The Reproductive Biology of *Clematis addisonii*

by

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(ABSTRACT)

Clematis addisonii Britton (Ranunculaceae) is a Virginia endemic restricted to calcareous soils in a four county region of the Ridge and Valley Province in Virginia. A two year study of the reproductive biology of this species reveals that it is self-compatible, showing no significant reduction in fecundity following self-pollinations.

Morphological observations indicate that this species is protogynous. In vivo pollen tube growth supports this conclusion. Field observations suggest that the morphological pistillate phase lasts significantly longer than the staminate phase and is sufficient enough in length that cross-pollination is likely to occur during the time period preceding the staminate phase. The secretion of nectar from the onset of anthesis enhances the probability that outcrossing will occur prior to the presence of self-pollen in flowers.

These findings suggest that, in spite of self-compatibility, populations of Clematis addisonii are capable of maintaining high levels of outcrossing by virtue of protogyny and nectar secretion from the onset of anthesis.

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Introduction

From collections made along the banks of the Roanoke River in Roanoke County, Virginia, Nathaniel Lord Britton, in 1890, first recognized Clematis addisonii Britton (Addison's Leatherflower) as a distinct species. Britton named the plant in honor of his friend and then president of the Torrey Botanical Club, Addison Brown (Vail 1891). Since then, only a small number of C. addisonii populations, ranging in size from less than 20 individuals to as many as 1000 (Van Alstine 1993), have been identified from a four county region within the Ridge and Valley Province of Virginia (Harvill et al. 1992; see Figure 1). Although it is one of the rarest members of its genus, and is extremely rare even within its restricted range in Virginia, C. addisonii has recently been removed from consideration as a candidate for federal listing as endangered or threatened (Ramsey 1991). It is currently ranked G2/S2 (i.e., very rare, susceptible to extirpation) by the Virginia Natural Heritage Program (Van Alstine 1993).

Taxonomic treatment

Clematis, a genus of the Ranunculaceae, comprises approximately 300 species whose distribution, although worldwide, is concentrated primarily in the temperate regions of the northern hemisphere (Lloyd and Bennett 1989). The

genus Clematis possesses a number of characteristics that are typical of the Ranunculaceae, such as apocarpous gynoecium and numerous, spirally arranged stamens and pistils, but is set apart by being woody (to various degrees), having opposite leaf arrangement, and valvate petaloid-sepals (Heywood 1979). The genus is said to be most closely related to Pulsatilla (Erikson 1943).

The most recent taxonomic treatment of Clematis divides the genus into four subgenera; Clematis, Atragene, Viticella, and Viorna (Keener and Dennis 1982). Subgenus Viorna, which includes C. addisonii, is distributed primarily over the central and southern United States (Keener and Dennis 1982). The members of subgenus Viorna are perennial, erect herbs or shrubby vines having one to several stems arising from a woody caudex, and simple to pinnately-compound opposite leaves (Dennis 1976). Their nodding flowers are large, hermaphroditic, and thick-sepalled.

Species biology

Clematis addisonii typically grows on dry, rocky slopes in soils high in magnesium and calcium. Its habitats range from mesotrophic forest and woodland to submesotrophic woodland and scrub and include a range of moisture regimes from floodplain edge and mesic forest to xeric outcrop (Van Alstine 1993).

Clematis addisonii is a morphologically distinct species with thin, broadly ovate glaucous leaves (see Figure 2). It achieves a height of approximately .5 m and is generally erect in its growth habit, although plants approaching a vine-like habit with stems of somewhat greater lengths are occasionally found. Most plants in shady habitats consist of a single, unbranched stem with simple leaves. Plants found in more open areas are often multi-stemmed with short, axillary branches, the uppermost leaves of which may be lobed or divided to

various degrees. Plants have been observed to cease above-ground growth in late spring or early summer, and during particularly dry summers they may die back to the woody caudex and not resume growth until the following spring.

Clematis addisonii begins flowering in mid to late April and continues to do so through early or mid June. Sporadic flowering may occur in late summer following a season of unusually high precipitation. The flowers of C. addisonii are terminal, although plants may also produce flowers laterally. Single-stemmed plants often produce only a single flower while multi-stemmed and branched plants usually produce one or more flowers per stem. The flowers are nodding and have four thick, glabrous sepals that are pink to mauve in color. The numerous stamens are distinct and spirally arranged on the receptacle. The apocarpous gynoecium comprises various numbers of pistils, the ovaries of which each contain a single ovule. The styles are persistent, and elongate and become strongly recurved in fruit.

The fruit of C. addisonii is a flattened, orbicular achene to which is attached a long, plumose "tail" formed by the style. Fruits mature in late summer or early fall. The nymphal stage of the hemipteran Euschistus tristigmus preyed on maturing fruits in both study populations. The insects pierce the pericarp with their beaks and consume the developing embryos. Following damage, the fruits, although appearing intact, stop developing. Close inspection of damaged fruits reveals the tiny pin-holes made by the insects, and the hollowed-out interior. Herbarium specimens of Clematis viticaulis exhibited similar fruit damage, indicating that fruit predation by this insect is not unique to Clematis addisonii.

Although the exact mode of seed dispersal is not known, casual observations indicate that, in spite of the presence of

long appendages on the fruits, the size and weight of the fruits preclude wind as a dispersal agent (see also Clematis fremontii var. Riehlii, Erikson 1945). It is more likely that fruits drop from the plant and are washed short distances downslope by runoff from precipitation. Seedlings discovered in the spring of 1994 in one of the study populations were growing in clumps near mature plants indicating that seeds fall and germinate in place or may sometimes gather and germinate in surface depressions or near the bases of rocks. Height measurements taken for 23 of these seedlings during mid-May and again in August of 1994 showed no additional above-ground growth for any of the seedlings during this time interval.

Apart from the information concerning habitat preferences and population status little else is known about Clematis addisonii. This study was undertaken to obtain the first information regarding its life history, specifically from the standpoint of its reproductive biology.

The questions that this investigation addresses are:

I. How does anthesis progress in Clematis addisonii? What are the periods of stigma receptivity and anther dehiscence and how do they correspond to each other?

II. Does Clematis addisonii require pollinator activity for seed set? If so, what is the mechanism of attraction?

III. Is Clematis addisonii self-compatible or self-incompatible? Are there mechanisms in place that promote cross-pollination?

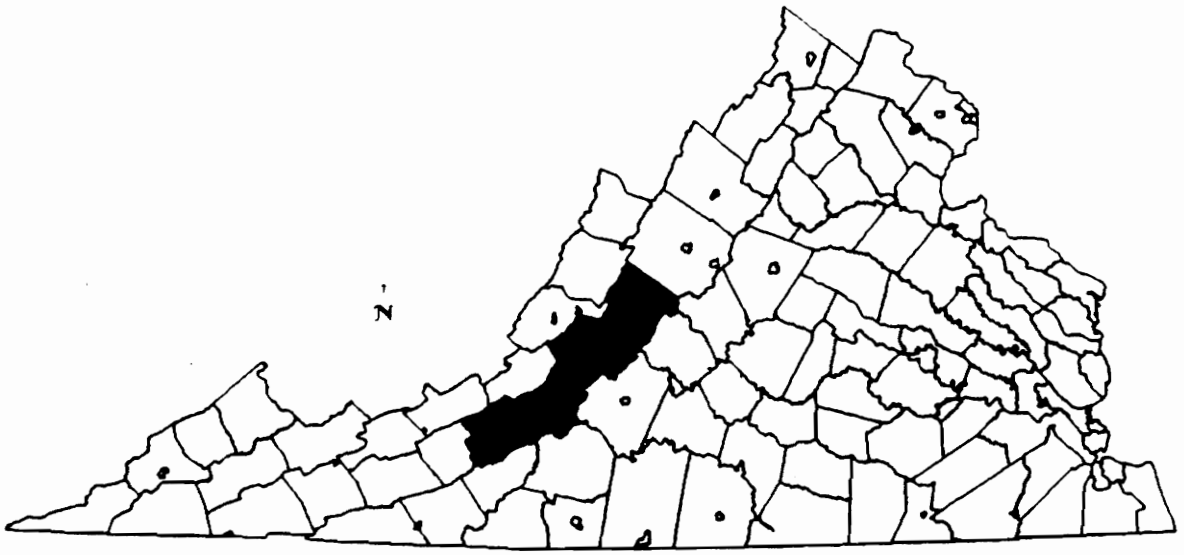


Figure 1. Geographical distribution of the Virginia endemic Clematis addisonii. Highlighted areas represent counties in which C. addisonii populations have been reported: Montgomery, Roanoke, Botetourt, and Rockbridge.

Figure 2. Flowering specimen of Clematis addisonii.

Chapter 1

The Intrafloral Phenology of *Clematis addisonii*

Literature Review

Gender expression in flowering plants is of interest because of its inherent role in breeding and pollination systems. Hermaphroditism, intrafloral bisexuality, is the predominant form of gender expression, found in an estimated 95% of all flowering plant species (Richards 1986). Although the bisexual flower has both stamens and pistils, there are few hermaphroditic plants in which the maturation and presentation of the sexes is simultaneous (Stout 1928). In most, the stamens and pistils mature at different times, a condition called dichogamy.

Dichogamy has been recognized in flowering plants for well over 200 years (Stout 1928). Its prevalence relative to homogamy, the synchronous maturation of sex organs, suggests that asynchronous maturation of sexes has adaptive value, this value customarily being assigned to outcrossing (Darwin 1876; Stout 1928; Proctor and Yeo 1972). Although homogamous taxa have been found to have lower outcrossing rates than dichogamous species, it has also been shown that dichogamy is

not necessarily more common in self-compatible than in self-incompatible species as would be expected, suggesting that dichogamy does not function exclusively as an outcrossing mechanism (Bertin 1993).

