Sex Expression in a Rainforest Understory Herb, Begonia urophylla

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UNIVERSITY OF MIAMI

SEX EXPRESSION IN A RAINFOREST UNDERSTORY HERB,

BEGONIA UROPHYLLA

By

John Cozza

A DISSERTATION

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of the University of Miami
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SEX EXPRESSION IN A RAINFOREST UNDERSTORY HERB,  
BEGONIA UROPHYLLA

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Monoecy, the production of distinct male and female flowers on the same plant, is an important, though little studied, sexual strategy in the rainforest understory. This study of a monoecious plant discovered a cue to induce flowering, explored the interplay of gender constraint vs. plasticity in a natural population, and tested possible causes of gender in two laboratory experiments.

An experiment in the lab found that reduced photoperiod for three weeks is an unambiguous cue for flowering. The remarkably long inductive period is followed by a long and variable period of floral initiation. This results in only partial synchronization of flowering among plants in a patch, which enhances mating opportunities in this protandrous plant.

Inflorescence architecture is highly constrained, and ideally produces a phenotypic gender (proportion female) of about 0.5. However, in the forest at Las Cruces, Costa Rica, most plants were less female than predicted, mostly through abortion of female buds. Plants showed gender plasticity between and within years. Large plants produced more flowers and were more female in gender, and less variable in gender, than small plants. Reproduction was poorly correlated with environmental resource availability, measured as canopy openness, soil moisture, pH, and soil P, NH₄ and NO₃.
Phenotypic selection analysis on seed production suggests an optimal gender of 50-60% female, yet plasticity to be less female than this optimum, and in particular to express only male function, has been maintained.

In a factorial experiment in the lab, high light or high nitrogen caused plants to produce more flowers and to be proportionally more female, and larger in weight, than low light or nitrogen. The effects of light and nitrogen on reproduction, plant size, and leaf greenness suggest an energy based determination of gender. Gender may be mostly influenced by plant size, but sometimes also opportunistically by environment.

Inoculation with mycorrhizas caused plants to be less female in gender, and smaller in weight, than plants that were not inoculated. This suggests a net cost of mycorrhizas under experimental conditions, and supports the emerging view of the mycorrhizal symbiosis as not necessarily mutualistic under all circumstances.
DEDICATION

I dedicate this dissertation to my parents, Ann (Hilinski) Cozza and John Cozza, who brought me into this world, raised me the best they could, and always made sure I did my homework.
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CHAPTER 1

Introduction: Sex expression in monoecious flowering plants

SUMMARY

Monoecy, the production of distinct male and female flowers by each individual in a plant population, may confer several advantages over the ancestral sexual system of hermaphroditism. Monoecy may enhance outcrossing, decrease wasteful pollen discounting and stigma clogging, allow adaptation of the floral sex ratio and specialization of male and female structures and functions, or even act as a relict bet-hedging strategy in dioecious species. In addition to these advantages that might favor its evolution and maintenance, monoecy enables the emergent strategies of pollination by deceit and gender plasticity. Many questions involving the evolution and maintenance of monoecy can be studied in taxa that comprise a large diversity of monoecious species, such as the large tropical genus Begonia.

Hermaphroditism, where each plant in the population can reproduce as both a male and female, and the sexes are combined in each flower, is the ancestral sexual strategy of eudicot and monocot flowering plants, after early experiments by the basal angiosperms with many sexual systems (Bateman et al. 2006). Hermaphroditism remains highly successful, with about 80% of flowering plant species today employing it (Proctor et al. 1996, p. 322). Given that success, many have wondered how it might be adaptive for individuals to give up one sex function, and be either male or female—the sexual system of dioecy (Darwin 1877, Renner and Ricklefs 1995, Freeman et al. 1997, Mitchell
and Diggle 2005, Scofield and Schultz 2006). Fewer have studied the separation of the sexes into distinct flowers on the same individual—the sexual system of monoecy, which has evolved in about 7% of flowering plants (Yampolsky and Yampolsky 1922). Monoecy may confer some of the advantages of dioecy, without the risk to reproductive assurance of giving up one sex function (Ågren and Schemske 1995, Aluri and Ezradanam 2002).

Monoecy may have evolved because it increases the efficiency of outcross pollination (Harder et al. 2000). The fundamental paradox of the hermaphrodite flower is that it is a sexual structure, presumably an adaptation to promote outcrossing, and yet the most proximate mating opportunity is selfing within the flower. Adaptations to promote outcrossing over selfing could work by rendering self-crosses genetically incompatible (Barrett 1988; Proctor et al. 1996, pp. 323-330), or by separating the presentation of the flower’s own ovules and pollen in space or time (Bawa et al. 1982, Lloyd and Webb 1986, Webb and Lloyd 1986). Monoecy is but one of these many adaptations (Barrett 2002a). In species that already evolved self incompatibility of pollen and ovules in hermaphroditic flowers, monoecy may still be advantageous, as a way of minimizing waste of pollen in self flowers (pollen discounting), or avoiding stigma clogging with self pollen (Bertin 1993, Harder et al. 2000, Barrett 2002b). Minimizing this waste of potential mating opportunities may especially benefit trees, shrubs, and large clonal plants that have many flowers in bloom at once.

Another way that monoecy can be adaptive is by allowing enhanced variation in gender, the relative allocation to male and female function (Lloyd 1980a). Gender may be varied in hermaphroditic flowers by partially curtailing or promoting the development
of the structures of one sex in the flower (Cox 1988), and monoecy may simply represent this process carried to its most efficient execution (Willson 1983, p. 70). In the evolution of monoecy, the developmental programs of male and female structures are decoupled, and gender may be determined simply by regulating the relative number of male and female flowers produced. Often, a male-biased gender is adaptive, due to resource budget limitations on the number of fruits or seeds that can be produced, contrasted with the low cost of pollen and the non-saturating male fitness gain from producing large amounts of it (Charlesworth and Morgan 1991). If gender is heritable, and is determined by one or just a few regulatory genes (Irish and Nelson 1989, Dellaporta and Urrea-Calderon 1993), the gender (or genders) that maximizes both male and female fitness under most circumstances can be selected for. In addition to selection for optimal allocation of resources (Sutherland and Delph 1984, Sutherland 1986, Delesalle and Mooreside 1995), pollinator preferences may drive selection on gender (Castillo et al. 2002). After convergence on an optimal gender, developmental or architectural mechanisms to constrain gender to that optimum might be adaptive.

Monoecy allows specialization of the sex functions. Development of male and female flowers can be segregated to different parts of the inflorescence, or even onto distinct single sex inflorescences. This segregation could increase the efficiency of pollination by wind (Cox 1988, Freeman et al. 1997) or animals (Jordan and Harder 2006), protect female structures from destructive pollen foragers (Cox 1988), or enable different male and female flowering strategies on the level of the inflorescence (Bawa 1977, Marten and Quesada 2001, Huang et al. 2006, Prusinkiewicz et al. 2007). Freed from having to develop together in the same flower, male and female structures can
become specialized for their functions morphologically, as well as in their spatial locations on the inflorescence or plant (Cox 1988).

Male and female functions may also be enhanced if they can develop at distinctly different points in time. Inflorescence- or plant-level protogyny, in which female flowers open before males, or protandry, in which males open first, may increase outcrossing and minimize the interference of male and female functions (Lloyd and Webb 1986). If the separation of male and female flowering is complete, *temporal dioecy* has evolved, (Cruden 1977, 1988). A consequence of temporal dioecy within a season is that if the flowering times of neighboring individuals are somewhat out-of-synchrony, their male and female phases will overlap, and their mating opportunities will increase (Thomson and Barrett 1981, Borges et al. 1997, Chapter 2: Rivera and Cozza 2008). Thus, mechanisms that promote semi-synchronous flowering of individuals in the population should be selected for. A long induction period and variable initiation period for flowering might be such a mechanism in temporally dioecious *Begonia urophylla*, and is the subject of Chapter 2.

Monoecy may be available as a bet hedging strategy in an otherwise mostly dioecious population. If long-lived male and female individuals are adapted to different microhabitats, they may encounter situations where their fitness is greater through the opposite sex function, and therefore a capacity for labile sex expression, called *leaky dioecy* or *subdioecy*, is selected for and retained (Freeman et al. 1997). Retaining the ability to develop at least some flowers of both sexes may be particularly adaptive in patchy or unpredictable environments (Freeman et al. 1980, McArthur et al. 1992, Dorken et al. 2002). In some cases, monoecious individuals may represent one of several
gender morphs in a population, including genetic males and females (Freeman et al. 1984, El-Keblawy et al. 1995, Ueno and Kadono 2001, Glawe and de Jong 2005). In other cases, male and female individuals may represent the extremes of a monoecious gender continuum (Yampolsky 1920, Schlessman 1986, 1988; Fellingham and Linder 2003). In some species, monoecious and dioecious populations are found in different habitats (Costich and Meagher 2001, Dorken et al. 2002) or circumstances. For example, monoecy favors colonization and spread in new habitats, compared to dioecy, because monoecious plants can be self-pollinated in the absence of a mate (Pannell et al. 2008). There is evidence that monoecy can re-evolve from dioecy (Kafkas et al. 2000, Zhang et al. 2006).

Once monoecy has evolved, it presents an opportunity for modified sexual systems to emerge. For example, segregating the developmental programs of male and female structures also may result in the segregation of rewards for pollinators, particularly if pollen is a reward. If nectar is not offered, then female flowers are often non-rewarding, and if so, they are strongly selected to be pollinated by deceit. Pollinators may be deceived by (and thus will select for) female flowers that are larger or more attractive than male flowers (Kawagoe and Suzuki 2002) or alternatively, that mimic male flowers (Willson and Ågren 1989, Ågren and Schemske 1991, Le Corff et al. 1998). On the inflorescence level, pollination by deceit may select for a gender or genders that maximizes the mistake visits to female flowers (Ferdy et al. 1998, Castillo et al. 2002), and thus may lead to constraint on gender.

An especially advantageous adaptation of monoecy is that, by segregating the sexes into distinct flowers, it facilitates gender plasticity. If gender plasticity has been
selected for, individuals can facultatively adjust their relative allocation to male or female function, according to plant size (de Jong and Klinkhamer 1994, Klinkhamer et al. 1997) or environment (Charnov and Bull 1977, Korpelainen 1998). Gender plasticity differs from adaptive gender constraint, in that gender change takes place during the lifetime of an individual, rather than in the population over evolutionary time. Gender plasticity may be selected for when individuals can gain more fitness from one sex than the other, and the relative fitness gain can change with plant condition, microhabitat, variation in environment over time, availability of potential mates, or pollination ecology (Charnov 1982, pp. 202-215; Bawa and Beach 1991). Unlike some of the other adaptations postulated to maintain monoecy, the advantages of gender variation (adaptive constraint or plasticity) may be maximized in monoecy compared to other sexual systems, and thus may be particularly important evolutionarily. The interplay of gender plasticity and constraint, and the correlates of gender in a natural population of Begonia urophylla, are the subjects of Chapter 3. In Chapters 4 and 5, laboratory experiments explore possible causes of gender in B. urophylla.

Samman et al. 2001, Sarkissian et al. 2001, Bertin 2007). Very few studies, however, have involved monoecious herbs of the forest understory (Cid-Benevento 1987, Schlessman 1987, Sato 2002), except for the celebrated Jack-in-the-pulpit, *Arisaema triloba*, and its close relatives (e.g. Lovett Doust and Cavers 1982, Clay 1993, Richardson and Clay 2001, Vitt et al. 2003). Even fewer of these studies have taken place in the tropics (Ågren and Schemske 1995, Vallejo 2001), despite the fact that monoecy can be a major sexual strategy of tropical understory plants, for example representing 15.5% of the understory flora at La Selva, Costa Rica (Kress and Beach 1994).

The genus *Begonia* comprises over 1500 tropical (and a few subtropical) species (Hughes and Hollingsworth 2008), with a variety of growth forms including shrubs, small trees, climbers, epiphytes, and many herbs of open places and, especially, of the forest understory. Almost all are monoecious, most are probably pollinated by deceit, and many are protandrous. The basic form of the inflorescence is a dichotomously branched cyme, but there are many variations of inflorescence architecture, gender, and size (Richardson 1993, Goulet et al. 1994, Golding and Wasshausen 2002). Flowers are usually large and insect pollinated, and thus relatively easy to study. The combination of stereotyped and variable features among species make begonias ideal for asking many questions about the evolution, ecology, and development of gender in monoecious plants.

To study sex expression using begonias, a species with a life history that narrows down the possible competing explanations of gender would be best. In most species, pollination by deceit could explain the evolution of gender constraint. However, there could be alternative explanations if, for example, plants are short-lived, produce many inflorescences, or otherwise have male and female flowers in bloom at the same time.
Fortunately, there are long-lived herbaceous species, such as *Begonia urophylla*, that produce just one inflorescence per season, and that have complete separation of male and female phases within the inflorescence. Morphological specialization of male and female flowers might complicate selection on gender, but there are species like *B. urophylla* with roughly similar male and female floral morphology. Likewise, studies of gender plasticity are simplified in small growing species like *B. urophylla* (making measurement of plant size easier) that live in variable habitat, and do not show obvious clonal growth or other forms of asexual reproduction (but see Chapter 6).

This is a story of one species, *Begonia urophylla*, but some of the findings may apply in general to long-lived herbs of the rainforest understory. It is hoped that this study will contribute to our understanding of plant reproductive ecology, inspire more research in the mysterious world of the forest understory, and encourage the use of the diverse and varied genus *Begonia* in such research.
CHAPTER 2

Reduced photoperiod induces partially-synchronous flowering in *Begonia urophylla*¹

SUMMARY

The monoecious understory herb *Begonia urophylla* blooms in the dry season at Las Cruces, Costa Rica, and bloom is partially synchronized. An experiment in the lab investigated reduced photoperiod as an unambiguous cue for flowering. The inductive period was found to be 3 weeks for flowers but less for bud formation. Variation in bloom time may be an adaptation to enhance fitness gain of plants, such as *B. urophylla*, with sequential male and female phases.

BACKGROUND

Synchronization of blooming within a plant population may increase a plant’s chances of mating with other individuals of the same species. It may improve pollination and seed set (Kelly 1994), satiate seed predators (Janzen 1974) and increase outcrossing and genetic recombination (Lin 2000). Partial synchronization, in which individual plants of the same species do not completely overlap in bloom, may benefit plants that have sequential male and female phases, such as begonias. To fully or partially synchronize their blooming, plants may use a signal from the environment as a cue. In tropical forests, cues such as a change in water status (Newberry et al. 2006, Borchert 1983, Opler et al. 1976, Reich and Borchert 1982), temperature (Appanah 1993, Yasuda et al. 1999, Ashton et al. 1988, Sakai et al. 1999), light intensity (Wright and van Schaik 1994,

Chapman et al. 1999, Yeang 2007), or photoperiod (Rivera and Borchert 2001) may be sensed by trees in the canopy. Cues used by canopy trees, however, may not be effective in the understory.

In the understory, photoperiod may be the most reliable cue to induce bloom. Other cues such as temperature, drought, rains, and light intensity may be too different across understory microhabitats to be reliable. Differences in elevation, slope, canopy density, and soil may modify these potential flowering cues, even over short distances. On the other hand, change in photoperiod is not modified by the topography or canopy, and should be consistent across microhabitats.

