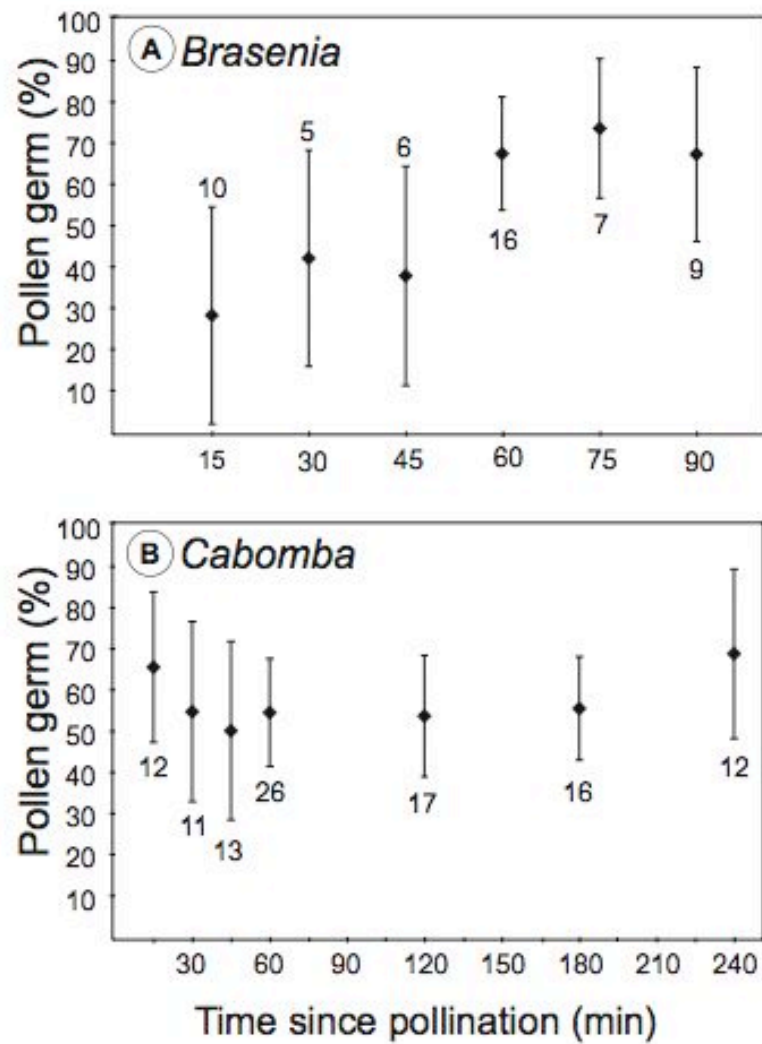


Figure 2.4. Timing of pollen germination in (A) *Brasenia* and (B) *Cabomba*. Pollen germination percentage for each time point. Bars are the 95 % confidence interval and numbers refer to the number of maternal plants sampled.

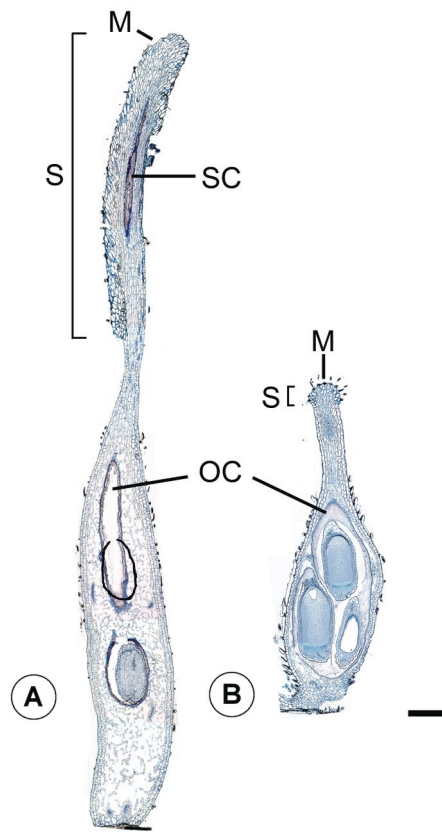


Figure 2.5. Median longitudinal section of (A) *Brasenia* and (B) *Cabomba* carpels. In *Brasenia*, the solid oval indicates the location of the apical ovule from another section. In both, the stylar canal is mostly out of section, but extends from the mouth of the carpel through the stylar neck to the apex of the ovarian cavity. Scale bar = 500 μm . Abbreviations: M = carpel mouth, OC = ovarian canal, S = stigmatic region, SC = stylar canal. Stain: toluidine blue O.

Figure 2.6. Pollen tube growth in *Brasenia*. (A) Pollen tubes (arrows) have grown within ground tissue to reach the stylar canal where they turn toward the ovary (four h after pollination [hap]). Note differential callose wall thickenings (white arrowhead) and fully developed callose plugs (black arrowhead). Scale bar = 100 μm . (B) Thin section through stigmatic surface showing a germinated pollen grain with a pollen tube (arrow) growing between two diagonally-oriented cells of the carpel ground tissue that comprises the transmitting tract (10 hap). Scale bar = 25 μm . (C) Thin section showing pollen tubes (arrow) in secretion-filled stylar canal (10 hap). Note diagonally-oriented parenchyma cells of the pollen tube transmitting tract, which corresponds to the region in Figure 2.6B. Scale bar = 100 μm . (D) Stylar canal filled with pollen tubes (arrow) showing abundance of callose plugs (arrowhead, many out of focal plane; 8 hap). Scale bar = 100 μm . (E) Micropyle entered by a pollen tube (arrow) with callose plugs (arrowhead; 8 hap). Scale bar = 100 μm . Abbreviations: F = funiculus, FG = female gametophyte, MP = micropyle, PG = pollen grain, SN = stylar neck, TT = transmitting tract. Stains: aniline blue (A, D, E); toluidine blue O (B, C).

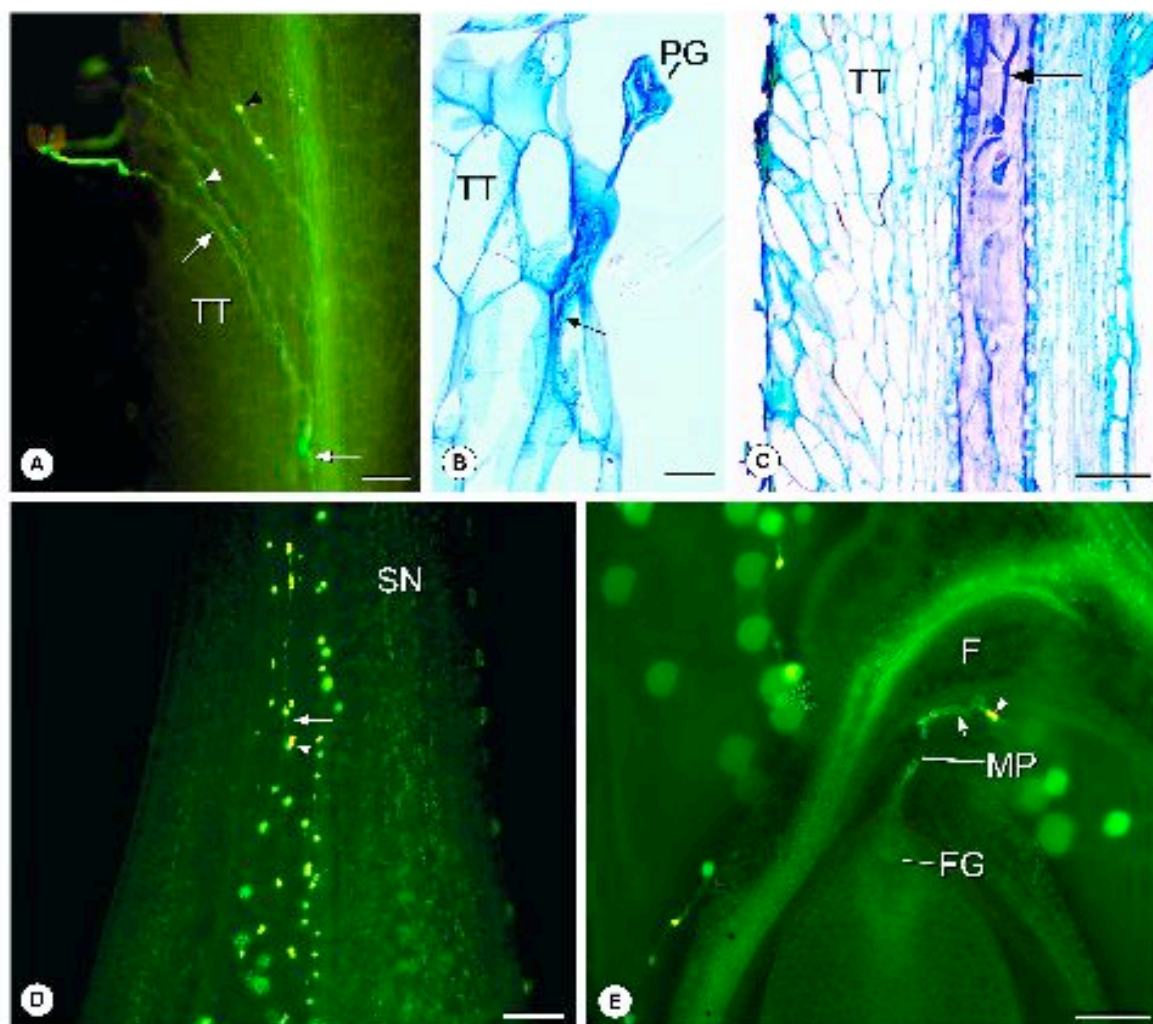


Figure 2.6

Figure 2.7. Pollen tube growth and timing of ovule penetration in (A) *Brasenia* and (B) *Cabomba*. Each symbol indicates the location of the leading pollen tube in a carpel relative to the tip of the carpel (0). In *Cabomba*, this distance corresponds closely to total pollen tube length, whereas in *Brasenia*, it does not. Black circles indicate that ovule entry has not occurred in the carpel, blue circles indicate that a pollen tube has entered the apical ovule but not the basal-most ovule; red circles indicate both ovules penetrated. A red circle with subscript indicates that the basal-most ovule was entered, but not the apical ovule. Line drawings of carpels show mean $\pm$ 95 % confidence interval distances from the carpel tip to the base of the stigmatic region ($n = 33$ [A], 52 [B]), the apical micropyle ($n = 33, 52$), the basalmost micropyle ($n = 11, 46$), and the base of the carpel ($n = 29, 42$; receptive surface of stigma illustrated with thicker line). Note differences in scale.

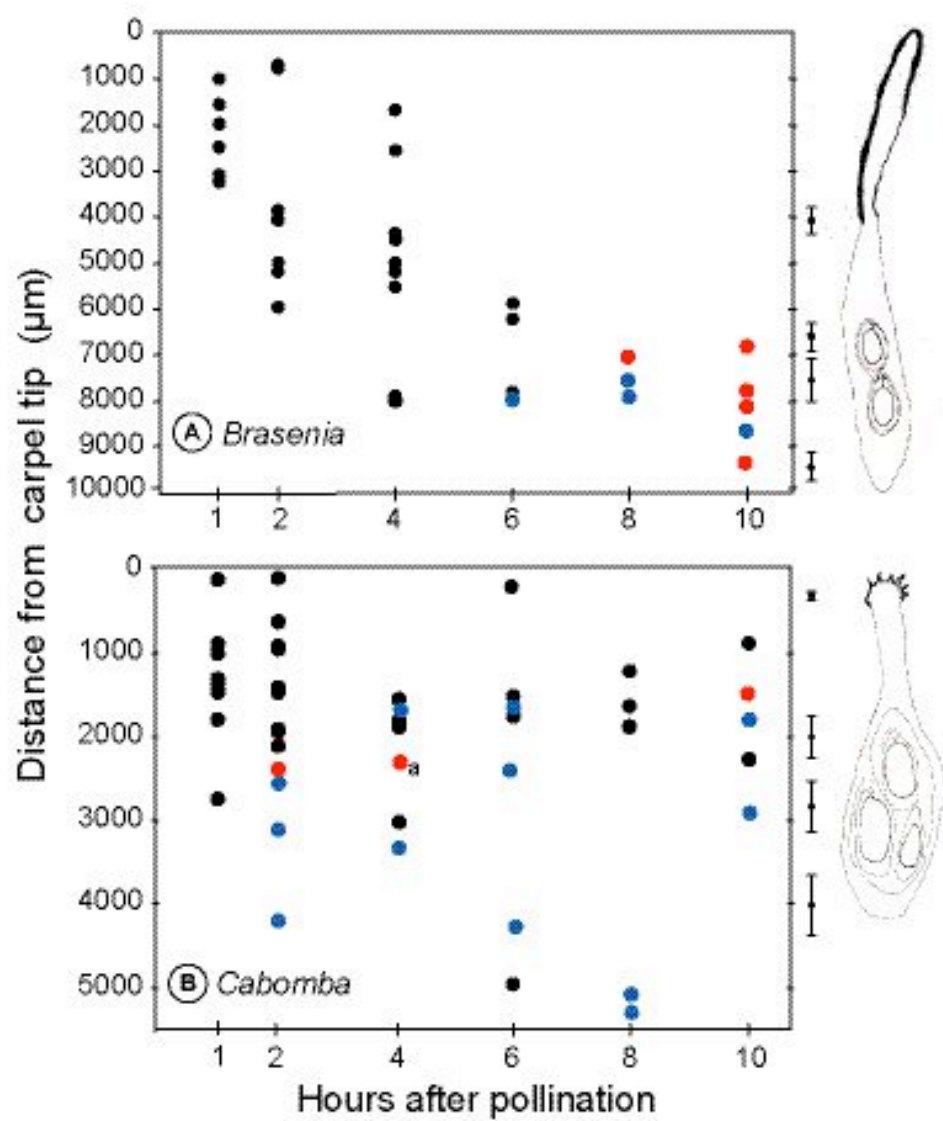


Figure 2.7

Figure 2.8. Pollen tube growth in *Cabomba*. (A) Germinated pollen showing most pollen tubes growing into the ground tissue beneath the stigma to reach the central stylar canal (2 h after pollination [hap]). Scale bar = 100 μm . (B) Thin section through the carpel tip showing the narrow mouth of stylar canal and obliquely-oriented cells of ground tissue. Scale bar = 100 μm . (C-E) Adjacent serial sections of stigma showing newly formed pollen tube penetrating cuticle and growing between cells of stigmatic surface (pollen tube cytoplasm indicated by arrows; 1 hap). Scale bar = 20 μm . (F) Hand-section of a carpel showing pollen tubes in the stylar canal and entering the ovarian cavity (2 hap). Note lack of callose plugs at this time. Scale bar = 200 μm . (G) Close-up of pollen tube in stylar canal showing telophase of mitosis II to form two sperm nuclei (2 hap). Scale bar = 20 μm . (H) Stigma of a second-day flower showing a band of callose at the base of the stigma. Scale bar = 200 μm . Abbreviations: CL = callose, CT = cuticle, M = carpel mouth, OC = ovarian cavity, P = stigmatic papilla; PG = pollen grain, PT = pollen tube, SC = stylar canal, SN = stylar neck, SP = sperm nucleus. Stains: aniline blue (A, F, H); toluidine blue O (B-E); aniline blue + DAPI (G).

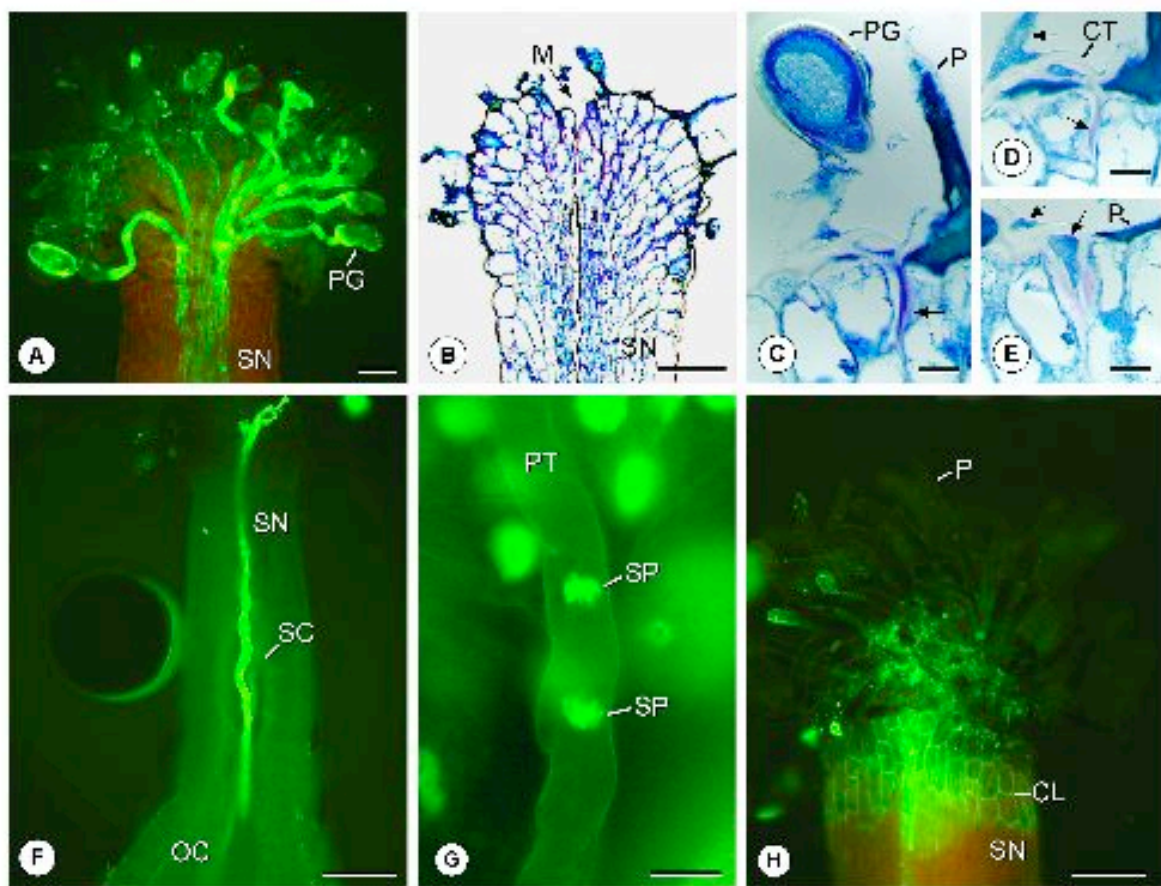


Figure 2.8

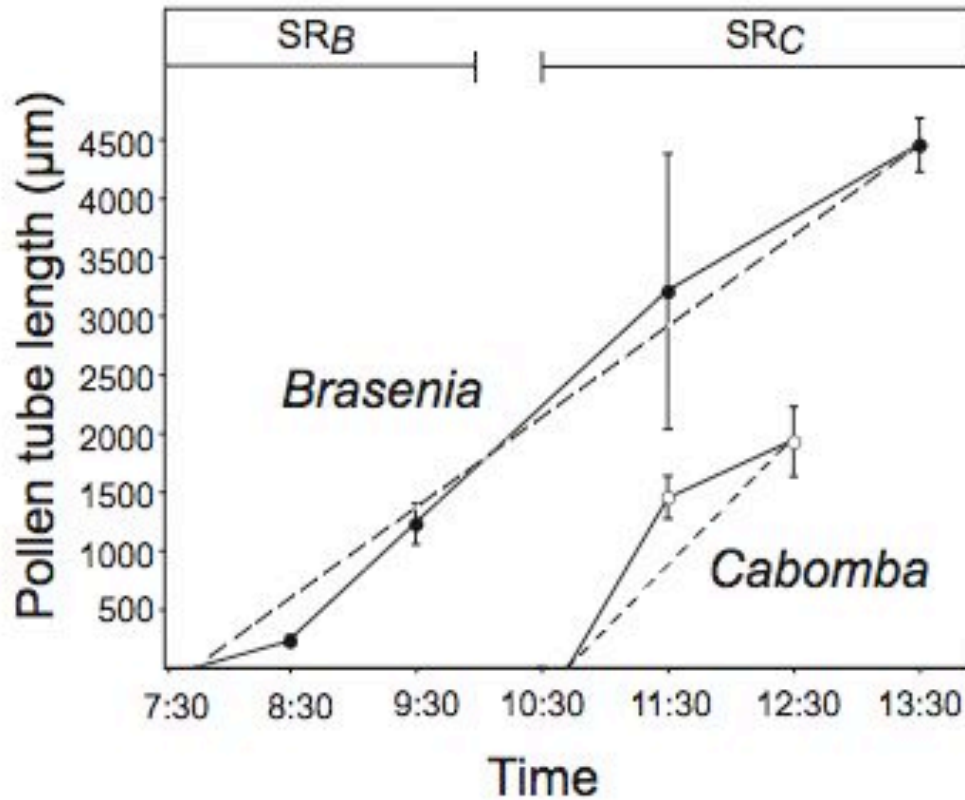


Figure 2.9. Pollen tube length over time in *Brasenia* and *Cabomba*. SR_B and SR_C indicate the stigma receptivity periods for *Brasenia* and *Cabomba*, respectively. Slopes of solid lines indicate growth rates within each time interval, and dotted lines indicate the maximum sustained growth rate. Trend lines begin at 15 minutes after pollination to account for time to pollen germination. Bars indicate standard errors.