While the occurrence of dichogamy in self-incompatible species would seem to preclude its role in outcrossing, it has been argued that self-incompatibility may sometimes be only partial and that dichogamy serves to augment the potential for outcrossing (Lloyd and Webb 1986; Bertin 1993). Because of the coexistence of dichogamy with other mechanisms presumed to promote outcrossing, alternative hypotheses for the selective role of dichogamy in hermaphrodites have been proposed (Bawa and Beach 1981; Lloyd and Yates 1982; Lloyd and Webb 1986; Palmer, et al. 1989).

The synchronous maturation of stamens and pistils can result in increased levels of self-pollen being deposited on stigmas (Palmer, et al. 1989). For outcrossers, self-pollen on the stigma can pose a physical obstacle to cross-pollen by occupying deposition and germination sites (Bawa and Opler 1975) as well as be a waste of pollen in self-incompatible plants whose reproductive success is pollinator limited (Wyatt 1983; Campbell and Motten 1985). Furthermore, the co-occurrence of self- and cross-pollen tubes in the same style may modify the growth of cross-pollen tubes and enhance the fertilization ability of self-pollen (Lloyd and Yates 1982; Barrett 1988). Dichogamy functions to alleviate the physical and physiological obstacles imposed by the presence of self-pollen by ensuring a period of time during anthesis when self-pollen is not available for deposition on stigmas.

Staggering the maturation times of stamens and pistils does not fully resolve the potential drawbacks imposed by bisexuality in outcrossers, however. The coupling of stamens and pistils within the same flower could be described as an

uncomplementary arrangement, given the functional dissimilarity of the two structures. The comparable positioning of stamens and pistils in hermaphroditic flowers, while being an efficient arrangement for pollen transfer by animal vectors (Baker and Hurd 1968), may result in conflict between the two processes of pollen delivery and removal. Consequently, Lloyd and Webb (1986) have proposed that avoidance of interference between stamens and pistils has been an important factor in the evolution of dichogamy. They argue that bisexuality can lead to conflict between stamens and pistils and diminish their ability to function successfully, particularly if they are simultaneously presented; the presence of one may obstruct access to the other or disrupt the normal transfer of pollen. Dichogamy provides a solution by delaying or modifying the growth of one gender or providing for its senescence following the completion of its function (Bock and Peterson 1975; Lloyd and Webb 1986; Jonsson, et al. 1991).

The two forms of dichogamy, protogyny and protandry, refer to the order of gender presentation during anthesis. Lloyd and Webb (1986) hypothesize that the order of presentation in hermaphrodites has been selected for based on effectiveness in avoiding selfing and interference between the genders, in facilitating prolonged pollen presentation, and positioning the genders for optimal transfer of pollen. Protogyny, the presentation and maturation of stigmas before pollen, is regarded as the less common form of dichogamy (Bawa and Beach 1981; Wyatt 1983; Lloyd and Webb 1986). However, the association of protogyny with wind and beetle pollination (Faegri and van der Pijl 1979; Bertin and Newman 1993) and its prevalence among primitive dicots such as the Magnoliidae (including its non-beetle pollinated members) (Bernhardt and Thien 1987) have been noted. Furthermore, protogyny is shown

to be common among early flowering species in north temperate and polar regions (Schemske, et al. 1978).

The evolution of protogyny has been attributed to several conditions; when intact stamens serve as the sole attractant to pollinators (Lloyd and Webb 1986; Richards 1986; but see van der Pijl 1978), under conditions of pollinator uncertainty (Schemske, et al. 1978; Bawa and Beach 1981), and when self-pollen is presented in a way that precludes the depostion of cross-pollen (Bawa and Opler 1975; Motten 1982; Galen, et al. 1986; Lloyd and Webb 1986). Furthermore, that protogyny has been shown to be more prevalent in self-compatible species than protandry suggests the avoidance of selfing has been an important factor in the evolution of intrafloral protogyny (Bertin and Newman 1993).

In this study, through a series of field and laboratory observations, the sequence of floral events during anthesis will be examined to establish the relationship between the pistillate and staminate phases in the hermaphrodite Clematis addisonii. The following questions will be addressed: 1) What is the pattern of pistil and stamen maturation? 2) What does this pattern imply about the breeding system of C. addisonii?

Materials and Methods

Floral Phenology

To determine the sequence of morphological events over the course of anthesis, in late April, 1994, fifteen individuals growing in a natural population in Montgomery County, Virginia were selected for observation. All individuals were single-flowered and selected when in bud stage. Each flower was observed daily from the beginning of anthesis until the end of anther dehiscence, and morphological changes were recorded and described. Day 1 of anthesis was assigned to flowers having

open calyxes at least 2 mm in diameter. Day 1 of the staminate phase was assigned to flowers in which one anther had begun to dehisce. The final day of the staminate phase was assigned when the last anther had dehisced, regardless of the presence of pollen in the flower. Ten of the monitored individuals were observed until sepal drop.

Time and duration of stigma receptivity

To determine the period of stigma receptivity, in vivo pollen tube germination and growth were observed. Fourteen individuals were tagged in the field and assigned a particular day of anthesis to be hand-pollinated: 1, 2, 3, 4, 5, and 8 days. Day 1 of anthesis was assigned when calyx openings were 2 mm in diameter. Nylon mesh bags were placed over the flowers in the bud stage. When the flowers had opened enough to expose the androecia, they were emasculated to prevent contamination of stigmas with self-pollen.

Flowers with attached peduncles were cut at the assigned day of anthesis, immediately placed in water, and taken to the laboratory for pollinations. All stigmas were pollinated once, using pollen taken from freshly dehisced anthers of at least two different individuals from the same population. The identity of pollen donors changed between pollination episodes, so that flowers collected on different days received pollen from different donors. Pollen was allowed to germinate and grow for 1, 3, 6, 10, 14, 18, and 24 hours, after which pistils were excised and immediately fixed in 70% ethanol to prevent further pollen tube growth (Aizen, et al. 1990).

Fixed pistils were then processed for fluorescence microscopy using the method described by Martin (1959). Pistils were removed from ethanol and softened in 8N NaOH at room temperature overnight. The following day, pistils were removed from the NaOH and placed in a vial of distilled water

for 1-3 hours. Pistils were then placed in 0.1% solution of water-soluble aniline blue dye decolorized in 0.1 N K_3PO_4, for at least 4 hours. After staining, the pistils were placed on glass slides and mounted in several drops of the dye solution. Coverslips were placed over the pistils. Using the eraser end of a pencil, pistils were squashed by gently pressing against the coverslip. Coverslips were sealed with wax.

Pistils were viewed using a Nikon SA Microphot with a U.V. 1B filter cube under epifluorescent light. Numbers of pollen grains and pollen tubes were counted, and individual tube lengths or tube fronts were measured using an ocular micrometer at 10X or 40X magnification.

Results

Floral Phenology

On separation of the perianth segments (day 1 of anthesis), the stigmas were, in all cases, exserted above the stamens (see Figure 1.1a). Flowers in this stage of development, with no signs of anther dehiscence were designated as pistillate phase. During the early portion of the pistillate phase, stigmas were moist and papillate in appearance and reflexed backward (Figure 1.1b). As flowers progressed through the pistillate phase, stigmas became less reflexed (Figures 1.1c, 1.1d). As flowers approached the staminate phase, there was a gradual elongation of the filaments so that differences in length between the stamens and pistils became less pronounced. The outermost stamens elongated more rapidly than those located closer to the pistils so that stamen elongation as well as anther dehiscence occurred sequentially in a centripetal pattern (see Figures 1.2a, b). In 13 of the 15 individuals sampled, anthers did not begin dehiscence until stamens had achieved a length equal to or exceeding the height of the stigmas. The anthers of the other two flowers dehisced

when at positions below that of the stigmas. Anther dehiscence continued for 3 to 5 days.

The mean number of days that plants remained morphologically pistillate was 5.53 days ± .192. The mean number of days in the staminate phase (interval between dehiscence of first and last anther) was 4.07 days ± .182 (Table 1.1).

Mean number of days from onset of anthesis to sepal drop in the 10 plants observed until senescence was 14.2 days ± 0.8 (Table 1.1).

Stigma Receptivity

Pollen germinated on all pistils pollinated on the first day of anthesis indicating that stigmas are receptive at the onset of anthesis (Figure 1.3). All pistils pollinated 2, 3, 4, and 5 days into anthesis also exhibited pollen tube germination (Table 1.2). The pistils of the one flower pollinated on the eighth day of anthesis also exhibited pollen tube germination, although pollen tube lengths tended to be shorter than those found in pistils of flowers pollinated earlier in anthesis (Tables 1.3 a-f, Appendix I).

One hour after pollination, pollen tubes had not yet reached the transmitting tissue of the style (Figure 1.3), although three hours after pollination, pollen tubes were present in the transmitting tissue of most of the styles. Pollen tubes were absent in the transmitting tissue of the day 8 pistil collected 3 hours after pollination, however. Tubes had not yet reached the ovaries of different aged pistils collected 18 and 24 hours after pollination.

The demonstration of stigma receptivity in pistils of flowers collected 8 days into anthesis indicates that flowers remain functionally pistillate beyond the morphological pistillate phase (which lasted up to 7 days in the fifteen

plants observed in the field) so that there is some degree of overlap between the pistillate and staminate phases (Figure 1.4).