METHODS

We studied photoperiodic bloom induction in *Begonia urophylla*, a monoecious rainforest herb that ranges from Chiapas, Mexico to Colombia and Peru (Solomon 2008). At Las Cruces, Costa Rica, it is found in the understory and begins to bloom in mid-December at the end of the wet season, and continues to bloom through much of the dry season. The bloom is partially synchronous and spread out over two months (Le Corff et al. 1998). One of us (Cozza) observed plants in the forest from early January – April 2003 and late December – April 2004. In 2004, bloom began about a month before the rainy season ended, making drought an unlikely cue for induction. At this latitude (8° 47' N; longitude 82° 57' W, elevation ~1200 m), the shortest day is about 11 hour and 25 minutes (Edwards 2001).

We propagated plants of *B. urophylla* from leaves collected at Las Cruces, and grew the plants in the lab at University of Miami. We potted the plants individually in 4”
geranium pots with Turface® (Profile Products LLC, Buffalo Grove, IL, USA), a coarsely ground montmorillite clay, as the growing medium. Nutrients were supplied weekly with Peters 20-20-20 fertilizer at 0.64g/L, supplemented with 0.16g/L NH₄NO₃. Plants were grown under 40W cool white fluorescent lights with a photoperiod of 12 hours light: 12 hours dark. Average photosynthetically active radiation (PAR) was 835 µmol/s/m² at 3 cm under the bulb and 636 µmol/s/m² at 8 cm under the bulb. The 3-8 cm range reflects the range of a plant’s leaf distances from the bulbs.

At the start of the experiment, about 1 year and 9 months after propagation, rhizomes were ~ 6-10 cm in length (this best reflects plant size since rhizomes creep along ground rather than growing upright). Three treatments of ten haphazardly selected plants each were placed under a lightproof, vented plastic tent with an 11 hour light: 13 hour dark cycle for three weeks, two weeks, or one week. This photoperiod, although it is shorter than the shortest day at Las Cruces, was chosen to ensure induction, especially since the photoperiod that the plants actually experience may be different in different microhabitats in the understory. Ten control plants were maintained under a 12 hour light: 12 hour dark cycle. Once removed from their respective inductive treatments, plants were placed back in 12 hour light: 12 hour dark cycles. They were checked weekly for buds and inflorescences from 40 to 120 days after induction. Temperature was checked almost daily during the treatment period. A paired sample t-test showed no temperature difference between the 11 hour treatments and the control ($t = 1.46, df = 16, P = 0.16$). Moisture was supplied to all plants in excess (by automatic mist every two hours), and thus did not differ between treatments.
RESULTS
In the 11 hour light: 13 hour dark treatments, a majority of the plants produced inflorescence buds (Figure 2.1). Whether or not these buds developed into blooming inflorescences depended on treatment. All of the plants bloomed in the 3 week treatment, while only 6 of 10 bloomed in the two week treatment, and none bloomed in the one week treatment. In the control, 3 of 10 plants produced buds but none bloomed. Buds first appeared about 40 days after the start of induction. Flowers first appeared about 80 days after induction. Buds and flowers were still present on some plants 125 days after induction, when final observations were made, however no new plants were induced to bloom after this time.

DISCUSSION
We conclude that reduction of photoperiod is an unambiguous cue for flowering in B. urophylla. Because all plants were kept constantly moist, and temperature did not vary between treatments, changes in water status and temperature can be ruled out as cues in our experiment. This supports observations at the field site of plants blooming partially-synchronously in moist streambeds, on dry hilltops, shaded slopes, exposed cliffs and in other microhabitats, where moisture and temperature cues might differ or be lost altogether.

We can estimate the threshold photoperiod for bloom induction in the forest by using the number of days observed between the start of induction and bloom in the lab, if we assume that induction in the forest has the same time course as it did in the lab. Plants
in the three-week treatment started blooming about 80 days after the start of induction. In the forest, the earliest bloomers started to bloom around December 10. Subtracting 80 days gives the start of induction for these plants on about September 21, when the day is exactly 12 hours long. Thus, we infer that plants should be induced to bloom when photoperiod decreases below 12 hours for a three week period. The induction of buds (but not flowers) in three of the control plants supports a threshold photoperiod of close to (but somewhat less than) 12 hours. Closer to the Equator, where seasonal variation in photoperiod is reduced, bloom might occur later in the year. Equatorial populations may exist and could help resolve how bloom induction can occur at the equator, where there is no appreciable difference in photoperiod throughout the year (Borchert et al. 2005, Yeang 2007).

Buds and flowers had different inductive periods in *B. urophylla*. The one week treatment induced inflorescence buds in most of the plants, but a longer period of induction (between two-three weeks) was needed for the buds to complete their development into flowers. Inflorescence buds that did not complete their development were lost by abscission.

Although photoperiodic induction usually functions to synchronize bloom, flowering in *B. urophylla* occurs only partially synchronously. Individual plants of *B. urophylla* begin blooming over the course of about two months (Le Corff et al. 1998), usually producing just one inflorescence. The male flowers of an inflorescence bloom before the females. Bloom times are staggered, so nearby plants may be in different sex phases, and thus there are increased opportunities for mating. The timing of bloom may affect the relative success of a plant’s male and female phases. Plants that bloom earlier
than their neighbors will function mostly as females (because they enter female phase just as neighbors are starting bloom as males), while the latest bloomers will function mostly as males.

Variation in bloom time could be caused either by differences in the actual photoperiod plants experience, or by differences in nutrients and energy available to individual plants. Although changes in photoperiod are the same across microhabitats, the actual photoperiod experienced by a plant that is more heavily shaded could be different from that of a plant that is less shaded. Alternatively, though induction is generally thought to be an all-or-nothing phenomenon, the lag period between induction and bloom could vary between individual plants (Coupland 1995). Most plants of *B. urophylla* in the forest have a single shoot which produces leaves continuously during the rainy season. After the inductive signal is received from the leaves, an inflorescence is formed as new growth at or near the shoot apex. Since the floral meristem is not pre-formed, there is a considerable and potentially variable lag period between induction and bloom. Large plants, or those growing in favorable conditions (such as high light or little moisture stress), may be able to obtain and store more nutrients and energy. These plants may be able to form the inflorescence bud more quickly once induced. Plants growing in marginal conditions may need more time to accumulate or mobilize the resources that they need to develop buds and flowers.

The ability to adjust the time-to-blooming after induction could have been favored by natural selection in *B. urophylla*. Female function generally requires more energy than male function does (Klinkhamer et al. 1997, Korpelainen 1998). If stronger plants bloom earlier, when there are more mating opportunities for females, female function
would be enhanced. Conversely, plants that are too weak to develop fruits and seeds would have their male function enhanced by blooming later. Adjusting bloom time based on energy or nutrient status may be an adaptation to enhance fitness gain from male vs. female function in this tropical understory herb.
Figure 2.1. Bud and bloom induction of *Begonia urophylla*. Bars represent the number of plants that budded (open) and bloomed (solid). Plants were exposed to 11 hour light and 13 hour dark cycles for one week, two weeks and three weeks. The control plants were exposed only to 12 hour light and 12 hour dark cycles. There were ten plants in each treatment. No plants in the control and one week treatments bloomed.
CHAPTER 3

Plasticity vs. constraint in the sex expression of *Begonia urophylla*

SUMMARY

Developmental constraint and phenotypic plasticity may interact to determine the sex expression of a monoecious plant. I studied *Begonia urophylla*, a perennial herb of the tropical rainforest understory, for two flowering seasons at Las Cruces, Costa Rica. Inflorescence architecture is highly constrained, and ideally produces a phenotypic gender (proportion of flowers that are female) of about 0.5. However, most plants were less female than predicted, mostly through abortion of female buds. Plants that bloomed in two successive years, or that produced two successive inflorescences in the same year, showed gender plasticity. Large plants produced more flowers, were more female in gender, set more fruits, and had a taller inflorescence than small plants. Large plants also were less variable in gender than small plants. Few correlations were detected of reproduction with environmental resource availability, measured as canopy openness, soil moisture, pH, and soil P, NH₄ and NO₃. There was a bimodal distribution of gender in the population. Phenotypic selection analysis on seed production suggests an optimal gender of 50-60% female, yet plasticity to be less female than this optimum, and in particular to express only male function, has been maintained.

BACKGROUND

Flowering plants have evolved a variety of sexual systems. In most of these systems, at least some individuals express both sexes. In such cosexual individuals, gender, the
relative expression of male and female function, may be developmentally constrained to an optimal gender or genders, or it may be variable and plastic during a plant’s lifetime. Gender constraint and plasticity are strategies that, under different evolutionary circumstances, may allow the fitness of each sex to be maximized (Charnov and Bull 1977, Freeman et al. 1984).

The interplay of gender constraint and plasticity may occur at different modular levels of reproductive development, depending on the sexual system (Cox 1988). Most species of flowering plants are hermaphroditic, with both sexes functioning in each flower. In hermaphrodites, gender is the relative allocation to male and female structures within the flower, but how to count petals and other shared structures is problematic (Charlesworth and Morgan 1991). In monoecious plants, male and female structures are segregated into separate flowers, and gender can be determined simply by the relative production of flowers of each sex, either on the level of the inflorescence or the whole plant.

Developmental constraint on gender is favored by selection if there is a gender (or genders) that maximizes both male and female fitness under most circumstances. Inflorescence architecture, through the branching pattern and locations of male and female flowers, determines and constrains gender in monoecious plants (Lovett Doust and Harper 1980, Bertin and Kerwin 1998, Arntz et al. 2002, Sun et al. 2006). A plant’s life history may favor constraint on gender, for example, in adaptation for wind pollination (Vollbrecht et al. 2005), self pollination (Arntz et al. 2002, Ågren and Schemske 1995), or when specific pollinator movements on the inflorescence enhance

Gender plasticity is a change in sex expression by individual plants, which is not solely a manifestation of the plant’s developmental program (Schlicting and Smith 2002, Diggle 2002). Plasticity is favored by selection if different genders maximize male or female fitness under different circumstances (Alpert and Simms 2002), and if local adaptation does not occur (Linhart and Grant 1996, Sultan and Spencer 2002). Gender may be modified according to environment (Charnov and Bull 1977, Korpelainen 1998) or plant size (de Jong and Klinkhamer 1994, Klinkhamer et al. 1997). In general, good conditions or large size will favor the sex that gains the most fitness from the increased resources. Often, that is the female sex, because seeds and fruits are more costly than pollen (Charlesworth and Morgan 1991). If this is the case, large plants or those in good conditions will be highly female (Charnov and Bull 1977, de Jong and Klinkhamer 1994, Klinkhamer et al. 1997).

Monoecious plants in the forest understory should experience the evolutionary conflict of gender constraint vs. plasticity. The understory is a heterogeneous habitat that might favor plasticity (Alpert and Simms 2002), while on the other hand, understory plants have a variety of specialized life histories and pollination strategies which might favor constraint. Few species have been studied, however, and trends have not been revealed (Lovett Doust and Cavers 1982, Clay 1993, Vitt et al. 2003, Sato 2002, Schlessman 1987, Cid-Benevento 1987, Ågren and Schemske 1995, Vallejo 2001). Very little is known about the sex expression of most monoecious understory plants, especially in the tropics.
Begonias, many of which live in the understory of tropical forests, offer a model system to study constraint vs. plasticity in sex expression. Almost all of the >1500 species are monoecious (Hughes and Hollingsworth 2008, Clement et al. 2004; see Shui et al. 2002 for some dioecious spp.). Branching patterns of the inflorescence and locations of male or female bud development are highly stereotyped within species, yet they vary between species, so inflorescence traits are presumably subject to selection (Goulet et al 1994).

The pollination system of begonias could select for constraint on gender. In most species, male flowers offer pollen that is gathered by small generalist bees, while female flowers offer nothing, and depend on the bees to land on them by mistake (Ågren and Schemske 1991, Le Corff et al. 1998). This system, pollination by deceit, could lead to different optimal genders, depending on pollinator behavior. In one scenario, stabilizing selection acts to produce a somewhat male biased gender that, on the level of the patch, most often leads to a visit to a male flower followed by a mistake visit to a female flower (Castillo et al. 2002). In an alternative scenario, the deceptive flowers are visited most often if they are either rare (so that bees don’t learn to avoid them), or common (so that naïve bees have a greater chance of making a mistake); disruptive selection may then produce a bimodal distribution of gender in the population (Ferdy et al. 1998).

Although pollination by deceit may tend to constrain gender in begonias, heterogeneity of the understory over space and time could select for gender plasticity (Alpert and Simms 2002). Resources for understory plants are usually very limited, but sunflecks and nutrient rich patches occur unpredictably (Chazdon and Pearcy 1991, Le Corff 1993, Grogan and Galvao 2006). In addition, the steady accumulation of meager
resources by older and larger plants could select for gender plasticity based on age or size. Gender plasticity could have several mechanisms in begonias, despite the developmental constraints on inflorescence architecture. Mechanisms of gender variability in cultivated *Begonia semperflorens* included change in the ratio of inflorescence branching (which increases the relative production of female flowers) to elongation (which increases the relative production of male flowers), and differential loss of male or female buds (Matzke 1938).

Developmental constraint and plasticity may interact to determine the gender of an individual *Begonia* plant. If adaptation for pollination by deceit is most important to fitness, then gender should be constrained. If conditional factors like understory microhabitat or plant size are most important, then gender should be plastic. To address this issue, I studied the rainforest herb *Begonia urophylla* to ask the following questions:

1. How much does gender vary from the constraints imposed by inflorescence architecture?
2. Do individual plants show gender plasticity between and within flowering seasons?
3. Does plant size or environment correlate with gender?
4. Is there evidence for an optimal gender in *B. urophylla*?

**METHODS**

*Study species and sites*—*Begonia urophylla* Hook. is a perennial understory herb. A mature plant has a single creeping rhizome (stem) with several (usually 3-8) fleshy leaves. Leaf size varies considerably depending upon overall plant size. Plants produce leaves during the rainy season and flower during the dry season, with a single
inflorescence (occasionally two or very rarely three or more) displayed an average of 30 cm above the rhizome, but sometimes much lower, even beneath the leaves. Each dichotomously-branching inflorescence is protandrous, producing male flowers first, and then (usually) female flowers. Typically, the male and female phases do not overlap in an inflorescence (Le Corff et al. 1998), making plants, in effect, temporally dioecious (Cruden 1977).

*Begonia urophylla* grows mostly in mountainous areas of tropical forests from Chiapas, Mexico to Venezuela and Peru (Solomon 2008). I studied *B. urophylla* at Las Cruces Biological Station, Costa Rica (8° 47' N, 82° 57' W), a premontane rainforest fragment on the Pacific slope near the Panamanian border. Elevations range from 1000-1350 m, and rainfall is about 4000 mm per year with strong seasonality, 95% falling from April-December (2005-2007 weather data, OTS 2008). The distinct dry season lasts from January to March (Hartshorn 1983, OTS 2008). There are no weather data from the study period (2003-2004).

Large numbers of plants grew at three sites, which I call Mixed Forest, Secondary Forest, and Primary Forest. The Mixed Forest site is near the station on the Rio Jaba trail at Quebrada Wilson, the Secondary Forest site is farther into the forest at Rio Jaba, and the Primary Forest site is farthest from the station (but close to the edge of the fragment) on the Lower Loop trail near Quebrada Nocaraca. The sites represent the range of environments in which *B. urophylla* is found, including trailsides and streamsides, natural or cut banks, cliffs, hilltops, and unstable slopes. Distances between the sites range from 625-690 m. Though geographically distinct, it is unlikely that the sites represent genetically isolated populations. Small stingless bees are the main pollinators of *B.*
urophylla (Le Corff et al. 1998); related bee species in Panama have flight ranges of up to 2.1 km (Roubik and Aluja 1983), and in Brazil of up to 950 m (Araujo et al. 2004). Data from all three sites were combined for analyses, except as noted.