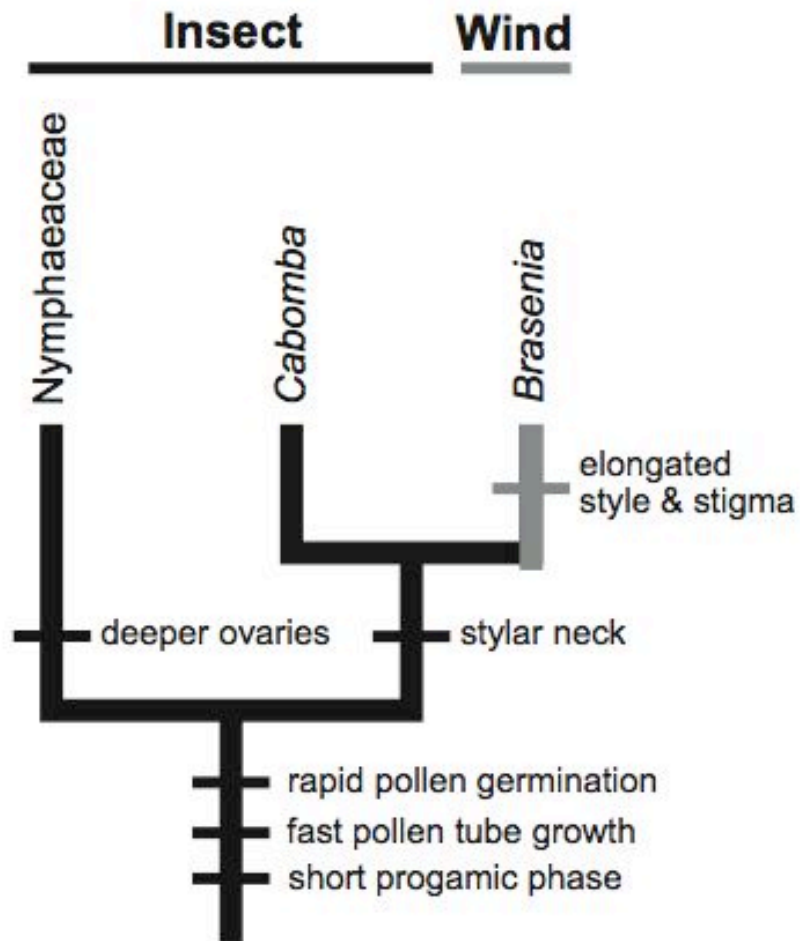


Figure 2.10. Evolution of pollination syndromes and progamic phase traits in Nymphaeales.

Character states of Hydatellaceae, the sister group to traditional Nymphaeales, are unknown.

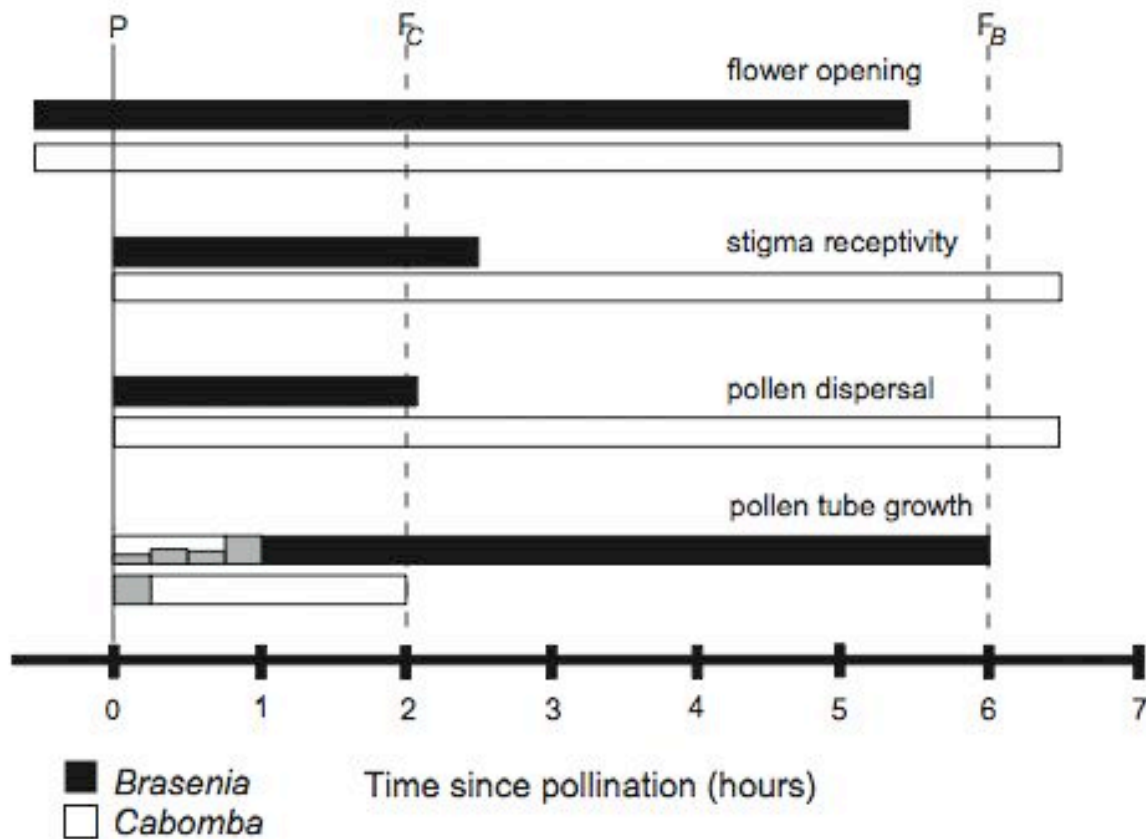


Figure 2.11. Relative timing of progamic phase ontogenies in *Brasenia* (solid bars) and *Cabomba* (open bars). Period of flower opening, stigma receptivity, pollen dispersal, pollen germination, and the progamic phase are arranged relative to the onset of stigma receptivity, at which time hand-pollinations (P) were performed (7:30 am in *Brasenia* and 10:30 am in *Cabomba*). F_B and F_C indicate fertilization, as determined by ovule penetration, in *Brasenia* and *Cabomba*, respectively. Note that gray areas in progamic phase bars reflect time to maximum pollen germination.

CHAPTER III: REPRODUCTIVE ECOLOGY OF THE BASAL ANGIOSPERM

TRITHURIA SUBMERSA (HYDATELLACEAE)

This chapter is a slightly modified version of an original research article published in the December 2010 issue of the journal *Annals of Botany*.

Taylor M.L., T.D. Macfarlane, and J.H. Williams. 2010. Reproductive ecology of the basal angiosperm *Trithuria submersa* (Hydatellaceae). *Annals of Botany* 106: 909-920.

In the following chapter, the words “we” and “our” refer to my co-authors and me. My contributions to this paper include (1) formation and further development of the original hypotheses (2) development of experimental pollination techniques and completion of all field experiments (3) preparation of materials to secure funding and permits (4) completion of all microscopical and statistical analyses (4) construction of figures, and (5) most of the writing.

ABSTRACT

Trithuria, the sole genus in Hydatellaceae, is an important group for understanding early angiosperm evolution because of placement within, or as sister to, the ancient lineage, Nymphaeales (water lilies). Although also aquatic, *Trithuria* differs from water lilies in that all species are extremely small, and most have an annual life form and grow in seasonal wetlands. Very little is known about their reproductive ecology. In this study, we report on reproductive timing, mode of pollination, and characteristics of the breeding system of *Trithuria submersa* in Western Australia. Mass collections of open-pollinated plants from different ecological settings were used to characterize the reproductive developmental sequence and natural pollen reception. Hand-pollination, caging, and emasculation experiments were used to measure outcross + geitonogamous pollen reception versus autonomous self-pollination in two populations over two field seasons. Natural outcross or geitonogamous pollination was by wind, not by water or insects, however pollen reception was extremely low. Pollen production was very low and pollen release was non-synchronous within populations. The pollen to ovule (P/O) ratio was 23.9, compared to 1569.1 in dioecious *Trithuria austinensis*. Stigmas became receptive before male phase and remained so until anthers dehisced and autonomous self-pollination occurred. Natural pollen loads are composed primarily of self pollen. Self- and open-pollinated plants had equivalent seed set (both > 70 %). Self-pollinated plants produced seed within 17 days. Autonomous self-pollination and self-fertilization are predominant in *T. submersa*. The low P/O ratio is not an artefact of small plant size and is inconsistent with long-term pollination by wind. It indicates that *T. submersa* has evolved a primarily autogamous breeding system. Selfing, along

with the effect of small plant size on the speed of reproduction, has enabled *T. submersa* to colonize marginal ephemeral wetlands in the face of unpredictable pollination.

INTRODUCTION

Nymphaeales is an ancient lineage that diverges from the basal-most, or next most basal node of the flowering plant phylogenetic tree (Qiu *et al.* 1999; APG III 2009) and is represented among the oldest known angiosperm macrofossils (Friis *et al.* 2001 2009; Wang and Dilcher 2006; Mohr *et al.* 2008; Taylor *et al.* 2008). *Trithuria* Hook.f., the sole genus within the family Hydatellaceae, has recently been placed as sister to the water lilies, Nymphaeales *sensu stricto* (Nymphaeaceae + Cabombaceae; Saarela *et al.* 2007; Borsch *et al.* 2008). A number of morphological and anatomical features unite these two ancient groups (e.g. Rudall *et al.* 2007; Friedman 2008; Endress and Doyle 2009). However, the reproductive ecology and life history of *Trithuria* species are likely to be quite different from water lilies and have not yet been studied in detail in the wild.

Water lilies are herbaceous perennials that typically inhabit stable, permanently inundated habitats. Only *Ondinea purpurea* (= *Nymphaea ondinea*, Löhne *et al.* 2009) and *Barclaya rotundifolia* (both in Nymphaeaceae) are known to occupy habitats that experience seasonal dry-down. Both are perennials that survive seasonal dry periods as persistent rhizomes and tubers (Schneider and Carlquist 1995; Williamson and Moseley 1989). In contrast, ten of twelve species of *Trithuria* occupy ephemeral aquatic habitats. All ten are reported to be annuals that survive the dry period as seeds (Hamann 1998; Gaikwad and Yadav 2003; Sokoloff *et al.* 2008a). Annuals are extremely rare among extant basal angiosperms and are found only in

Trithuria, and possibly in two species of Nymphaeaceae, *Euryale ferox* (Kadono and Schneider 1987) and *Victoria cruziana* (C. Magdalena, Royal Botanic Gardens, Kew, UK ‘pers. comm.’).

Water lilies are large plants, with large floating leaves, extensive rhizomes and flower sizes that range from 1-2 cm in diameter in Cabombaceae (Williamson and Schneider 1993) to 30-50 cm wide in *Victoria* (Schneider and Williamson 1993). In contrast, whole plants of *Trithuria* are often less than 1 cm in diameter and bear tiny “flowers” (Figure 3.1A-B; all figures referenced in this chapter are found in Appendix 3). These small reproductive structures have characteristics of both flowers and inflorescences and they may represent a transitional, pre-floral stage in the evolution of the flower (Endress and Doyle 2009; Rudall *et al.* 2009). Hereafter we refer to them as “reproductive units,” occasionally abbreviated as “RU” (Rudall *et al.* 2007; 2009).

Given its phylogenetic position as sister to the rest of water lilies, *Trithuria* may offer important clues to the evolution of reproductive function among early angiosperms and Nymphaeales *sensu lato* in particular. To date, studies of *Trithuria* have concentrated on characterizing vegetative morphology (Edgar 1966; Gaikwad and Yadav 2003), pollen morphology (Bortenschlager *et al.* 1966; Remizowa *et al.* 2008), and developmental aspects of the reproductive unit (Rudall *et al.* 2007, 2009), shoot (Sokoloff *et al.* 2009), female gametophyte (Friedman 2008; Rudall *et al.* 2008), seeds (Tuckett *et al.* 2010a, b) and seedlings (Cooke 1983; Tillich *et al.* 2007; Sokoloff, *et al.* 2008b).

All *Trithuria* species are thought to be abiotically pollinated. Wind pollination has been hypothesized for *T. konkanensis* and other species with emergent reproductive units, on the basis of floral morphology (Hamann 1998; Gaikwad and Yadav 2003). Water pollination has also been

hypothesized as a possibility, particularly in the two permanently submerged species (Rudall *et al.* 2007). Nothing is known of breeding systems, apart from the fact that four species are dioecious, and hence obligately outcrossing (Yadav and Janarthanam 1995; Sokoloff *et al.* 2008a). There are no data on relative timing and duration of male and female function, a potentially important aspect of the breeding system of the other species that are monoecious or have bisexual reproductive units.

The objective of this study was to understand the reproductive ecology of *Trithuria submersa* Hook.f., a species found in seasonal, rain-fed wetlands of southwestern Western Australia, as well as parts of southern New South Wales, South Australia, Victoria, and Tasmania (Sokoloff *et al.* 2008a). *Trithuria submersa* was chosen because it is a widespread species with emergent, bisexual reproductive units. Our goals were, (1) to determine the primary pollen vector, (2) to describe the relative timing and duration of anther dehiscence and stigma receptivity and (3) to determine if self pollination and self seed-set occur. We also report the pollen/ovule ratio of dioecious *T. austinensis* and pollen load size in *Brasenia schreberi* to enable a discussion of alternative life history strategies among wind-pollinated Nymphaeales.

METHODS

Reproductive biology of Trithuria submersa

Fieldwork on *Trithuria submersa* was undertaken in November/December 2008 at Kulunilup Swamp (Figure 3.1A), Kulunilup Nature Reserve, Western Australia (34° 19' S, 116° 46' E). A second field season was undertaken in November/December 2009 at Kulunilup Swamp and nearby Frying Pan Swamp (34° 16' S, 116° 42' E). Laboratory work was conducted at the

Department of Environment and Conservation Science Division facility in Manjimup, WA and at the University of Tennessee, Knoxville. Voucher specimens have been deposited in the University of Tennessee herbarium (TENN).

Southwestern Western Australia experiences cool, wet winters and hot, dry summers and exhibits a vast network of wetlands that undergo a strong seasonal hydrological cycle of winter flooding and summer drawdown (Hill *et al.* 1996). “Swamps,” or “sumplands,” are characterized by shallow standing water in late winter through spring, followed by complete evaporation of water over the course of a few days to a few weeks (Hill *et al.* 1996). These swamps can also be considered vernal pools (Holland and Jain 1988; Rheinhardt and Hollands 2008). Seeds of *T. submersa* germinate in swamps and plants mature while entirely submerged. Reproductive units become gradually exposed as the water level falls and flowering and fruit set must be completed before the swamp dries out. *Trithuria austinensis*, *T. australis*, and *T. bibracteata* are also found in this region, with *T. australis* and *T. bibracteata* sometimes growing alongside *T. submersa*.

Reproductive development

Because of the small size of *Trithuria* plants, reproductive development was characterized by relative stages rather than by absolute time. A developmental sequence was reconstructed from a mass collection of open-pollinated *T. submersa* reproductive units (made without knowledge of developmental stage). To understand the effect of environment on reproductive development, the mass collection comprised equal numbers of haphazardly collected RUs in four distinct ecological settings: (1) reproductive units *entirely submerged*, (2) reproductive units *newly emergent* with between 50-75 % of the reproductive unit above water

level, (3) reproductive units *fully emergent* but plants still partially submerged, and (4) plants completely emergent (hereafter termed *long emergent*; see Figures 3.1A, B).

Reproductive units were fixed in FAA (2:1:10 40 % formaldehyde, glacial acetic acid, 95 % ethanol) or 3:1 (95 % ethanol: glacial acetic acid) for 24 h and then stored in 70 % ethanol. Carpels were removed, stained with aniline blue for 4-8 h, and viewed under UV light to visualize pollen grains (methods in Taylor and Williams 2009). Onset and duration of pollen reception and stigma receptivity were assessed by recording the number of germinated pollen grains on each stigmatic hair at each developmental/ecological stage and the proportion of carpels exhibiting pollen germination and pollen loads were compared. Since ungerminated pollen grains can wash off stigmatic hairs during fixation, only germinated grains were compared. If data were non-normally distributed, a non-parametric Wilcoxon Rank Sums test was performed. If variances were not equal, an unequal variance *t*-test was used (Ruxton 2006). Analyses were performed in JMP v.7.0.2 statistical software (SAS Institute Cary, NC). When comparing three or more means after a significant one-way ANOVA, a Games-Howell post-hoc test was performed with SPSS 16.0 (SPSS Inc., Chicago, IL). Individual plants were the experimental unit. All measures of variance in the text are standard deviations.

A second experiment used hand-pollinations of stage two and three reproductive units to determine if stigmas were receptive before anther dehiscence. Foreign pollen was excluded from haphazardly selected, fully submerged plants by covering them with clear plastic cups staked into the ground with wire. All reproductive units except the focal unit were first removed to prevent geitonogamous pollination within the “cage.” After the reproductive unit emerged within the cage, but before anthers dehisced, carpels were pollinated by gently brushing a dehiscent

anther across stigmatic hairs. The pollen donor plant was 1-5 m distant from the caged plant and there was no evidence for rhizome connections between plants. Hand-pollinated reproductive units were collected within 6 h after pollination and fixed in 3:1. Carpels were scored for number of germinated pollen grains.

Pollination syndrome

To determine if entomophily occurred, insect behaviour in the population was assessed by direct observation (~ 75 h). Twenty plants were marked with coloured thread and their reproductive units were observed periodically from emergence until anther opening and then continually until anthers were judged to be empty. The number of events in which insects contacted reproductive units and the activity of the insect (resting or foraging) was recorded. To test for anemophily, 11 glass slides were thinly coated with petroleum jelly and placed at 1 m intervals in a transect through the Kulunilup Swamp population to trap wind-borne pollen. As anther dehiscence was common in late morning and early afternoon, slides were set at 10 am and collected after 6 h to prevent exposure to afternoon rain. Pollen grains were counted and typed (no other *Trithuria* species in the population were reproductive at the time).

To determine the potential for hydrophily, 20 plants with developing reproductive units were kept submerged in the laboratory for 15 days. Anthers were monitored periodically, and RUs were collected well after bracts reflexed and scored for pollen reception and anther opening.

Breeding system

A caging experiment tested for autonomous self-pollination and self-fertilization. Thirty plants with a single reproductive unit at Kulunilup swamp were covered with cups as above to prevent cross-pollination and geitonogamy (cups also excluded wind as a pollen vector). Twelve plants were collected 3-5 days after emergence, fixed in 3:1, and the germinated pollen load was determined. The remaining 18 plants were collected three weeks later, after seeds were mature. Seed-set was calculated as the number of developed seeds divided by the total number of ovules and seeds (ovules could be easily seen through the carpel wall with a stereomicroscope). A developed seed was conspicuously larger than a mature ovule in an unpollinated reproductive unit and had a hard seed coat.

An emasculation experiment was designed to determine pollen load sizes with and without self-pollination. Immature anthers were removed from 23 reproductive units, which were then allowed to naturally receive pollen, including geitonogamous pollen. The 23 emasculated reproductive units and 23 untreated reproductive units were collected 15 days after emasculation or anther abscission to ensure maximum pollen reception. Carpels were fixed and stained with aniline blue and pollen was counted.

For analysis of pollen production, anthers of *T. submersa* were each macerated in 200 μ l of 1 % polyethylene glycol (PEG) in 95 % ethanol and gently vortexed for 30 seconds. One tenth (20 μ l) of the pollen mixture was placed on a glass slide and the entire cover slip was scanned. The number of observed pollen grains was multiplied by 10 to estimate total number of grains per anther and this was multiplied by the number of anthers in the reproductive unit to estimate the total number per unit. The number of pollen grains per reproductive unit was divided by the number of carpels in the unit (one ovule/carpel) to obtain the pollen to ovule (P/O) ratio.

For comparison, pollen production and P/O ratio in dioecious *T. austinensis* D. D. Sokoloff, Remizowa, T. D. Macfarl. & Rudall were calculated using 11 male and 11 female mature reproductive units collected at Branchinella Lake, shire of Manjimup, Western Australia (34° 21' S, 116° 43' E). One anther from each male reproductive unit was macerated in PEG and pollen production per anther and reproductive unit was determined as above. As *T. austinensis* is dioecious, average pollen production per male reproductive unit was divided by the average number of carpels (one ovule/carpel) in female reproductive units to obtain the P/O ratio. Also for comparison, stigmatic pollen loads were determined for open-pollinated flowers of *Brasenia schreberi* J.F. Gmel ($n = 16$ plants; methods and location in Taylor and Williams 2009).

RESULTS

Reproductive development

Individual plants produced from 1-18 reproductive units (mean = 4.8 ± 3.7 in Kulunilup Swamp, 5.4 ± 4.4 in Frying Pan Swamp) over the course of the season. Reproductive units were borne singly on peduncles of different heights and emerged at different times (Figure 3.1B). Most reproductive units possessed a single stamen (Figure 3.1C), but 3 % from Kulunilup swamp and 24 % from Frying Pan swamp had two (Figure 3.1D; $n = 180, 67$, respectively). Reproductive units contained an average of 19.3 ± 6.4 carpels ($n = 280$, range: 3-37) each with a single ovule and three uniseriate stigmatic hairs.

Buds enlarged under water and mature bracts partially reflexed (Figure 3.1B-D), whether or not the reproductive unit had emerged. Once they reflexed, the bracts did not close again. The

stamens and carpels continued to develop whether or not they were under water, but anther dehiscence did not occur until the reproductive unit had emerged.

Within open reproductive units, five distinct developmental stages could be characterized with respect to stamen development (Table 3.1). In stage 1, anthers and carpels were positioned at similar heights. Ovaries and their ovules had already attained their mature size (cf. Figure 3.1E, F). Each of the three uniseriate stigmatic hairs was fully formed, but the cells had not expanded (mean height to width ratio of 5th cell = 0.31; $n = 10$; Figure 3.1E).