Discussion

Field observations indicate that Clematis addisonii is protogynous. A sequence of morphological stages similar to those observed in C. addisonii was noted by Bock and Peterson (1975) in the protogynous Pulsatilla patens (Ranunculaceae) and by Jonsson, et al. (1991) in three other species of Pulsatilla.

Variation was evident, however, in the duration of the morphological sexual phases. Similar variation has been observed in other taxa. Schemske (1977) found that the pistillate phase of Claytonia lasted between 1 and 8 days, depending on temperature. Pistillate phase longevity has also been observed to vary as a result of the amount of time stigmas remain unpollinated. It has been suggested that plants may have the ability to adjust the duration of the pistillate phase according to the amount of time required for sufficient pollen deposition to occur (Richardson and Stephenson 1989). Hand-pollinations were shown to decrease the duration of the pistillate phase in Pulsatilla (Jonsson, et al. 1991). Pollen deposition and removal were found to accelerate both sexual phases in Lobelia cardinalis (Devlin and Stephenson 1984). Furthermore, the pistillate phase of Campanula rapunculoides was influenced by both the source and amount of pollen applied to the stigmas, with pistillate phases being extended in flowers receiving no pollen, self-pollen, or low levels of cross-pollen (Richarson and Stephenson 1989). Even though all Clematis addisonii individuals in this study produced fruit, indicating that pollination did occur, it is not known whether the variation

in pistillate phase longevity reflects any of these phenomena, however. It is also unclear whether pollinator activity modified the rate of anther dehiscence in flowers.

The prolonged pistillate phase of Clematis addisonii may be an important factor in the level of fecundity a plant experiences in that it allows sufficient time for most stigmas to receive pollen. Unlike syncarpous species which may require only a single pollinator visit for seed set (i.e. Salvia reflexa and Quamoclit coccinea; Cruden, et al. 1983), Clematis addisonii may require multiple visits for sufficient seed set because it is apocarpous and all stigmas may not receive pollen during the first pollinator visit. Because the outer whorl of pistils is situated lower on the receptacle and deeper in the calyx, these stigmas may not be contacted by pollinators until later in anthesis when the calyx is fully separated, unlike pistils residing on the crest of the receptacle which may receive pollen when the calyx opening is only 2 mm in diameter. Therefore, pollination is likely to be asynchronous, with the stigmas of a single C. addisonii flower receiving pollen from numerous donors as a result of multiple pollinator visits taking place over several days.

Douglas and Cruden (1994) suggest that the prolonged pistillate phase of A. canadensis reduces the liklihood of pollination failure during periods of pollinator inactivity due to unfavorable weather conditions. Because there were periods of cold, rainy conditions during the early part of this study when pollinator activity was noticeably lower, the same may also apply to C. addisonii. But for the earliest blooming individuals, a prolonged pistillate phase may confer additional benefits. Female reproductive success in out-crossing insect-pollinated plants is limited not only by pollinator activity but also by pollen availability. The proportion of flowers in pistillate and staminate phases in

dichogamous species will vary over time (Pellmyr 1987) such that the sex ratio of protogynous species is expected to be female biased early in the flowering season during which pollen may be of limited availability (Webb 1984; Jennesten, et al. 1988). Furthermore, earlier flowers may receive fewer visits from pollinators than later ones because of a delay in assessment by pollinators of newly available resources (Barrett 1980). Prolonged pistillate phases and long periods of stigma receptivity in early flowering individuals of C. addisonii would then be essential to their receiving sufficient pollinator visitation and pollen deposition for adequate seed production.

Many plant taxa release pollen in stages, either by successive dehiscence of different anthers or by the gradual or repeated release of pollen from single anthers or anther tubes (Harder and Thomson 1989). The sequential opening of the numerous anthers of C. addisonii enhances male reproductive success in two ways. First, pollen waste is minimized by spreading anther dehiscence out over a period of several days, thereby restricting the amount of pollen that is available to pollinators in a single day. Harder and Thomson (1989) found that only 0.6% of the pollen removed in one visit to Erythronium americanum reached conspecific stigmas. Second, the gradual presentation of pollen would not only minimize pollen wastage, but would also increase the number of occasions that pollen could be picked up by different pollinators. This would, therefore, ensure the dissemination of pollen to a number of different mates (Lloyd and Yates 1982; Lloyd 1984). In addition, "intrasexual interference" between the sequentially dehiscing anthers is minimized by delaying the elongation of later-functioning stamens so that they do not obstruct pollinator access to functioning stamens (Lloyd and Webb 1986).

Studies of dichogamous plant taxa often do not distinguish between the morphologically defined pistillate and staminate phases and the functional pistillate and staminate phases. That stigmas of flowers 8 days into anthesis were shown to be receptive demonstrates that flowers are functionally pistillate at least into the early portion of the staminate phase (the morphological pistillate phase lasted a maximum of 7 days in field observations). Clematis addisonii is, therefore, incompletely protogynous. Incomplete protogyny is said to provide better protection against self-fertili zation than incomplete protandry, while still permitting it if cross-pollination fails to occur (Faegri and van der Pijl 1979; Bawa and Beach 1981). This "delayed self-fertilization" is thought to be selected for over "prior self-fertilization" which occurs with incomplete protandry (Lloyd 1979). Because C. addisonii has been shown to be self-compatible (see Chapter 3), continued stigma receptivity into the staminate phase may lead to self-fertilization in pistils that did not receive cross-pollen during the morphological pistillate phase. The position of the stamens during the staminate phase may increase the liklihood that unpollinated, receptive stigmas will receive self-pollen instead of cross-pollen. For stigmas receiving mixtures of both self- and cross-pollen, the potential for self-fertilization will depend on the growth rate of self-pollen tubes, which, in some plants, has been shown to be inferior to cross-pollen tube growth rates (Bateman 1956; Bowman 1987). In flowers whose stamens do not elongate beyond and obstruct the stigmas, cross-pollination would still be possible during the staminate phase.

Several hypotheses have been used to explain the evolution of protogyny. For C. addisonii two of these hypotheses seem to be particularly relevant. First, for

single-flowered individuals, protogyny ensures a period of time during which stigmas can receive only cross-pollen and, therefore, serves as an effective outcrossing mechanism. The potential for outcrossing is augmented by the longevity of the pistillate phase which allows sufficient time for cross-pollen to be transferred to the flower before the onset of the staminate phase. It is not known, however, whether dichogamy effectively prevents geitonogamous pollinations (interfloral self-pollination) in individuals producing multiple flowers with overlapping antheses. Preliminary observations of self-pollen tube growth in five and six day old pistils show that self-pollen germinates as quickly on the stigma as cross-pollen. Therefore, protogyny may not be as effective an outcrossing mechanism in multi-flowered plants as it appears to be for single-flowered plants.

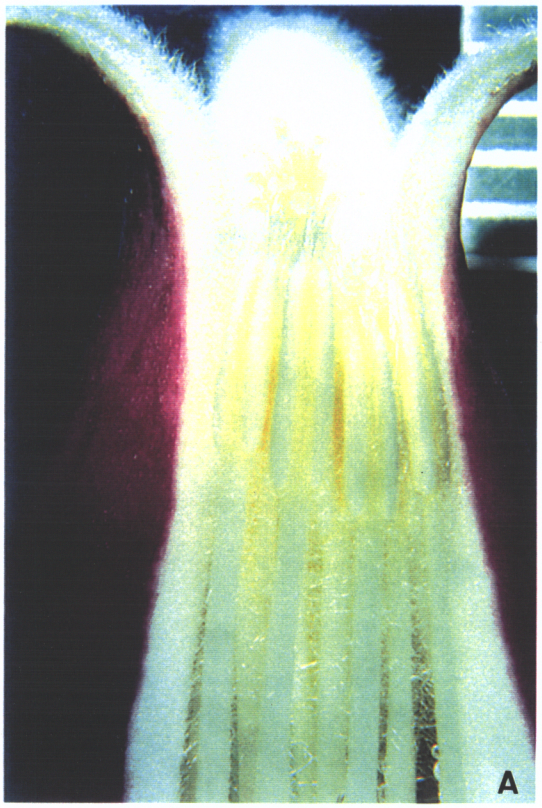
Second, protogyny provides a mechanism by which interference between the stamens and pistils can be avoided. The flower of C. addisonii has numerous stamens and pistils that are closely arranged on the receptacle and surrounded by a thick, bell-shaped calyx. Pollinator access to the stigmas is facilitated by delaying the elongation of the filaments during the early portion of anthesis. Stigmas are exserted above the stamens for at least four days so that their contact with pollinators is not obstructed by stamens. During the pistillate phase, the filaments slowly elongate, with the outer whorl growing more rapidly, so that by the first day of the staminate phase, the outermost dehiscing anthers are sufficiently elevated in the flower for contact with pollinators. Stamen extension beyond the height of the pistils prevents their being obstructed by the pistils and the centripetal, sequential elongation of the stamens prevents "intrasexual interference" between stamens.

Uncertainty of cross-pollination because of weather

induced pollinator inactivity is another condition that is suggested as leading to the evolution of protogyny (Bawa and Beach 1981). The occurrence of protogyny among many spring flowering species appears to substantiate this hypothesis (Schemske, et al. 1978). This hypothesis may pertain to C. addisonii only during the early portion of the flowering season in mid to late April when conditions may be cool enough to significantly retard pollinator activity. May and early June conditions are generally warm enough so that pollinator activity is not severly limited by weather. Because C. addisonii is shown to be incapable of self-pollinating in the absence of pollinators (see Chapter 3), protogyny can provide for self-pollination only in the presence of pollinator activity, however.