Sixty-five plants flowered at the Mixed Forest site in 2003 and 122 in 2004. In the Secondary Forest site, 51 plants flowered in 2003 and 113 in 2004. In the Primary Forest site, 94 plants flowered in 2003; only these same individuals were studied in 2004, of which 67 flowered again.

**Phenotypic gender**—Phenotypic gender is defined as the sex expression of each individual plant, without regard to the availability of potential mates in the population (Lloyd 1980a). I calculated phenotypic gender in *B. urophylla* as the proportion of flowers on an inflorescence that were female. At the Mixed Forest site in 2003, I censused flowers and fruits every 2-3 days. At the Mixed Forest site in 2004 and the Secondary Forest site in both years, I censused 1-2 times a week. The Primary Forest site was censused only once in 2003 and twice in 2004. I was, however, able to infer the sex of buds and flowers that I missed by the positions and freshness of the scars they left on the inflorescence, and whether or not they had flowered by the size of the scar and the presence of withered floral structures. For example, female buds are produced in a different geometric orientation than the preceding males, and frequently leave a dangling pedicel if they flower without setting a fruit. I used the first (and usually only) inflorescence of each plant for all comparisons (except for gender plasticity within a season).
In addition to gender, I considered reproductive effort at several stages of development. To estimate reproductive effort during initial floral development, I calculated what I call the *inflorescence branching index*. This index is the sum of the maximum and minimum numbers of levels of branching, on both sides of the dichotomously branched inflorescence (Figure 3.1). The inflorescence branching index reflects the number of flower buds initiated, before any bud losses or damage to the inflorescence occur. I assumed that terminal female buds were initiated as the final level of branching on all inflorescence axes of all plants, even if they could not be observed. As measures of reproductive effort at subsequent stages of development, I counted the number of male and female flowers an inflorescence produced, and the number of fruits it set. I defined fruit set as the initiation of a young fruit from a female flower, and considered a fruit to be set if the ovary was retained after petals were lost, the stigma withered, and it started to turn green. Finally, to consider indirect reproductive effort, I measured inflorescence height as the length of the stalk, from the point of its attachment on the rhizome, to the scars left by the bracts that originally enclosed the entire set of flower buds. The inflorescence stalk is a secondary sexual characteristic that can contribute to fitness (Mendez and Diaz 2001).

Number of flowers, gender, and number of fruits set often were not normally distributed, or showed heterogeneity of variance. Accordingly, to correlate these variables with each other, or with measures of resource availability, I used Spearman’s Rank Correlation ($r_s$) unless otherwise specified. For statistical analyses, I employed spreadsheets for Spearman’s correlation and quadratic regression (J. McDonald, University of Delaware); Mann-Whitney U-tests, Wilcoxon signed ranks tests, and Chi-
squared tests (R. Lowry, Vassar College); and Pearson’s correlations, linear regressions, and t-tests on Excel (Microsoft Corporation, Redmond, WA, USA).

**Developmental constraint** —If gender is wholly developmentally constrained in *B. urophylla*, a stereotyped or idealized inflorescence architecture would determine gender. This idealized architecture, based on field and laboratory observations (Figure 3.2), shows symmetrical dichotomous branching, with a male flower produced at the first branch point and at each succeeding branch point. The final branching on each axis produces two female flowers, one to each side of each final male flower. Thus, the population of flowers on the inflorescence doubles at each level of branching, and because the last level of branching yields female flowers, the phenotypic gender (proportion female) of the idealized inflorescence approaches 0.5 (Table 3.1). To determine whether gender was constrained by architecture, I compared the observed proportion female with that expected from the idealized architecture, and analyzed the correlation of proportion female with the total number of male and female flowers each plant produced.

**Gender plasticity**—I tested for directional gender change at the population level over two successive years. For plants that flowered in both years, I compared the proportion female in 2003 to that in 2004 using Wilcoxon signed ranks tests. To determine whether change in gender was associated with change in plant size (measured as total leaf area as described below), I categorized plants as becoming larger or smaller, and more or less female, and performed a chi-squared test of association.
I also checked for changes of sex expression within seasons, by focusing on plants that produced two inflorescences in the same season. I compared flower production, proportion female, and fruit set in the successive inflorescences using Wilcoxon signed ranks tests.

**Plant size and gender**—I measured plant size non-destructively as total leaf area at the end of each flowering season in 2003 and 2004. To enable calculation of leaf area from the width of the assymetrical leaves, I collected a series of leaves, representing the full range of leaf sizes. I then used a leaf area meter (Model AM100, Analytical Development Company, Hoddesdon, Herts, UK) to measure the area and the width of each leaf. Leaf width accurately predicted leaf area ($r^2 = 0.996$, $N = 71$, $P < 0.001$) using the power regression equation:

$$\text{leaf area in cm}^2 = 1.3704 \times (\text{leaf width in cm})^{1.8693}$$

I analyzed the correlation of total leaf area with measures of reproduction (inflorescence branching index, total flowers, proportion female, fruits set, and inflorescence height), and Bonferroni corrected the P values. To assess a possible trade-off between reproductive vs. vegetative growth, I correlated measures of reproduction with the size ratio of the youngest leaf (leaf #1, produced just before flowering) to an old leaf (leaf #4, produced about a year earlier). Mature leaf size in this species is highly plastic, ranging from 5-240 cm$^2$. The single rhizome produces new leaves one at a time,
so that leaf size represents most of the allocation to above ground vegetative growth, at the time when each leaf was produced.

To test if plant size affects gender variability, I ordered plants by total leaf area for all sites combined, and divided them into groups of 20. I calculated the coefficient of variation (CV) of the proportion female for each group of 20, and used Pearson’s correlation to test for a relationship between CV and average total leaf area. I also grouped the plants by intervals of 50 cm$^2$ of leaf area (or 100 cm$^2$ if there were fewer than 3 plants in an interval), and again used Pearson’s correlation to test for a relationship between CV and average total leaf area.

**Environment and gender**—I correlated resource availability in 2003 (canopy openness, soil moisture, pH, P, NH$_4$, and NO$_3$) with reproduction in 2003 and in 2004 (inflorescence branching index, total flowers, proportion female, fruits set, and inflorescence height), and Bonferroni corrected the P value.

To estimate light availability to each plant, I took canopy openness readings using a convex spherical densiometer (Lemmon 1956) held just above each plant (or as close as possible) at the end of the flowering season in 2003. Because the leaves of each plant clearly faced in one direction, I took the densiometer readings facing only in that direction.

To measure soil moisture, pH, N and P, I took a small soil sample (about 50 cm$^3$) near each plant at the end of the flowering season (and therefore also close to the end of the dry season) in 2003. Each sample comprised 4 sub-samples from the top 2-3 cm of the soil surface, 5-10 cm from the plant. I dried each sample to constant weight at 60°C,
and calculated percent soil moisture. Soil moisture data were not included from the Primary Forest site, as it was sampled the day after the other two sites, after it may have rained. The lab at the Centro Agronómico Tropical de Investigación y Enseñanza (CATIE) performed soil nutrient analyses (pH in water, ammonium N, nitrate N, and available P using the Olsen method) for plants that had the most male- and female-biased genders at all three sites in 2003. The remaining samples were stored in a refrigerator and analyzed in 2004 for pH and available P, but not for N.

To explore possible correlations of reproduction with the internal concentration of nutrients in the plants, I harvested a series of 23 plants representing the full range of genders at the Secondary Forest site, after they had flowered in 2004. I dried the above-ground shoots (rhizome + leaves) at 60°C. CATIE analyzed the samples for concentration of N, P, K, Ca, Mg, Cu, Zn, Mn, and Fe. I correlated the concentration of each nutrient with reproduction (total flowers, proportion female, and fruits set) and Bonferroni corrected for a total of 39 comparisons.

Because arbuscular mycorrhizal fungi are involved in nutrient uptake, the percent colonization of roots by mycorrhizas might be correlated with gender. Fine root samples were collected from each of the harvested plants, cleared and stained with Trypan blue (Vierheilig et al. 2005), and observed at 400x under the compound microscope for vesicles of arbuscular mycorrhizas using the line intersect method (Giovannetti and Mosse 1980). I correlated percent mycorrhizal colonization with measures of reproduction (total flowers, proportion female, and fruits set).
Optimal gender— I constructed frequency distributions of gender for plants at the Mixed Forest and the Secondary Forest sites, where every flowering plant was counted in both years. In addition, at the Mixed Forest site in 2003, I was able to determine when the first flower opened on each inflorescence. I compared the sex expression of early flowering plants (first flower opened before Jan. 18) to that of late bloomers (first flower opened Jan. 18 or later) using a Mann-Whitney U-test. I also tested for differences in plant size (leaf area), canopy openness, and soil moisture between early and late bloomers, using t-tests.

To estimate female fitness, I collected seeds from all plants at the Secondary Forest site in 2004. I harvested fruits from each plant’s first inflorescence as they ripened (fruit turning brown and dehiscing) and weighed the seeds without further drying. I correlated total seed weight with total number of flowers produced, number of female flowers, and number of fruits set using Pearson’s correlation, and seed weight with proportion female using Spearman’s rank correlation. To control for plant size, I calculated seed weight per cm² of total leaf area, and correlated it with proportion female using Spearman’s rank correlation. A phenotypic selection analysis was performed to test for stabilizing selection on gender. To estimate relative female fitness for each individual, I divided each plant’s total seed weight by the average seed weight for the site, and regressed relative fitness on gender (proportion of flowers that were female) using quadratic regression. I repeated the analysis using relative seed weight per cm² of total leaf area as the measure of relative female fitness.
RESULTS

**Gender constraint and plasticity**—Inflorescence architecture usually set an upper limit on proportion female, but not a lower limit (Figure 3.3). In 2003, only 2% of plants were more female than predicted by architecture; most were less female than predicted. In 2004, 17% of plants were more female predicted by architecture, but most plants were still less female than predicted. Proportion female was more tightly correlated with the number of female flowers a plant produced, than with the number of male flowers (Figure 3.3).

A total of 113 plants that flowered in 2003 flowered again in 2004. Plants produced more flowers in 2004 than in 2003 (Wilcoxon’s $W = -3145, N = 113, P < 0.001$). Plants also were proportionally more female in 2004 than they were in 2003 (Figure 3.4A; $W = -4399, N = 113, P < 0.001$).

In 2003, 19 plants (9.0% of those that flowered) produced a second inflorescence; in 2004, 59 plants (19.5%) did so (Figure 3.4B). In both years, the second inflorescence was proportionally less female than the first (in 2003: $W = 78, N = 19, P = 0.046$; in 2004: $W = 656, N = 59, P < 0.01$). In 2004, the second inflorescence also produced fewer flowers ($W = 896, N = 59, P < 0.001$) and fewer fruits ($W = 486, N = 43, P < 0.001$) than the first. Although 64% of the 113 plants that flowered in both years became either larger and more female, or smaller and less female, this association was not significantly different than would be expected by chance ($\chi^2 = 0.60, df = 1, P = 0.47$).

**Plant size, environment, and gender**—Large plants, as measured by total leaf area, had a more highly branched inflorescence, produced more flowers, were proportionally more
female, produced more fruits, and had a taller inflorescence than small plants in both years (Table 3.2). The only exception was number of fruits set in 2003, which was not significantly correlated with total leaf area. No trade-off of reproduction with vegetative growth (expressed as the size ratio of young leaf to old leaf) was detected. Small plants were more variable in gender, as measured by coefficient of variation (CV), than large plants were in 2003 (sorted into groups of 20 plants: $r = 0.71$, $N = 10$, $P = 0.02$; sorted into size intervals of 50 cm$^2$: $r = 0.48$, $N = 14$, $P = 0.08$), and more strongly in 2004 (Figure 3.5A, sorted into groups of 20 plants: $r = 0.81$, $N = 15$, $P < 0.001$; Figure 3.5B, sorted into size intervals of 50 cm$^2$: $r = 0.88$, $N = 18$, $P < 0.001$).

Measures of reproduction in 2003 and 2004 were only weakly correlated with environmental resource availability in 2003 (Table 3.2). Number of fruits set in 2003 was negatively correlated with soil moisture (which ranged from 9 to 55% of wet soil weight), and inflorescence height in 2004 was positively correlated with soil pH (which ranged from 5.1 to 7.9) in 2003. No significant correlations were detected between reproduction in either 2003 or 2004, and canopy openness (range: 5 – 43%), soil phosphorus (range: 2 – 30 mg/Kg), soil nitrogen as NH$_4$ (range: 4 – 117 mg/Kg), or soil nitrogen as NO$_3$ (range: 2 – 57 mg/Kg) in 2003.

In the harvested gender series, calcium concentration in the shoot was positively correlated with total number of flowers ($r_s = 0.65$, $N = 23$, $P = 0.03$). When only those plants that were $\leq 50\%$ female were considered, calcium concentration also was positively correlated with proportion female ($r_s = 0.78$, $N = 14$, $P = 0.04$). Flower and fruit production, and proportion female, were not significantly correlated with the concentrations of N, P, K, Mg, Cu, Zn, Mn, or Fe in the shoot. Roots were colonized by
arbuscular mycorrhizas, but no correlation of percent colonization by vesicles with number of flowers, proportion female, or number of fruits was detected. There was no correlation of total leaf area with reproduction (total flowers, proportion female and fruits set) in the gender series sample. Total leaf area also was not correlated with concentration of any of the nutrients in the shoot. Total leaf area was, however, a good predictor of leaf biomass ($r = 0.81$, $N = 23$, $P < 0.001$) and leaf + rhizome biomass ($r = 0.77$, $N = 23$, $P < 0.001$).

**Optimal gender** — There was a bimodal distribution of gender at the Mixed Forest and Secondary Forest sites combined, in both years (Figure 3.6). Peaks of proportion female were at 0 (all male) and 0.3-0.5 (partly female). In 2004, there was a third peak at 1 (> 90% female flowers); these highly-female plants were all at the Secondary Forest site. The all-male peak had more plants than the partly-female peak in 2003, while the partly-female peak had the most plants in 2004.

At the Mixed Forest site in 2003, plants that flowered later in the season were proportionally less female than those that had flowered earlier (Mann-Whitney’s $U = 266$, $n_{\text{early}} = 39$, $n_{\text{late}} = 25$, $P = 0.002$). Although there was no difference in plant size (total leaf area) or soil moisture between early and late bloomers, early bloomers experienced an average of 17% greater canopy openness than late bloomers ($t = 1.67$, $n_{\text{early}} = 38$, $n_{\text{late}} = 24$, $P_{\text{one-tailed}} = 0.05$).

Plants that were about 50% female had the highest values of total seed weight at the Secondary Forest site in 2004 (Figure 3.7A). Plants that were less than 40% female, as well as those that were more than 90% female, produced only relatively low seed
weights. Total seed weight was strongly and positively correlated with total number of flowers \( r = 0.81, N = 60, P < 0.001 \), female flowers \( r = 0.79, N = 60, P < 0.001 \), and fruits set \( r = 0.92, N = 60, P < 0.001 \). Total seed weight was also strongly correlated with total leaf area \( r_s = 0.69, N = 59, P < 0.001 \). Controlling for plant size, plants that were about 50% female still had the highest values of seed weight per cm\(^2\) of leaf area (Figure 3.7B). A plant’s total seed weight, relative to the site average, was predicted by its proportion female according to the parabolic function: 

\[
y = -8.26x^2 + 9.94x - 1.47
\]

(Figure 3.7A; \( r^2 = 0.33, N = 60, P < 0.001 \)). A plant’s total seed weight per unit leaf area, relative to the site average, also was predicted by its proportion female by the parabolic function: 

\[
y = -6.12x^2 + 7.08x - 0.69
\]

(Figure 3.7B; \( r^2 = 0.26, N = 59, P < 0.001 \)).