In stage 2, anther filaments had elongated but had not reached their full length, and the anthers protruded above carpels, but not the stigmatic hairs (Figure 3.1C). The stigmatic hairs had partially elongated via cell expansion, with one hair typically longer than the others. Cells near the base of the stigmatic hair elongated first, whereas cells at the tip rarely expanded (Figure 3.1C, F).

In stage 3, the anthers were positioned above most or all of the stigmatic hairs. The longest stigmatic hairs had more than doubled in length to their mature size (mean height to width ratio = 0.76; $n = 15$; Figure 3.1F). The second stigmatic hair often remained slightly shorter than the first whereas the third stigmatic hair did not elongate in any of the carpels observed (Figure 3.1F).

Stage 4 was characterized by anther dehiscence (Figure 3.1D). Anthers opened along two longitudinal lines of dehiscence that extended the length of the anther (Figure 3.2A-B). Dehiscence was observed at all times of day, and anthers generally emptied within a few minutes after opening. At this stage stigmatic hairs often exhibited one or more collapsed cells, causing the stigmatic hair to bend or curl. Cells in the top half of the hair were more prone to collapse

than those near the base (Figure 3.1D, F). Occasionally, filaments were also observed to bend, lowering the dehiscent anther toward the stigmatic hairs (Figure 3.1D). After dehiscence, anthers abscised, leaving the filament— this indicated the onset of stage 5 (Table 3.1).

In contrast to other water lilies, in which the entire perianth closes after anthesis, bracts of *T. submersa* remained reflexed throughout fruit development. Fruits appeared mature and were falling out of reproductive units within 17 days of dehiscence. Seeds from reproductive units that were naturally pollinated 10-16 Nov. 2008 were collected on 3 Dec., stored dry at room temperature (~21° C) and planted in saturated soil (18° C), on 15 Mar. 2009. These germinated while submerged and began flowering on 6 Oct. 2009. Seeds collected on 1 Dec. 2009 were stored as above and planted in chilled (10° C), saturated soil on 1 July 2010. These first germinated 30 days later.

Pollination syndrome

Two indications that *T. submersa* might experience pollination by water were, 1) buds opened, stigmatic hairs and stamen filaments elongated, and pollen matured under water, and 2) aerenchyma was present in the connective tissue of the anther (Figure 3.2A). However, anthers were never dehiscent in either naturally or experimentally submerged reproductive units. Furthermore, submerged reproductive units (ecological stage 1) never received pollen (Figure 3.3A-B), despite having fully elongated stigmatic hairs. Even among newly emergent reproductive units (ecological stage 2) only a single stigmatic hair received pollen (Figure 3.3B). Only emergent reproductive units received pollen (Figure 3.3).

Nine insect visits to *Trithuria* plants were recorded over the course of two years of field observations. In all nine cases insects only rested on bracts. No foraging behaviour within reproductive units was ever observed, although two of 264 anthers in the fixed material appeared to have been partially eaten.

Sixty-four of 427 pollen grains on glass slides were from *T. submersa*. Anthers were also observed shedding pollen in the wind. Pollen was not sticky and exhibited a smooth exine (Figure 3.2D). On stigmas, pollen grains measured $18.5 \mu\text{m} \pm 1.7 \mu\text{m}$ by $15.8 \mu\text{m} \pm 1.8 \mu\text{m}$. A dehiscent anther(s) is held above the reproductive unit, which is in turn held above the vegetative body by a long slender peduncle (0.5 - 3.0 cm long and 0.4 mm wide; Figure 3.1A, B; 3.2B). Anther morphology of *T. submersa* was nearly identical to that of wind-pollinated water lily, *Brasenia schreberi* (Cabombaceae; Figures 3.2B-C), including the presence of robust endothelial bands (Figure 3.2A; see also Taylor and Osborn 2006).

Breeding system

In the mass collection experiment, only one emergent reproductive unit received pollen during developmental stages one and two and no pollen germination was observed (Figure 3.4A-B). In stage 3, 25 % of the reproductive units and 6 % of carpels per reproductive unit exhibited stigmas with germinated pollen (Figure 3.4A). However, the average germinated pollen load in stage 3 was small (0.08 grains/carpel; Figure 3.4B). Within reproductive units that received pollen, 23 % of carpels received pollen and the average pollen load per carpel was 1.2 ± 0.5 . The maximum pollen load was three. In the hand-pollination experiment, 9 of 21 reproductive units, all in stage 3, had growing pollen tubes. Since, anthers were indehiscent when stigmatic hairs

became receptive in stage 3, the onset of female function occurs before the onset of male function in the bisexual reproductive unit in *T. submersa*.

Reproductive units with dehiscent anthers (stages 4 and 5) received much more pollen than those prior to dehiscence (stages 1-3). In 2008, almost all post-dehiscent reproductive units had received pollen; the percentage of pollinated carpels/RU was significantly higher than that of pre-dehiscent RUs (Figure 3.4A); and pollen loads were also higher (Figure 3.4B). In 2009, 6 % of reproductive units received pollen prior to anther dehiscence, whereas 91 % received pollen after dehiscence (Figure 3.5A), and both the percentage of carpels pollinated and pollen loads were significantly higher (Figure 3.5A-B).

In the caging experiment, outcross or geitonogamous self-pollination by wind was prevented so that only autonomous self pollination by gravity could occur. In caged plants, 100 % of reproductive units and a mean of 87 % of carpels/RU received germinable pollen, a significantly higher percentage than that of open-pollinated plants (Figure 3.6A). The average stigmatic pollen load was 5.8 ± 1.8 on caged plants versus 2.3 ± 2.7 on stage 5 open-pollinated plants. Mean seed set of the 18 caged plants was not significantly different than the open-pollinated control (Figure 3.6B).

The emasculation experiment showed that reproductive units received outcross or geitonogamous pollen. These plants received significantly fewer pollen grains (14 % versus 64 % of carpels/RU; Figure 3.7A) and had smaller pollen loads than the open-pollinated control (0.38 ± 0.9 versus 2.5 ± 1.7 pollen grains/carpel; Figure 3.7B). For comparison, the mean pollen load per carpel of *Brasenia schreberi*, which receives only outcross or geitonogamous pollen from a separate inflorescence, was 3.0 ± 3.1 .

Mature anthers in *T. submersa* contained 426.0 ± 149.4 pollen grains and the average pollen to ovule (P/O) ratio was 23.9 ($n = 27$). Reproductive units of *T. austinensis* had similar numbers of ovules as *T. submersa* (17.1 versus 19.3), but more anthers (7.9 versus 1.1) and much greater pollen production of 3,526 grains/anther and 27,650 grains/RU. Its P/O ratio was 1569.1 ($n = 11$). Anthers in *T. austinensis* measured 1.96 mm long x 0.52 mm wide x 0.29 deep, compared to 0.71 x 0.32 x 0.22 mm in *T. submersa* ($n = 5$ each).

DISCUSSION

Trithuria species are similar to other Nymphaeales in that they begin development while totally submerged. However, unlike most water lilies that occur in more or less permanent aquatic environments, nearly all *Trithuria* species are found in ephemeral wetlands. Vernal pool plants are typically small and exhibit fast vegetative and reproductive development (Zedler 1990). Plants of *Trithuria submersa* are quite small (< 1 cm in diameter) and their reproductive function, triggered by water drawdown, was short and locally unpredictable. The period between fertilization and seed dispersal was also short, only 17 days in 2008. Small plant size and the brief window for reproduction underlie many aspects of *T. submersa*'s reproductive biology. Below we discuss the reproductive development of *T. submersa* in light of its probable aquatic, perennial ancestry and the evolution of its ephemeral wetland ecology.

Pollination syndrome

Hydrophily in *T. submersa* was a distinct possibility because reproductive units often opened under water, and both stamens and carpels reached mature sizes while submerged. At

least two other *Trithuria* species may carry out their entire life history underwater (Edgar 1966; Pledge 1974; Rudall *et al.* 2007). However, two observations indicate desiccation was necessary for anther dehiscence. First, only emergent anthers dehiscenced, whether in collected material or in plants that were kept artificially submerged for up to 15 days and second, anthers possessed endothelial bands, which are known to facilitate anther opening upon desiccation and are not present in submerged anthers of water-pollinated plants (D’Arcy 1996; Endress 1996). Our data also show that emergence was necessary for pollen reception. Stigmatic hairs from mass collected submerged reproductive units received no pollen and artificially submerged plants set no seed.

Emerging reproductive units created an indentation in the water surface that might serve to draw floating pollen or abscised anthers toward the stigmatic hairs (Figure 3.1B). Many aquatic taxa, including several seagrasses (Cymodoceaceae; Hydrocharitaceae; Zosteraceae), achieve pollination via floating pollen or anthers that physically contact receptive stigmas (Cox 1988; Cox and Humphries 1993). Among early-divergent lineages of angiosperms, both the basal eudicots *Ceratophyllum* (Ceratophyllaceae) and basal monocot *Lepilaena cylindrocarpa* (Potamogetonaceae) have anthers that abscise underwater and float to the surface to release pollen (Cox 1988). Anthers of *T. submersa* also abscise and the large lacunae in the connective tissue suggest anthers could float on the water surface. However, in *T. submersa* pollen was dry, anthers emptied before they abscised and partially emergent reproductive units received very little pollen. We conclude *Trithuria submersa* is not water-pollinated.

We also ruled out insect pollination. Extensive observation over the course of two field seasons in several populations indicated that insects did not interact with *Trithuria* reproductive

units, and there was very little evidence of pollen scavenging. Pollinivory is quite common in many basal angiosperms (Thien *et al.* 2009), but in *T. submersa* the reward would be quite small, given how little pollen was present in an anther. Under insect pollination, large pollen loads might be expected within at least some RUs, but on emasculated RUs, no stigma received more than three pollen grains and most received no pollen at all.

Experimental, observational and anatomical evidence indicate that *T. submersa* at least occasionally exhibits wind-pollination. Pollen traps captured wind-borne pollen and small numbers of viable pollen were received on stigmas of emasculated and female phase reproductive units. Pollen in *T. submersa* is not sticky, lacks ornamentation, and is at the small end of the size range of wind-dispersed pollen (Friedman and Barrett 2009). Reproductive units lack a perianth or showy bracts, dehiscent anthers are elevated on elongated filaments, and receptive stigmatic hairs often extended beyond the bracts. Organ placement that reduces interference is common in wind-pollinated plants (Whitehead 1969; Friedman and Barrett 2009).

Pollination and the breeding system

Trithuria submersa can be characterized as self-pollinated, self-compatible and autogamous. Stigmas are receptive before and during anther dehiscence. Thus, stigmas can receive outcross or geitonogamous (self) pollen prior to autonomous self-pollination by the overarched anther. Outcross pollination was exceptionally rare – when reproductive units were emasculated more than 86 % of carpels received no pollen at all. Yet in unemasculated plants that could outcross and self, over 60 % of carpels received pollen. Pollen reception was even greater on caged plants in which only autonomous self-pollination could occur. These results

indicate autonomous self-pollination compensates for the lack of outcross pollen reaching stigmas of *T. submersa*.

Open-pollinated plants had 71 % seed set, a result inconsistent with the extremely low levels of cross-pollination, unless self-pollination is ubiquitous and plants are self-compatible. Self-compatibility was confirmed by the 83 % seed set of self-pollinated plants in the caging experiment. Germination of open-pollinated seeds in the greenhouse indicates seeds have high viability, as also found by Tuckett *et al.* (2010b) for the same species. Our data show that such seeds were likely self-fertilized, and therefore have low levels of inbreeding depression. Another indication of long-term inbreeding is the low P/O ratio of 24. Such a low P/O ratio is consistent with obligate autogamy (Cruden 1977).

It could be argued that the exceptionally low P/O ratio of *T. submersa* evolved in large part because extreme reduction in plant size caused reduced pollen production, leading to the subsequent evolution of autogamy. Its reproductive units are only 2 mms wide and have a mean of 1.1 anthers with 426 pollen grains per anther. However, *T. austinensis*, a close relative that is of similarly small size, produces about eight times more anthers and eight times more pollen per anther, even though it has slightly larger pollen than *T. submersa*. Thus, the extremely low pollen production of *T. submersa*, the proximate cause of its low P/O ratio, cannot be due to small plant size alone. To underscore the point, the P/O ratio of 1569 of *T. austinensis* is within the low end of the range of those of other dioecious and wind-pollinated species (Cruden 2000; Michalski and Durka 2010). Thus, we interpret the low P/O ratio of *T. submersa* as being causally linked to the evolution of an obligately autogamous breeding system.

Given our conclusions, traits typically associated with cross-pollination, such as the onset of stigma receptivity prior to anther dehiscence, pollination by wind and a somewhat sequential maturation of RUs, now seem to have little function. Perhaps one of the best-supported conclusions of recent comparative studies of basal angiosperms is that bisexual flowers were ancestrally protogynous (Endress 2010). As such, the prior onset of stigma receptivity in *T. submersa* is likely an historical effect and if so, would indicate a similar level of developmental integration within a bisexual RU (*Trithuria*) as within a bisexual flower (other basal angiosperms). Its retention in present-day *T. submersa* may be less related to facilitating cross-pollination than to its function in ensuring self-pollination. Early onset of a long period of stigmatic receptivity is currently maintained because of the uncertainty in timing of anther dehiscence and the predominant mode of pollen reception - autonomous self-pollination.

Reproductive strategies of wind-pollinated Nymphaeales

Many of the structural features associated with wind pollination in *T. submersa* (pollen, anthers and reproductive units) are also present in other *Trithuria* species. It may be that all but the two perennial species with submerged reproductive units have some degree of wind-pollination, as hypothesized by Hamann (1998). Within the sister lineage to Hydatellaceae, Nymphaeales *sensu stricto*, insect pollination is generally thought to be plesiomorphic (e.g. Friis et al. 2001; Borsch et al. 2008) and the only documentation of wind-pollination in the group is in *Brasenia schreberi* (Osborn and Schneider 1988). Here we compare ecological and historical aspects of the evolution of anemophily in *Trithuria* and *Brasenia*, as well as the subsequent shift

from a primarily wind-pollinated to a primarily autonomously self-pollinated reproductive system in present-day *T. submersa*.

There are strong indications that the common ancestor of Nymphaeales *sensu lato* was aquatic and perennial, with homoeocious (bisexual or monoecious) reproductive units in which female organs matured before male organs (Friis *et al.* 2001; Crepet *et al.* 2004; Wang and Dilcher 2006; Mohr *et al.* 2008; Taylor *et al.* 2008; Endress and Doyle 2009; Endress 2010). The major ecological difference between Nymphaeales *sensu stricto* (Cabombaceae + Nymphaeaceae) and Hydatellaceae involves their aquatic environment – the former are large perennials that occupy permanently inundated habitats (Williamson and Schneider 1993), whereas the latter are extremely small and most are annuals that live in a seasonal aquatic environment (only two species of *Trithuria* occur in permanently inundated habitat and both retain the ancestral perennial habit)(Edgar 1966; Pledge 1974; Sokoloff *et al.* 2008a).

Wind pollination is favored in dry, open environments (Whitehead 1969; Culley *et al.* 2002; Friedman and Barrett 2009), yet wind can also be a reliable pollen vector in the large, open habitats of many aquatics that flower above water. Long-term persistence in predictable environments with seasonal cues can also enable a high degree of synchronization of flowering within a population to evolve (Whitehead 1969). Synchrony of flowering is much stronger in *B. schreberi* (closely coincident onset timing and of much shorter duration) than in other insect-pollinated Nymphaeales (Osborn and Schneider 1988; Taylor and Williams 2009). It also occurs in the context of the strict dichogamy typical of Nymphaeales – female phase occurs on the first day of anthesis and male phase begins on the second day, a pattern present in most basal angiosperms (Endress 2010). Floral buds also open sequentially on *B. schreberi* shoots,

minimizing the potential for geitonogamy. We found that outcross pollen transfer by wind was successful in *B. schreberi* and we have observed high seed set in several populations, both in the southeastern USA and in Australia (Taylor and Williams 2009; personal observations). The species has a cosmopolitan distribution (Williamson and Schneider 1993).

The evolution of wind pollination in *Trithuria* has a quite different history. Extreme reduction in plant size occurred before the common ancestor of extant species. Subsequently, the annual species have shifted from their ancestral perennial habit in permanent wetlands to seasonal wetlands. *Trithuria austinensis* reflects one outcome of such a shift. It typically grows in shallow wetlands in a very dense carpet, forming large populations that are fairly constant in size from year to year. Gradual water drawdown causes many neighbouring plants along the receding wetland margins to emerge and to flower simultaneously in an open environment.

Pollen production and P/O ratios were relatively high, consistent with wind pollination and outcrossing. Large population size, dioecy, and predictable environmental cues all favour the origin and maintenance of outcrossing by wind pollination (Friedman and Barrett 2008, 2009). An alternative, and perhaps more derived strategy is represented by *T. submersa*, which has bisexual reproduction units, but in which dichogamy has been lost. In *T. submersa* cross-pollination via wind can occur but was rare in both years. *T. submersa* is found in disturbed, early-successional wetland habitats, such as in roadside ditches or in shallow depressions in recently burned areas. Relative to *T. austinensis*, its populations are small and ephemeral, and individuals are not as densely distributed. Furthermore, the topography and surrounding vegetation in their small patchy habitats forms a more closed environment for these small plants, greatly reducing exposure to wind. Though plants matured fairly synchronously in both years,

their reproductive units did not. Small differences in plant height or relative ground level often caused neighbouring reproductive units to emerge, and anthers to dehisce, hours or even days apart. Thus, low population density, non-synchrony of pollen dispersal, and the often closed habitat are all causes of ineffective outcross pollen transfer by wind. The shift to an effective system of autonomous self-pollination and primarily autogamous reproduction has exacerbated the effect by causing lower pollen production.

Self-fertilization has long been associated with the ability to colonize and succeed in pioneer habitats (Stebbins 1970) and one indicator of success is that *T. submersa* is one of the most widespread *Trithuria* species. Pioneer habitats are not typically colonized by obligately outcrossing *T. austinensis*. On the other hand, *T. bibracteata* is considered to be a pioneer species, and although its breeding system is unknown, it has bisexual reproductive units like *T. submersa*. Understanding the significance of the great reproductive diversity in *Trithuria* in the context of early angiosperm evolution will certainly require a greater appreciation of the intimate connection between their reproductive biology and their natural ecological settings.

Conclusion

Nymphaeales is thought to be ancestrally aquatic, perennial and probably insect pollinated. In contrast, most species of *Trithuria* are annuals and inhabit seasonal wetlands. Among these, *T. submersa* has evolved to colonize disturbed, early-successional habitats. Protogyny and prolonged stigma receptivity may have initially promoted outcrossing with reproductive assurance via delayed selfing. The overlapping male and female phases now seem

to function primarily to ensure autonomous self-pollination and high seed set in an unpredictable and heterogeneous pollination environment.

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APPENDIX 3

Table 3.1: Developmental sequences of reproductive units in *Trithuria submersa*.

Developmental Stage	Stamen	Carpel
1	Filament not elongated, anther not above carpels	Stigmatic hairs short (mean of longest hair = $253 \pm 58 \mu\text{m}$; $n = 7$)
2	Filament partially elongated, anther beginning to emerge above carpel body, but not above stigmatic hairs	At least one stigmatic hair per carpel has begun elongating
3	Filament fully elongated, anther above carpel tops and many of the stigmatic hairs. Anther not dehiscent.	Typically two stigmatic hairs per carpel have elongated (mean = $463 \mu\text{m} \pm 172$; $n = 15$)
4	Filament fully elongated as in stage 3. Anther dehiscent.	Stigmatic hairs as in stage 3, some hairs bent with collapsed cells
5	Anther abscised.	Stigmatic hair length as in stage 3. (mean = $480 \mu\text{m} \pm 239$; $n = 10$).

Figure 3.1. Morphology and development of *Trithuria submersa*. (A) Habit of *Trithuria submersa* at Kulunilup Swamp, Kulunilup Nature Reserve. *T. submersa* plants are red (bright green leaves are immature *Goodenia claytoniacea*). (B) Reproductive units at various stages of emergence; completely submerged (stage 1; arrow), at the water level creating a depression (stage 2; arrowhead), and fully emergent (stage 3; asterisk). bar = 5 mm. (C) One reproductive unit showing four bracts surrounding carpels with elongating stigmatic hairs and a single central stamen comprised of a partially elongated filament and non-dehiscent anther (developmental stage 2). Note that one stigmatic hair is typically longer than the others. bar = 500 μm . (D) One reproductive unit with two dehiscent anthers (developmental stage 4). Some stigmatic hairs have received pollen and cells have collapsed, causing stigmatic hairs to bend (arrowhead). bar = 500 μm . (E) A single carpel from a stage 1 reproductive unit. Stigmatic hairs are short and cell length is much greater along the axis perpendicular to the stigmatic hair. bar = 100 μm . (F) A composite image (two focal planes) showing a single carpel from a mature reproductive unit (stage 4) with two elongated and one short stigmatic hair. Stigmatic cells have elongated and some have collapsed (arrowheads). Gray line indicates the border between images. bar = 100 μm . Abbreviations: A, anther; B, bract; C, carpel; F, filament; H, stigmatic hair; M, carpel mouth; O, ovule.