Protogyny in Clematis addisonii, therefore, appears to be multi-functional. First, it promotes outcrossing by ensuring a period of time during which stigmas can receive only foreign pollen. Protandry fails to provide for this since self-pollen may still be present in the flower at the time the stigmas become receptive. Protogyny also provides a tactic by which the multiple stamens and pistils can avoid interfering with one another during their respective periods of maturation. For outcrossers, simultaneous presentation and maturation of stamens and pistils would lead to conflict between their functions as well as result in higher rates of self-pollination.

Figure 1.1. Morphological changes of Clematis addisonii flowers through the pistillate phase of anthesis. A. Pistillate phase flower early in anthesis, stigmas exserted above stamens; note nectar droplets at base of flower. B. Stigmas of early pistillate phase flower; recurved and papillate. C. A later pistillate phase flower with stigmatic area less recurved. D. Late pistillate phase flower showing straightening stigmas and elongating stamens.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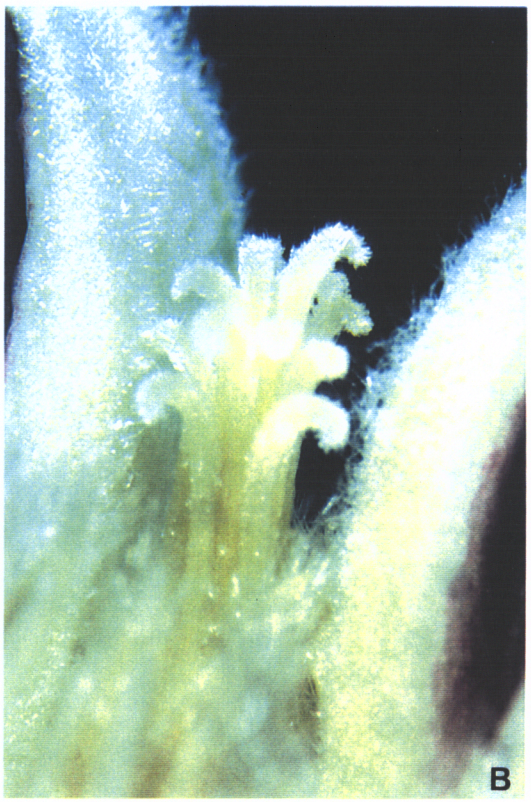
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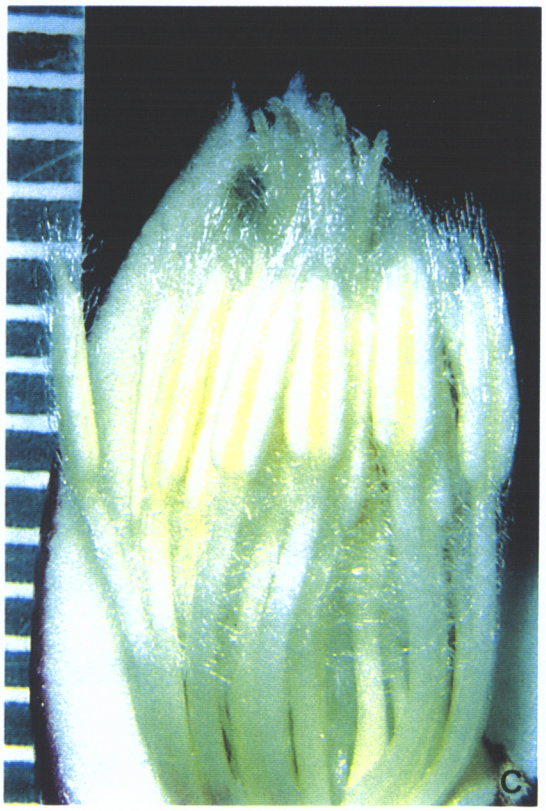
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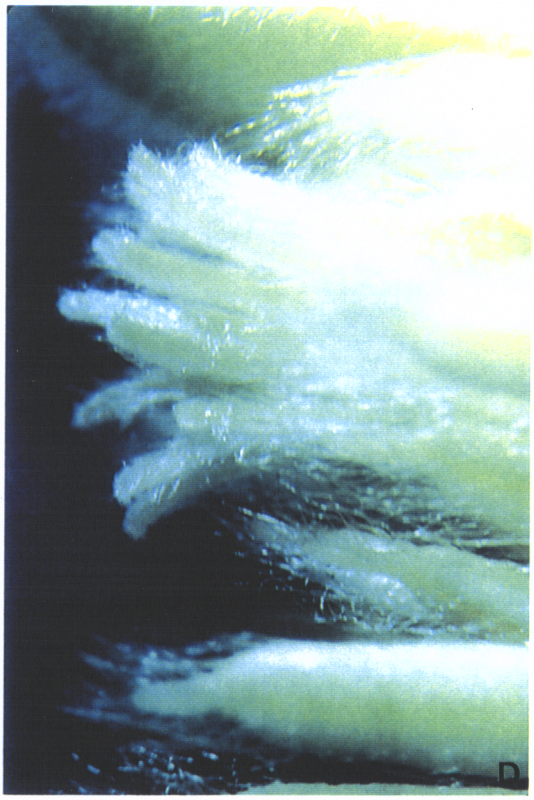

D

Figure 1.2. Clematis addisonii flowers in the staminate phase of anthesis. **A.** Early staminate phase flower with outermost anthers dehiscing and overtopping stigmas. **B.** Early staminate phase flower showing centripetal elongation and maturation of stamens; inner stamens are not fully elongated and have not begun dehiscence; note nectar droplets at base of flower.

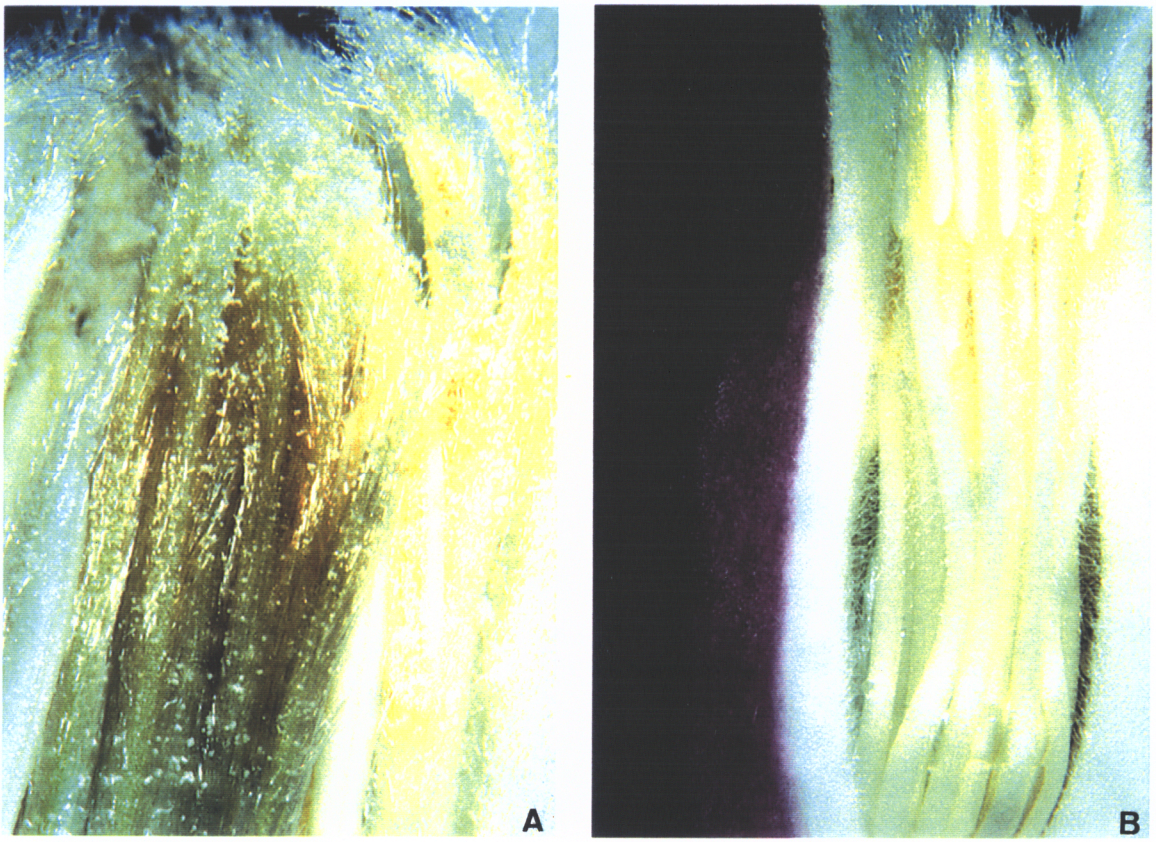
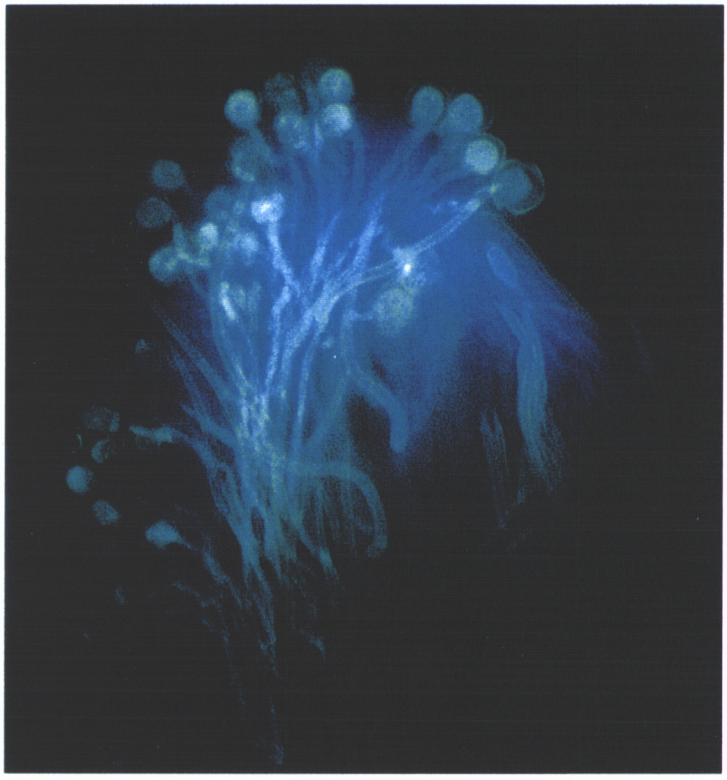

A
B

Figure 1.3. Pollen tubes in a stigma of Clematis addisonii one hour after hand pollination made on the first day of anthesis; viewed under fluorescence.