**DISCUSSION**

**Gender constraint and plasticity**—Despite the rigidity of the developmental program, inflorescence architecture only partially constrained gender in *B. urophylla*. Relatively few plants were more than 50% female, the gender predicted by architecture in all but the smallest inflorescences. However, there seemed to be no constraint on being less female than predicted by architecture. It was common for there to be fewer than the predicted two female flowers branching from the last male flower on an inflorescence axis. Although often not visible under field conditions, in the lab it was observed that two female buds per final male bud are almost always produced, but then sometimes aborted, often at a very tiny size. Although abortion of mature female buds after male buds have flowered could be explained simply as resource depletion, abortion of tiny immature female buds before male buds have flowered suggests resource reallocation, and thus
plasticity. Female bud abortion is the main mechanism by which plants become less female than predicted by architecture. The high correlation of female flower number, but not male flower number, to gender supports this conclusion (Figure 3.3). Male buds also were aborted, resulting in plants that were more female than predicted by architecture. Although developmental variations did occur in the branching pattern and sites of male or female bud formation, they caused relatively minor changes in gender, compared to bud abortion.

Individuals of *B. urophylla* showed gender plasticity from year to year, but the correlates of this plasticity were not clear. There was no association of size change and gender change in plants that flowered in both years, despite the strong correlation of size with gender observed in the population. Microsite changes may explain the gender transitions of some plants. For example, a canopy opening could make more energy available for reproduction, and the gender of a plant could be shifted to female, as occurs in the orchid *Catasetum viridiflavum* (Zimmerman 1991) or the palm *Attalea funifera* (Voeks 1988). Conversely, a plant could have its reproductive budget cut by canopy closure, shading from competitors, environmental stress, herbivory, or pathogens (Bertin 1982, Allison 1992, Cobb et al. 2002). Such local changes would be expected to cause some plants to become more female and others to become less female. However, the overall transitions observed were directional, with more plants flowering in 2004 than in 2003, and plants producing more flowers and becoming proportionally more female. The year preceding the 2004 flowering season may have been a better one for begonia growth and resource provisioning than the year preceding the 2003 season. Unfortunately, there are no weather data to test this prediction.
Plants that made a second inflorescence showed gender plasticity within a season. The second inflorescence was proportionally less female than the first, perhaps because resources available for reproduction had been depleted. A similar occurrence was seen in cultivated squashes (El-Keblawy and Lovett-Doust 1996). In other monoecious plants, however, such as the shrub *Croton bonplandianum*, and the herbs *Arum italicum* and *Sagittaria* spp., late inflorescences were more female than the first (Uma Shaanker and Ganeshaiah 1984, Mendez 1998, Huang et al. 2002, Dorken and Barrett 2003a). This contradiction may be explained by the sexual system of these latter species. All are protogynous, producing female flowers first, then male flowers. Because female flowers on the first inflorescence may have few or no potential mates in the population (as male flowers have not opened yet), plants that minimize allocation to these early female flowers would have more resources available for subsequent flowers with better mating prospects, and would be favored by selection (Brunet and Charlesworth 1995). Squashes and most begonias (including *B. urophylla*) are protandrous, the opposite sexual system.

**Plant size, environment, and gender**—Large plants (measured by total leaf area) produced more flowers, were proportionally more female, and set more fruits than small plants. Large leaf area allows for more photosynthesis; the amount of photosynthate available may be the main determinant of sex allocation in begonias. In a lab experiment, inflorescences of *B. franconis* were grown *in vitro* under various hormonal and nutritional environments. Low sucrose levels inhibited maturation of female buds, and could presumably lead to a more male-biased gender *in vivo* (Berghoef and Bruinsma 1980). In the forest understory, light is most often the limiting resource, and it is patchy
in space and time (Chazdon and Fetcher 1984). Accumulation of sufficient
photosynthate for reproduction, particularly fruit and seed production, could take years.
In the temperate forest understory, annuals are poorly represented, and perennial species
of understory herbs may not reproduce until they are 7-10 years old (Bierzychudek 1982).
Size may be the most reliable predictor of the reproductive budget, and thus the sex
allocation, of a long-lived plant in the forest understory (Lovett Doust and Cavers 1982,
Bierzychudek 1984).

In addition to its correlation with the direct costs of reproduction, plant size was
strongly correlated with an indirect cost, the height of the inflorescence. Inflorescence
height may contribute to both sex functions. A tall inflorescence increases the visibility
of the display, and may enhance pollinator visitation (Peakall and Handel 1993).
Investment in display is thought to mostly benefit male mating success, because female
flowers may be fully pollinated by a few visits, while male flowers achieve matings
through additional visits (Stanton et al. 1986, Willson and Price 1977). In B. urophylla,
female flowers are pollinated by deceit, and have thousands of ovules, so a showy display
may also benefit female mating success (Schemske 1980, Kawagoe and Suzuki 2003,
Parra-Table and Vargas 2007). Furthermore, seeds of B. urophylla are wind dispersed, so
a tall inflorescence should increase dispersal distances, enhancing female fitness by
freeing more offspring from local resource competition with their mother (de Jong and
Klinkhamer 1994). Although the cost of building a structure increases disproportionately
with its height (Cooley et al. 2004), selection may favor the plasticity to build a single,
maximally tall inflorescence, rather than several shorter, cheaper ones.
Evidence for effects of environmental resources on the reproduction of *B. urophylla* is suggestive but inconclusive. The negative correlation of number of fruits set to soil moisture is unusual; most studies have found increased female function in moist sites (reviewed in Korpelainen 1998). However, in moist temperate forest, *Arisaema triphylla* plants growing at a wet site were predominantly male (Lovett Doust and Cavers 1982). Since moist microsites at Las Cruces also tended to be shady (*r* = −0.22, *N* = 139, *P* = 0.01), any negative effect of moisture might have been compounded by a lower availability of light energy. The positive correlation of inflorescence height in 2004 with pH is noteworthy; correlations of pH with reproduction have been reported only rarely (Lovett Doust and Cavers 1982, Korpelainen 1998). Low pH (especially below pH 5.3) in tropical rainforest soils is associated with sequestering of base metal cationic nutrients (e.g. K, Ca, Mg) and Al toxicity (Sollins 1998). Although most plants in this study were growing at pH > 5.3 as measured at the soil surface, soil pH values at Las Cruces were found to be much lower (3.95 to 5.36) in a previous study, when 15 cm deep core samples were taken (Jin et al. 2000). Roots of *B. urophylla* appeared to be mostly at or near the surface (pers. obs.), but any deeper roots may encounter a different, more hostile nutrient environment than my data show. Soil moisture, pH, and P may be more strongly correlated with reproduction than this study revealed, as suggested by significant correlations before Bonferroni correction.

In the harvested plants, the correlation of calcium concentration with flower number and gender is intriguing. Calcium helps regulate cell membrane permeability, cell wall structure and expansion, enzyme and hormone activity, and pollen tube growth
(Pilbeam and Morley 2007). How calcium may affect sex expression is unknown, but it can be a limited nutrient in tropical soils (Grubb, 1989, p. 422).

**Protandry and gender**—Male and female buds in a begonia inflorescence are probably initiated at the same time (Pastrana 1932), and thus the total number of buds initiated might reflect the resource status of the plant at the earliest stages of reproduction. In protandry, male buds mature and flower before the females. Thus, the plant’s resource status may be different when female flowers develop, compared to what it was when male flowers developed. The sequential nature of male and female flowering makes it likely that any short term resource deficiency will result in proportionately fewer female flowers on an individual.

Small plants were more variable in gender than large plants (Figure 3.5). Because small plants are less able to gather and store resources than large plants, they are more vulnerable to resource deficiency, and thus to being proportionately less female (Schlessman 1987). However, the patchy nature of resource distribution in space and time means that small plants may escape resource deficiency (Voeks 1988). Thus, on the population level, small plants could be quite variable in their sex expression. Large plants, on the other hand, may have accumulated enough resources to buffer against environmental or biotic patchiness and stochasticity, and would thus be less variable in sex expression than small plants.

**Optimal gender**—The bimodal distribution of gender observed at two sites in both years is not explained by pollination by deceit (Ferdy et al. 1998), because plants at the all-
male peak lack deceptive female flowers altogether. A gender threshold, where small plants are male and must reach a certain size before producing female flowers, could produce a bimodal distribution of gender (Lovett Doust and Cavers, 1982; Condon and Gilbert, 1988; Delesalle, 1989; Sarkissian et al., 2001; Vitt et al., 2003). In *B. urophylla*, however, distinct thresholds were not evident in plots of gender vs. plant size (data not shown).

The all-male peak of gender observed in the *B. urophylla* population probably does not represent a stable gender morph as seen in some species (Lloyd 1980a, Freeman et al. 1981), because 89% of plants that were all-male in 2003, and flowered again in 2004, changed gender. Instead, the all-male gender is probably just an endpoint of the distribution of gender (Schlessman 1986). Fewer resources for reproduction result in fewer female flowers for a given number of male flowers, until there are no female flowers; still fewer resources then produce only “male” plants with fewer flowers. At the only site and year where flowering time was recorded (Mixed Forest in 2003), the all-male plants were concentrated at the end of the flowering season. This could reflect marginal resource status, manifested both as a delay in initiation of flowering (while resources are gathered to form reproductive structures) and as a loss of female function. Less canopy openness, and thus presumably lower light levels, above late flowering plants could potentially explain both their late flowering time and their less female gender.

Is there an “optimal” gender in *B. urophylla*? Total seed weights, though only obtained for one site in one year, suggest that the highest potential for female fitness is when the inflorescence is about 50% female, which matches the partly female peak of
gender in the population, as well as the expression of the idealized architecture. When plant size is taken into account (seed weight per cm$^2$ of leaf area), the greatest potential for female fitness remains at a gender of about 50% female. This is evidence for stabilizing selection on gender in *B. urophylla*, through a component of fitness that indicates female success.

Potential female fitness diminished to either side of the 50% female gender. Plants that were < 50% female aborted female buds, and thus did not produce the maximum output of female flowers; fewer female flowers predictably led to lower total seed weight. Plants that were > 50% female aborted male buds, and would thus seem to have more resources available for female function. However, seed weights were very low for highly (> 90%) female plants. Perhaps the loss of all or most male buds indicates some environmental stress that also reduced seed weight, even if the female phase seemed normal. Alternatively, the female flowers of these plants may have not have been pollinated. The highly-female plants flowered at the beginning or middle of the season, when there were available mates (male phase plants) in the population, but they may not have been visited by pollinators. Pollination by deceit is often thought to be the mistake of naïve pollinators (Le Corff et al. 1998), but pollinator learning may also play a role. During the rewarding male phase of an inflorescence, which can last 2-3 weeks, stingless bees from the same hive(s) may visit each day, and they may become trained to it and recruit hive mates (Hubbell 1978, Breed et al. 2002, Makino and Sakai 2007). Suddenly, just after its peak production of male flowers, the inflorescence becomes female, and the bees are deceived. Without a male phase to train the bees, the female phase might not be
visited, because experienced bees can discriminate against non-rewarding female flowers (Ågren and Schemske 1991, Le Corff et al. 1998).

**Evolution of gender**—Constraints on gender in *B. urophylla* may be maintained by the selective advantage of having different male and female mating strategies. The production of male flowers at successive branch points along the inflorescence is a sequential strategy that extends the male phase, and may provide maximum opportunities for mating with female flowers on different plants. On the other hand, the simultaneous opening of all of a plant’s female flowers is an explosive strategy, giving a maximum and concentrated display that attracts the greatest number of pollinators to the non-rewarding flowers (Prusinkiewicz et al. 2007). A similar mix of male and female strategies occurs in the monoecious understory trees *Cupania guatemalensis* (Bawa 1977) and *Geonoma epetiolata* (Marten and Quesada 2001). The ability to evolve distinct male and female mating strategies on the inflorescence or plant level may contribute to the maintenance of monoecy.

Within the constraints of inflorescence architecture, reproductive plasticity acts at several points in development. The total number of buds produced by an inflorescence is determined by the number of times the inflorescence branches during early development. Inflorescences ranging in potential output from three to over a hundred buds are produced. After this early allocation decision point, the developmental program of bud identity is highly constrained, but reproductive output and gender can be adjusted by abortion of male or female buds. During inflorescence growth and the male phase, the energy budget for reproduction can be reassessed. If resources are depleted to a level that
might not allow fruit and seed production by the entire cohort of female buds, further
development of some or all female buds can be stopped (Lloyd 1980b). The mechanism
could be hormonal or nutritional (Stephenson 1981, Berghoef and Bruinsma 1980).
Female bud loss follows non-random patterns. For example, one female bud of each pair
is often aborted, or an asymmetrical pattern of bud loss on a branch is “mirrored” on the
opposite branch. This is evidence that resources are reallocated on the inflorescence level
in *B. urophylla*, as in, for example, *Solanum hirtum* (Diggle 1994).

Gender in *B. urophylla* is related strongly to plant size. Selection might favor the
plasticity for large and old plants to be maximally and optimally female. With the limited
availability of light in the understory, or the limited availability of nutrients in tropical
rainforest soils, accumulating enough resources to form fruits and seeds may be a slow
process, best enabled by the large leaf area and high nutrient storage capacity of a large
plant. Short term correlations of reproduction with available resources from the
environment may be evident only if the resources are unusually abundant or scarce.
Developmental constraint based on architecture (and perhaps ultimately on pollinator
selection) interacts with gender plasticity based on plant size to determine sex expression
in this rainforest herb.
Table 3.1. Idealized inflorescence architecture and sex expression of *Begonia urophylla*. Patterns of branching and production of flowers of each sex are shown in Figure 3.2, and are described in the text.

<table>
<thead>
<tr>
<th>number of levels of branching</th>
<th>number of male flowers</th>
<th>number of female flowers</th>
<th>total flowers ((m + f))</th>
<th>Proportion female</th>
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<td>1</td>
<td>1</td>
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<td>3</td>
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<td>3</td>
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<td>4</td>
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<td>5</td>
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<td>32</td>
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<td>6</td>
<td>63</td>
<td>64</td>
<td>127</td>
<td>0.50</td>
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</table>
Table 3.2. Correlation of resource availability with reproduction over two years. For each comparison, the top row shows Spearman’s rank correlation (significant values of r_s after Bonferroni correction are in bold), the middle row shows the original P value of each correlation before Bonferroni correction (original values of \( P \leq 0.05 \) are in italics), and the bottom row shows the number of plants as (N). The table is continued on the next page.