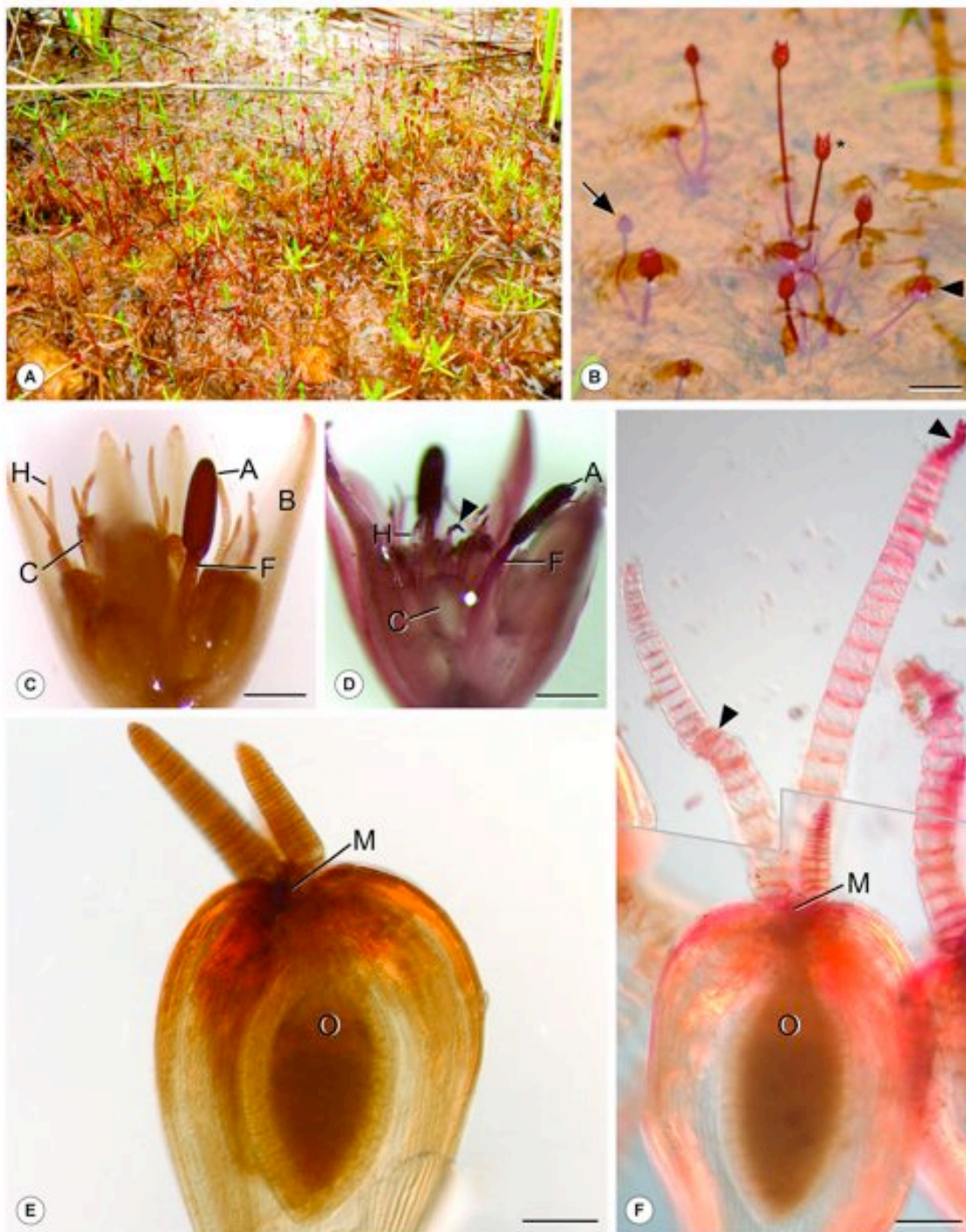


Figure 3.1

Figure 3.2. Pollination and pollen reception in *Trithuria submersa*. (A) Cross section of a dehiscent anther showing numerous endothecial bands (arrow), aerenchyma ground tissue, and the two stomia just beginning to open (one indicated by arrowhead). bar = 50 μ m. (B) Mature reproductive units of *Trithuria submersa* with fully elongated filaments supporting dehiscing anthers (developmental stage 4). The stigmatic hairs are visible above and between the reflexed bracts (arrowhead). bar = 5 mm. (C) Staminate flower of wind-pollinated *Brasenia schreberi* with dehiscing anthers supported by long, slender filaments. Compare to 2B. bar = 5 mm. (D) Receptive stigmatic hair (from a developmental stage 3 reproductive unit) with germinating pollen grains (two in plane of focus). bar = 25 μ m. (E) Carpel in which cross-pollination was prohibited, with self-pollen tubes successfully reaching the carpel mouth. bar = 150 μ m. Abbreviations: A, anther; F, filament; L, aerenchyma; M, carpel mouth; H, stigmatic hair; P, peduncle; PG, pollen grain; PT, pollen tube. Stains: toluidine blue O (A), aniline blue (E).

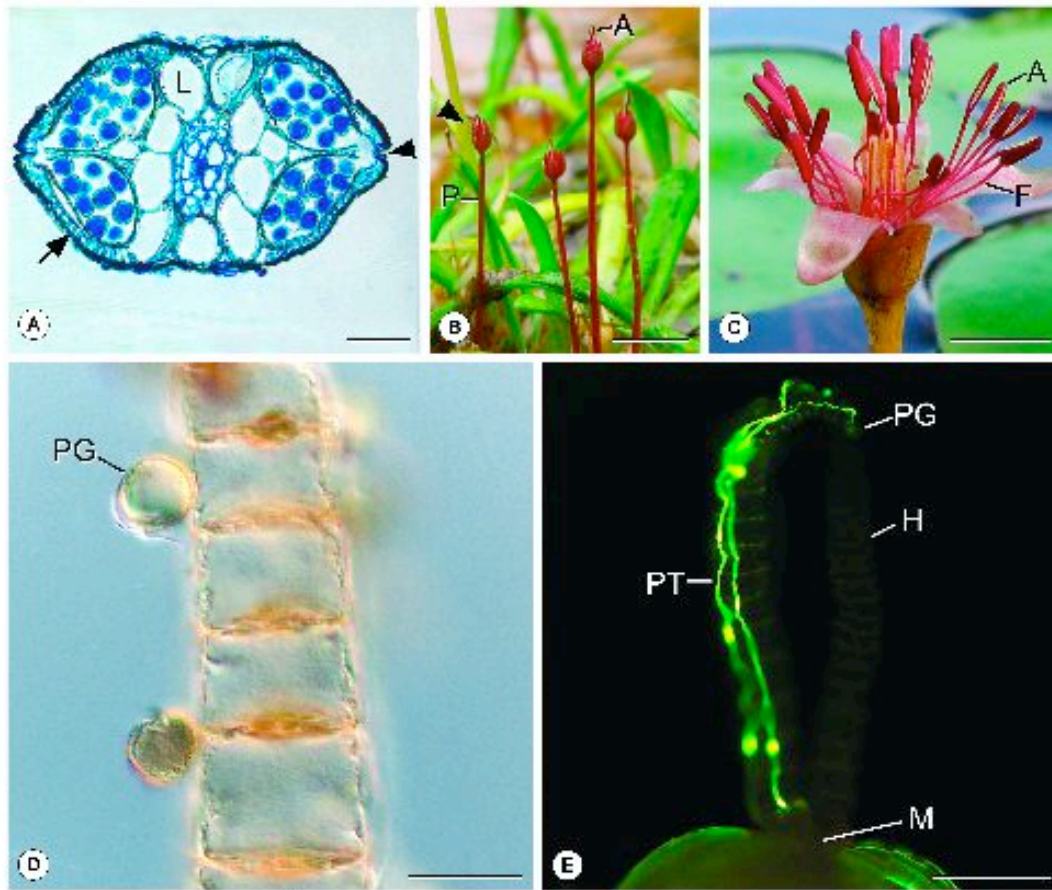


Figure 3.2

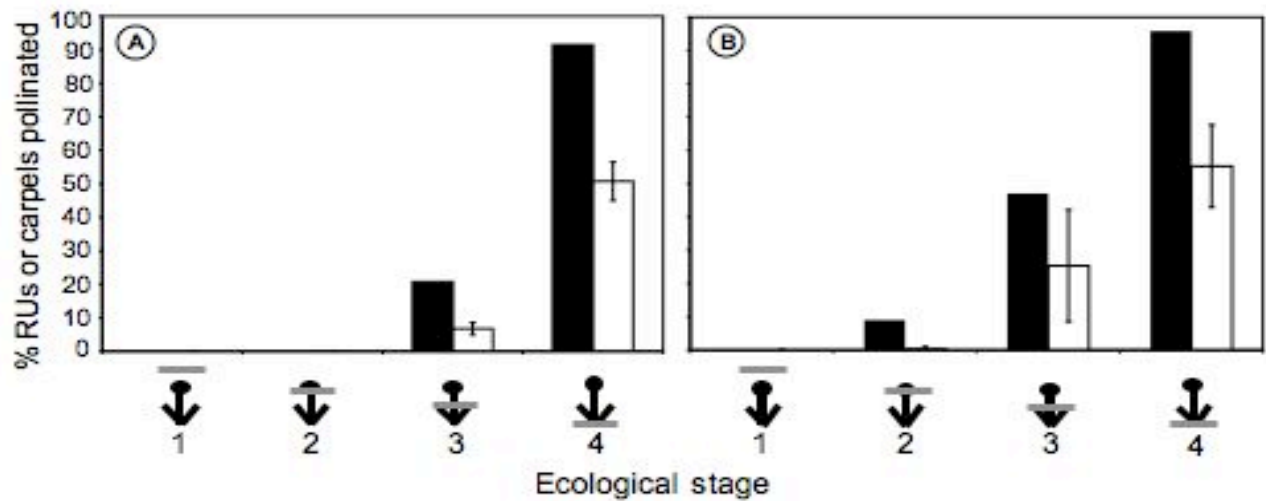


Figure 3.3. Natural pollen reception by ecological stage of *Trithuria submersa*. Pollen reception occurs only in partially or fully emergent reproductive units. The percentage of reproductive units (black) or carpels per reproductive unit (white) that were naturally pollinated at each of the four ecological stages (1-4) is shown for each population. (A) Kulunilup Swamp (2008; $n = 25, 25, 82, 35$). (B) Frying Pan Swamp (2009; $n = 15, 11, 17, 13$). On the x-axis, the position of the water level (gray bar) is represented relative to the reproductive unit (black circles). Error bars = 95 % CI

Figure 3.4. Natural pollen reception by stamen developmental stage of *Trithuria submersa* at Kulunilup Swamp. Outcross pollen reception can occur during developmental stages 1-3, whereas potential for self pollination occurs during stages 4 and 5 (grey background). Developmental stages are categorized by the position of non-dehiscent anthers (black ovals) or dehiscent anthers (grey ovals) relative to that of the carpels (white ovals). (A) Percent reproductive units (black) and carpels per reproductive unit (white) that received pollen at each developmental stage (1-5). The percentage of carpels pollinated is significantly higher in stages 4 and 5 than in stages 1-3 (ANOVA/Games-Howell: $F = 32.32$, $df = 4$, $p < 0.0001$). (B) Pollen load per carpel in emergent reproductive units at each developmental stage. Pollen loads are significantly higher in stages 4 and 5 than in stages 1-3 (ANOVA/Games-Howell test: $F = 11.58$, $df = 4$; $p < 0.0001$). Error bars = 95 % CI. $n = 15, 28, 28, 18, 22$, respectively. Submerged and water level reproductive units (ecological stages 1 and 2) were excluded from analyses of pollen reception by developmental stage.

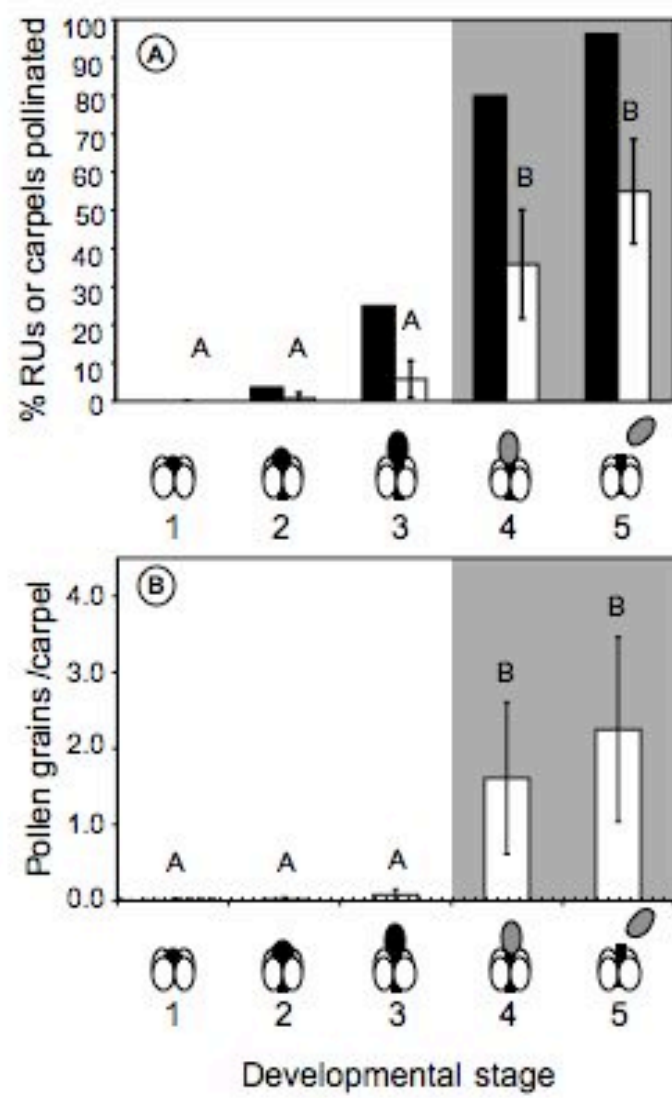


Figure 3.4

Figure 3.5. Natural pollen reception by stamen developmental stage in *Trithuria submersa* at Frying Pan Swamp. Reproductive units with dehiscent anthers could have received autonomous self pollen, as well as geitonogamous-self and outcross pollen (grey background), whereas those without dehiscent anthers could only have received outcross or geitonogamous-self pollen (white background). (A) Percent of reproductive units (black bars) or carpels per reproductive unit (white bars) that received pollen (Wilcoxon Rank Sums: $\chi^2 = 30.21$; $df = 1$; $***p < 0.0001$). (B) Pollen load per carpel in emergent reproductive units pre- or post anther dehiscence (Wilcoxon Rank Sums: $\chi^2 = 30.20$; $df = 1$; $***p < 0.0001$). Error bars = 95 % CI. $n = 18, 23$, respectively. Submerged reproductive units (ecological stage 1) were excluded from this analysis.

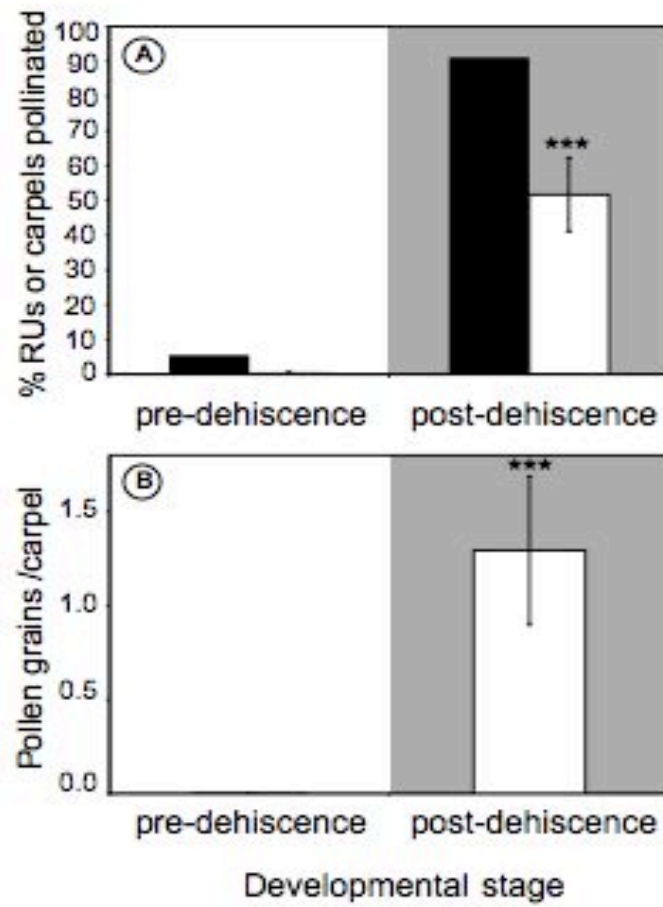


Figure 3.5

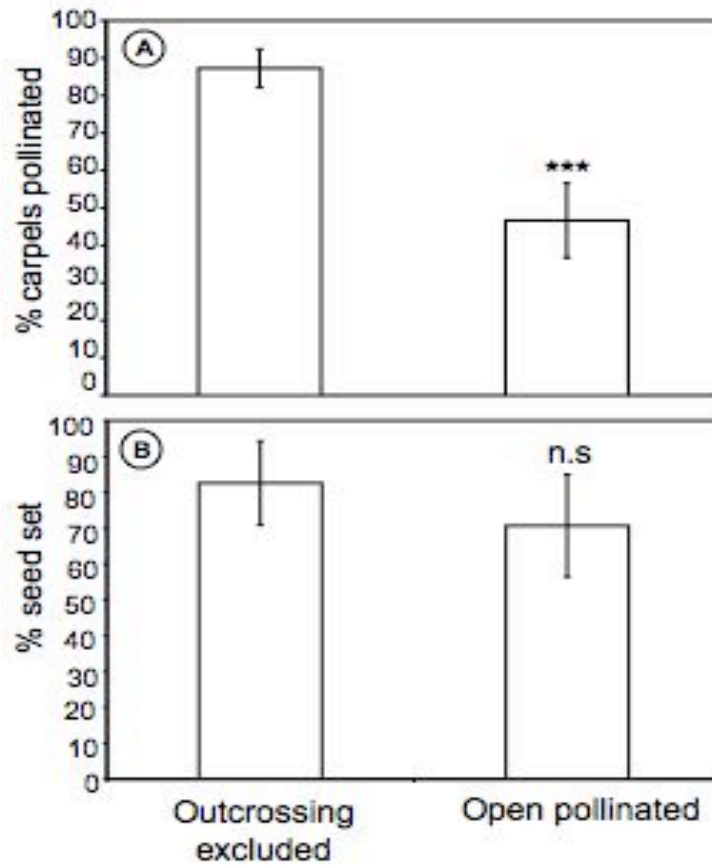


Figure 3.6. Self-pollen reception by caged and uncaged plants. (A) The percentage of carpels per reproductive unit that received pollen when cross-pollination was either experimentally excluded to allow only autonomous self-pollination ($n = 12$) or allowed to occur naturally ($n = 46$). Treatments were significantly different (Wilcoxon Rank Sums: $\chi^2 = 14.82$; $df = 1$; $***p < 0.0001$). (B) Seed set in reproductive units in which cross-pollination was experimentally excluded ($n = 18$) or not ($n = 14$). Treatments were not significantly different (Wilcoxon Rank Sums: $\chi^2 = 3.40$; $df = 1$; $p = .065$). Error bars = 95 % CI.

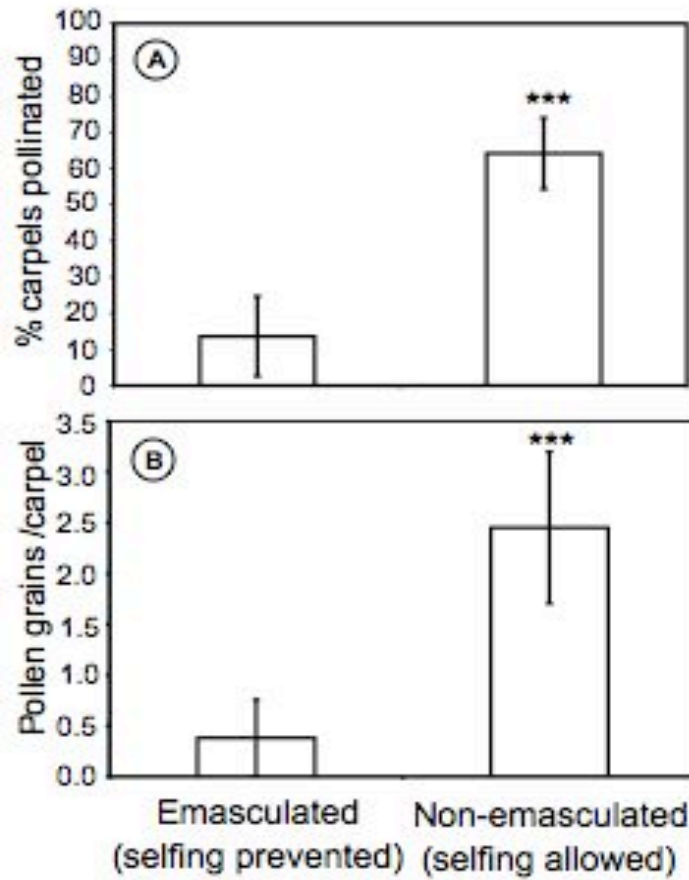


Figure 3.7. Outcross pollination in emasculated reproductive units. (A) The percent of carpels per reproductive unit that received pollen when self pollination was prevented by emasculation ($n = 23$) compared to untreated reproductive units in which self-pollination could occur ($n = 23$). Treatments were significantly different ($t = 7.07$, $df = 44$, $***p < 0.0001$). (B) Pollen load per carpel in emasculated reproductive units ($n = 23$) compared to open-pollinated reproductive units ($n = 23$). Treatments were significantly different (unequal variance t -test: $t' = 5.12$, $df = 32.42$, $***p < 0.0001$). Error bars = 95 % CI.

CHAPTER IV: POST-POLLINATION BIOLOGY IN *TRITHURIA* (HYDATELLACEAE):
CONSEQUENCES OF BREEDING SYSTEM DIVERGENCE.

This chapter is a modified version of an original research article to be submitted for publication by M.L. Taylor and J.H. Williams.