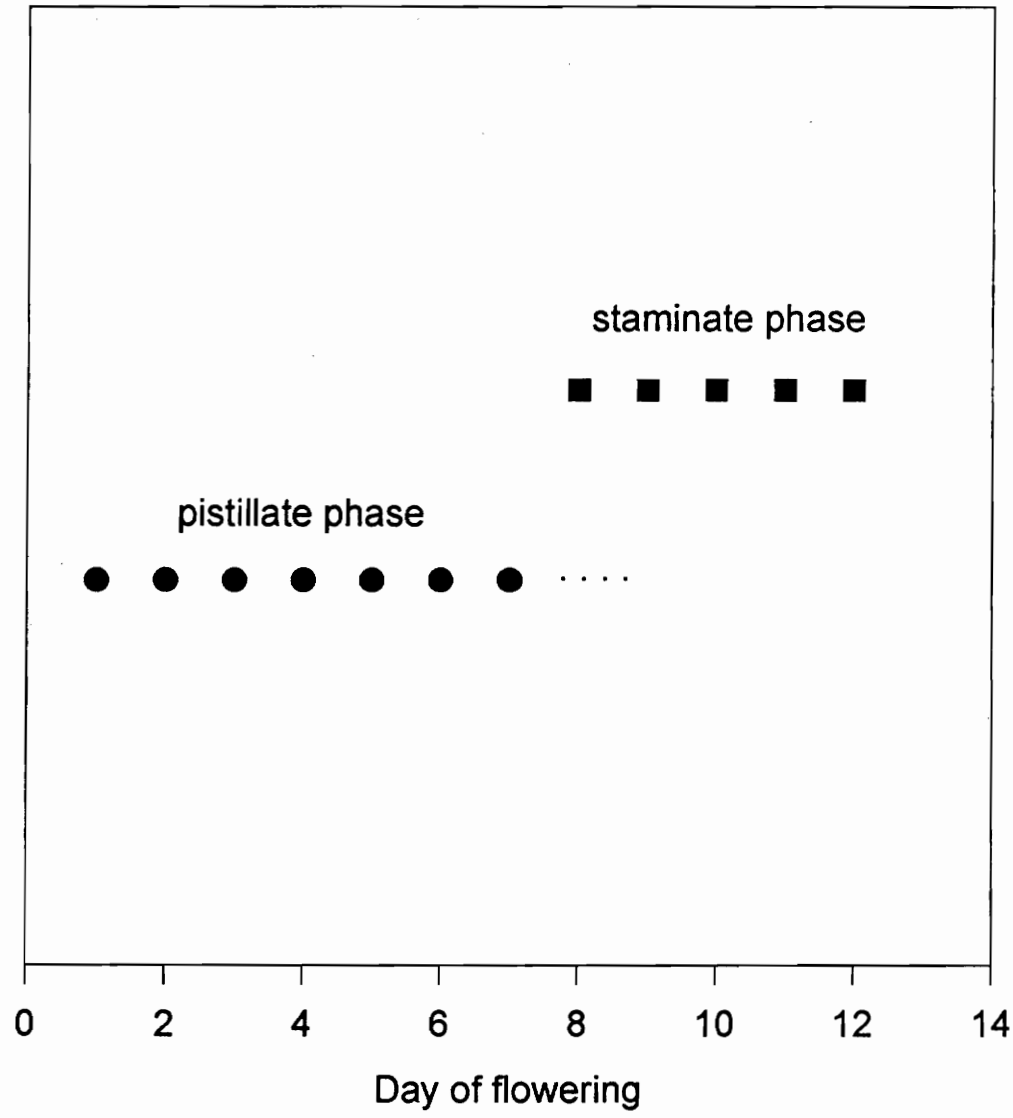


Figure 1.4. Order of gender maturation with potential longevity of the sexual phases (in days) in Clematis addisonii; morphological pistillate phase (large dots), functional pistillate phase (small dots), and morphological staminate phase (squares).

Table 1.1. Means and standard errors for duration of pistillate and staminate phases (n=15) and anthesis (n=10).

Length of pistillate phase (n=15)	Length of staminate phase (n=15)	Days to senescence (n=10)
$\bar{x}$ = 5.553 s.e.= .192	$\bar{x}$ = 4.067 s.e.= .182	$\bar{x}$ = 14.2 s.e.= .8

Table 1.2. Means and standard errors of pollen tube lengths (μm) in pistils 1, 2, 3, 4, and 5 days into anthesis, one hour after hand-pollinations; and for pistils 1, 2, and 4 days into anthesis, three hours after pollination.

Day of anthesis	Hours after pollination	Mean pollen tube length
1	1	77.99 ± 2.14
2	1	86.13 ± 40.39
3	1	102.85 ± 51.56
4	1	57.15 ± 27.15
5	1	16.62 ± 6.88
1	3	2283.33 ± 365.54
2	3	1021.25 ± 382.85
4	3	375.00 ± 225.03

Chapter 2

The Nectar of Clematis addisonii

Literature Review

Pollen dispersal is a primary component of gene flow in plant populations and for most flowering plants is accomplished through visitation by animals seeking the rewards that are present in flowers. Nectar is the principle floral reward presented to animal pollinators and is the most common reward offered by hermaphroditic plants (Simpson and Neff 1983).

Although nectar contains a number of non-sugar constituents (Baker and Baker 1981), it is the sugar constituents that impart the bulk of the food value to pollinators (Heinrich 1975). Analyses demonstrate that a number of sugar types may be present in nectar, although the dissacharide sucrose and the monosaccharides glucose and fructose are the most common (Baker and Baker 1983). Because nectar is derived from phloem sap which does not contain glucose or fructose, the presence of these sugars in nectar indicates a degree of sucrose modification en route from sieve cell to secretory cell (Zimmerman and Brown 1977). The sugar constituents of nectar and their ratios (Percival 1961; Baker and Baker 1983), variations in the concentration of nectar (Wells, et al. 1992) and the pattern of nectar production

(Pleasants 1983; Cruden, et al. 1983; Devlin, et al. 1987) have received considerable attention because of their role in pollinator attraction and the effect they have on gene flow by modifying the foraging behavior of these pollinators (Levin 1979). The evolution of the nectar properties of a plant species will be influenced by its breeding system, which ultimately determines the need for pollinator services and the need for rewards to attract them (Baker and Baker 1983).

It has been suggested that the constraints imposed by both phylogeny and pollination biology are the primary determinants of nectar composition (Baker and Baker 1983). Pollinators have been shown to have taste preferences (Percival 1961; Baker and Baker 1983) and the "taste" of nectar, which varies with the proportion of constituent sugars, may affect, in part, the relative attractiveness of a nectar to a particular pollinator (Baker and Baker 1983; Southwick 1990), such that an association exists between nectar type and pollinator. Furthermore, nectar types tend to remain fairly constant across family lines, so that certain nectars are characteristic of particular plant families (Percival 1961; Baker and Baker 1983). The significance of these consistencies is assumed to be that plant species are providing pollinators with nectars having distinct tastes (Baker and Baker 1983). Although shifts in nectar sugar composition during anthesis have been reported (Percival 1961; Walker, et al. 1974; Loper, et al. 1976), nectar sugars, unlike nectar volumes and concentrations (see Corbett 1978), have a tendency to remain uniform even with changing environmental conditions (Wykes 1953; Percival 1961; Baker and Baker 1981; 1983; 1990; but see Walker, et al. 1974). Therefore, not only are pollinators presented distinct tasting nectars from different plant species, but they are also presented nectars that are uniform and relatively consistent

in their taste over time.

In addition to the evidence that pollinators have taste preferences which may influence their decision to exploit the nectar of a particular species, the caloric value of that nectar may also influence their decision to forage as well as their foraging behavior. Pollinator foraging is driven primarily by "economics" (see Heinrich 1979), and one of the factors determining visitation by a potential pollinator is the relationship between its energy demands and the resources it can effectively harvest from the flower (Heinrich and Raven 1972). Bumblebees forage in a manner that maximizes the net rate of caloric intake relative to their energy investment (Marden 1984). The caloric value of nectar is dependent upon the volume and concentration of that nectar (Heinrich 1976) both of which have been found to vary with environmental conditions (Corbett 1978) as well as with the age of the flower (Pyke 1978). Dilute nectars place a limit on the amount a pollinator can ingest during a single visit (Heinrich 1975). Nectars with high sugar concentrations are assumed to be more attractive to pollinators (Inouye, et al. 1980) because they provide the highest net intake of calories per unit time (Heinrich 1976). The demonstration that bees have the ability to differentiate between nectar concentrations has prompted the argument that the caloric value of nectar, rather than its sugar composition, is the primary determinant of foraging behavior (Wells, et al. 1992).