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<td>Size ratio of young leaf to old leaf</td>
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<td>Resource availability variable</td>
<td>Inflorescence branching index</td>
<td>Number of flowers</td>
<td>Proportion female</td>
<td>Number of fruits set</td>
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<td>Soil P</td>
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<td>0.34</td>
<td>−0.10</td>
<td>0.23</td>
<td>0.05</td>
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<td>−0.33</td>
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<td>(76)</td>
<td>(118)</td>
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<tr>
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<td>−0.35</td>
<td>0.13</td>
<td>−0.19</td>
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<td>Soil NO₃</td>
<td>−0.05</td>
<td>−0.15</td>
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*P ≤ 0.05, ** P < 0.01, *** P < 0.001; after Bonferroni correction for 40 comparisons per year.
Figure 3.1. Architecture of an inflorescence of *Begonia urophylla*. An “m” indicates a male flower and an “f” indicates a female flower. The inflorescence branching index is the sum of the maximum and minimum number of branch levels on each side of the inflorescence.

\[
\text{Inflorescence branching index} = 4 + 3 + 3 + 2 = 12
\]
Figure 3.2. Idealized architecture and resulting gender (proportion female) of two small inflorescences of *Begonia urophylla*. An “m” indicates a male flower and an “f” indicates a female flower.
Figure 3.3. Correlations of proportion female to the number of male flowers, and to the number of female flowers, in 2003 (A, B) and 2004 (C, D). Each cross (×) represents the number of male flowers produced by one plant, and each diamond (●) represents the number of female flowers. The curve represents the proportion female predicted by the idealized production of male or female flowers. Spearman’s rank correlations (rs) of proportion female to the numbers of female or male flowers are given at the upper right of each panel. For A and B, N = 209; for C and D, N = 303.
Figure 3.4. Gender changes of individual plants over time. (A) Proportion female of the first inflorescence of plants that flowered in both 2003 (open box) and 2004 (stippled box) at all sites combined (N = 113). The horizontal gap in each box shows the median proportion female, and the diamond shows the mean. Each box shows the 25th and 75th percentiles, and the whiskers show the range. (B) Proportion female of the first and second inflorescences of plants that produced two inflorescences, in 2003 (N = 19) and in 2004 (N = 59). Box plots are as described in (A). For inflorescence #2 in 2003, the median proportion female was 0.
Figure 3.5. Correlation of variability in gender (proportion female) with plant size in 2004, considered two ways. In (A), each diamond (▲) represents 20 plants, except for the rightmost diamond, which represents 21 plants. In (B), each square (■) represents a 50 cm² interval of total leaf area, except for the rightmost 5 squares, which each represent a 100 cm² interval. Numbers above the squares show the number of plants in each interval.
Figure 3.6. Frequency distributions of gender in 2003 (A) and 2004 (B), at the Mixed Forest and Secondary Forest sites combined. The gender classes from 0.1 to 1.0 represent plants up to and including that proportion female. The gender class 0 represents plants that produced only male flowers.
Figure 3.7. Effect of gender on relative fitness, measured two different ways at the Secondary Forest site in 2004. (A) Effect of proportion female on the total weight of seeds produced, relative to the mean total weight of seeds. Each circle represents one plant (N = 60). (B) Effect of proportion female on the total weight of seeds produced per cm² of leaf area, relative to the mean total weight of seeds per cm². Each circle represents one plant (N = 59). In both (A) and (B), the curve represents the quadratic regression of relative fitness on proportion female.
CHAPTER 4

Light and nitrogen affect sex expression of Begonia urophylla

SUMMARY
Monoecious plants in the rainforest understory may adjust their gender (proportion of flowers that are female) based on plant size and environment. In a factorial experiment in the lab, we grew Begonia urophylla under two levels each of light, nitrogen, and phosphorus. High light or high nitrogen caused plants to produce more flowers and to be proportionally more female. High light and nitrogen also caused plants to be larger (by weight), compared to low light and nitrogen. Treatment differences in leaf greenness suggest that the nitrogen effect on gender may act indirectly through chlorophyll metabolism. The effects of light and nitrogen on reproduction and plant size support an energy based determination of gender in this understory rainforest herb, with the flexibility to employ either size dependent sex determination, or environmental sex determination.

BACKGROUND
Many flowering plants express both sexes on each individual, and show plasticity in gender, the relative expression of male and female function. Gender plasticity may be particularly adaptive in monoecious (separate male and female flowers on the same individual) and perennial plants, compared to hermaphrodites or annuals (Charnov and Bull 1977, Korpelainen 1998, Willson 1983, pp. 70-71). Gender plasticity may be affected by plant size (de Jong and Klinkhamer 1994, Klinkhamer et al. 1997) or

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2 Co-authors: Astrid Alfaro, Jeanette Rivera, Diane Toledo, and Michelle Hershman.

Resources that are limited and patchy in distribution, and that affect the relative fitness of the sex functions, should have the greatest effect on an individual’s gender (Charnov and Bull 1977, Alpert and Simms 2002). In the rainforest understory, light is often the most limited resource, and it is patchy in space and time (Chazdon and Fetcher 1984). The sex that requires the most energy should be favored in high light microhabitats, compared to low light. In insect pollinated plants, that is usually the female sex, because seeds and fruits are energetically more costly than pollen (Charlesworth and Morgan 1991). Soil nutrients could affect gender either directly or indirectly. Male and female functions may have different direct requirements for nutrients, such as nitrogen or phosphorus, as evidenced by the nutrient content of the reproductive structures of each sex (Ashman and Baker 1992, Ishida et al. 2005). Alternatively, nutrients involved in energy metabolism could have an indirect effect on gender, through the amount of energy made available for reproduction. For example, nitrogen is a major component of chloroplast proteins and chlorophyll, and if deficient, reduces chlorophyll content, and thus photosynthetic capacity (Luttge 1997, p. 94; Barker and Bryson 2007). Phosphorus has both structural and metabolic roles (Sivak and Walker...
1986, Sanchez 2007), and may especially be limited in tropical rainforest soils (Luttge 1997, p. 73).

Environmental resources affect reproductive development at different modular levels (e.g. flower, inflorescence, or whole plant), depending on the sexual system (Cox 1988). Most angiosperms are hermaphroditic, with both sexes functioning in each flower. In this sexual system, gender can be defined as the relative allocation to male and female structures within the flower, but quantifying the relative contribution to each sex of petals and other shared structures is problematic (Charlesworth and Morgan 1991). In monoecious plants, male and female structures are segregated into separate flowers, and gender can be determined simply by the relative number or biomass of flowers of each sex, either per inflorescence or for the whole plant.

Although the effects of environment on sex expression have been widely studied (Korpelainen 1998), few species of monoecious herbs from the forest understory have been studied (Lovett Doust and Cavers 1982, Clay 1993, Vitt et al. 2003; Schlessman 1987, Cid-Benevento 1987, Sato 2002); even fewer such species have been from the tropics (Ågren and Schemske 1995, Vallejo 2001). Light and soil nutrients may affect sex expression, but the direction and magnitude of their effects, and their interaction, is unknown for tropical monoecious understory plants. In this laboratory experiment with the tropical understory herb, *Begonia urophylla*, we ask the following questions:

1. Does availability of light, nitrogen, or phosphorus affect gender?
2. Does availability of light, nitrogen, or phosphorus affect plant size, which could in turn affect gender?
METHODS

**Study species**—*Begonia urophylla* Hook. is a perennial understory herb of the Neotropical rainforest. In the forest, a mature plant has a single creeping rhizome (stem) with several (usually 3-8) fleshy leaves. Plants produce new leaves during the rainy season and flower in the dry season, usually producing a single inflorescence (occasionally two or very rarely three or more). Each dichotomously-branching inflorescence is protandrous, producing male flowers first, and then (usually) female flowers (Le Corff et al. 1998). See Chapter 3 for a thorough description of the species and its habitat.

**Plant culture**—We propagated *B. urophylla* from leaves collected at Las Cruces Biological Station, Costa Rica, and grew them in the lab at University of Miami. After establishing the plants in a commercial peat-based mixture, we potted them individually in 4” pots with Turface® (Profile Products LLC, Buffalo Grove, IL, USA), a calcined (baked), coarsely ground montmorillonite clay (arcillite), as the growing medium. Nutrients were supplied weekly with Peters 20-20-20 fertilizer (including micronutrients) at 0.64g/L, supplemented with 0.16g/L NH₄NO₃. This nutrient solution contained 69 mg/L of N (available as NH₄ and NO₃), and 56 mg/L of P, each measured as the actual elemental concentration. Plants were grown under 40W cool white fluorescent lights with a photoperiod of 12 h light: 12 h dark. Average photosynthetically active radiation (PAR) was 835 µmol/s/m² at 3 cm under the bulbs, and 636 µmol/s/m² at 8 cm under the bulbs. The 3-8 cm range reflects the range of a plant’s leaf distances from the bulbs. We
rearranged the pots weekly between edge and interior positions. Water was supplied to all plants in excess, by automatic misting with distilled water every two hours.

**Experimental design**—We began experimental treatments 26 months after propagation. Plants were randomly assigned to eight treatments (n = 100 plants per treatment), with 3 factors fully crossed in a three-way (2 × 2 × 2) design. Factors (independent variables) and their levels were: high light (0% shade) or low light (50% shade), high N (69 mg/L measured as elemental N in a nutrient solution) or low N (15 mg/L), and high P (56 mg/L measured as elemental P in a nutrient solution) or low P (5.6 mg/L). For each factor, the high level represented the conditions under which plants had already been growing vigorously for the previous 26 months, and the low level represented an intended moderate deficiency, based on the ranges of light and soil nutrient availability measured in the field (Chapter 3). Dependent variables were number of flowers produced, proportion of flowers that were female, and plant size measured as total leaf area or as biomass.

We set up the experimental treatments using commercially available 50% shade cloth and four nutrient solutions that we formulated, in place of the Peter’s-based nutrient solution that we had been using before the experiment. In all four nutrient solutions, we kept the ratio of NH₄ to NO₃ forms of available nitrogen the same as it had been in the Peter’s-based solution, but we did not include any urea nitrogen. All nutrient solutions contained the same concentrations of other macronutrients (K, Mg, Fe), and micronutrients (B, Cu, Mn, Mo, Zn) as the Peter’s-based solution. Additional calcium was not supplied, because it was not present in the Peter’s-based solution, and was
available to plants from the Turface growing medium (Carlile and Bedford 1988, Johnson 2006). In formulating experimental nutrient solutions, the concentration of one ion cannot be controlled; that ion was SO₄ in this study. In particular, the low P solutions had about 3 × the concentration of SO₄ as the high P solutions. The pH values of the nutrient solutions were between 6.0 and 6.3. We applied 70 mL of the appropriate nutrient solution to each plant weekly.

During the experiment, photoperiod, misting, and pot rearranging continued as before. Occasionally, plants produced inflorescence buds; because these buds were premature and not intentionally induced by us, we removed them promptly. Eleven weeks after treatments began, we induced the plants to flower by reducing the photoperiod to 11 hours light: 13 hours dark for 3 weeks (Chapter 2: Rivera and Cozza 2008). Mass flowering began 2 months later. During flowering, we continued to apply the nutrient solutions, but stopped rearranging the pots to avoid damaging the inflorescences.

**Data collection and harvest**—To count the number of flowers of each sex produced, we diagrammed inflorescences at two points in time: at the end of the sequential opening of male flowers, and during the nearly simultaneous opening of female flowers. The sex identity and fate of buds that were not directly observed could usually be inferred (Chapter 3). Because some plants did not flower, and others that did flower were (haphazardly) not diagrammed at or near the two critical times, sample sizes were reduced and unequal (range of n = 23 to n = 55). Although many plants produced multiple inflorescences under lab conditions, we used the first inflorescence for all
analyses, because most plants growing naturally in the forest produce a single inflorescence.

After flowering ended, 3 months after it began, we measured leaf greenness of each plant in SPAD units, as an estimate of chlorophyll concentration (Richardson et al. 2002). We measured a leaf of the same age on each plant (which had been the youngest mature leaf at the start of flowering) using a portable SPAD meter (Model SPAD-502, Konica Minolta, Tokyo, Japan), taking the average of 3 readings per leaf.

We harvested the plants, and took a digital image of each leaf. Total leaf area of each plant was calculated using the free program ImageJ (http://rsbweb.nih.gov/ij/; Reinking 2001). We dried the leaves and rhizome (stem) of each plant to constant weight at 60°C, and weighed leaves and rhizomes separately using analytical balances. Roots, which had been stored in a refrigerator while leaves and rhizomes were being processed, were rinsed to remove all planting media, dried at 60°C, and weighed.

**Data analysis**—We calculated the phenotypic gender (Lloyd 1980a) as the proportion of flowers on an inflorescence that were female. To examine the effect of light, nitrogen, or phosphorus on total number of flowers, gender, or plant size, we used 3-way ANOVA on SPSS 16.0 (SPSS Inc., Chicago, IL, USA). To test for a correlation of gender to plant size, we used Pearson’s correlations on Excel (Microsoft Corporation, Redmond, WA, USA). Because SPAD values might help to explain effects of light and nitrogen on gender, we performed a 2-way ANOVA on the effects of light \(\times\) nitrogen on SPAD using SPSS. The approximately normal distribution of gender in this experiment allowed the use of parametric statistics (Zar 1999, p. 185), and the Type III Sum of Squares
ANOVA as performed by SPSS is recommended for unbalanced designs (Shaw and Mitchell-Olds 1993).

RESULTS
Plants grew much faster under experimental conditions in the lab than they did in the forest. Plants growing naturally in the forest often produced 3-5 leaves in a year; most plants in the lab produced that many leaves in four months (data not shown).

Plants produced significantly more female flowers, and more total flowers, under high light, high nitrogen, and high phosphorus than under low light, nitrogen, or phosphorus (Table 4.1, Figure 4.1). No interactions of light, nitrogen, or phosphorus were detected. Because of the greater production of female but not male flowers, plants were proportionally more female under high light and under high nitrogen, than under low light or nitrogen. No effect of phosphorus on proportion female was detected, nor were any 2- or 3-way interactions of light, nitrogen, or phosphorus.

The effect of plant size on gender was not explicitly tested experimentally. However, gender (proportion female) was positively correlated with plant size, whether measured as total dry weight ($r = 0.39$, $P < 0.001$, $N = 309$) or total leaf area ($r = 0.17$, $P = 0.003$, $N = 315$).

Plant size was affected by light and nutrients (Table 4.2, Figure 4.2). Overall, total vegetative dry weight was greater under high light and high nitrogen, than under low light or low nitrogen, with no main effects of phosphorus. Rhizome (stem) dry weight was greater under high light and high nitrogen, than under low light or low nitrogen, but light had a greater effect on rhizome weight than N did. Dry weight of leaves was greater
under high nitrogen than under low nitrogen, but light had no effect on leaf weight. The combined effects of light and nitrogen influenced the relative biomass allocation to leaves and rhizome. High light mostly increased the relative biomass allocation to rhizome, compared to low light ($F_{1,301} = 203, P < 0.001$), while high nitrogen mostly increased the relative biomass allocation to leaves, compared to low nitrogen ($F_{1,301} = 148, P < 0.001$). Belowground, root dry weight was affected by light and nitrogen, but depended on interactions with phosphorus. At low phosphorus, root weight increased under either high light or high N (compared to low light or low N), but at high phosphorus, root weight only increased under the combination of high light and high N. The shoot: root ratio was higher under high nitrogen than under low nitrogen.

Interaction effects of light, nitrogen, and phosphorus modified their simple effects on leaf and rhizome weight (Table 4.2, Figure 4.2). Leaf weight had a two-way interaction with light and nitrogen, in which leaf weight at low N was slightly less at high light than at low light. There was a subtle three-way interaction effect of light, nitrogen, and phosphorus on leaf weight. Rhizome weight also was affected by a complex but subtle two-way interaction of light and nitrogen, and a three-way interaction of light, nitrogen, and phosphorus.