In the following chapter, the words “we” and “our” refer to my co-author and me. My contributions to this paper include (1) formation of the original hypotheses (2) development of experimental pollination techniques and completion of all field experiments (3) preparation of materials to secure funding and permits (4) completion of all microscopical analyses (4) construction of figures, and (5) all of the writing.

ABSTRACT

Breeding system, reproductive morphology, and pollen tube growth traits are closely linked. However, relatively little is known about the consequences of breeding system evolution on post-pollination development, particularly in early-divergent angiosperm lineages. *Trithuria* (Hydatellaceae) is unique among basal angiosperms in that it exhibits considerable variation in sexual system and breeding system. To determine the consequences of breeding system divergence on pollen tube growth and other reproductive traits, post-pollination development was investigated in three Western Australian *Trithuria* species (*T. austinensis*, *T. australis*, and *T. submersa*). These species exhibit dioecy, monoecy, and hermaphroditism, respectively, and at least two different breeding systems (*T. austinensis* = outcrossing; *T. submersa* = selfing). In the species studied, the time to fertilization was < 1 h, pollen tube pathways were short (*T. submersa* = 0.48; *T. austinensis* = 1.95 mm) and pollen tube development was rapid. Outcrossing *T. austinensis* exhibited more male investment, slower pollen germination (15-45 vs. 5-15 min), faster pollen tube growth rates (499 vs. 321 $\mu\text{m/hr}$), and higher pollen tube attrition ($\sim 70\%$ vs. $< 10\%$) than selfing *T. submersa*. *Trithuria australis* exhibits selfing and both low pollen production (350 grains/anther) and a low P/O ratio (32:1), typical of plants that experience high levels of selfing. The patterns of divergence in post-pollination biology observed in *Trithuria* matched theoretical predictions based on experiments in derived angiosperms. This is evidence that post-pollination development responds to similar selective forces across the angiosperm phylogeny.

INTRODUCTION

In seed plants, pollination and fertilization are separated by a life history stage known as the progamic phase. In angiosperms, the progamic phase takes place within the closed carpel and involves a set of specific interactions among the male gametophyte, carpel, and female gametophyte that determine the success of male gametes (Cresti *et al.* 1992; Herrero and Arbeloa 1989; Williams *et al.* 1999; de Graaf *et al.* 2001; Herrero 2000, 2003). There is tremendous variation in the structures and ontogenies involved in the progamic phase and the selective forces that shape this diversity are of considerable interest in studies of angiosperm evolution (Maheshwari 1950; Mulcahy and Mulcahy 1987; Williams 2008).

Progamic phase development is intimately linked with breeding system (e.g. Barrett *et al.* 1996; Mazer *et al.* 2010). Outcrossing results in greater genetic diversity of stigmatic pollen loads and a greater opportunity for pollen competition than selfing (Willson and Burley 1983; Mulcahy and Mulcahy 1987; Mazer *et al.* 2010). This is hypothesized to drive the evolution of longer styles (Goodwillie and Ness 2005), faster pollen tube growth rates (Kerwin and Smith-Huerta 2000; Smith-Huerta 1996), and greater pollen tube attrition (Plitmann 1993, 1994; Smith-Huerta 1997), as well as to have an affect on pollen germination rate (Plitmann and Levine 1990; Smith-Huerta 1996; Kerwin and Smith-Huerta 2000). In addition, outcrossing is associated with greater investment in male reproductive function, including larger pollen and larger pollen to ovule ratios (P/O; Cruden 1977; Barrett *et al.* 1996). Mazer *et al.* (2010) have recently developed predictions for the trajectory of male gametophyte evolution due to breeding system divergence.

Novel angiosperm progamic phase traits, including the closed carpel, accelerated pollen tube growth, and double fertilization, have been hypothesized to play a critical role in

angiosperm diversification (Stebbins 1974; Doyle 1978; Doyle and Donohue 1986; Williams 2008). The evolutionary success of flowering plants has also been attributed to their increased ability to discriminate between potential mates prior to fertilization, which is an component of breeding system (Whitehouse 1950; Stebbins 1957). Despite the putative importance of these traits in angiosperm evolution, relatively few studies have explicitly investigated the consequences of breeding system evolution on progamic phase development (but see Mazer *et al.* 2010), or addressed these phenomena in early-divergent angiosperm lineages.

Trithuria (Hydatellaceae) has recently been placed in the ancient angiosperm lineage Nymphaeales (water lilies; Saarela *et al.* 2007), which originated from the basalmost or next most basal node of the extant angiosperm phylogenetic tree (e.g. Qiu *et al.* 1999, 2006; Löhne and Borsch 2005) and is well represented in the oldest angiosperm macro fossil record (Friis *et al.* 2001, 2003, 2009, 2010; Wang and Dilcher 2006; Mohr *et al.* 2008; Taylor *et al.* 2008). *Trithuria* is of particular interest in evolutionary studies because it exhibits several unique reproductive traits, including traits associated with cotyledon morphology (Sokoloff *et al.* 2008b), seed provisioning strategy (Friedman 2008), and reproductive structure morphology (Rudall *et al.* 2009; Rudall and Bateman 2010). Reproductive structures of *Trithuria* are composed of bracts surrounding stamens and/or carpels which, because of their uncertain homology, are typically referred to as reproductive units (Rudall *et al.* 2007, 2009). *Trithuria* may also represent an extreme in progamic phase modification because of its highly reduced size and shorter overall life history than any other water lily – it is the only basal angiosperm known to possess an annual life cycle (Sokoloff *et al.* 2008a; Taylor *et al.* 2010).

Trithuria exhibits great diversity in sexual system: four of the twelve species of *Trithuria* typically have bisexual reproductive units, four are dioecious, and four are typically cosexual (monoecious), with unisexual male and female reproductive units produced on the same plant (Yadav and Janarthanam 1995; Sokoloff *et al.* 2008a). Among early-divergent angiosperm lineages, such variation in sexual system is rare. Most other basal angiosperms, including all of Nymphaeaceae and Cabombaceae, and many Austrobaileyales (*Austrobaileya*, *Illicium*, *Trimenia*) exhibit bisexual flowers, whereas other taxa, including *Amborella*, *Hedyosmum*, *Ascarina*, and Myristicaceae (Magnoliales) are dioecious (Endress 2001, 2010). Variation in sexual system in *Trithuria* has set the stage for divergence in breeding system. The dioecious species, including *T. austinensis*, are almost certainly obligately outcrossing, but at least one of the bisexual species, *T. submersa*, is primarily selfing (Taylor *et al.* 2010).

The goal of this study was to investigate the progamic phase in *Trithuria*. We describe life history, breeding system, and aspects of pollen tube growth in three *Trithuria* species that exhibit the range of diversity in sexuality found in *Trithuria*: *T. austinensis*, *T. submersa* and cosexual (monoecious) *T. australis*. For *T. austinensis* and *T. submersa*, we also comprehensively describe post-pollination development, documenting timing of pollen reception, germination and ovule entry, as well as pollen tube growth rates. We discuss how evolution in sexual system and breeding system is reflected in progamic phase development in *Trithuria* and address this in the context of other basal angiosperms.

METHODS

Field sites

The focal *Trithuria* species occur in ephemeral wetlands that are characterized by standing water in the wet season, a brief period of water drawdown lasting a few days to a few weeks, and a period of complete desiccation during the dry season (Hill *et al.* 1996). *Trithuria* seeds germinate and plants grow vegetatively while completely submerged. As the water level drops, plants become exposed and must complete flowering and fruit-set before the habitat dries out completely (Taylor *et al.* 2010).

Experimental pollinations and collections of *Trithuria austinensis*, *T. australis*, and *T. submersa* were undertaken in southwest Western Australia (shire of Manjimup) during November-December of 2008 and 2009. Laboratory work was conducted at the Department of Environment and Conservation Science Division facility in Manjimup, WA and at the University of Tennessee, Knoxville. Voucher specimens have been deposited in the University of Tennessee Herbarium (TENN). Experimental pollinations and collections were conducted at Branchinella Lake (*T. austinensis* D.D.Sokoloff, Remizowa, T.D.Macfarl. & Rudall; 34° 21' S; 116° 43' E; Figure 4.1A; all figures referenced in this chapter are found in Appendix 4), Frying Pan Swamp (*T. australis* (Diels) D.D.Sokoloff, Remizowa, T.D.Macfarl. & Rudall and *T. submersa* Hook.f; 34°16' S, 116° 42' E; Figure 4.1B), and Kulunilup Swamp, Kulunilup Nature Reserve (*T. submersa*; 34°19' S, 116°46' E; Figure 4.1C). All three localities are within 11 km of each other.

Reproductive morphology

Plants of all three species were collected in mass and fixed whole in FAA (2:1:10 40 % formaldehyde, glacial acetic acid, 95 % ethanol) for 24 h and stored in 70 % ethanol for analysis of reproductive morphology. For *T. australis*, correlations of plant size and the proportion of female reproductive units were measured using Pearson's correlation test in SPSS 16.0 (SPSS Inc., Chicago, IL; $n = 20$ plants). In a subset of 12 plants, the pollen production per anther was measured by macerating one anther per plant in 20 μ l of 1 % polyethylene glycol in 95 % ethanol (PEG). The pollen mixture was vortexed for 30 s, placed on a drop of glycerine on a glass slide, and covered. The entire cover slip was scanned and all pollen grains were counted. The number of pollen grains per anther was used to calculate the pollen production and P/O ratio per reproductive unit and per plant. The P/O ratio of all plants were averaged to determine the mean P/O ratio. All measures of variance in the text are standard deviations unless otherwise noted.

Pollen reception

To determine when plants received pollen and the size of natural pollen loads in *T. austinensis*, reproductive units were haphazardly collected in mass from four ecological stages: (1) plants entirely *submerged*, (2) plants submerged, but reproductive units *newly emergent* with 50-75 % of the unit above the water line (Figure 4.2A), (3) plants submerged but reproductive units *fully emergent* (Figure 4.2A), and (4) whole plants completely emergent (see also Chapter III). *Trithuria australis* reproductive units were haphazardly collected in mass for the same purpose. However, due to extremely rapid water drawdown and an overall 'flattened'

plant morphology, plants collected represented only two ecological stages (1) whole plants submerged and (2) whole plants emergent. Reproductive units were fixed in FAA for 24 h and stored in 70 % ethanol. Carpels were dissected out of reproductive units stained 4-8 h with 0.1 % aniline blue and viewed under UV light with a Zeiss (Carl Zeiss, Oberkochen, Germany) Axioplan II compound microscope for visualization of pollen grains and tubes.

A caging experiment was conducted with *T. australis* to test for within plant self-pollination. 15 submerged plants were covered with pollen exclusion cages constructed from clear plastic cups staked into the ground with flexible wire. Plants were collected three days after plant emergence, fixed in FAA, and stored in ethanol. Carpels were dissected out, stained with aniline blue, and viewed under UV light for visualization of pollen grains and tubes.

Pollen tube development

Experimental pollinations were conducted in both *T. austinensis* and *T. submersa* to document the timing of pollen tube developmental events. Submerged female plants of *T. austinensis* were covered with pollen exclusion cages to prevent external pollination. Emergent anthers were collected and stored briefly (< 1 h) on filter paper to promote anther opening. As female reproductive units became emergent and stigmatic hairs mature, stigmatic hairs were gently brushed with dehiscing anthers. Over the course of the study period, 243 reproductive units were pollinated between 930 and 1400 h and collected at one of the following time points: 5, 10, 15, 20, 30, 45 min or 1, 1.5, 2, 2.5, or 3 h after pollination.

Trithuria submersa anthers were removed from immature reproductive units with closed bracts and plants were covered until mature. Emergent anthers were collected from nearby plants

and briefly stored until fully open (< 1 h), then brushed gently over the mature stigmatic hairs.

115 reproductive units were pollinated between 830 and 1130 h over the course of the study and collected at 5, 15, 30 min or at 1, 1.5, 2, or 3 h after pollination. Naturally pollinated reproductive units of all species were also collected.

All reproductive units collected were fixed in FAA or 3:1 (95 % ethanol: glacial acetic acid) for 24 h and stored in 70 % ethanol. Carpels were removed, stained 4-8 h with aniline blue, rinsed in distilled water, stained with 4',6-diamidino-2-phenylindole (DAPI) for 4 h, and viewed under UV light for simultaneous viewing of callose and nuclei. For histological analysis, carpels were dehydrated to 95 % EtOH, then infiltrated and embedded in JB-4 polymer (Polysciences, Inc., Warrington, PA, USA) following standard protocols. Serial-sections (5 μ M) were cut with a Sorvall Dupont JB-4 microtome (Newtown, Connecticut, USA), using glass knives, mounted on glass slides and stained with 0.1 % toluidine blue O (TBO) for general histology, 0.1 % aniline blue for visualization of callose, or 0.01 % Auramine O for visualization of the cuticle. Specimens were imaged with a Zeiss Axioplan 2 compound microscope.

Average sustained pollen tube growth rate was determined for each pollen tube by dividing the length of the pollen tube by the time since pollination. In *T. austinensis*, 15 min was subtracted from the time since pollination to account for time to pollen germination (see “Results”). Rates were compared with a one-way ANOVA conducted in SPSS 16.0.

RESULTS

Reproductive morphology and ecology

Trithuria austinensis – Plants grew at very high population densities in a monoculture carpeting the entire bottom of Branchinella Lake. The habitat is very open due to little surrounding vegetation and no canopy. Water evaporated from the lake margin so that large patches of adjacent *T. austinensis* plants became emergent at once (Figure 4.1A). However, not all reproductive units in a plant emerged simultaneously (Figure 4.2A).

Plants of *T. austinensis* are dioecious. Multiple reproductive units were supported by long peduncles (9.0 ± 2.9 mm; $n = 15$; Figures 4.2A-B). Female plants produced more reproductive units than male plants (Table 4.1). Female reproductive units measured 3.3 ± 0.3 mm from the base to the tip of the bracts and contained an average of 14.8 uniovulate carpels (Table 4.1) that each exhibited 5.5 uniseriate stigmatic hairs (Table 2; Figures 4.2D, 4.3B).

Anthers or carpels were completely enclosed in bracts of immature reproductive units. As female reproductive units matured, the stigmatic hairs elongated and emerged between the bracts of female reproductive units (Figure 4.2D). Mature stigmatic hairs were 2.06 mm long (maximum length = 3.89 mm; Table 4.2) and extended far beyond the tips of the bracts (Figure 4.2D). As male reproductive units matured, filaments elongated and pushed anthers out of the bracts which did not ever strongly reflex (Figures 4.2B, E). Male reproductive units contained 7.9 anthers that matured consecutively and produced over 3500 pollen grains each (Table 4.1; Taylor *et al.* 2010). Anthers abscised after they dehisced, leaving behind a persistent filament (Figure 4.2E). The pollen to ovule ratio was 1569 at the reproductive unit level (pollen produced

by male units to ovules produced by female units; Taylor *et al.* 2010) and 1138 at the plant level (pollen produced in male plants / ovules produced in female plants; Table 4.1).

Plants of *T. austinensis* did not receive pollen while submerged (Table 4.3) but began receiving pollen that successfully germinated as soon as stigmatic hairs emerged above the water surface (ecological stage 2; Table 4.3). Pollen loads increased over 3 times in stage 3 (fully emergent reproductive units) and by stage 4 (long emergent units), over 86 % of carpels per reproductive unit had received pollen (Table 4.3). The average pollen load in stage 4 plants was just over 29 grains per reproductive unit or 2.1 pollen grains per ovule (Table 4.2). *Trithuria austinensis* anthers opened via a two longitudinal slits that extended the entire length of the anther (Figure 4.2B) and we observed pollen being released directly into the air. We did not observe any insects landing on reproductive units or any anthers with signs of pollinivory.

Trithuria australis - Plants of *T. australis* grew at medium to high densities throughout Frying Pan Swamp (Figure 4.1B). This locality was less open than Branchinella Lake and *T. australis* plants grew intermixed with plants of slightly taller *Centrolepis*, as well as other vegetation.

Trithuria australis plants produced both male and female reproductive units aggregated together in a central head, with male units located in the center (Figures 4.2I-J). Reproductive units were either sessile or borne on very short peduncles (0.4 ± 0.3 ; $n = 15$), giving the plant a distinctly flattened morphology. Frying Pan Swamp was fairly flat, so plants near each other became emergent at approximately the same time. The sessile reproductive units were exposed simultaneously, unlike those of *T. austinensis* or *T. submersa*.

Plants produced an average of 16.7 ± 7.7 reproductive units (range = 5-30). Plants continue to produce reproductive units while submerged and have been observed to produce over 240 reproductive units in years with delayed water drawdown (TD. Macfarlane 'pers. comm.'). $85.5 \% \pm 8.0 \%$ of reproductive units in plants were female (Table 4.1) and plant size (total number of reproductive units) had no effect on this proportion (Pearson's Correlation; $r = -0.037$; $p = 0.876$). Bracts of female reproductive units were 2.3 ± 0.2 mm long and enclosed 10.1 uniovulate carpels (Figure 4.2F; Table 4.1). Each carpel exhibited 5.7 stigmatic hairs that were 2.0 mm long at maturity (maximum length = 3.3 mm; Figure 4.3C; Table 4.2). Male reproductive units produced 6.9 anthers that developed consecutively and produced 350 pollen grains (Figure 4.2G; Table 4.1). Anthers abscised after dehiscing (Figure 4.2G). The pollen to ovule ratio was 248 within reproductive units and 32 within plants (Table 4.1).

In *Trithuria australis*, no pollen was evident on submerged reproductive units (Table 4.3) and none of these reproductive units was developmentally mature. Bracts were completely closed, filaments not elongated, and stigmatic hairs were very short. During the study season, the water rapidly evaporated from a few cm deep (still completely submerging plants), such that no plants with reproductive units at the water level were observed. In addition, no plants were collected in a female-only phase with mature stigmatic hairs but indehiscent anthers. Every emergent plant collected had reproductive units with at least one dehiscent anther. This is evidence that reproductive development occurs extremely quickly in *T. australis*.

In emergent plants, 75 % of carpels per reproductive unit had received pollen (Table 4.3) and pollen load per reproductive unit was 38, with an average of 3.2 pollen grains per ovule (Table 4.2). Pollen loads were likely a mixture of outcross and self-pollen. Plants caged to

prevent out-crossing had an equal percentage of pollinated carpels and pollen loads higher than those observed in uncaged plants (Table. 4.2). Self-pollen produced tubes that entered the carpel mouth.

Trithuria submersa –*T. submersa* was investigated in two populations: Frying Pan Swamp and Kulunilup Swamp. Adjacent *T. submersa* plants became emergent at different times and the surrounding vegetation created a closed environment (Figure 4.1C; Taylor *et al.* 2010).

Trithuria submersa plants produced bisexual reproductive units that were borne on long peduncles (26.7 ± 6.8 mm; $n = 15$; Figures 4.2C, H; Table 4.1). Bracts measured 1.8 ± 0.3 mm in length and enclosed 19.3 carpels and 1.1 anthers (Figure 4.2H; Table 4.1). Carpels exhibited exactly three uniseriate stigmatic hairs that radiated from the carpel mouth and were 0.56 mm long at maturity (maximum length = 1.05 mm; Figure 4.3A; Table 4.2). Anthers each produced 426 pollen grains and the pollen to ovule ratio was 24 (Table 4.1; Taylor *et al.* 2010).

Plants of *T. submersa* do experience a brief female phase in which only outcross pollen is received, but resulting pollen loads are quite small. The majority of pollen received on stigmatic hairs of *T. submersa* is self-pollen (Taylor *et al.* 2010).

Pollen and pollen germination

Pollen grains of all three species were small (< 25 μ m in diameter), nearly spherical, and monosulcate (Figures 4.4F-I). Pollen was bicellular in dehiscing anthers of all three species and on receptive stigmas of *T. submersa* and *T. austinensis*. Pollen germinated along the entire length of the stigmatic hair and the aperture did not have to be in direct contact with the stigmatic

surface for germination to occur (Figures 4.4F-I). At germination, the inner layer of the pollen wall fluoresced following aniline blue staining, indicating the presence of callose (1,3- β -glucan; Stone and Clarke 1992; Figure 4.4G)

Trithuria austinensis pollen first germinated between 10 and 15 min after pollination. No individuals collected at 5 or 10 min exhibited germination, compared to 26 % of individuals collected at 15 min. Percent germination remained relatively low at 20 and 30 min after pollination (24 % and 19 %), but rose to 44 – 48 % between 45 min and 2 h after pollination. *T. submersa* pollen germinated even earlier, with 13 % of individuals exhibiting germination at 5 min after pollination. Percent germination rose to 33 % after 15 min and afterward, was between 25 and 38 %. Microscopical evaluation in both species revealed that stigmatic hairs in several individuals were very short, and thus likely not receptive at the time of hand-pollination (Taylor *et al.* 2010). This lack of receptivity probably accounts for the rather low (< 50 %) maximum percent pollen germination observed.

Pollen tube growth and structure of the pollen tube pathway

The general pattern of pollen tube growth and structure of the pollen tube pathway was the same in all three species. However, the length of both the potential pollen tube pathway and the distance that pollen tubes actually traveled differed considerably (Figure 4.3; Table 4.4). *T. austinensis* pollen tubes grew between 0.33 to 3.24 mm to the carpel mouth (mean = 1.92 mm), *T. submersa* pollen tubes grew between 0.10 and 1.00 mm (mean = 0.48 mm), and *T. australis* pollen tubes grew 1.07 to 2.54 mm (average = 1.95 mm).

Pollen tubes emerged from the aperture and grew around the grain to reach the surface of the stigmatic hair (Figures 4.4F-I). Pollen tubes commonly branched shortly after germination, at the place where the tube first came into contact with the stigmatic surface (Figure 4.4I). Branching was not observed at any other point and one branch was always much shorter than the other.