The quality of a plant's nectar reward has been shown to influence the amount of time a pollinator spends on a flower (Zimmerman 1983a) which, in turn, affects both pollen deposition (Thomson and Plowright 1980) and pollen removal (Waddington 1981; Mitchell 1993). As such, fecundity may be considered a function of nectar production. Furthermore, the distance flown between pollinator visits has been found to be

inversely related to the quality of the reward encountered at the previous flower visited so that nectar production may also exert an influence on the pattern and degree of gene flow that occurs in a plant population (Morse 1980; Real 1981). While various other components of a plant species' reproductive biology will affect neighborhood size and population genetic structure, Zimmerman (1982) has demonstrated that nectar producing plants tend to have larger neighborhood sizes relative to non-nectar producers as a result of increased pollinator flight distances between them.

Reference to nectar secretion in Clematis has been infrequent, although Clematis fremontii Watson (Erikson 1945) and C. vitalba L. (Jones 1939) are two species for which nectar secretion has been described. Clematis viorna L. and C. coactilis (Fernald) Keener have also been observed to produce nectar (Edwards, personal observations). Apart from the observation of nectar secretion in these species, neither the composition of their nectar nor the pattern of its production have been investigated.

Clematis addisonii, a self-compatible hermaphrodite, has been found to secrete nectar (personal observation). This study was conducted to 1) Determine the pattern of nectar production in C. addisonii. Is nectar produced for the duration of anthesis or only during a certain portion of anthesis? 2) Assess the properties of nectar over the period of nectar production as measured in terms of total sugars, sugar concentration and sugar composition. 3) Interpret the secretion of nectar in C. addisonii within the context of its breeding system.

Materials and Methods

During May 1994, one flower from each of seven randomly chosen

plants at Site 1 (main study population in northern Montgomery County) was bagged shortly before anthesis. From day 1 of anthesis (assigned when calyx opening was 2 mm in diameter), each flower was checked daily for nectar secretion. Nectar was collected each morning throughout the time of its secretion. With each collection, flowers were completely drained of the nectar that had accumulated over the 24 hour period. The day of anthesis and the volume of nectar collected were recorded at each collection.

In August and early September 1994, a population approximately .5 km northeast of Site 1 produced a second flush of flowers. Each of the six flowering individuals from this small population was sampled according to the same schedule and procedure as those from the May sampling.

Nectars were analyzed for total sugars and sugar constituents using high performance thin layer chromatography following the method described by Fell (1990).

Nectar collections

Nectar collections were made using Drummond microcapillary tubes. Volumes were calculated by the following formula:

$$\frac{\text{mm of nectar in tube}}{\text{mm total length of tube}} \times \text{calibrated vol. of pipette} = \text{vol. of nectar}$$

Nectar was aspirated from the microcapillary tubes into 1 ml 75% EtOH and frozen until analysis.

Nectar analysis

I. Pretreatment

Merck pre-coated silica gel plates (10 cm X 10 cm) were used for the chromatographic procedure. Prior to nectar spotting, plates were prewashed in methanol and dried for 1 minute using a hair dryer. Plates were then pretreated with a spray of 0.1 M sodium bisulphite solution and dried for 1 minute. This was

followed by a second spray of citrate buffer (1:10 dilution of Sigma citrate buffer: water, pH 4.8). Pretreated plates were then dried at 100° C for 1 hour. Following heat activation, plates were transferred to a dessicator until use.

II. Spotting procedure

Prior to spotting, all nectar samples were placed in a sandbath to evaporate the liquid from them, and then resuspended in a known volume of 95% EtOH to facilitate calculations of total sugars.

Samples were applied at 1.0 µl volumes using a Camag Nanomat 1 applicator and 1.0 µl Drummond microcapillary tubes. Four or five standards of mixtures of sucrose, glucose, and fructose ranging in concentration from 2.0 µg/µl to .25 µg/µl were spotted on each plate. Each plate was spotted with the nectar collected from one individual, with each day's sample being spotted twice consecutively. Because plants sampled in May yielded a greater number of samples than a single plate could accomodate, a subset of the these samples over the duration of nectar secretion was analyzed. Since the duration of nectar production for plants sampled in August/September was shorter, all daily samples from a single plant could be accomodated on a plate.

After spotting, plates were dried for 1 minute using a hair dryer.

III. Developing procedure

After drying, plates were developed 3 times by running in an 85:15 mixture of acetonitrile and water (Gauch et al. 1979) in a covered CAMAG Twin Trough Chamber. The solvent was run 7 cm from the bottom of the plate each time. Between runs, plates were dried for 1 minute with a hair dryer. After the final drying plates were gently brushed to remove small particles of

dust that could char during heating and interfere with quantification.

IV. Charring and visualization procedure

After developing and drying, plates were dipped into a ceric/sulfuric acid solution. The solution is made by diluting 1 part of 0.100 N ceric sulfate in 2 N sulfuric acid into 10 parts of 15% sulfuric acid. After dipping, the plates were heated at 110° C for 15 minutes to char the sugars. Depending on their concentration, sugars char as light brown to dark brown spots.

V. Quantification

All quantitative measurements were performed using a CAMAG TLC Scanner II interfaced to an Sp-4270 intergrator. Plates were scanned at a wavelength of 440 nm. Peak areas of absorbance were utilized for quantification.

Values (µg/µl) obtained from the concentrations of mixed standards spotted on each plate were used to construct a standard curve for each sugar from the samples. Because each sample was spotted twice, the concentration given for that sample represents the average value of the two concentrations. Total sugars for each sample are the product of the resuspended volume of each sample and the micrograms of sugar in one microliter of solution. Total sugars are presented in milligrams. Concentrations for each sample were calculated by dividing total sugars by the total volume of nectar collected from the flower.

Results

The flowers of Clematis addisonii began secreting nectar on the first day of anthesis (when calyx was open 2 mm in diameter) and continued to do so throughout the pistillate and

staminate phases (see Figures 1.1a and 1.2b, Chapter 1). Nectar secretion during the May sampling took place over a greater number of days than during the August/September sampling because of the longer life span of flowers in the spring. Eleven days was the longest period of nectar secretion during May (Appendix II; Tables 2.1 c,f) and 7 days was the longest period of nectar secretion during the August/September sampling (Appendix II; Table 2.2 d). Flowers for which nectar data are not presented for day 1 of anthesis showed evidence of nectar secretion, but amounts were so scanty that collection was not possible.

Chromatographic analyses demonstrate that the nectar of Clematis addisonii consists primarily of sucrose (Tables 2.1 and 2.2). Fructose and glucose were found in small quantities in some samples, but they were never detected in nectar collected on the first day of anthesis (Appendix II; Tables 2.1 a-g, 2.2 a-f). Hexoses were not present until day 6 or day 8 of anthesis in the May nectar samples (Appendix II; Tables 2.1 b-g) with the exception of one flower for which fructose was found in nectar collected on the second day of anthesis (Appendix II; Table 2.1 a). Hexoses were detected on the second or third day of anthesis in the August/September nectar samples (Appendix II; Tables 2.2 a,c,d,e). Fructose was the only sugar detected in samples collected on the last day of nectar secretion in five flowers (Appendix II; Tables 2.1 b, c, f, g; Table 2.1 a).

Total sugar production (sucrose + glucose + fructose) peaked between day 3 and 6 of anthesis in the May nectar samples (Figure 2.1 a) and on day 2 of anthesis in the August/September nectar samples (Figure 2.1 b). Differences in sugar production over anthesis were not statistically significant during either sampling period, however (One Way Repeated Measures ANOVA; $p > .05$).

Nectar volumes during anthesis follow a pattern similar to that found for total sugars. In the May samples, nectar volumes peaked between days 3 and 6 of anthesis (Figure 2.2 a) with significantly different nectar volumes found between flowers 1 and 3 days into anthesis (One Way Repeated Measures ANOVA; $p < .05$). August/September nectar volumes peaked on the third day of anthesis with no significant difference in volumes found between days (Figure 2.2 b).

Unlike sugar levels and nectar volumes which tended to fluctuate somewhat over time, increasing sugar levels accompanied by increasing nectar volumes maintained the nectar at relatively stable concentrations throughout both sexual phases (Figures 2.3 a and b). No significant difference in nectar sugar concentrations was found between days during either sampling period (One Way Repeated Measures ANOVA; $p > .05$). Mean sugar concentrations ranged between .27 (s.e. $\pm$ .08) and .29 µg/µl (s.e. $\pm$ .03) through day 8 of anthesis and fell to .22 µg/µl (s.e. $\pm$ .02) on day 10 of anthesis in the May samples (Figure 2.3 a). Mean sugar concentrations in the August/September nectar samples ranged from .24 (s.e. $\pm$ .11) to .29 µg/µl (s.e. $\pm$ .08) during anthesis, with the exception of day 3 when concentrations fell to .16 (s.e. $\pm$ .07) as a result of decreasing sugar levels and increasing nectar volumes (Figure 2.3 b). Although sugar concentrations will change as nectar volumes fluctuate with environmental conditions (Corbett 1978), the thickness of its sepals and the downward-facing bell-shaped flowers of Clematis addisonii may buffer against drastic changes in nectar sugar concentrations.