Light and nitrogen had opposing effects on leaf area (Table 4.2, Figure 4.3). Leaf area was greater under high N than under low N, but less under high light than under low light. There was also an interaction effect of light and N on leaf area: the effect of light was greater at high N than low N. There were no main or interaction effects of phosphorus on leaf area.
Light and nitrogen also affected leaf greenness in opposite directions. SPAD readings revealed that leaves were greener under low light than under high light, and under high nitrogen than under low nitrogen, with no significant interaction (Figure 4.4; for light: \( F_{1,261} = 52.9, P < 0.001 \); for N: \( F_{1,261} = 30.4, P < 0.001 \)). When included in the analysis, phosphorus had no main or interaction effects on leaf greenness (data not shown).

DISCUSSION

Light and nutrients affected sex expression in *B. urophylla*. Light intensity and nitrogen availability both affected the number of female flowers (but not male flowers) a plant produced, and thus its gender. The effect of light on gender could be direct or indirect. Light affected gender directly in the genetically monoecious orchid *Catasetum viridiflavum*, with the intensity of light shined on the developing inflorescence determining whether flowers were male or female (Zimmerman 1991). In *B. urophylla*, light might instead affect gender indirectly, through the amount of photosynthate made available for reproduction. This hypothesis is supported by experiments involving *Begonia franconis* (Berghoef and Bruinsma 1980). On inflorescences of *B. franconis* grown *in vitro* at high sucrose levels, more female buds matured into flowers, and gender was more female, than on inflorescences grown at low sucrose levels.

Nitrogen might affect gender through its role in photosynthesis. This hypothesis is supported by the effects of nitrogen on leaf greenness. At both high and low light intensity, leaves were greener at high nitrogen than at low nitrogen. Because leaf greenness is proportional to chlorophyll concentration in the leaf (Richardson et al.
2002), plants under high nitrogen had a higher chlorophyll concentration than those under low nitrogen. We assume that the correlation between leaf color and chlorophyll concentration described in other species applies to *B. urophylla*, and that the leaf of each plant we measured was representative of the entire plant. Because the total leaf area of the high nitrogen plants was almost double, on average, the total leaf area of the low nitrogen plants (Figure 4.3), it then follows that high nitrogen plants not only had higher chlorophyll concentration than low nitrogen plants, but higher chlorophyll content as well. With more chlorophyll, and more leaf area and leaf biomass, plants growing under high nitrogen would be able to make and accumulate more photosynthate to fuel the more expensive female function, than plants growing under low nitrogen. Female function is expected to be more expensive than male function in insect pollinated plants, owing to the greater cost of making fruits and seeds, compared to making stamens and pollen (Lovett Doust and Cavers 1982, Zimmerman 1991, Charlesworth and Morgan 1991). In *B. urophylla*, seeds are tiny and fruits, being green and thus photosynthetic until they are almost ready to disperse seeds, may pay some of the cost of the female function. Green fruits recycle 10% or more of respiratory carbon through their own photosynthesis; the winged fruits of maple (*Acer platanoides*) can even achieve a net carbon gain during the daytime (Aschan and Pfanz 2003). However, photosynthesis by fruits did not pay most of the costs of female function in most of the species that were studied, and thus female function would still be expected to be more costly than male function.

The marginally significant effect of phosphorus on numbers of female flowers and total flowers suggests that under greater phosphorus limitation than we tested, an effect of phosphorus on proportion female might emerge. Soils in the natural habitat of
B. urophylla at Las Cruces were found to be very low in available phosphorus, with high potential for P immobilization (Jin et al. 2000). Furthermore, in this protandrous species, female flowers mature after resources have already been spent on male flowers, so being highly female should require additional photosynthate and minerals (including phosphorus) that are not needed by slightly female or all-male plants. The additional cost of female function would apply even if it were not more costly than male function, so long as there is a net cost of female function.

Although this experiment did not explicitly test the role of plant size on gender, it did reveal several effects of environment on plant size, and thus perhaps indirectly on gender. Light and nitrogen both affected plant size, even in this short term experiment with plants of the same age. In long-lived understory plants, the effects of environment on plant size, reproduction, and gender may be cumulative over the long term (Rodríguez-Buriticá 2005). Plant size mostly determines reproduction and gender in understory Arisaema spp., with small plants either not reproducing or doing so as males, and large plants becoming female (Lovett Doust and Cavers 1982, Clay 1993, Vitt et al. 2003). Plant size was correlated with gender in B. urophylla in this experiment, as well as in a natural population growing in the forest (Chapter 3).

In this experiment, there was no tradeoff between allocation to growth and reproduction—high light or high nitrogen increased both. This is consistent with a reproductive budget based mostly on plant size, and its effect on the ability to accumulate photosynthate or mineral nutrients in storage tissues, as seen in two species of understory palms (Mendoza and Franco 1998, Cunningham 1997). However, there were differences in allocation to different vegetative organs of B. urophylla, depending on light and
nitrogen levels. This suggests a mechanism for the synergistic effect of light and nitrogen on gender. By contributing most heavily to leaf growth, and to the synthesis of chlorophyll and the photosynthetic apparatus, nitrogen enhances the plant’s ability to capture light energy (Lüttge 1997, pp. 88-99). The rate of photosynthesis in shade-adapted plants is correlated with leaf N concentration. However, shade adapted plants are also highly responsive to increases in light intensity (Lüttge 1997, pp. 96-100). High light intensity could lead to increased photosynthesis and accumulation of photosynthate (= biomass) in the rhizome, increasing the energy budget for reproduction, and enabling a highly female gender. In our experiment, the same leaf weight, but less leaf area, under high light (compared to low light) means that the plant is maintaining fewer or smaller, yet thicker (or denser) leaves, as seen in sun adapted leaves of the temperate understory tree *Asimina triloba* (Young and Yavitt 1987), with potential reallocation of energy savings from leaf growth and maintenance to reproduction.

The interactions of phosphorus with the effects of light and nitrogen on plant weight suggest a role for P in sex expression, acting indirectly through plant size over the long term. Because phosphorus was never limiting under experimental conditions in the lab, its role in gender determination under natural conditions may not have been revealed.

Despite the effects of light and nitrogen on reproduction in this experiment, light and N availability did not correlate with gender in a natural population in the forest (Chapter 3). Nitrogen is not a limiting resource in most lowland tropical soils (Sollins 1998), where instead, phosphorus is usually limiting (Luttge 1997, p. 73). However, in the forest understory, light may most often be limiting (Chazdon and Fetcher 1984). The amount of light energy available in the understory over a flowering season may be
smaller, under most circumstances, than the cost of reproduction in this species, particularly the high cost of the female function, as in the cycad Zamia skinneri (Clark and Clark 1988). If so, then short term light, nitrogen, and phosphorus effects would be obscured much of the time, accumulation of resources for reproduction would be slow, and plant size would usually have the greatest effect on gender. Ordinarily, smaller and younger plants might only be able to accumulate enough photosynthate to express the cheaper male sex (if they can reproduce at all), while larger and older plants, with ample provisions, maximally express the more expensive but reliable female sex.

The frequency distribution of gender was different in the lab experiment than in the natural environment of the forest, suggesting that alternative mechanisms of gender determination are available, depending on circumstances. The distribution of gender in the forest was strongly bimodal, with a prominent all-male peak, and up to 52% of plants were all-male (Figure 3.6A in Chapter 3). Under experimental conditions in the lab, there was only a tiny all-male peak, and only 2.5% of plants were all-male. This contrast in gender distribution between forest and lab suggests that whatever conditions caused the all-male gender in the forest were largely absent in the lab experiment. In a similar result, monoecious populations of Ecballium elaterium showed a unimodal or weakly bimodal distribution of gender (with 0-18% all-male plants) in moist, favorable sites, and a strongly bimodal distribution of gender (with 50% all-male plants) in a dry, stressful site (Costich 1995). For B. urophylla, vegetative growth and inflorescence production were greater, in most treatments, in the lab than in the forest. In the lab, lack of pollination and subsequent fruit development may have diverted resources to production of additional inflorescences. When fruit development was prevented in a species of
ancestral wild squash and two varieties of garden squash, flower production increased (Avila-Sakar et al. 2001, El-Keblawy and Lovett Doust 1996). Even after considering this reallocation of resources, conditions experienced by most B. urophylla plants in the lab were probably more favorable to reproduction than those usually experienced by plants in the forest. The physiological plasticity to respond to favorable conditions by becoming proportionally more female has evidently been selected for in B. urophylla. Sex expression may thus have two mechanisms, one based on a slow accumulation of resources under the marginal conditions typical in the rainforest understory, and the other based on an opportunistic response to favorable conditions when they are available. A resource windfall, such as a canopy opening, dispersal to an exposed microhabitat like a cliff face, proximity to a nutrient rich patch of soil, or a particularly favorable growing season, may be a common, though unpredictable, occurrence. The impact of such a windfall on gender would be greatest for small plants, possibly explaining why small plants in the forest are more variable in gender than large plants (Chapter 3).

A synthesis of the results of this laboratory experiment, and the findings from the forest (Chapter 3), suggests that understory plants can combine environmental sex determination (ESD) and size dependent sex determination (SDS). When the scale of resource income from the environment is comparable to the cost of reproduction (in the currency of, for example, photosynthate), environmental sex determination may be observed, as it was in the lab. If, on the other hand, the scale of resource income is much smaller than the cost of reproduction, size dependent sex determination may be observed, as it was in the forest (Chapter 3). Under circumstances particularly favorable to
reproduction, environment can trump size, and become the main determinant of gender in a long-lived herb of the forest understory.
Table 4.1. $P$ values for the effects of light, nitrogen, and phosphorus on the numbers of male and female flowers, total flowers, and proportion female, from three-way ANOVAs. Significant uncorrected values of $P$ are in bold. $N = 315$.

<table>
<thead>
<tr>
<th>Source</th>
<th>$df$</th>
<th>Male flowers</th>
<th>Female flowers</th>
<th>Total flowers</th>
<th>Proportion female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light</td>
<td>1</td>
<td>0.31</td>
<td>$&lt; 0.001$</td>
<td>0.003</td>
<td>$&lt; 0.001$</td>
</tr>
<tr>
<td>N</td>
<td>1</td>
<td>0.16</td>
<td>$0.001$</td>
<td>0.011</td>
<td>$&lt; 0.001$</td>
</tr>
<tr>
<td>P</td>
<td>1</td>
<td>0.14</td>
<td>$0.029$</td>
<td>0.053</td>
<td>0.17</td>
</tr>
<tr>
<td>Light $\times$ N</td>
<td>1</td>
<td>0.31</td>
<td>0.76</td>
<td>0.50</td>
<td>0.81</td>
</tr>
<tr>
<td>Light $\times$ P</td>
<td>1</td>
<td>0.94</td>
<td>0.67</td>
<td>0.85</td>
<td>0.78</td>
</tr>
<tr>
<td>N $\times$ P</td>
<td>1</td>
<td>0.59</td>
<td>0.25</td>
<td>0.37</td>
<td>0.18</td>
</tr>
<tr>
<td>Light $\times$ N $\times$ P</td>
<td>1</td>
<td>0.10</td>
<td>0.33</td>
<td>0.17</td>
<td>0.56</td>
</tr>
<tr>
<td>Residual</td>
<td>307</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.2. *P* values for the effects of light, nitrogen, and phosphorus on plant size, from three-way ANOVAs. Significant uncorrected values of *P* are in bold. N = 315, except for root weight, total weight, and shoot: root weight ratio, for which N = 309.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Leaf area</th>
<th>Leaf weight</th>
<th>Rhizome weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light</td>
<td>1</td>
<td>&lt; 0.001</td>
<td>1.0</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>N</td>
<td>1</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>0.026</td>
</tr>
<tr>
<td>P</td>
<td>1</td>
<td>0.51</td>
<td>0.34</td>
<td>0.20</td>
</tr>
<tr>
<td>Light × N</td>
<td>1</td>
<td>0.018</td>
<td>0.022</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Light × P</td>
<td>1</td>
<td>0.25</td>
<td>0.62</td>
<td>0.53</td>
</tr>
<tr>
<td>N × P</td>
<td>1</td>
<td>0.31</td>
<td>0.41</td>
<td>0.66</td>
</tr>
<tr>
<td>Light × N × P</td>
<td>1</td>
<td>0.55</td>
<td>0.62</td>
<td>0.026</td>
</tr>
<tr>
<td>Residual</td>
<td>307</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Root weight</th>
<th>Total weight</th>
<th>Shoot: root weight ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light</td>
<td>1</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>0.67</td>
</tr>
<tr>
<td>N</td>
<td>1</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>0.006</td>
</tr>
<tr>
<td>P</td>
<td>1</td>
<td>0.95</td>
<td>0.30</td>
<td>0.29</td>
</tr>
<tr>
<td>Light × N</td>
<td>1</td>
<td>0.19</td>
<td>&lt; 0.001</td>
<td>0.27</td>
</tr>
<tr>
<td>Light × P</td>
<td>1</td>
<td>0.011</td>
<td>0.13</td>
<td>0.059</td>
</tr>
<tr>
<td>N × P</td>
<td>1</td>
<td>0.72</td>
<td>0.48</td>
<td>0.51</td>
</tr>
<tr>
<td>Light × N × P</td>
<td>1</td>
<td>0.027</td>
<td>0.028</td>
<td>0.37</td>
</tr>
<tr>
<td>Residual</td>
<td>301</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.1. Effects of light, nitrogen, and phosphorus on flower production and proportion female. Panels show: A) low phosphorus and B) high phosphorus. Stippled bars show average number of male flowers, and open bars show average number of female flowers. \(N = 315\).
Figure 4.2. (p. 73). Effects of light, nitrogen, and phosphorus on the relative dry weight of plant organs. Panels show: A) low phosphorus and B) high phosphorus. For each stacked bar, the open portion at the top shows average dry weight of leaves, the lightly shaded portion in the middle shows average dry weight of the rhizome (stem), and the solid portion on the bottom shows average dry weight of roots. Note that root weights are positive, but are shown below the x axis to facilitate comparison of above- and below-ground allocation of biomass. The entire stacked bar represents the average total dry vegetative weight of the plant. N = 315 for leaves and rhizome, and N = 309 for roots.
Figure 4.3. Effects of light, nitrogen, and phosphorus on the total area of leaves. Stippled bars show low phosphorus, and open bars show high phosphorus. $N = 315$. 
Figure 4.4. Effect of light and nitrogen on the greenness of a same-age leaf. Error bars represent SE. N = 315.
CHAPTER 5

Mycorrhizas affect sex expression of *Begonia urophylla*

SUMMARY

Mycorrhizas are vital to the mineral nutrition of most terrestrial plants, yet their role in plant sex expression is almost unknown. In an experiment in the lab, we inoculated plants of *Begonia urophylla* with arbuscular mycorrhizal fungi, and compared their sex expression to plants that were not inoculated. Inoculation with mycorrhizas caused plants to be less female in gender (proportion of flowers that were female) than plants that were not inoculated. Inoculated plants were also smaller in dry weight than non-inoculated plants. The results suggest a net cost of mycorrhizas under experimental conditions, and, if female function is more costly than male, a tradeoff between allocation to reproduction and allocation to mycorrhizas. Our results support the emerging view of the mycorrhizal symbiosis as dynamic, and not necessarily mutualistic under all circumstances.