Upon reaching the stigmatic surface, pollen tubes penetrated the cuticle of the stigmatic hair cell (Figure 4.4H). Following auramine O staining, the stigmatic hair wall fluoresced brightly, indicating the presence of a lipid cuticle (Heslop-Harrison 1977; Figures 4.4J-K). The stigmatic cell wall was distinctly bi-layered, with the inner opaque layer continuous beneath pollen tubes associated with the stigmatic hair (Figures 4.4K-M). The outer layer, which is translucent under light but stains strongly for cuticle, became thickened at pollen tube edge and stretched thinly over the pollen tube wall (Figures 4.4J-K). Callose was present in the pollen tube wall (Figures 4.4E, I, M) and occasionally in the stigmatic hair cell walls (Figure 4.4E). Most pollen tubes walls maintained a round shape in cross-section (Figures 4.4H, J-M), even near the growing tip.

Pollen tubes never pulled away from the stigmatic hairs (Figures 4.4D-E). Although the ends of stigmatic hairs in *T. austinensis* and *T. australis* became entangled and had to be physically pulled apart during dissections, pollen tubes never crossed from one stigmatic hair to another (Figure 4.4C) and loose pollen tubes were never observed in dissected material. Pollen tubes did not grow laterally around stigmatic hairs (Figures 4.4A-C), but often grew away from the carpel mouth and over the top of the hair before continuing growth toward the carpel mouth.

In all three species, multiple stigmatic hairs per carpel often supported pollen tubes and each hair often supported multiple pollen grains.

Pollen tube walls were strongly callosic near the grain (Figures 4.4G, I) and along the length of the pollen tube (Figure 4E), but lacked callose at the growing tip (Figure 4.4G). The inner layer of the pollen wall continued to fluoresce, as well. Pollen tubes were narrow, $4.3 \pm 0.9 \mu\text{m}$ in *T. austinensis* ($n = 98$), $3.9 \pm 0.7 \mu\text{m}$ in *T. submersa* ($n = 38$), and $4.0 \pm 0.7 \mu\text{m}$ in *T. australis* ($n = 28$). Numerous callose plugs formed in each species through thickening of internal pollen tube walls (Figures 4.5A-B, E, G).

Upon reaching the base of the stigma, pollen tubes turned at sharp angles and grew laterally to enter the carpel mouth (Figures 4.5A, C). Once pollen tubes entered the open carpel mouth, they grew through a short, extremely narrow canal that extends through a differentiated region of carpel cells. The cells that form the transmitting tract are small, slightly elongated, and more densely cytoplasmic than those of the surrounding carpel (Figures 4.5H-M). In *T. submersa*, multiple pollen tubes were often observed entering the carpel mouth (Figure 4.5A). This occurred less frequently in *T. austinensis*.

Trithuria austinensis carpels have a short elongated neck and the transmitting tract is 5-10 cell layers thick (Figures 4.5J, M), whereas *T. submersa* lacks this elongated region and the transmitting tract is less than 5 cells thick (Figure 4.5K). In both species, the canal is very narrow (Figures 4.5H-I) and we never observed more than one pollen tube growing through it.

As soon as pollen tubes reached the ovary they entered the micropyle (Figure 4.5J). Pollen tubes growing freely in the ovarian cavity were never observed. Pollen tubes grew through a region of elongated cells in the inner integument that likely physically directs them to

the nucellus, which is 2-3 layers thick (Figure 4.5J). At pollination, the four-celled/ four-nucleate female gametophyte is mature, with the single polar nucleus oriented near the micropylar pole (Figure 4.5J).

Developmental timing of T. austinensis and T. submersa pollen tubes

Callose plugs – In *T. austinensis*, callose plugs were first observed at 45 min after pollination, but only in the one pollen tube that had reached an ovule. At 1 h after pollination, callose plugs were present in 39 % of pollen tubes, but were typically few per tube. Callose plugs were more abundant at 1.5 h (in 43 % of pollen tubes) and 2 h after pollination (88 %). All pollen tube that had reached the carpel mouth exhibited callose plugs. The first callose plug developed $205.9 \pm 85.2 \mu\text{m}$ from the grain and plugs were fairly regularly spaced along the length of the tube, $153.8 \pm 55.1 \mu\text{m}$ apart on average (range 58 – 302 μm).

In *T. submersa*, callose plugs appeared as early as 30 min after pollination and were present in every tube by 2 h after pollination, as well as in every tube that had reached an ovule. Callose plugs are much closer together in *T. submersa*, first forming $84.9 \pm 56.0 \mu\text{m}$ from the grain and occurring every $85.67 \pm 34.3 \mu\text{m}$ (range 33.5-181.3).

Pollen cell mitosis - Nuclei were not visible in every pollen tube observed in either species. Mitotic division of the generative cell occurred between 45 min and 2 h after pollination in *T. austinensis*. Sperm nuclei were never observed in pollen tubes before 1 h, whereas undivided generative cells and sperm nuclei (Figure 4.5D) were observed in pollen tubes at 1 and 1.5 h after pollination. At 2 h after pollination and after, only post-mitotic sperm nuclei were observed. In *T. submersa*, 2 sperm nuclei were observed in pollen tubes as early as 15 min after

pollination, but undivided generative cells were also observed in pollen tubes up to 1 h after pollination. Mitosis occurred while pollen tubes were growing along the stigmatic hair in both species.

Carpel and ovule entry – *Trithuria austinensis* pollen tubes first entered the carpel mouth at 30 min after pollination and reached an ovule at 45 min, although only one pollen tube (4.0 %) had entered the micropyle (Figure 4.6B). At 1 h, 6.5 % of pollen tubes had entered a micropyle and after 1.5 h, between 21 and 32 % of pollen tubes had reached an ovule (Figure 4.6B).

Trithuria submersa pollen tubes had first entered the carpel mouth at 15 min after pollination and by 30 min, 42.9 % of pollen tubes had entered the micropyle (Figure 4.6B). However, only one pollen tube (10.0 %) was observed entering an ovule at 1 h and only 42.3 % at 1.5 h. By 2 h after pollination, over 90 % of pollen tubes had entered an ovule (Figure 4.6B).

Pollen tube growth rate – Average growth rate in *T. austinensis* pollen tubes was $499.4 \pm 431.1 \mu\text{m/hr}$ (Figure 4.6A). However, pollen tubes that were successful in reaching a micropyle within 3 h grew over twice as fast (mean = $1046.7 \pm 604.5 \mu\text{m/hr}$; Figure 4.6A). Average pollen tube growth rate in *T. submersa* was $321.1 \pm 281.0 \mu\text{m/hr}$ over the first 2 h, the period in which most pollen tubes reach the ovule (Figure 4.6).

DISCUSSION

Trithuria austinensis, *T. australis*, and *T. submersa* exhibit different sexual and breeding systems and this, in turn, is associated with divergence in reproductive morphology and progamic phase development. Here we first discuss the evidence for divergent breeding systems

in these species and concomitant modifications in resource allocation and reproductive morphology. We then describe pollen tube development and progamic phase timing in *Trithuria* species and discuss reproductive traits in light of breeding system differences. Finally, we address the evolution of the progamic phase in *Trithuria* in the context of the other water lilies and other basal angiosperms.

Breeding system in T. austinensis and T. submersa

Trithuria submersa is a primarily selfing species (Taylor *et al.* 2010). Self-pollen loads and seed set are high, whereas out-cross only pollen loads are extremely small. Plants do experience a short female-only phase and any outcrossing is accomplished through wind-pollination (Taylor *et al.* 2010).

Trithuria austinensis is dioecious, and therefore, is very likely obligately outcrossing. In this study, outcrossing was efficient in the *T. austinensis* population studied, with over 86 % of carpels per reproductive unit receiving pollen and having average pollen loads of two grains per ovule. Furthermore, *T. austinensis* exhibits traits characteristic of outcrossing wind-pollinated plants. Plants were densely packed in an open habitat with few barriers to wind-borne pollen and large numbers of plants became emergent and flowered in synchrony. Anthers opened via two large slits and were borne on flexible filaments, which likely facilitates anther emptying. Pollen was within the size range expected for efficient wind-pollination, was not sticky, and lacked significant ornamentation. In addition, carpels exhibited a relatively large stigmatic surface area, with a mean of 5.7 stigmatic hairs each (Whitehead 1969; Friedman and Barrett 2009).

We observed no evidence of insect visitation and reproductive units lacked morphological adaptations associated with insect pollination, such as a perianth. We consider it unlikely that insects play a significant role in pollination of *T. austinensis*.

Water was also a potential pollen vector in *T. austinensis* because stigmatic hairs elongated underwater and often floated on the water surface as reproductive units became emergent. However, submerged reproductive units received no pollen, indicating that pollination did not occur underwater. Reproductive units at the water level received much less pollen than those that were fully emergent and this indicates that pollen is, at the very least, not primarily transferred via the water surface.

Evidence for selfing in cosexual T. australis

Trithuria australis exhibits the potential for high levels of selfing. The caging experiment clearly demonstrates that high pollen loads (> 5 grains per ovule) can result from self-pollination and that self-pollen tubes successfully enter ovules. Furthermore, mass collected *T. australis* plants exhibited no female-only phase, suggesting that carpels have no opportunity to receive cross-pollen before self-pollen is available.

More work is needed to determine the proportion of selfing vs. outcrossing in *T. australis*, but these results indicate that whole plant morphology significantly affects breeding system in *T. australis*, and that this species may have evolved a primarily selfing breeding system. Cosexuality (monoecy) is hypothesized to evolve in some species as a mechanism to prevent selfing (Lloyd 1972; Charlesworth and Charlesworth 1978; Charlesworth 1993), but this is unlikely in *T. australis*, in which high levels of selfing can occur. Unisexual reproductive

structures are also hypothesized to evolve as a result of selection for more efficient pollen removal and receipt in a wind-pollinated system (Friedman and Barrett 2008a, 2008b) and we consider this to be more probable in *T. australis*.

Resource allocation

Trithuria austinensis is outcrossing, whereas *T. submersa*, and possibly *T. australis*, exhibit selfing (Taylor *et al.* 2010; this study). Sex allocation theory predicts that plants will allocate fewer resources to male function as higher levels of selfing evolves because fewer pollen grains are exported to outcross ovules and the fitness gain from pollen production is reduced (Charnov 1982; Brunet 1992). Thus, selfing populations or species should ultimately exhibit less male investment than related outcrossing ones (Charnov 1982; Barrett *et al.* 1996; Sato and Yahara 1999). Male investment in dioecious species has been shown to approximate or even underestimate, male investment in outcrossing, hermaphroditic species (Philbrick and Rieseberg 1994; Zunzunegui *et al.* 2006). As predicted, selfing *T. submersa* exhibited much less investment in male function than *T. austinensis* (Table 4.1). Compared to *T. austinensis*, plants of *T. submersa* produced fewer anthers per plant and fewer pollen grains per anther, resulting a 50-fold difference in pollen production per plant and a much smaller pollen to ovule ratio (Table 4.1). *Trithuria australis* exhibited male investment that was an order of magnitude smaller than that of *T. austinensis* and had a much smaller pollen to ovule ratio, as well (Table 4.1). Reduced male investment is additional evidence that *T. australis* maintains high levels of selfing.

Reproductive morphology

Reproductive structures are highly reduced in *Trithuria*, consisting of only bracts surrounding stamens and/or carpels. Despite this simplicity, the three species studied exhibited strikingly different reproductive unit morphologies due to differences in bract length and carpel packaging (*c.f.* Figures 4.2D, F, H). In *T. austinensis*, the reproductive unit has a distinctly vertical orientation, with carpels overtopping of each other. The long stigmatic hairs extend through overtopping carpels to emerge from the bracts. In contrast, the carpels of *T. submersa* are positioned in the same plane and only slightly overtop each other. As a result, the reproductive unit is rather bowl shaped. *Trithuria australis* reproductive units are intermediate. Bracts are shorter and carpels overtop each other to a lesser degree than in *T. austinensis*, but also produce long stigmatic hairs.

Different carpel packaging strategies are likely advantageous in different pollen reception environments. The vertical orientation in *T. austinensis* may increase the stigmatic surface area that is in contact with pollen in the airstream. In contrast, in *T. submersa*, the bowl shaped reproductive unit likely facilitates capture of pollen falling from the overarching anthers in the same reproductive unit.

We hypothesize that the entire reproductive head in *T. australis* functions like the bisexual reproductive unit of *T. submersa*. Short bracts and long stigmatic hairs increase the horizontal receptive surface area extending over the reproductive head and increase the likelihood that stigmatic hairs will receive pollen from the overarching anthers.

Rates of pollen tube germination

Pollen germination in both *Trithuria submersa* and *T. australis* occurred very shortly after pollen reception. *Trithuria submersa* exhibited faster pollen germination than *T. austinensis*, with germination occurring within 5 min after pollination and reaching the observed maximum by 15 min, compared to 15 and 45 minutes in *T. austinensis*. Outcrossing species might be expected to have faster germination, as it is thought to result in more pollen competition and, thus, stronger selection for rapid pollen germination (Mazer *et al.* 2010). However, pollen identity and pollen-stigma interactions also play a role in pollen germination and pollen tube growth rates (Mulcahy 1971; Snow and Spira 1991; Acar and Kakani 2010). In selfing populations, stigmas across generations receive pollen from the same single donor (itself) and pollen-stigma interactions can become specialized, resulting in greater pollen germination success and faster pollen germination. In contrast, stigmas in outcrossing populations must interact with a variety of pollen genotypes and are not as likely to evolve epistatic pollen-stigma interactions (Plitmann and Levine 1990; Kerwin and Smith-Huerta 2000; Mazer *et al.* 2010). *Trithuria* exhibits the same pattern as Polemoniaceae genera and *Clarkia tembloriensis* populations: higher germination success and faster germination rates in selfing populations (Plitmann and Levin 1990; Kerwin and Smith-Huerta 2000).

Pollen tube growth rates

In contrast to pollen germination, *T. austinensis* exhibited significantly faster pollen tube growth rates than *T. submersa* (499 $\mu\text{m/hr}$ vs. 321; $p = 0.01$). *Trithuria austinensis* pollen tubes that were successful at reaching a micropyle over the first 3 h grew even faster, at rates well

above the average (mean = 1046 $\mu\text{m/hr}$). Outcrossing populations are predicted to have faster pollen tube growth due to pollen competition (Mazer *et al.* 2010) and faster growth rates have been documented in outcrossing vs. selfing populations of *Clarkia tembloriensis* (Kerwin and Smith-Huerta 2000). As multiple stigmatic hairs per carpel in *T. austinensis* typically received pollen and each hairs often supported more than one pollen tube, pollen competition potentially occurs in *T. austinensis*.

Most basal angiosperms exhibit growth pollen tube rates of 80 – 300 $\mu\text{m/hr}$ (Williams 2008). These are faster than pollen tube growth rates exhibited by gymnosperms ($< 20 \mu\text{m/hr}$), but slower than those of more derived angiosperms (1000 – 40,000 $\mu\text{m/hr}$) and these moderate rates are thought to be plesiomorphic in angiosperms (Williams 2008).

Water lilies exhibit faster mean pollen tube growth rates than woody basal angiosperm species ($\sim 300 - 1050 \mu\text{m/hr}$) and acceleration in pollen tube growth rate likely occurred with the transition to the aquatic habitat, before the origin of Hydatellaceae (Williams 2008; Williams *et al.* 2010; this study). Furthermore, *T. austinensis* exhibits pollen tube growth rates that are considerably faster than those of *T. submersa* and that are among the fastest observed in Nymphaeales. Therefore, *T. austinensis* likely exhibits acceleration in pollen tube growth rate compared to the *Trithuria* ancestor. Fast pollen tube growth in *T. austinensis* results in a short fertilization interval, in which the fastest pollen tubes entered the ovule within 45 min, and may have evolved to maintain a short progamic phase in the face of carpel elaboration. Ovule entry in *T. austinensis* occurred only 15 min later than in *T. submersa*, despite pollen tubes having grown over three times as far. However, many pollen tubes grew at rates similar to those exhibited by *T. submersa* and the maximum percentage of ovule entry did not occur until 2 h after pollination.

This suggests that acceleration in pollen tube growth rate may lag behind stigmatic hair length and that elaboration of the carpel occurred first. This is the pattern observed in Cabombaceae, in which the pollen tube pathway lengthened in *Brasenia* without acceleration in pollen tube growth rate (Taylor and Williams 2009).

Pollen tube growth

The ultrastructure of the pollen tube pathway was quite similar in all three species. After germination, pollen tubes travel down the length of the stigmatic hair. Pollen tube branching was observed in all three species, but only when pollen tubes first contact the stigmatic surface. Branching of *Trithuria* pollen tubes was also observed by Prychid *et al.* (in press). Pollen tube branching is rare in angiosperms and typically occurs in the ovary near the micropyle (e.g. *Butomus*, Fernando and Cass 1997; *Oenothera*, Sniezko 1996; *Spinacia*, Wilms 1974). Branching at the micropyle also occurs in conifers (Wilms 1974; Owens *et al.* 2005 Fernando *et al.* 2005).

Prychid *et al.* (in press) report that pollen tubes of *T. submersa* penetrate the cuticle and grow deep into the outer wall and that the primary stigmatic hair wall serves as transmitting tissue. We clearly observed pollen tubes penetrating the cuticle, as well as a cuticular layer of the stigmatic hair that appeared to extend over the pollen tube wall. We did not examine stigmatic hairs at the resolution of Prychid *et al.* (in press), but we did not see deformation of the pollen tube wall. Therefore, if the pollen tubes are growing deeply in the stigmatic cell wall, then the stigmatic hair cell wall must be rebuilt to accommodate pollen tubes.

After entering the carpel mouth, *Trithuria* pollen tubes grow through a narrow canal. This canal is extremely narrow and the secretory cells of the inner carpel surfaces are in close proximity to each other. However, the secretory cells do not become interlocked to form a solid tissue like that of *Nymphaea* (Williams *et al.* 2010). Furthermore, pollen tubes grow only through the zone of secretion and not within the secretory tissue.

Pollen tube attrition

In *Trithuria austinensis*, we observed no increase in the percentage of pollen tubes that reached a carpel mouth after 2 h, indicating that pollen attrition rate is high. In no case did every pollen tube supported by stigmatic hairs *T. austinensis* individuals reach the carpel mouth. In contrast, over 90 % of pollen tubes had entered the carpel mouth by 2 h after pollination in *T. submersa*. Pollen tube attrition rates are predicted to be higher in outcrossing versus selfing populations because mixed pollen loads and pollen competition increase the likelihood that some pollen genotypes will be unsuccessful (Mazer *et al.* 2010). Higher pollen tube attrition has been documented in outcrossing vs. selfing populations of Brassicaceae (Plitmann 1993), Polemoniaceae (Plitmann 1994), and *Clarkia* (Smith-Huerta 1997).

Pollen tube guidance and arrest have shown to be controlled, in part, by signaling molecules in the transmitting tract of *Arabidopsis* (Hulskamp *et al.* 1995; Ray *et al.* 1997; Palanivelu and Preuss 2006). The finding that *T. austinensis* pollen tube growth is arrested on, or within the wall of the stigmatic hair supports the hypothesis that pollen-stigma interactions occur as pollen grows along the stigmatic hair and that these stigmatic cells function as a transmitting

tract (Prychid *et al. in press*). *Amborella* also exhibits reduction of pollen tube number in the stigmatic tissue, before pollen tubes reach the mouth of the stylar canal (Williams 2009).

In *T. submersa*, little to no reduction occurred on the stigmatic hair, but only one pollen tube was observed in each canal. Physical constraint likely prevented more than one pollen tube from entering this narrow tissue. No pollen tube cohort reduction has been observed in *Brasenia* or *Cabomba* (Taylor and Williams 2009) and in *Nymphaea*, many pollen tubes reach the ovary (Williams *et al.* 2010).

Evolution of breeding system and reproductive development in Trithuria

The common ancestor of Nymphaeales is typically thought to have been perennial, homoecious (with hermaphroditic or monoecious reproductive structures), and insect pollinated (Friis *et al.* 2001; Crepet *et al.* 2004; Wang and Dilcher, 2006; Borsch *et al.* 2008; Mohr *et al.* 2008; Taylor *et al.* 2008, Endress and Doyle 2009, Endress 2010). However, the ancestral states within *Trithuria* are difficult to elucidate, in great part, because there is no fossil record for Hydatellaceae.

Plants of all twelve *Trithuria* species are small, aquatic to semi-aquatic, and are hypothesized to be abiotically pollinated. The most parsimonious explanation is that the common ancestor of extant *Trithuria* exhibited these character states, as well. If this is the case, a transition from biotic to abiotic pollination likely occurred after the origin of Hydatellaceae, but before the radiation of the extant crown group.

At least ten of the twelve *Trithuria* species are annuals (Sokoloff *et al.* 2008a) and the annual habit may have evolved independently in several *Trithuria* lineages (Iles W. and Graham

SW. ‘unpub.’). Alternatively, this transition may have occurred once in the *Trithuria* common ancestor, with the putative perennial species *T. filamentosa* and *T. inconspicua* secondarily evolving the perennial habit. Secondary evolution of the perennial life history is the most parsimonious explanation; however, we consider independent evolution of the annual life history equally as likely because life history and habitat are tightly associated in *Trithuria*. Both perennial species are both found in permanently inundated habitats, whereas all annual species are found in ephemeral wetlands (Edgar 1966; Pledge 1974; Sokoloff *et al* 2008a). If the transition to ephemeral habitats occurred multiple times, then independent transitions to the annual habit are probable.