Discussion

This study supports the prediction that taxonomically related plants may secrete nectar of similar sugar composition. The sucrose dominance of Clematis addisonii nectar is typical of

the Ranunculaceae, a family characterized by sucrose-rich or sucrose-dominant nectars (Baker and Baker 1983). The shift observed in the nectar composition of C. addisonii from sucrose only to the additional presence of fructose and glucose later in anthesis has also been reported in Pulsatilla vulgaris and garden varieties of Delphinium (Percival 1961). The reasons for the changes in sugar composition seen in this study are not known, although yeast contamination and the action of invertase have been implicated in such changes in other studies (Frey-Wyssling, et al. 1954; Gilliam 1975). In this study, however, yeast contamination is unlikely since at no time was nectar cloudy in appearance. Percival (1961) suggests that the nectar of all species may vary in composition within certain limits because of the presence of enzymes, but that these variations have no significant impact with regard to pollinators. However, Loper, et al. (1976) found that as citrus flowers aged they became less attractive to foraging honey bees because of a decrease in sucrose content in the nectar of older flowers. Because fructose and glucose were usually detected in such small quantities in the nectar of C. addisonii it seems unlikely that their presence would modify pollinator foraging to any great extent.

Because pollinators have been shown to have different "taste" preferences for nectar, the influence of pollination biology on nectar composition has also been pointed out (see also Percival 1961). The nectar of Clematis addisonii supports the hypothesis that sugar constituents may be used as an indicator of pollinator type. Sucrose-rich or sucrose-dominant nectars have been found to be secreted by plants pollinated by long-tongued bees and butterflies (Baker and Baker 1983). Two seasons of field observations have shown that C. addisonii is visited primarily by bumblebees and butterflies. Butterflies may play only a minor role in the

pollination of C. addisonii, however, because they forage for nectar by clinging to the recurved tips of the sepals, inserting their probosci into the base of the flower without actually entering it. Consequently, they may obtain nectar without picking up or depositing substantial loads of pollen. Small butterflies, whose probosci are too short to reach the base of the flower where the nectar accumulates, may act more as nectar thieves. Rather than entering the flower, they insert their probosci from the outside, between the valvate margins of the sepals to obtain nectar. Bumblebees, which are much more aggressive in their foraging, appear to be the primary pollinator of C. addisonii because they crawl inside the flower and insert their heads far down into it to obtain nectar.

Unlike those species in which nectar secretion is discontinuous over anthesis, the data show that the flowers of Clematis addisonii secrete nectar throughout most of anthesis, making them attractive to nectar-seeking pollinators during both pistillate and staminate phases. Although sex differential nectar secretion has been described in various dichogamous plant taxa (Metrosideros collina, Carpenter 1976; Delphinium nelsonii, Cruden, et al. 1983; Helleborous foetidus, Herrera and Soriguer 1983; Lobelia cardinalis, Devlin, et al. 1987), statistically significant differences in the volumes of nectar secreted over time were not apparent in Clematis addisonii (the exception being between nectar volumes of day 1 and day 3 of anthesis in May samples). Failure to identify significant differences may be due to the small sample sizes of the study and the heterogeneity among individuals in nectar secretion or simply because such differences do not exist in C. addisonii. However, despite the absence of statistically significant differences, there was an apparent pattern to nectar secretion over the course of

anthesis in both sampling periods, and certain inferences can be drawn from this pattern.

Nectar volumes from both sampling periods peaked on the third day, during the pistillate phase of anthesis. A two or three day delay in full nectar secretion may be favored in Clematis addisonii because it allows sufficient time for the calyx to open wide enough so that pollinators are provided better access to the inside of the flower to obtain nectar. The calyx opening of flowers one day into anthesis may be too narrow for pollinators, primarily bumblebees, to fully access nectar (as well as pollen and stigmas) which would consequently reduce pollinator effectiveness. The initiation of nectar secretion on the first day of anthesis may, however, function to attract nectar-seekers and accustom them to visiting the flowers.

The pattern of nectar secretion has been shown to be closely integrated with a plant's breeding system (Cruden, et al. 1983). The pattern of nectar secretion, in addition to the protogynous development of its flowers (see Chapter 1), suggests that Clematis addisonii is adapted for outcrossing. Nectar secretion at the onset of anthesis would facilitate cross-pollination by attracting insects to pistillate phase flowers, before self-pollen has been shed in the flower. Whereas nectar secretion in some species ceases after the first pollinator visit (presumably because one visit is sufficient for adequate seed yields; see Cruden, et al. 1983), the secretion of nectar over the entire pistillate phase would encourage the multiple pollinator visits that are likely required for high seed yields in the apocarpous C. addisonii. Therefore, not only does the pattern of nectar secretion in C. addisonii facilitate cross-pollination, it is also an important component of its fecundity.

Unlike the protogynous Helleborous foetidus which ceases

nectar secretion early in the staminate phase (Herrera and Soriguer 1983), C. addisonii continues to secrete nectar throughout the staminate phase. Continued nectar secretion in staminate phase C. addisonii flowers would ensure visitation by pollinators and dispersal of a plant's pollen into the population. Pollen dispersal may provide the principle means of gene flow in C. addisonii populations since seed dispersal appears to be somewhat limited. Although pollen would also function as an attractant and food source to pollinators in staminate phase flowers, pollens have been shown to differ with respect to nutritional value (Levin and Haydak 1957). Nectar secretion during the staminate phase may be necessary for meeting the energy demands of its pollinators, if the pollen of C. addisonii is of low nutritional value (see Simpson and Neff 1983).

In summary, because Clematis addisonii relies on the activity of insects to facilitate pollination (see Chapter 3), nectar secretion is an important component of its fecundity. Prolonged nectar secretion enhances seed yields by ensuring that the flowers, which are apocarpous, receive multiple visits from pollinators. Furthermore, nectar secretion at the onset of anthesis favors seed set via outcrossing in the protogynous flowers. Continued nectar secretion during the staminate phase ensures that plants remain attractive to pollinators and experience further reproductive success through the male function. Pollen dispersal by nectar-foraging insects may be the principle means of gene flow in C. addisonii populations.

Table 2.1. Mean total sucrose (mg) and total sugars (mg) with standard errors in nectar of Clematis addisonii during anthesis. May, 1994. Site 1.

Day of anthesis	Mean total sucrose	Mean total sugars
1	.23 ± .03	.23 ± .03
2	.60 ± .08	.62 ± .11
3	1.06 ± .16	1.06 ± .16
6	.71 ± .22	.94 ± .21
8	.52 ± .31	.84 ± .41
10	.14 ± .07	.41 ± .08

Table 2.2. Mean total sucrose (mg) and total sugars (mg) with standard errors in nectar of Clematis addisonii during anthesis. August, September, 1944. Site 2.

Day of anthesis	Mean total sucrose	Mean total sugars
1	.99 ± .24	.99 ± .24
2	2.78 ± .66	2.78 ± .65
3	1.66 ± .48	1.83 ± .47
4	.44 ± .39	.64 ± .49
5	.44 ± .23	.66 ± .30
6	.63 ± .32	1.05 ± .50

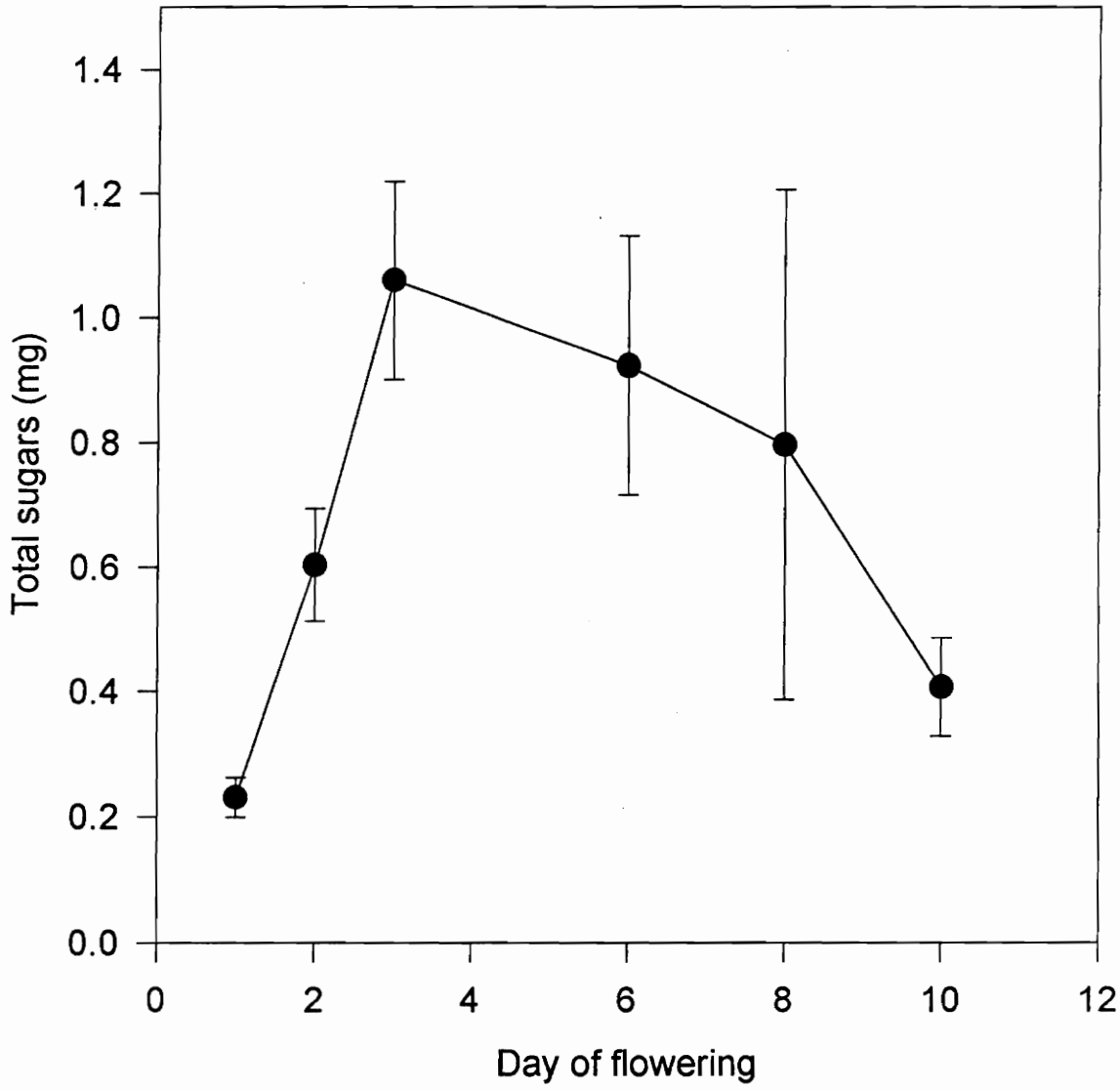


Figure 2.1a. Mean total sugar levels (mg), with standard error bars, in the nectar of Clematis addisonii 1, 2, 3, 6, 8, and 10 days into anthesis (differences among days not significant; $p > .05$, One Way Repeated Measures ANOVA). May, 1994. Site 1.