BACKGROUND

The mycorrhizal symbiosis is a key driver of the ecology and evolution of land plant diversity. Arbuscular mycorrhizal fungi benefit their host plants in many ways, including increased uptake of phosphorus (Bolan 1991) and other soil nutrients (Hodge et al. 2001, Clark and Zeto 2000, Janos et al. 2001), resistance to drought (Augé 2001, Augé 2004), and protection from pathogens and herbivores (Cardoso and Kuyper 2006, Bi et al. 2007). It is even possible (though controversial) that tree seedlings in the forest understory are nurtured by the common mycorrhizal network (Simard and Durall 2004). In return for

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3 Co-authors: Michelle Hershman and David Janos.
these services, host plants provide the fungi with photosynthate (Kiers and van der Heijden 2006). An emerging view of the mycorrhizal symbiosis is that it is dynamic and complex, and not necessarily mutualistic under all circumstances (Fitter 1991, Janos 2007). In particular, the cost of mycorrhizas may exceed their benefit while mycorrhizal colonization is establishing, or if phosphorus is readily available from the soil (Johnson et al. 1997).

The effects of the mycorrhizal symbiosis on plant growth and biodiversity have been well-studied (Lekberg and Koide 2005, Wang and Qiu 2006, Tawaraya 2003). Little is known, however, about the effects of mycorrhizas on plant reproduction and sex expression (Pendleton 2000, Poulton et al. 2002). If female function is more costly than male function (Charlesworth and Morgan 1991), mycorrhizas might favor expression of female function by making mineral resources available for reproductive development and photosynthesis. Alternatively, the cost of maintaining mycorrhizas could decrease the energy budget for reproduction, and favor the less expensive male function.

The effect of mycorrhizas on the sex expression of tropical understory herbs is unknown, and ripe for study in plants such as begonias. The genus *Begonia* comprises over 1500 species (Hughes and Hollingsworth 2008), many of which live in the forest understory. Most species are monoecious (Clement et al. 2004), bearing separate male and female flowers on the same individual, so they may vary their gender simply by adjusting the proportion of flowers that are female (Chapter 3; Matzke 1938). In this study of a tropical rainforest herb, *Begonia urophylla*, we ask the following questions:

1. Does inoculation with mycorrhizas affect gender?
2. Does inoculation with mycorrhizas affect plant size, which might indirectly affect reproduction and gender?

**METHODS**

*Study species—* *Begonia urophylla* Hook. is a perennial understory herb of the Neotropical rainforest. In the forest, a mature plant has a single creeping rhizome (stem) with several (usually 3-8) fleshy leaves. The plants produce leaves during the rainy season and flower during the dry season, with a single inflorescence (occasionally two or very rarely three or more). Each dichotomously-branching inflorescence is protandrous, producing male flowers first, and then (usually) female flowers (Le Corff et al. 1998). Roots from plants in the forest are colonized by arbuscular mycorrhizal fungi (Chapter 3). See chapter 3 for a detailed description of the species and its habitat.

*Plant culture—* We propagated plants of *B. urophylla* from leaves collected at Las Cruces Biological Station in Costa Rica, and grew the plants in the lab at University of Miami. After establishing the plants in a peat based mixture, we potted them individually in 4” geranium pots with Turface® (Profile Products LLC, Buffalo Grove, IL, USA), a baked, coarsely ground montmorillite clay (arcillite), as the growing medium. Nutrients were supplied weekly with Peters 20-20-20 fertilizer at 0.64g/L, supplemented with 0.16g/L NH₄NO₃. Plants were grown under 40W cool white fluorescent lights with a photoperiod of 12 h light: 12 h dark. Average photosynthetically active radiation (PAR) was 835 µmol/s/m² at 3 cm under the bulb, and 636 µmol/s/m² at 8 cm under the bulb. The 3-8 cm range reflects the range of a plant’s leaf distances from the bulbs. We
rearranged the pots weekly between edge and interior positions. Water was supplied to all plants in excess, by automatic misting with distilled water every two hours.

**Experimental design**—We began experimental treatments 26 months after propagation. Plants were randomly assigned to two treatment groups (n = 100): one group to receive mycorrhizal inoculum, and the other to remain non-inoculated. Both treatments received high light (0% shade), high nitrogen (69 mg/L in a nutrient solution we formulated), and low phosphorus (5.6 mg/L). The high levels of light and nitrogen represented the conditions under which plants had already been growing vigorously (with N concentration, and NH₄: NO₃ ratio, the same as in the original Peter’s-based solution), and the low level of phosphorus represented an intended moderate deficiency. Other macronutrients (K, Mg, Fe) and micronutrients (B, Cu, Mn, Mo, Zn) were supplied in the same concentrations as in the Peter’s-based solution. See Chapter 4 for details.

To prepare mycorrhizal inoculum, we modified the method of Janos et al. (2001). We collected, from about 3 m² of lawn, whole plants of live grasses (which facultatively form arbuscular mycorrhizas), shook off loose soil, cut the roots off by hand, rinsed off the adhering soil, and soaked the roots in water overnight. The next day, we cut the roots into ~1 cm pieces, divided them into roughly equal portions, and applied a 1 cm ‘mulch’ to the surface of the medium of each plant in the mycorrhizal treatment group, in contact with the living begonia roots. As a control, we autoclaved rinsed grass roots twice, 24 hours apart, and applied them to all the plants in the non-mycorrhizal group, as above. We then applied 10 mL of the water used to soak the live grass roots, after filtering
through Whatman #4 paper, to each plant to act as a microbial (but non-mycorrhizal) inoculum.

During the course of the experiment, photoperiod, misting, and pot rearrangement continued as before. We applied 70 mL of the nutrient solution to each plant weekly. Occasionally, plants produced inflorescence buds; because these were produced prematurely and not as the result of intentional induction by us, they were removed promptly. Ten weeks after mycorrhizal inoculation, we induced the plants to flower by reducing the photoperiod to 11 hours light: 13 hours dark for 3 weeks (Chapter 2: Rivera and Cozza 2008). Mass flowering began 2 months later. During flowering, we continued to apply nutrient solutions, but stopped rearranging the pots to avoid damaging the inflorescences.

**Data collection and harvest**—To count the number of flowers of each sex produced, we diagrammed inflorescences at two points in time: at the end of the sequential opening of male flowers, and during the nearly simultaneous opening of female flowers. The sex identity and fate of buds that were not directly observed could usually be inferred (Chapter 3). Because some plants did not flower, and others that did flower were (haphazardly) not diagrammed at or near the two critical points in time, sample sizes were reduced. Although many plants produced multiple inflorescences under lab conditions, we used the first inflorescence for all analyses, because most plants growing naturally in the forest produce a single inflorescence.

After flowering ended, 3 months after it began, we harvested the plants. We dried the leaves and rhizome (stem) of each plant to constant weight at 60°C, and weighed
them using analytical balances. Roots, which had been stored in a refrigerator while leaves and rhizomes were being processed, were rinsed to remove all planting media, dried at 60°C, and weighed. The roots of 14 plants were sampled to verify mycorrhizal colonization as described below. The dry weights of these roots were adjusted using the proportion:

$$\text{dry weight}_{\text{adj}} = \text{dry weight}_{\text{rem}} * \frac{\text{wet weight}_{\text{whole}}}{\text{wet weight}_{\text{rem}}}$$

where weight$_{\text{whole}}$ was the weight of the root system before sampling, and weight$_{\text{rem}}$ was the weight of the roots remaining after the sample was taken.

To verify mycorrhizal colonization, we sampled fine roots from 10 plants that were inoculated with mycorrhizas, and 4 plants that were not inoculated with mycorrhizas. We randomly collected root tips, cleared them in 10% KOH, fixed them in 1% HCl, stained them using Trypan blue, destained them in acid glycerol, mounted them on slides, and observed them for mycorrhizal structures at 400x under the compound microscope (Vierheilig et al. 2005). Because of the low level of mycorrhizal colonization observed, all roots on the slide (~16 cm total root length per plant) were surveyed. A plant was counted as colonized by mycorrhizal fungi if one or more unambiguous vesicles with attached hyphae were observed.

While examining root samples, we discovered that they were colonized by what appeared to be a holocarpic, endobiotic chytrid. For each root slide, ten random microscope fields were surveyed, and the number of chytrid zoosporangia was recorded.
Data analysis—To test whether inoculated roots were more likely than non-inoculated roots to be colonized by mycorrhizas, we set up a $2 \times 2$ contingency table, with number of plants inoculated vs. not inoculated as rows, and number of plants showing presence vs. absence of mycorrhizal vesicles as columns. We tested for a difference in colonization between inoculated and non-inoculated roots using a one-tailed Fisher’s Exact Test on Statistix 7.0 (Analytical Software, Talahassee, FL, USA).

Colonization of roots by chytrids was tested in a similar way. We set up a $2 \times 2$ contingency table, with number of plants inoculated vs. not inoculated with mycorrhizas as rows, and number of plants showing presence vs. absence of chytrids as columns. We tested for a difference in colonization by chytrids using a two-tailed Fisher’s Exact Test on Statistix 7.0. To test if chytrid load (in roots showing chytrid colonization) differed between roots inoculated vs. not inoculated with mycorrhizas, we used a Kruskal-Wallis one-way analysis of variance on Statistix 7.0.

We calculated phenotypic gender (Lloyd 1980a) as the proportion of flowers on an inflorescence that were female. To determine whether mycorrhizas affected gender, we compared the gender of plants with and without mycorrhizal inoculation, using a t-test on Excel (Microsoft Corporation, Redmond, WA, USA). We also tested for the effect of mycorrhizal inoculation on plant size using a t-test on Excel.

RESULTS

Roots of plants that were inoculated with arbuscular mycorrhizas were significantly more likely to be colonized by mycorrhizal fungi, than plants that were not inoculated ($n_{\text{myc}} = 10$, $n_{\text{no myc}} = 4$, $P_{\text{one-tail}} = 0.035$). There was no difference, however,
in the likelihood of colonization by chytrids, between roots of plants that were inoculated with mycorrhizas and those that were not inoculated (n_{myc} = 10, n_{no_myc} = 4, P \text{two-tail} = 0.57). For roots that showed colonization by chytrids, the chytrid load did not differ between plants that were inoculated with mycorrhizas and those that were not inoculated.

Inoculation with mycorrhizas had no effect on total flower production. However, plants that were inoculated with mycorrhizas were proportionally less female than those that were not inoculated (Figure 5.1A; t = 2.5, n_{myc} = 43, n_{no_myc} = 47, P = 0.015). Plants that were inoculated with mycorrhizas were also smaller in total dry weight than those that were not inoculated (Figure 5.1B; t = 2.8, n_{myc} = 43, n_{no_myc} = 47, P = 0.006).

DISCUSSION

Mycorrhizal inoculation affected sex expression in B. urophylla. Although the difference in gender attributable to mycorrhizas was small, it nonetheless demonstrates that mycorrhizas can affect sex expression, which has been shown in only one other species that we are aware of. In buffalo gourd (Cucurbita foetidissima), mycorrhizal plants produced more male, but not female, flowers than initially non-mycorrhizal plants (Pendleton 2000). In pot-grown tomato plants (Lycopersicon esculentum), mycorrhizal plants produced more flowers of both sexes than non-mycorrhizal plants, but gender was not affected (Poulton et al. 2002).

Mycorrhizas may have affected gender of B. urophylla by altering the energy budget for reproduction. High light and high nitrogen caused plants to be proportionally more female, compared to low light or nitrogen, suggesting that determination of gender is energy based in this species (Chapter 4). Because of protandry, female flowers impose
an additional energy cost after male flowers have already bloomed, even if they do not develop further into fruits with seeds. Mycorrhizal fungi, under the conditions of this experiment, may have reduced the energy available for flowering, thus causing their host plants to be proportionally less female than plants that were not mycorrhizal.

Mycorrhizas were a net cost to host plants under experimental conditions, as evidenced by the lower average dry weight of mycorrhizal plants, compared to non-mycorrhizal plants.

Alternatively, the mycorrhizal effect on gender may have had a more indirect mechanism. The cost of mycorrhizas could have reduced the energy available for vegetative growth, thus causing inoculated plants to be smaller than non-inoculated plants, as observed. Smaller plants are less able to obtain or store resources for reproduction (Lovett Doust and Cavers 1982), indirectly causing inoculated plants to be less female than non-inoculated plants. Parasitism by chytrids could also have affected sex expression. However, we did not detect any difference in colonization by chytrids that could explain the difference in gender between plants that were inoculated with mycorrhizas, and those that were not inoculated.

The net cost of mycorrhizas observed in this experiment may be explained by phosphorus availability, the spatial arrangement of begonia roots in the pots, or the physical properties of the growing medium. Although the nutrient solution we used was intended to be deficient in phosphorus, it may have not been. In the plants’ natural habitat in the Las Cruces forest, soils sampled to 15 cm deep were extremely low in available phosphorus (Jin et al. 2000), although the soil surface (top 2 cm) may have had higher available P (Chapter 3). *Begonia urophylla* evidently is able to extract scarce
phosphorus from these soils, perhaps with the help of mycorrhizas. Not only may our experimental nutrient solution have been effectively high in phosphorus, but begonia roots had easy access to it. Roots grew very densely in the pots, forming a solid 2 cm thick mat at the surface of the medium. Because roots were very close to or even touching each other, much of the phosphorus in the nutrient solutions was available to roots by direct interception, and there was no opportunity for a phosphorus depletion zone to develop between them (Bolan 1991). The large particle size and microporosity of the Turface medium may have not presented many intermediately-sized spaces for phosphorus to adsorb, where only fungal hyphae, but not plant roots, could find it (Bolan 1991). Thus, mycorrhizas may not have obtained any more phosphorus for roots than they could obtain themselves, leaving only a cost of maintaining the fungus, but no benefit from it.

The benefits of mycorrhizas in obtaining immobilized phosphorus might be much higher, or more variable, under natural conditions than in this experiment. In the forest, root systems of *B. urophylla* are diffuse and spreading (pers. obs.), making phosphorus depletion likely (Bolan 1991). Soils were granular or rock-like in some microsites in the forest, which could decrease the benefit of mycorrhizas in those sites, compared to sites with deeper or finer grained soils. However, available phosphorus is scarce regardless (Jin et al. 2000), and the ability of mycorrhizal fungi to obtain phosphorus from immobilized forms (Bolan 1991), and even from leaf litter (Aristizabál et al. 2004), could be a major source of phosphorus for plants and their reproductive requirements. Other environmental conditions that affect sex expression, such as light intensity or nitrogen availability (Chapter 4), as well as phosphorus availability at lower levels than we tested
in this experiment, may interact with the mycorrhizal effect. The experiment highlighted

the dynamic nature of the mycorrhizal symbiosis, which need not be a mutualism under

all circumstances.
Figure 5.1. Effect of inoculation with mycorrhizas on (A) gender (proportion female) and (B) total plant weight, under the same levels of light, nitrogen, and phosphorus. In both A and B, the open bar represents plants that were not inoculated with mycorrhizal fungi (n = 47), and the stippled bar represents plants that were inoculated with mycorrhizal fungi (n = 43). In both A and B, there was a significant effect of mycorrhizas ($P \leq 0.05$). Error bars represent SE.
CHAPTER 6

Conclusions and new questions about sex expression in *Begonia urophylla*, a long-lived monoecious herb of the rainforest understory

Within the constraint of a highly stereotyped inflorescence architecture, individuals of *Begonia urophylla* showed gender plasticity. An idealized architecture would produce a phenotypic gender of 50-60% female flowers, and that was a peak of the distribution of gender in a natural population, but many plants were proportionally less female as well. A gender of 50-60% female maximized potential fitness through the female function, measured as total seed weight, supporting the adaptive value of the architectural constraint on gender. In the natural population in the forest, plant size was strongly correlated with gender, while environmental resources (measured as light availability, soil moisture, soil pH, and soil nutrients including nitrogen) were not (Chapter 3). In laboratory experiments, however, light intensity, nitrogen availability, and mycorrhizas affected gender (Chapters 4 & 5). It would be interesting to test further the effect on gender of factors that approached significance in this study: calcium availability, pH, and phosphorus, the latter at more limited availability (with and without mycorrhizas) than was used in the lab experiment.