As *Trithuria* species moved into more ephemeral habitats, they may have experienced greater amounts of outcross pollen limitation (Stebbins 1957). Outcross pollen limitation is observed in *T. submersa* (Taylor *et al.* 2010). There are two common evolutionary trajectories that are thought to relieve pollen limitation: the evolution of selfing (Stebbins 1970; Eckert *et al.* 2006) and the evolution of strategies to maximize outcross efficiency, such as wind-pollination, dichogamy, or dioecy (Culley *et al.* 2002; Friedman and Barrett 2008a). *Trithuria* species could evolve selfing through a relaxation of their ancestral condition of protogyny in bisexual species (*T. submersa*) or by bringing the male and female unisexual units into close proximity (*T. australis*). Alternatively, selection to maximize pollen removal and reception via wind, which are independent events with different structural optima (Niklas 1985; Friedman and Harder 2007), and minimize interference, may have resulted in the evolution of dioecy (see Friedman and Barrett 2008a).

The evolution of selfing in *T. submersa* was apparently followed by a reduction in male investment and changes in reproductive morphology. Stigmatic hairs may have shortened, resulting in a short pollen tube pathway and a time to fertilization that ranks among the shortest in angiosperms. In contrast, in *T. austinensis* outcrossing likely drove the evolution of longer stigmatic hairs, and thus a longer pollen tube pathway. Pollen tube growth rates accelerated and the time to fertilization increased slightly.

Conclusion

Trithuria exhibits a short life history compared to other basal angiosperms, including other water lilies. Plants are small, typically annual, and inhabit ephemeral habitats. They also exhibit a degree of divergence in sexual system that is not observed in any other early-divergent angiosperm lineage and which may be correlated to their habitat. Dioecy and selfing in *Trithuria* species may have evolved as different strategies for maximizing pollination efficiency in habitats that require rapid reproduction and are often not particularly well-suited for wind-pollination.

Breeding system divergence in *Trithuria* has had consequences for stigmatic hair length and carpel packaging, as well as for developmental rates. The observed differences support the predictions for the trajectory of male gametophyte evolution in selfing vs. outcrossing populations (Mazer *et al.* 2010). This is evidence that post-pollination developmental processes in early-divergent lineages experience similar selective pressures and exhibit a similar evolutionary response to those of more derived angiosperms. Estimates of divergence dates and investigations of heretofore unstudied *Trithuria* species may help us further elucidate the timing, direction, and number of evolutionary transitions in *Trithuria*.

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APPENDIX 4

Table 4.1. Comparative male and female reproductive investment of *Trithuria* species.

Species	RUs per plant	Carpels per RU	Anthers per RU	Pollen grains per anther	Pollen grains per RU	Pollen to ovule ratio
<i>T. austinensis</i>	M: 5.4 ± 2.4 F: 8.6 ± 4.6	14.8 ± 4.2^b	$7.9 \pm 1.7^{a,c}$	$3525.9 \pm 1609.3^{a,d}$	$26818 \pm 9193^{a,d}$	RU: 1569.1^a PL: 1137.8
<i>T. australis</i>	M: 2.5 ± 2.1 F: 14.3 ± 6.7	10.1 ± 1.9	6.9 ± 2.7	349.8 ± 127.3^e	2490 ± 1013^e	RU: 248.4 PL: 31.7
<i>T. submersa</i>	4.3 ± 4.0^f	$19.3 \pm 6.4^{a,g}$	$1.1 \pm 0.3^{a,g}$	$426.0 \pm 149.4^{a,h}$	468.6 ± 164.4^h	23.9^a

Mean $\pm$ SD; ^aData in Taylor *et al.* (2010); *n* = 20 unless otherwise noted (*n*= ^b60; ^c10; ^d17; ^e12; ^f85; ^g280; ^h27). Abbreviations: RU = reproductive unit; P = plant.

Table 4.2. Comparative success of pollen reception in *Trithuria* species.

Species	No. of stigmatic hairs	Stigmatic hair length (mm)	Open-pollinated		Caged (no outcross pollination)	
			No. grains captured per RU	No. grains captured per ovule	No. grains captured per RU	No. grains captured per ovule
<i>T. austinensis</i>	5.5 ± 0.9 ^a	2.06 ± 0.59 ^b	29.1 ± 14.1	2.1 ± 1.5	—	—
<i>T. australis</i>	5.7 ± 0.7	2.00 ± 0.50	37.8 ± 42.9	3.2 ± 2.9	44.5 ± 37.3 ^c	5.3 ± 4.4 ^c
<i>T. submersa</i>	3.0 ± 0.0	0.56 ± 0.23 ^d	34.0 ± 42.3 ^e	3.0 ± 2.5 ^e	105.8 ± 59.4 ^f	5.8 ± 1.8 ^f

Mean ± SD; $n = 30$ unless otherwise noted ($n =$ ^a103; ^b66; ^c14; ^d64; ^e40; ^f10). Abbreviations: RU = reproductive unit

Table 4.3. Timing of pollen reception as measured by the percentage of carpels per reproductive unit with pollen at each ecological stage in *Trithuria* species.

Species	Submerged (eco stage 1)	Newly Emergent (eco stage 2)	Fully Emergent (eco stage 3)	Long Emergent (eco stage 4)	Self – only
<i>T. austinensis</i>	0 ± 0	19.16 ± 27.26	62.52 ± 35.39	86.51 ± 12.73	—
<i>T. australis</i>	0 ± 0	—	—	74.88 ± 28.48	76.77 ± 33.15 ^b
<i>T. submersa</i> ^a	0 ± 0	0 ± 0	6.75 ± 16.74	50.75 ± 34.21	87.28 ± 8.00

Percent ± SD; Cells left empty if the stage is not applicable (see text). ^aData for *T. submersa* from Taylor *et al.* (2010). *n* = 30, except at ^b*n* = 14 or as noted for *T. submersa* in Taylor *et al.* (2010).

Table 4.4. Pollen tube pathway length in *Trithuria* species.

Species	Mean stigmatic hair length ($\mu\text{m} \pm \text{SD}$)	Mean distance from grain to carpel mouth ($\mu\text{m} \pm \text{SD}$)	Mean distance from carpel mouth to FMG ($\mu\text{m} \pm \text{SD}$) ^a
<i>T. austinensis</i>	2062.8 $\pm$ 593.4 ^b	1917.9 $\pm$ 575.9 ^b	160.9 $\pm$ 19.5
<i>T. australis</i>	1195.5 $\pm$ 500.0 ^c	1948.7 $\pm$ 377.1 ^d	Not measured
<i>T. submersa</i>	556.2 $\pm$ 223.4 ^e	460.6 $\pm$ 219.9 ^f	175.6 $\pm$ 11.5

$n =$ ^a10, ^b66, ^c30, ^d15, ^e64, ^f38. Abbreviations: FMG = female gametophyte.

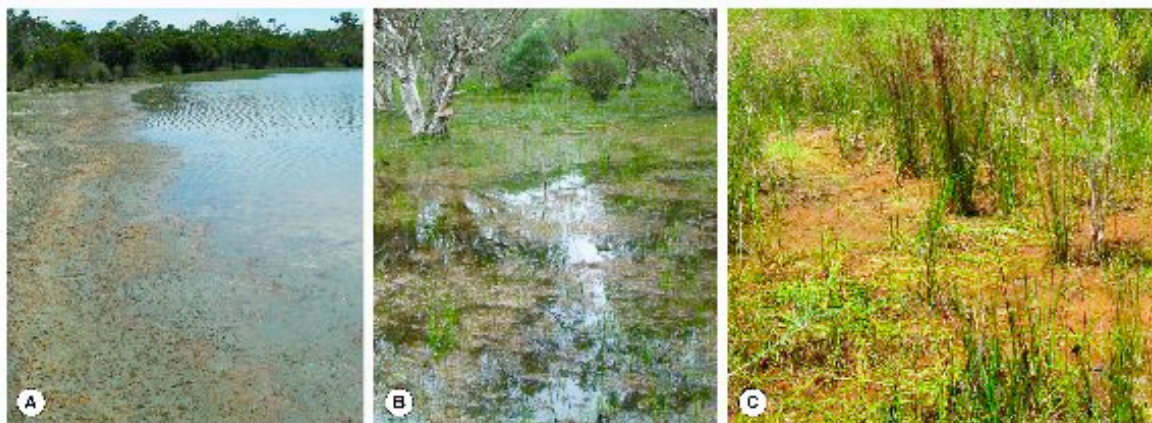


Figure 4.1. *Trithuria* habitats. (A) Branchinella Lake with water receding from the lake margin. *Trithuria austinensis* plants (not visible at this scale) are present in the saturated soil at the lake edge and across the entire bottom of the basin. (B) Frying Pan Swamp with water 1-5 cm deep. *Trithuria australis* plants are found throughout the swamp and are completely submerged. *Trithuria submersa* plants are restricted to a few small patches. (C) Kulunilup Swamp shortly after water has completely evaporated. *T. submersa* plants (not visible at this scale) are completely emergent, but not desiccated.

Figure 4.2. Reproductive morphology of *Trithuria* species. (A) *Trithuria austinensis* female plant mostly submerged, but with one fully emergent (arrow) and one newly emergent reproductive unit (asterisk). Stigmatic hairs are emerging from bracts (arrow). A few linear leaves are visible. Scale bar = 2 mm. (B) *Trithuria austinensis* male plant with an emergent reproductive unit. Multiple anthers (arrowhead) are dehiscing. Scale bar = 2 mm. (C) *Trithuria submersa* plants shortly after water drawdown. Stigmatic hairs (arrow) are emerging from bisexual reproductive units. Scale bar = 2 mm. (D) *Trithuria austinensis* female reproductive unit with many uniovulate carpels. Each carpel exhibits several elongated stigmatic hairs (arrow) that extend beyond bracts. Scale bar = 0.5 mm. (E) *Trithuria austinensis* male reproductive unit with several stamens of varying developmental stages. Several filaments have elongated and one anther has abscised (arrowhead), while at least one more is dehiscent. Immature stamens are enclosed in bracts. Scale bar = 0.5 mm. (F) *Trithuria australis* female reproductive unit with many uniovulate carpels. Elongated stigmatic hairs (arrow) extend far beyond bracts. Scale bar = 0.5 mm. (G) *Trithuria australis* male reproductive unit with several stamens. Several filaments are elongated and one anther has abscised (arrowhead). Scale bar = 0.5 mm. (H) *Trithuria submersa* reproductive unit with two central stamens (anthers have abscised) and several uniovulate carpels that are peripheral to the stamens. Each carpel has three stigmatic hairs (arrow). Scale bar = 0.5 mm. (I) *Trithuria australis* plant viewed from above, with linear leaves radiating from a central aggregation of male and female reproductive units. A few

male reproductive units, with elongated filaments and dehiscent anthers (arrowhead), are present in the center of the aggregation. Many female reproductive units (arrow) surround the male reproductive units. Scale bar = 2 mm. (J) *Trithuria australis* plant side view. Anthers in central reproductive units arch above the entire reproductive head and stigmatic hairs (arrow) emerge from female reproductive units and become intertwined with each other. Scale bar = 2 mm. Abbreviations: A = anther, B = bract, F = filament, L = leaf, C = carpel, P = peduncle.



Figure 4.2

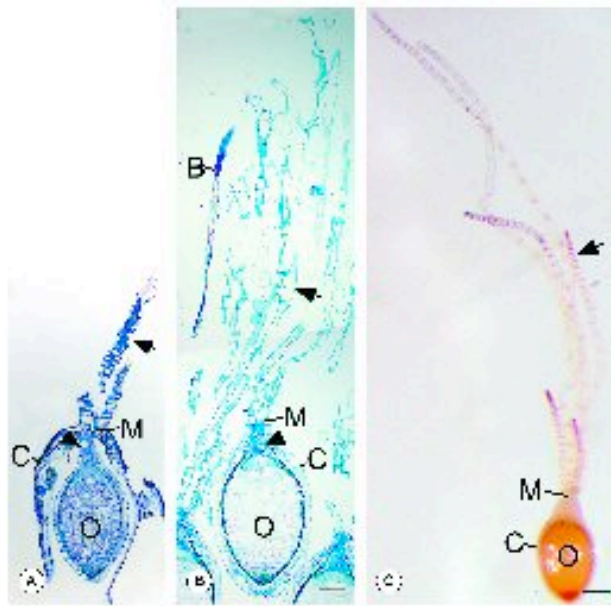


Figure 4.3. Carpel morphology in *Trithuria*. (A) Section through *Trithuria submersa* carpel with two stigmatic hairs (arrow) in the plane of section. A single ovule is enclosed within the carpel wall, with the micropyle (arrowhead) oriented near the carpel mouth. (B) Section through *Trithuria austinensis* carpel with four to five stigmatic hairs (arrow) at least partially in the plane of section. As in *T. submersa*, the micropyle (arrowhead) of the single ovule is oriented near the carpel mouth. (A) and (B) are at the same scale. Scale bar = 100 μm . (C) Whole *Trithuria australis* carpel with six stigmatic hairs (arrow). A single ovule is enclosed within the translucent carpel wall. Scale bar = 200 μm . Abbreviations: B = bract, C = carpel wall, M = carpel mouth, O = ovule. Stain: toluidine blue O (A,B).

Figure 4.4. Pollen germination and pollen tube growth. (A) *Trithuria austinensis* carpel with pollen tubes (arrow) growing along stigmatic hairs. Callose plugs (arrowhead) are present and at least one pollen tube has entered the carpel. Scale bar = 200 μm (B) Self-pollinated *Trithuria submersa* carpel with multiple pollen tubes (arrow) entering the carpel. Callose plugs are present (arrowhead). Scale bar = 20 μm . (C) Self-pollinated *Trithuria australis* reproductive unit with several carpels and many intertwined stigmatic hairs. Most stigmatic hairs support 1-2 pollen tubes (arrow) and none cross between hairs. Scale bar = 200 μm . (D) *Trithuria austinensis* stigmatic hair with a tightly associated pollen tube (arrow). Scale bar = 20 μm . (E) Stigmatic hair from D following aniline blue staining. Pollen tube walls (arrow) are callosic and a callose plug is developing (arrowhead) at 1 hour after pollination (hap). Faint fluorescence in the stigmatic hair cell wall indicates callose is present. Scale bar = 20 μm . (F) Germinated *T. austinensis* pollen grains with a relatively thick pollen wall (exine) at 1 hap. Pollen tubes have emerged from the aperture and the pollen tube tip (arrow) has reached the stigmatic hair. Scale bar = 10 μm . (G) Grains from F following aniline blue staining. Callose is present in the pollen tube wall and also in the inner pollen grain wall (arrowhead), but not at the growing tip (arrow). Scale bar = 10 μm . (H) Germinated *T. austinensis* pollen grain at 45 minutes after pollination. The pollen tube (arrows) has grown around the grain to contact the stigmatic hair and the tip has penetrated the cuticle of the stigmatic hair cell (arrowhead). Scale bar = 10 μm . (I). *Trithuria austinensis* pollen tube at 1 hap. Walls are callosic (arrow) and branching occurred at the point where the pollen tube contacted the stigmatic hair. Scale bar = 10 μm . (J) Section through a *T. austinensis* stigmatic hair stained with auramine O with one pollen tube in cross

section. The fluorescing outer layer of the stigmatic hair cell wall thickens (arrowhead) at the edge of the pollen tube and thins to surround the pollen tube. Scale bar = 5 μm . (K) Close-up of a *T. austinensis* stigmatic hair cell and pollen tube cross section stained with auramine O. Two layers in the stigmatic cell wall are apparent: the inner layer continuing beneath the pollen tube (arrow) and the outer one surrounding the pollen tube (arrowhead). The lipid layer that encloses the pollen tube is continuous with the cuticle of the stigmatic hair cell. Scale bar = 5 μm . (L) Cross-section of a *T. austinensis* stigmatic hair supporting two pollen tubes. Both the inner layer of the stigmatic hair cell wall (arrow) and the translucent outer layer that corresponds to the cuticle in *K* are visible (arrowhead). Scale bar = 10 μm . (M) Cross-section in *L* stained with aniline blue, indicating the presence of callose in the pollen tube only. The two layers of the stigmatic cell wall are faintly visible (arrow, arrowhead). Scale bar = 10 μm . Abbreviations: A = aperture, B = bract, C = carpel, E = exine, M = carpel mouth, PT = pollen tube, S = stigmatic hair. Stains: aniline blue (A-C, E, G, I, M), auramine O (J, K).

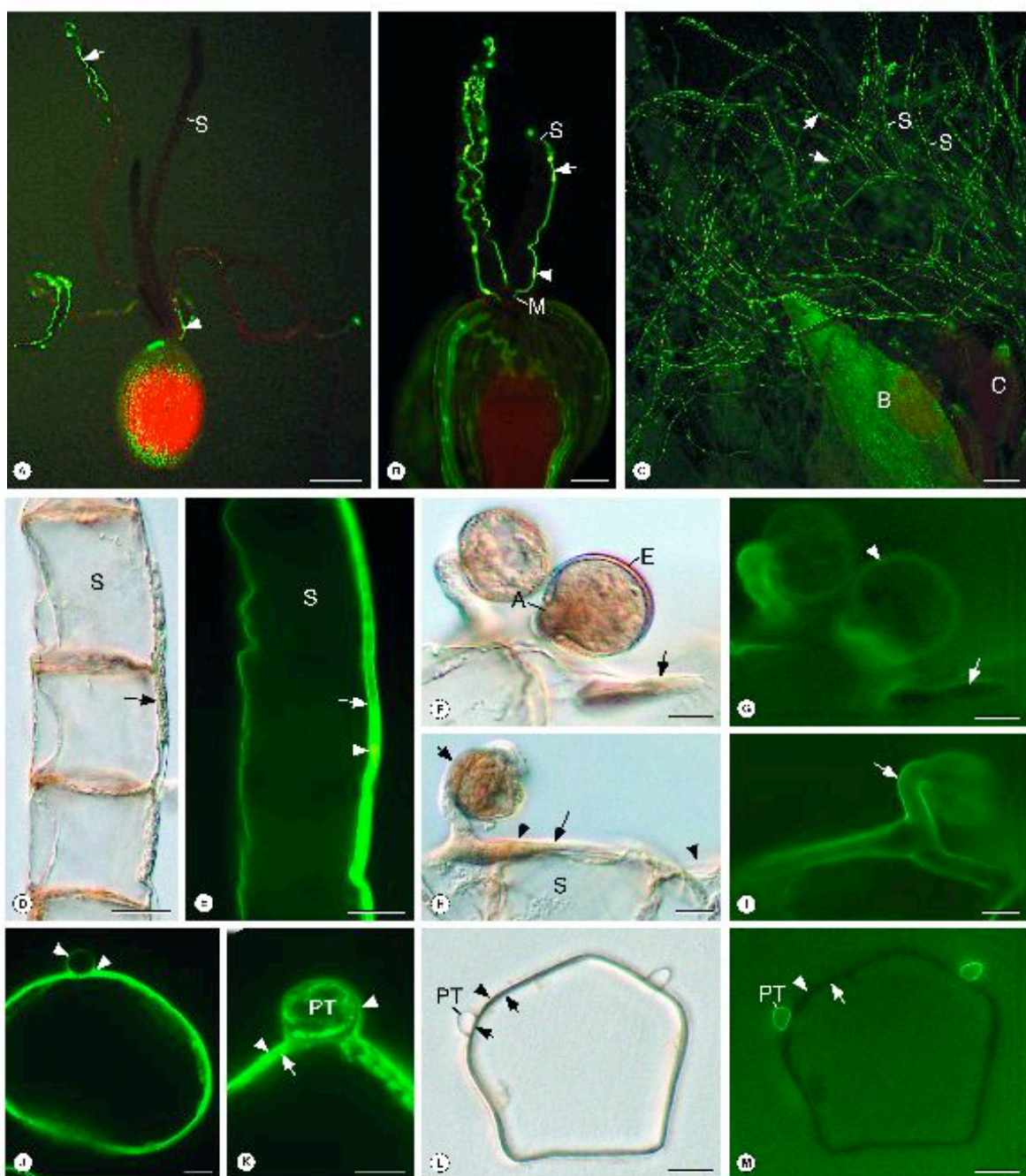


Figure 4.4

Figure 4.5. Late pollen tube growth. (A) *Trithuria submersa* carpel at 30 minutes after pollination (map). Three pollen tubes (arrows) have grown down the stigmatic hair and entered the carpel mouth. Callose plugs are present (arrowheads). Scale bar = 20 μm . (B) Longitudinal section through a *T. submersa* carpel mouth with one pollen tube (arrow) in section. Scale bar = 20 μm . (C) Longitudinal section through a *T. submersa* carpel. A pollen tube (arrow) has turned 90° to enter the carpel. Scale bar = 20 μm . (D) *Trithuria austinensis* pollen tube at 1.5 hours after pollination (hap). The second mitotic division has occurred and two sperm nuclei are present. Scale bar = 10 μm . (E) *Trithuria australis* pollen tube with two callose plugs (arrows). Scale bar = 20 μm . (F) *Trithuria austinensis* pollen tube with a callosic thickening of the pollen tube wall (arrow) indicating early callose plug formation at 2.5 hap. Scale bar = 5 μm . (G) Fully developed callose plug in *T. austinensis* at 45 map. Scale bar = 5 μm . (H) Section through a *T. submersa* carpel just at the base of stigmatic hairs. Large cells at the base of stigmatic hairs surround the carpel mouth and the zone of secretion is visible as a narrow canal (arrow). Scale bar = 10 μm . (I). Section through a *T. austinensis* carpel below the mouth. The transmitting tract (arrow), composed of small, densely cytoplasmic cells with comparatively large amounts of extracellular space, runs through the center and is surrounded by larger cells of the outer carpel wall. Scale bar = 10 μm . (J) Longitudinal section of a *T. austinensis* carpel at 30 map. The carpel mouth opens into the transmitting tract and zone of secretion. The micropyle is positioned very near the top of the ovarian cavity. Several layers of nucellar tissue are present above the

female gametophyte. Scale bar = 50 μm . (K) Longitudinal section of a *T. submersa* carpel at 30 map. The carpel mouth opens into a very short transmitting tract and the micropyle (arrowhead) is positioned near the top of the ovary. The nucellus is similar to that of *T. austinensis*. (L) Longitudinal section of a naturally collected *T. austinensis* carpel with two pollen tubes present along the length of the stigmatic hair and in the transmitting tract of the carpel (arrows). Only one pollen tube is present in the carpel tissue. Scale bar = 25 μm . (M) Close up of the transmitting tract of *T. austinensis* at 30 map. A pollen tube (arrow) has entered the carpel mouth and is growing through the zone of secretion between the densely cytoplasmic cells of the transmitting tract. Scale bar = 25 μm . Abbreviations: C = carpel, M = carpel mouth, N = nucellus, S = stigmatic hair, SN = sperm nucleus, TT = transmitting tract. Stains: aniline blue (A-C, E-G, L), DAPI (D), toluidine blue O (H-K, M).

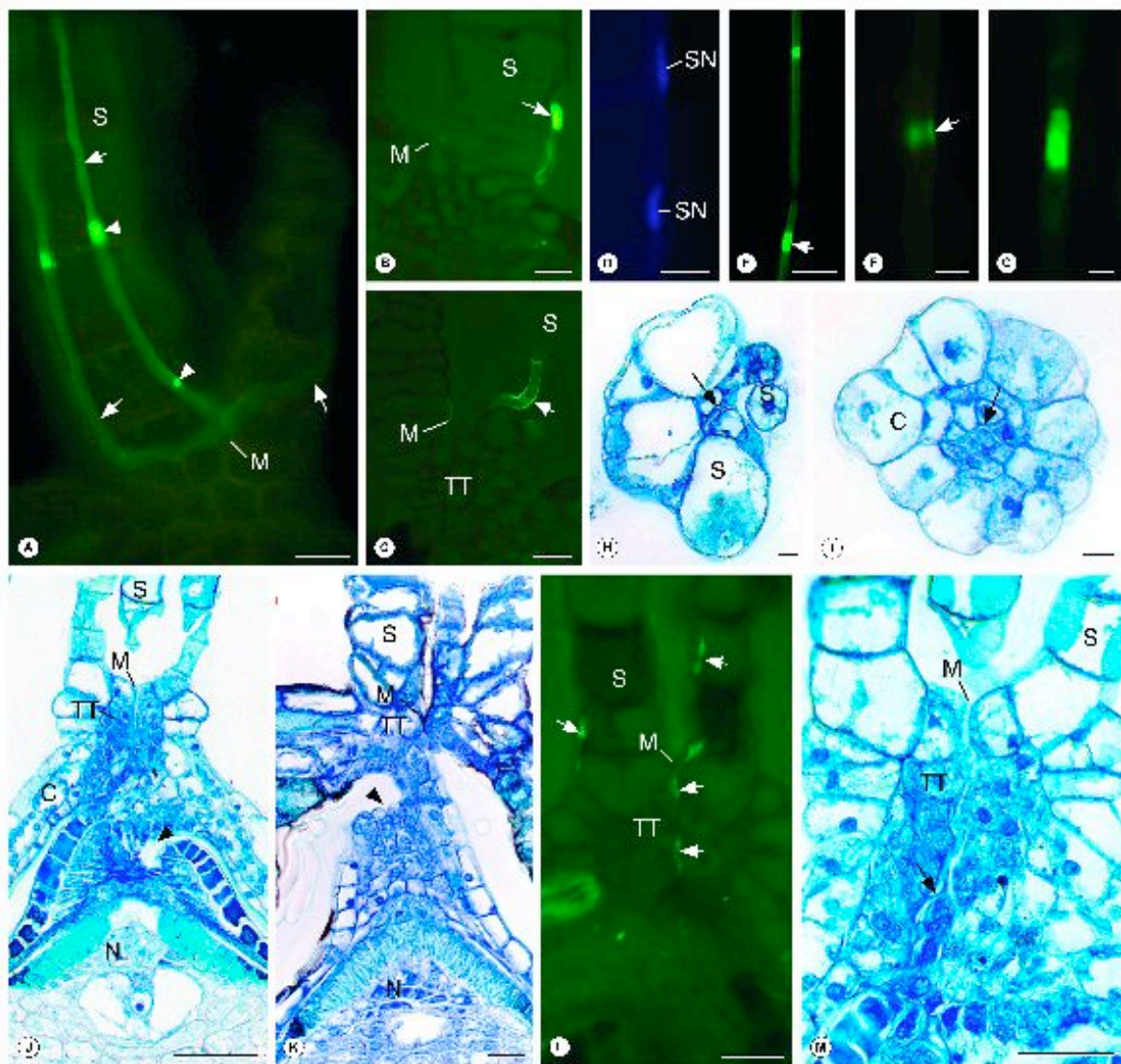


Figure 4.5

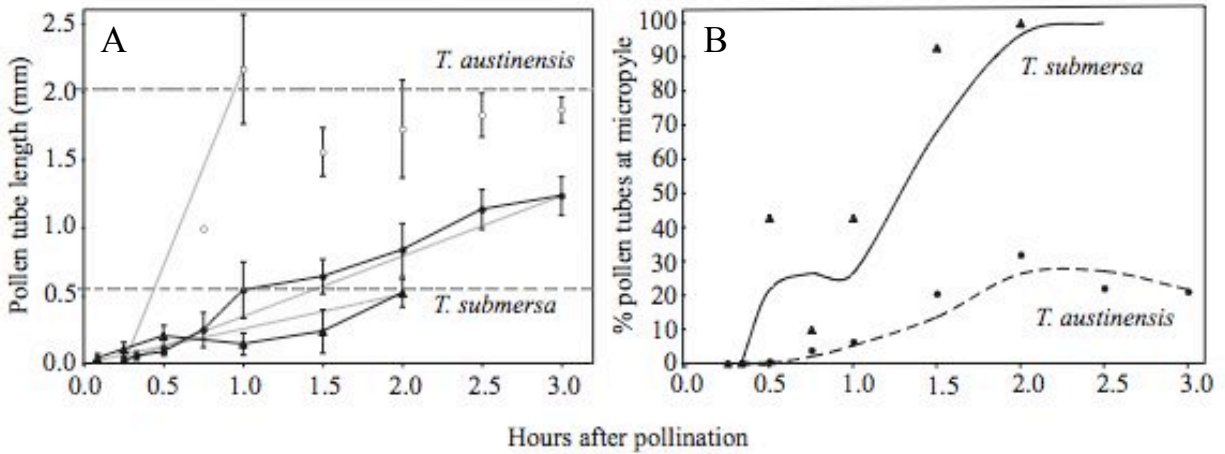


Figure 4.6. Comparative pollen tube growth in *Trithuria austinensis* and *Trithuria submersa*. (A) Average mean length of pollen tubes in individuals ($\pm$ SE) of *T. submersa* (triangles; $n = 45$ pollen tubes in 40 individuals) and *T. austinensis* (closed circles; $n = 192$ pollen tubes in 73 individuals), as well as in only those individuals of *T. austinensis* that had entered the micropyle (open circles; $n = 32$ pollen tubes). Slopes of black lines indicate growth rate within each time interval and slopes of grey lines indicate the average sustained growth rate. Dark grey dashed lines represent the average length of the realized pollen tube pathway. (B) Percent of pollen tubes that had entered the micropyle in *T. submersa* (triangles) and *T. austinensis* (circles) and at each collection point. A moving average trendline is shown for *T. submersa* (black line) and *T. austinensis* (dashed line).

CHAPTER V: CONCLUSIONS AND SYNTHESIS

The progamic phase is a critical life history stage in angiosperms in which reproductive structures and developmental programs from four genetic individuals (male and female gametophyte and two sporophytes) interact to achieve fertilization (Cresti *et al.* 1992; de Graaf *et al.* 2001; Herrero 2003). There is considerable diversity in progamic phase traits (Williams 2008); however, the forces that drive evolution in these traits and the consequences for the progamic phase as an integrated whole are not well understood.

The objective of this research was to investigate the progamic phase in Nymphaeales, an early-diverging angiosperm lineage that exhibits great variation in pollination syndrome, carpel morphology, and breeding system. I described reproductive structures and documented the timing of developmental events in five water lily species, representing three genera and two families: Cabombaceae (*Brasenia schreberi* and *Cabomba caroliniana*; Chapter II) and Hydatellaceae (*Trithuria austinensis*, *T. australis*, *T. submersa*; Chapter III, IV). Concurrently, progamic phase development was investigated in *Nymphaea odorata* (Nymphaeaceae; Williams *et al.* 2010) and selected results from that study are discussed in this chapter.

Divergence in pollination biology and breeding system has had consequences for progamic phase development in Nymphaeales. However, many progamic phase traits were shared by all taxa studied and are either conserved, or have evolved in parallel within Nymphaeales. Since they are similar despite divergence in other reproductive traits, it is likely that they were present in the water lily common ancestor. Below, I discuss some characteristics of the progamic phase in all water lilies and the evolutionary implications of these traits.

Pollen tube ultrastructure

Pollen tubes in all water lily taxa studied were constructed from callose (1,3- β -glucan; Stone and Clark 1992), which was present the entire length of pollen tubes, except at the growing tips (Figures 2.6D, 2.8F, 4.4A-B; Williams *et al.* 2010). A callosic pollen tube wall is a synapomorphy of angiosperms that has been linked to their evolutionary success. Callose can be synthesized rapidly and it is thought that callose walls can be constructed faster than the cellulose-based walls typical of gymnosperms, which allows for faster pollen tube growth (Knox 1984; Stone and Clark 1992; Williams 2009). Callose plugs were also present in each species studied, forming via thickening of the inner pollen tube walls (Figures 2.6A, 4.5E-G). Callose plugs are a synapomorphy of angiosperms, as well, and may function to seal pollen tubes from pathogen attacks (Williams 2009). The presence of callosic walls and callose plugs in all water lily taxa studied supports the hypothesis that these traits originated in an angiosperm common ancestor.

Pollen tube developmental rates

Pollen germination was rapid (< 1 h) in all water lily taxa investigated (Table 5.1; all figures referenced in this chapter are found in Appendix 5). Rapid pollen germination is often associated with pollen that is tricellular at dispersal (Mulcahy and Mulcahy 1983). In tricellular pollen, the generative cell undergoes mitosis to form the two sperm nuclei before pollen dispersal (Brewbaker 1967). However, this work indicates that bicellular pollen in water lilies is able to germinate extremely rapidly (within 5 min in *T. submersa*; Chapter IV). Therefore, other pollen physiological traits may be more important for achieving rapid germination than pollen

cell number. One such trait is a high water content at pollen dispersal, which is also thought to enable rapid pollen germination (Nepi *et al.* 2001; Franchi *et al.* 2002). Indeed, both *Brasenia* and *Cabomba* exhibit grains with >70 % water content at dispersal and *Trithuria* pollen grains exhibit morphological characteristics typical of partially hydrated pollen, as well (see Franchi *et al.* 2002).

Pollen tubes growth rates in all water lily taxa studied are faster than those in woody perennial basal angiosperms (> 300 vs. < 90 $\mu\text{m/hr}$; Figure 5.1). Growth rates in extant Nymphaeales almost certainly represent an acceleration from the ancestral pollen tube growth rate (Williams 2008). This acceleration can be inferred to have occurred early in water lily history, before the origin of Hydatellaceae, and it likely accompanied the transition to the aquatic habit. Rapid pollen tube growth rates have repeatedly evolved in early-divergent aquatic lineages, including Alismatales (basal monocots) and Nelumbonales (basal eudicots; Williams 2009).

One consequence of faster pollen tube growth rates in water lilies is a shortening of the entire progamic phase. Time to fertilization in every water lily species is less than 10 hours, despite a 13-fold range in pollen tube pathway lengths (Table 5.1). As a result, although Nymphaeales taxa exhibit pollen tube pathways that are both shorter (*T. submersa*) and longer (*Brasenia schreberi*) than those of woody basal angiosperms, they all have a shorter time to fertilization (Figure 5.2).

Pollen tubes growth through solid tissue

Carpels in *Brasenia* and *Cabomba* are ascidiate, forming as a hollow tube open at the mouth and sealed only by secretion (Endress 2005). Pollen tubes in these genera might be expected to enter the stylar canal only through the open mouth and reach ovules without ever growing between cells. This is observed in *Amborella*, in which pollen tubes only enter the stylar canal through the open mouth (Williams 2009). *Brasenia* and *Cabomba* pollen tubes, however, do not necessarily grow through the open carpel mouth and are able to reach the stylar canal by penetrating the cuticle of stigmatic cells and growing between cells that comprise the solid sub-dermal tissue of the stigma or stigmatic crest (Chapter II).

In contrast to those of Cabombaceae, carpels of Nymphaeaceae become partially sealed late in development when the inner carpel surfaces become interlocked (Igersheim and Endress, 1998; Endress and Igersheim, 2000). The evolution of complete carpel closure in *Nymphaea* represents an independent origin of true ‘angiospermy’ in flowering plants (Endress and Doyle 2009). In Nymphaeaceae, therefore, pollen tubes must grow through solid tissue in order to reach the ovary (Williams *et al.* 2010). Pollen tubes growth rate through zone of post-genital fusion is only slightly slower than growth rate through the secretion filled ovarian cavity (972 ± 470 vs. 1323 ± 486 $\mu\text{m/hr}$; Williams *et al.* 2010)

Trithuria pollen tubes do not grow through solid tissue in the same sense as those of Cabombaceae and Nymphaeaceae. Pollen tubes grow only through the open carpel mouth and proceed through a zone of secretion to reach the micropyle. However, pollen tubes clearly penetrate the cuticle of stigmatic cells and may grow within the stigmatic hair cell wall (Chapter IV, Prychid *et al.* in press), which might involve the same physiological processes as growth through a transmitting tract (Prychid *et al.* in press).

These results indicate that water lily pollen tubes evolved the ability grow between solidly packed cells before the origin of truly closed carpels. Thus, pollen tube innovations arose first and this allowed the modification of carpel morphology (e.g. through stylar elongation and post-genital fusion) without disrupting the timing of the progamic phase.

Male and female function in Nymphaeales: shifting boundaries

The timing of male and female function is critical to progamic phase development because it determines the onset of the progamic phase and provides a setting for this life history stage. Dichogamy, or the separation of male function (anther dehiscence) and female function (stigma receptivity) in time, is nearly ubiquitous among bisexual basal angiosperms (Endress 2010). Dichogamy can be advantageous in bisexual flowers if it reduces the chance of within flower selfing, limits interference between floral organs, or allows respective sexual function to be optimized during each phase (Lloyd and Webb 1986). Among dichogamous basal angiosperms, 21 of 23 families exhibit protogyny, with female function preceding male function (e.g. Gottsberger *et al.* 1980; Bernhardt and Thien 1987; Endress 2001, 2010).

Synchronous dichogamy, in which flowers in a population open and close at once, has been well documented in Nymphaeales (Prance and Arias 1975; Schneider and Jeter 1982; Osborn and Schneider 1988; Wiersema 1988; Williams *et al.* 2010; this study). However, this work has revealed that there is considerable variation in both the absolute and relative timing of anther dehiscence and stigma receptivity among species of Nymphaeales. This variation primarily arose through shifts in the timing of the offset of stigma receptivity or in the onset of anther dehiscence. For example, in *Brasenia*, the period of stigma receptive has been truncated

so that it ends well before flowers close. As the window of pollen transport is also short due to wind pollination, this maintains tight coordination between male and female function in the population (Chapter II). In contrast, male and female function must overlap in *Euryale* and *Barclaya*, as these two taxa exhibit autonomous selfing (Kadono and Schneider 1987; Williamson and Schneider 1994). The exact timing of stigma receptivity and pollen release has not been studied in these species; however, work in *Nuphar* suggests that offset in stigma receptivity may be delayed. Flowers of *N. pumila* experimentally pollinated on the 2nd and 3rd day, even after stigmatic fluid has dried up, still produce seeds, albeit fewer than experimentally pollinated 1st day flowers (Zhou and Fu 2007).

Trithuria also exhibits overlap in female and male function, although in *Trithuria*, precocious anther dehiscence is the more likely mechanism for overlapping phases. This overlap allows *T. submersa* to exhibit a primarily selfing breeding system (Chapter III) and the female-only phase appears to be drastically reduced, if not eliminated completely in *T. australis* (Chapter IV).

Water lilies exhibit modifications to a protogynous program that permit a host of pollination and breeding systems, including selfing, to evolve. This suggests that there is not strong selective pressure to maintain strict protogyny in these species. Many of the modifications exhibited, such as truncated or delayed stigma receptivity in *Brasenia* and *Nuphar*, are not apparent without experimental work, and this underscores the necessity of experimental data in determining onset and offset of reproductive function.

CONCLUSION

Nymphaeales is one of the oldest independent angiosperm lineages and water lilies have been evolving in parallel to all other angiosperm lineages, other than *Amborella*, for nearly all of documented angiosperm history. Therefore, it is not surprising that water lilies exhibit great diversity in reproductive traits. True carpel closure, wind-pollination, and a primarily selfing breeding system have all arisen independently in various water lily taxa and the progamic phase in Nymphaeales has evolved in concert. Pollen tube pathway length, timing of anther dehiscence, duration of stigma receptivity, and pollen tube growth rate have undergone conspicuous modification associated with shifts in pollination biology and breeding system. The post-pollination developmental program in Nymphaeales appears to experience selective pressures similar to those experienced by more recently-derived angiosperms and to evolve in similar ways. Nymphaeales exhibits rapid pollen germination, fast pollen tube growth, and in many cases, a relatively short pollen tube pathway. Consequently, the fertilization interval in Nymphaeales is short, which may be advantageous in the aquatic environment. The pollen tube innovations that underlie developmental flexibility were already in place before the divergence of Nymphaeales, giving credence to the hypothesis that innovations in male gametophyte development were instrumental in facilitating diversification of flowering plants.

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APPENDIX 5

Table 5.1. Comparative progamic phase timing in Nymphaeales species.

Species	Min to pollen germination	Time to ovule entry (h)	Mean pollen tube growth rate ($\mu\text{m/h} \pm \text{SD}$)	Mean pollen tube pathway length ^a (mm $\pm$ SD)
<i>Brasenia schreberi</i>	15-60	6	742.1 $\pm$ 76.5	6.62 $\pm$.087
<i>Cabomba caroliniana</i>	> 15	2	963.4 $\pm$ 514.2	2.04 $\pm$ 0.88
<i>Nymphaea odorata</i> ^b	5-45	2.5	1066 $\pm$ 548	2.04 $\pm$ 0.15 (95 % CI)
<i>Trithuria austinensis</i>	15-30	0.75	499.4 $\pm$ 435.1 1046.7 $\pm$ 604.5 ^c	2.06 $\pm$ 0.59 + 0.16 $\pm$ 0.02 ^d
<i>Trithuria submersa</i>	5-15	0.50	321.1 $\pm$ 281.0	0.56 $\pm$ 0.22 + 0.18 $\pm$ 0.01 ^d

^a Measured from the tip of the stigmatic surface to the closest micropyle; ^bData from Williams *et al.* 2010; ^cSuccessful pollen tubes only, see Chapter IV; ^dDistance from stigmatic hair tip to carpel mouth + distance from carpel mouth to micropyle.

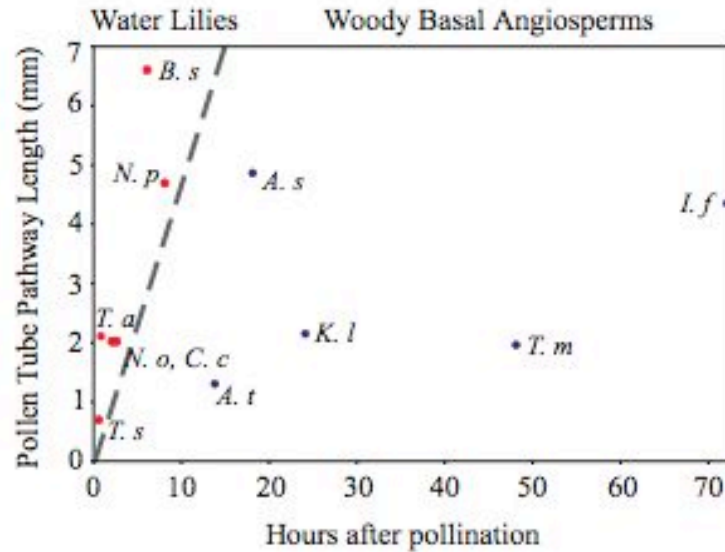


Figure 5.1. Comparative pollen tube pathway lengths and time to fertilization in basal angiosperms. Water lilies (red circles) exhibit greater variation in pollen tube pathlength than species of woody basal angiosperms (blue circles), but the time to fertilization is much shorter due to accelerated pollen tube growth rates. Species represented are *Austrobaileya scandens* (A. s), *Amborella trichopoda* (A. t), *Brasenia schreberi* (B.s.), *Cabomba caroliniana* (C. c), *Illicium floridanum* (I. f), *Kadsura longipedunculata* (K. l), *Nuphar polysepala* (N. p), *Nymphaea odorata* (N. o), *Trimenia moorei* (T. m), *Trithuria austinensis* (T. a), and *Trithuria submersa* (T. s). *Trithuria* species exhibit the shortest time to fertilization, a result of a shorter pollen tube pathway in *T. submersa* and of accelerated pollen tube growth rates in *T. austinensis*. Data from this study (B. s, C. c, T. a, T. s); Williams *et al.* 2010 (N. o); and Williams 2008 (all other taxa).

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