To synthesize results from the forest and from the lab, sex expression in *B. urophylla* may depend mostly on availability of photosynthate. In the forest understory, where light is limiting, energy for reproduction probably accumulates slowly much of the time, and depends on plant size (size dependent sex allocation, SDS). However, a sudden windfall of light or of a limited nutrient could affect gender (environmental sex allocation, ESD). In the forest, this might occur with an opening in the canopy, or upon
dispersal to an exposed microhabitat. The determination of gender according to size, but with the flexibility to respond to the environment, may be a general mechanism of sex expression in long-lived understory herbs. Further work could test the hypothesis that understory plants employ SDS as their default strategy of gender determination, but can facultatively switch to ESD. To test the responses of plants of a given size to the environment, resource availability could be manipulated (e.g. by shading or nutrient enrichment) for plants matched by size in the forest. Manipulations in the lab could be more comprehensive, and plant size could be controlled better. Another approach might be to manipulate plant size (e.g. by removing leaves) in field or lab experiments.

Monoecy, and the enhanced opportunity for gender plasticity it enables, may generally be adaptive in long-lived plants of the forest understory. Light levels are reduced and heterogeneous in the understory, compared to the canopy or other exposed environments (Chazdon and Fetcher 1984). Under such conditions, the reproductive budgets of understory plants could be unpredictable, and gender plasticity could maximize fitness of both sexes under various circumstances. The ability for a small plant to express a male or mostly male gender allows it some opportunity for short term fitness gain, while at the same time increasing its probability of long term survival and future fitness by avoiding the high cost of female function (Charlesworth and Morgan 1991). On the other hand, a large plant (or a small plant that experiences particularly favorable conditions) might maximize its fitness by expressing a mostly female gender.

Under continued disruptive selection on gender, monoecy may evolve to dioecy (separate male and female individuals), yet monoecy has been maintained in many plant lineages, including almost all *Begonia* species. In some cases, monoecy may be
maintained by the reproductive assurance it can provide in new or changeable habitats. For example, seeds of the squirting cucumber (*Ecballium elaterium*) are dispersed inefficiently by sticking to animals, but a new population may be started by just one (self-compatible) monoecious individual (Costich 1995). Similarly, monoecious populations of the arrowhead (*Sagittaria latifolia*) inhabit ephemeral freshwater habitats, and colonization is again favored by the reproductive assurance of monoecy (Dorken and Barrett 2003b). In *B. urophylla*, male and female phases are completely distinct in most inflorescences (Le Corff et al. 1998), precluding reproductive assurance, but overlap of the sex phases does occur occasionally (per. obs.).

Alternatively, monoecy may be maintained by inefficient pollination. The evolution of dioecy may require faithful pollination, because it affords no opportunity for plants to self pollinate (except in subdioecy, where flowers of the opposite sex are sometimes produced). A generalist pollination syndrome, or inefficient pollination, may favor the reproductive assurance of monoecy over dioecy as a bet-hedging strategy (Gross 2005). In the forest at La Selva, Costa Rica, 37% of the understory species are pollinated by beetles, small bees, or other small insects (Kress and Beach 1994). Of these, beetles are notoriously inefficient pollinators (Gross 2005), and so may be small generalist bees (Ramalho 2004), particularly when they learn to avoid deceptive flowers like (female flowers of) begonias (Le Corff et al. 1998). The sheer abundance of small generalist bees may outweigh their inefficiency as pollinators, however; fruit set of *B. urophylla* averaged almost 80% over two years in this study (data not shown). High fruit set, despite the non-overlap of male and female phases, suggests that reproductive assurance is not a factor in the maintenance of monoecy in *B. urophylla* (at least under
the conditions of this study). Instead, the maintenance of monoecy (though not necessarily its evolution) in this species may be better explained by the advantage of gender plasticity in a patchy and unpredictable environment.

This study focused on the expression of phenotypic gender, which can help answer questions about sex allocation. Although gender was quantified by counting male and female flowers, it is not known whether all flowers contribute equally to their respective sex function. For example, anther number is variable in this species (Burt-Utley 1985), and a pilot study in the lab revealed considerable variation in anther number within the inflorescence (data not shown). If pollen production is correlated with anther number (Mazer and Delesalle 1995, Huang et al. 2004), or if anthers produce different amounts of pollen under different conditions (Avila-Sakar et al. 2003), then the male flowers produced by a plant are not equally male in potential function. Male flowers of *B. urophylla* varied in size, too, with the earliest male flowers produced by an inflorescence tending to be the largest, but with male display collectively the largest towards the end of the male phase, when many flowers were blooming at once. Male function depends on intensity and quality of pollinator visitation, which in turn is affected by flower or display size (Ishii and Sakai 2002). Male and female function may also be affected by the spatial location of a plant in the patch (Nilsson 1992), and by pollinator abundance and community composition, which can change over the flowering season (Le Corff et al. 1998). Thus, the story of sex expression in *B. urophylla* may be much more complex than presented in this study.

*Functional gender* takes into account the potential or realized mating success of male and female flowers, in addition to their relative numbers (Lloyd, 1980a). In this
study, female success was estimated as the total weight of seeds produced by each plant at one site, but male function was not estimated for these plants. In a pilot study, I applied different colors of fluorescent dust to the male flowers of four plants at another site, allowed pollinators to visit, and then looked for the dust on the stigmas of all female flowers in bloom at the site. On average, each male-phase plant could have sired seeds in at least seven fruits total, on four different female-phase plants. It would be interesting to see if male success is influenced by gender, male display size, plant size, or environment. Because each female flower contains thousands of tiny ovules, paternity analysis to assess male success on a larger scale may be quite complicated.

Life history may enable alternative mechanisms of gender determination, besides relative allocation to male or female structures. For example, in this protandrous, temporally dioecious species, functional gender is affected by flowering time (Chapter 2: Rivera and Cozza, 2008). Plants that flower at the beginning of the season may function mostly as females, while those that flower at the end of the season may function mostly as males. Despite the use of photoperiod as a cue for flowering, flowering is only semi-synchronous, and its timing may depend on plant size or environment. In the lab, plants that received low light flowered later than those that received high light (data not shown), potentially enhancing their male function. This could be adaptive; plants that had less energy available for reproduction might gain more fitness through the male function than through the more costly female function (Charlesworth and Morgan 1991).

Many questions about sex expression in *B. urophylla* remain unanswered or unexplored. Observations of flower growth and morphology lead to new questions about pollination ecology, and thus functional gender. In the forest, repeated measurements of
flower size revealed that (at least) male flowers increased in size, each day after they opened (data not shown). Even flowers that had been castrated (anthers eaten) by herbivores were retained, and they continued to increase in size. Could continuous flower growth be a mechanism for increasing the size of the male display, and thus its attractiveness to pollinators? A plant at one site had flowers twice the size of those of other plants. Do larger flowers lead to greater visitation rates by pollinators in this species, as was observed in artificial flowers of *B. oaxacana* (Schemske et al. 1996)? Is there a tradeoff between flower size and number of flowers produced, and what is the effect of these two components of floral display on pollinator visitation to male and female flowers? Observed anecdotally, female flowers seemed to have several adaptations for increasing their attractiveness to pollinators. The mimicry of male flowers by female flowers as studied by Le Corff et al. (1998) is based on a head-on approach by a bee, looking directly into the flower face. Viewed from the side, however, the female flower is much showier than the male, because of a “fin” on the ovary that later aids in wind dispersal of the seeds. Does this fin, which is colored white or pink like the petals during flowering, act as a “supernormal” stimulus to bees? How much does it aid in “mistake” pollination? In addition to this potential morphological stimulus, female flowers seemed to open more slowly and less fully than males. Could they be mimicking a just-opening flower, which if it were male, would have the maximum amount of rewarding pollen? Are partly open flowers more attractive to flower visitors (small bees) than fully open flowers?

At Las Cruces, at least three other understory species of *Begonia* flowered at the same time as *B. urophylla*, including *B. tonduzii*, which was abundant and grew at 2 of
the 3 sites I studied. What is the effect of these other species on the pollination ecology of *B. urophylla*? Is there competition for pollination (Campbell 1985, Campbell and Motten 1985, Rathcke 1988), are pollinators partitioned between species (Stone et al. 1998), or is pollination facilitated by the mixed species display (Schemske 1981)? If the diversity of the understory community, or disturbance of it, help determine the sexual system, the gender, and the reproductive success of begonias, there may be applications to studies of forest fragmentation, conservation, and reforestation.

Comparative studies involving other species of *Begonia* with different inflorescence architectures could reveal larger patterns of gender constraint and plasticity. Inflorescences among different *Begonia* species differ widely in size (= number of flowers), degree of branching symmetry, default (or idealized) gender (including species with separate male and female inflorescences), degree of overlap of male and female phases, and sex of terminal flowers (Goulet et al. 1994). How might inflorescence architecture be related to plant form, growth strategy, habitat, or pollinator assemblage? How do different architectures affect pollinator behavior (Jordan and Harder 2006)? Do parameters of inflorescence architecture (symmetry, branching angles, spatial separation of flowers, sequence and overlap of sex phases) correlate with the degree of gender constraint or plasticity, or the determinants of gender? How does architecture vary within a species’ geographic range, as well as among co-occurring species?

Floral herbivory had a potentially large impact on sex expression in *B. urophylla* that was not explored in this study. The main herbivores were several species of small flea beetles (Coleoptera: Chrysomelidae) and weevils (Coleoptera: Curculionidae) that preferentially attacked male flowers. Within 1-2 days, beetles could eat all the anthers of
a flower, effectively castrating it. A loss of male function may occur before this, because pollinators might avoid flowers that are occupied by herbivores (Canela and Sazima 2003); one such encounter was anecdotally observed. What is the effect of floral herbivory on functional gender? Might such sex-differential herbivory contribute to the maintenance of monoecy (Cox 1988)? Several other types of herbivores were observed, including webworms (Lepidoptera) that bound and consumed a large portion of the inflorescence, treehoppers (Hemiptera) that parasitized the inflorescence at the stalk, and an unknown herbivore (mollusk?) that scraped the inflorescence stalk, often destroying it. What is the effect of the community of herbivores on reproduction and sex expression, especially when the entire inflorescence is attacked, in contrast to flowers of one sex?

Plants of *B. urophylla* invested considerable effort on sexual reproduction, and dispersed many seeds, yet seedlings were rarely observed in the forest. I only observed seedlings at three microsites: on a large boulder, on a cut bank, and on a cliff face—all exposed habitats—and never on the forest floor. Forest floor plants were observed reproducing on several occasions—but clonally. A healthy leaf would break off (or be shed?), and root about 0.5 m from the apparent “mother” plant. Intriguingly, many plants grew in clumped patches, with individuals separated by about this distance (pers. obs.). Asexual reproduction is common among understory plants (Kinsman 1990, Whigham 2004). In the lady slipper orchid (*Cypripedium acaule*), sexual reproduction may only occur after a rare event, such as a fire, even though flowers are produced every year (Gill 2001). What are the demographics of sexual vs. asexual reproduction in *B. urophylla*, and how does this affect population genetics? Do populations reproduce asexually most
of the time, with effective sexual reproduction and seed dispersal confined to colonization of exposed microhabitats, or rare events?

Mutant phenotypes of *B. urophylla* observed in the forest may inform our understanding of the evolution of monoecy, and determination of gender, in begonias. According to a recent phylogeny of the Curcurbitales order, to which the Begoniaceae belong, begonias might have evolved from a dioecious ancestor (Zhang et al. 2006). In that case, a residual presence of genetically male and female individuals in the population, or occasional reversions to the dioecious phenotype, might be expected, just as leaky dioecy is the rule in dioecious species that presumably evolved from monoecious ancestors (Freeman et al. 1997). Entirely male and female plants were observed in the forest. Although most expressed a different gender in the other season that plants were observed, a small percentage of individuals were male in both years. It is possible that genetic males and females exist in the population. However, two plants in the forest that expressed mutant phenotypes did not support the hypothesis of ancestral dioecy. These plants, which were about 0.5 m apart, produced strange flowers with characteristics of both sexes, at the transition point from male to female flowers on the inflorescence. In one mutant phenotype, transitional flowers had both anthers and stigmas, with a superior, but undeveloped, ovary (the ovary is normally inferior). Does this mutant phenotype suggest the form of a hermaphroditic ancestor? What is the mechanism of this rare, imperfect transition from male to female phase, and how does this transition normally occur without mishap?

The development of gender in *B. urophylla* or other species may reveal novel mechanisms of sex and gender determination. In a finding apparently not followed up
on, Pastrana (1932) found that male and female flowers of *B. schmitiana* had different numbers of chromosomes: males have 12 and females have 13. Is this unique mechanism of floral sex determination by a lone sex chromosome also found in *B. urophylla* or other species of *Begonia*? If not, what is the developmental mechanism of floral sex determination in other species? Because male and female flowers have different phenotypes, begonias may lend themselves to studies of the developmental genetics of floral identity in monoecious plants, bolstering findings from other genera (Dellaporta and Urrea-Calderon 1993, Talamali et al. 2003). Begonias may also help reveal the hormonal basis of gender in monoecious plants, especially in other Cucurbitales. In garden cucumbers (*Cucumis sativus*), ethylene causes femaleness, and gibberellins cause maleness, but ultimately sex may be determined by varying concentrations of, and target tissue sensitivities to, a single hormone (Yin and Quinn 1995). In an alternative view, Khryanin (2002) postulates cytokinins, not ethylene, as the ultimate female hormone. This raises the possibility that root: shoot ratio might affect gender, because cytokinins are produced in the roots and gibberellins in the leaves. What is the hormonal mechanism for gender determination and its connection to inflorescence architecture in begonias?

It is hoped that this story of a *Begonia* will augment our understanding of sex expression in monoecious plants of the rainforest understory. Because of their diversity in and around tropical forests, begonias may help reveal general trends of sex expression in understory herbs, and help us to synthesize theories of gender. Begonias may also help answer questions in population and community ecology, evolution of sexual systems and gender in plants, plant reproductive development and genetics, and conservation biology.
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VITA

John Cozza grew up in Cold Spring Harbor, Long Island, New York, a New England town outside New York City. He spent much of his youth wandering around in the oak-hickory forest that (he now realizes) surrounded his neighborhood, an experience of nature that made a huge difference in his life. At University of Pennsylvania, he was inspired by Dr. Daniel Janzen’s stories of the rainforest. After graduation in 1980 he worked as a lab technician, tutor, and interpretive gardener in Philadelphia. Moving on to New York City, he was Director of the Barnard College greenhouse from 1991 – 1997, during which time he developed a diverse plant collection of over 1000 taxa, and used it extensively for teaching and outreach. He continued his work with plants and teaching as Coordinator of Horticulture and Environmental Science at the Madison Square Boys and Girls Club from 1997 – 1999. He also taught biology lecture and lab at New York City Technical College for three years, and wrote curriculum for their online Great Thinkers in Science course. Then he went to University of Miami with the dream of doing research in the tropical rainforest. He got his wish, starting with participation in the Organization for Tropical Studies’ Tropical Biology 1 field course in 2001. Along the way, he had the good fortune to be a resource person for OTS, to teach labs at UM, and to work for the Gifford Arboretum, where he helped select, find, and grow 300 new trees, and use them for teaching. In the future, he hopes to combine his passion for growing plants and teaching about them, as well as helping to preserve their habitats.

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