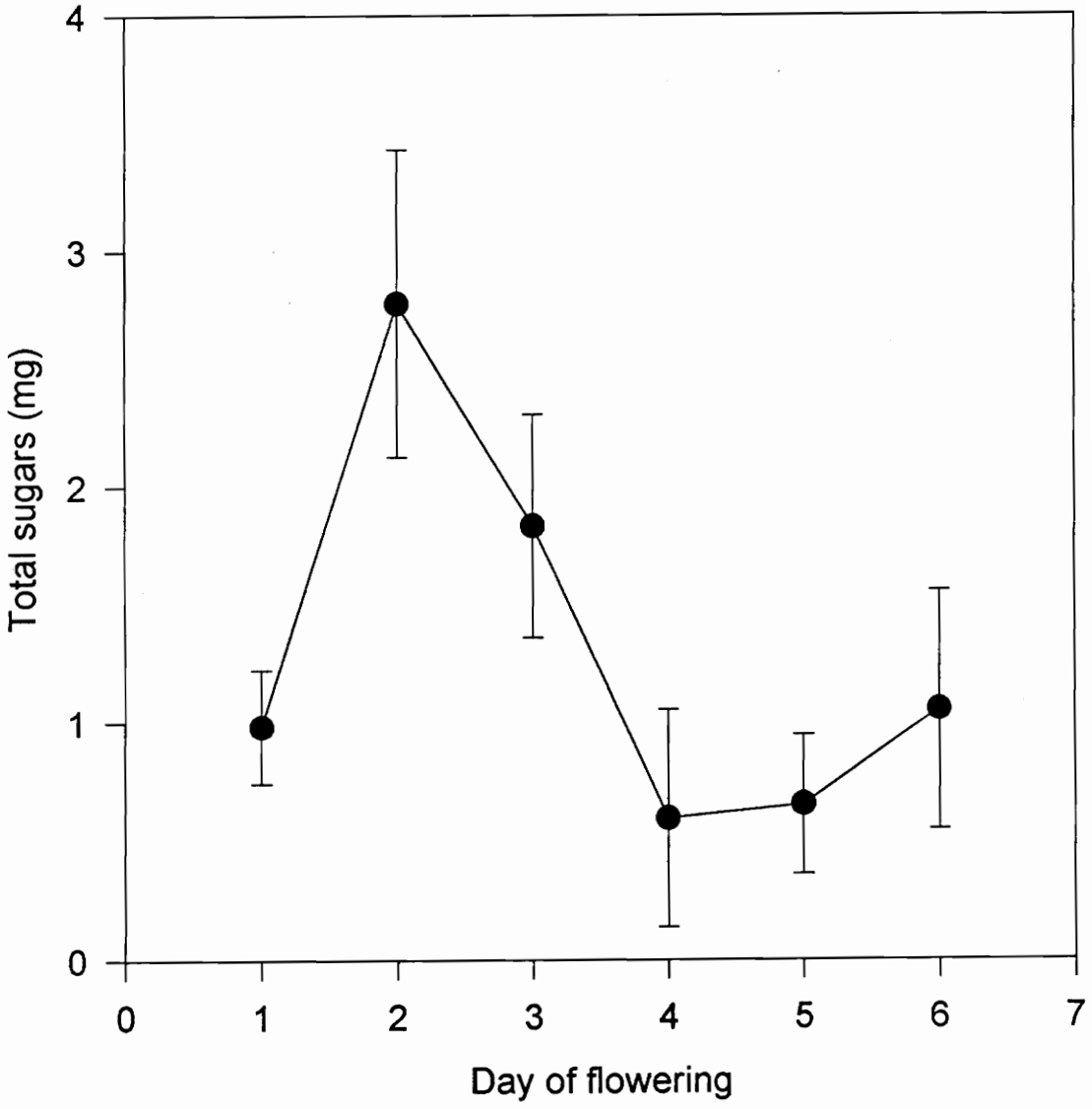


Figure 2.1b. Mean total sugar levels (mg), with standard error bars, in the nectar of Clematis addisonii 1, 2, 3, 4, 5, and 6 days into anthesis (differences among days not significant; $p > .05$, One Way Repeated Measures ANOVA). August/September, 1994. Site 2.

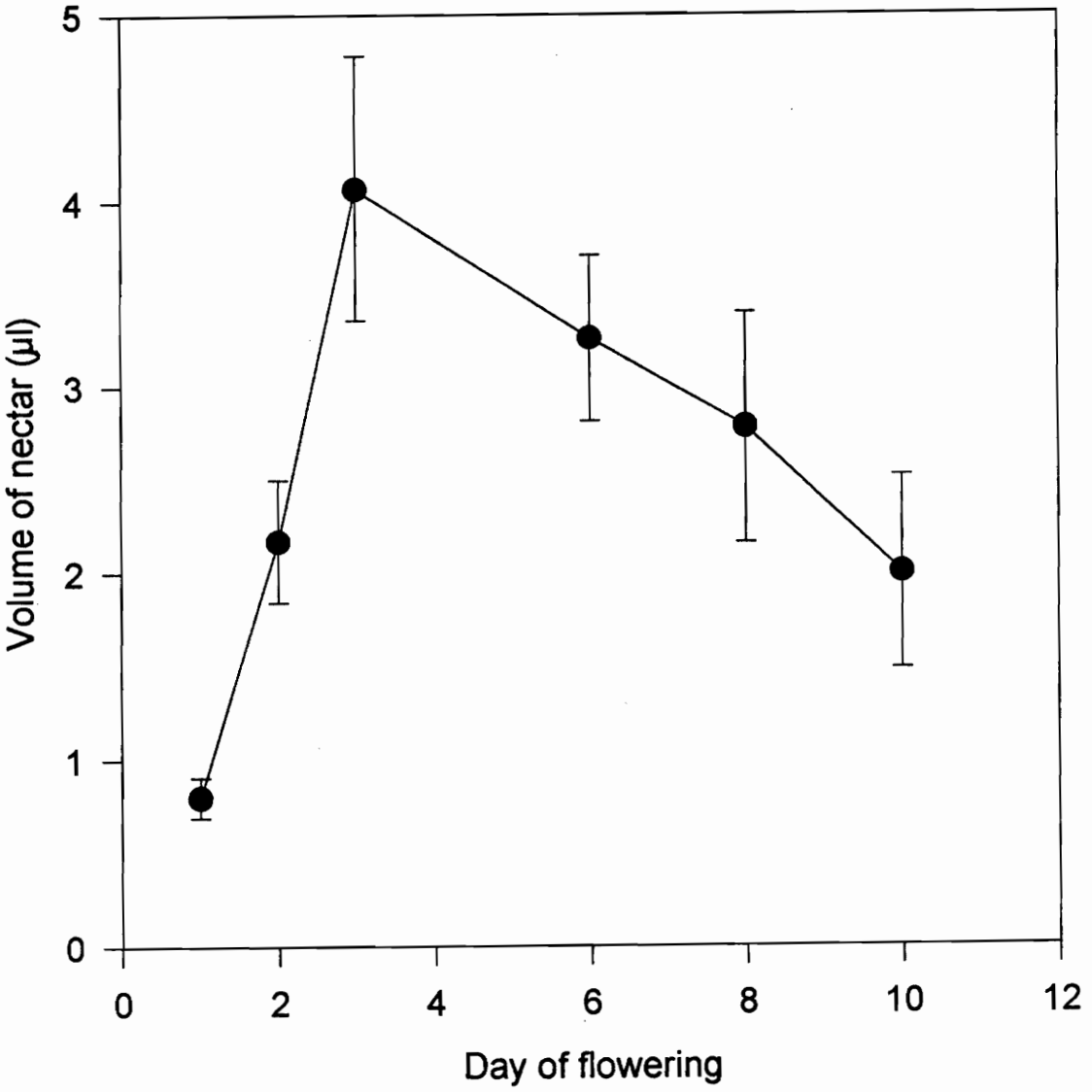


Figure 2.2a. Mean nectar volumes (µl), with standard error bars, secreted by Clematis addisonii flowers 1, 2, 3, 6, 8, and 10 days into anthesis (significant difference between day 1 and day 3; $p < .05$, One Way Repeated Measures ANOVA). May, 1994. Site 1.

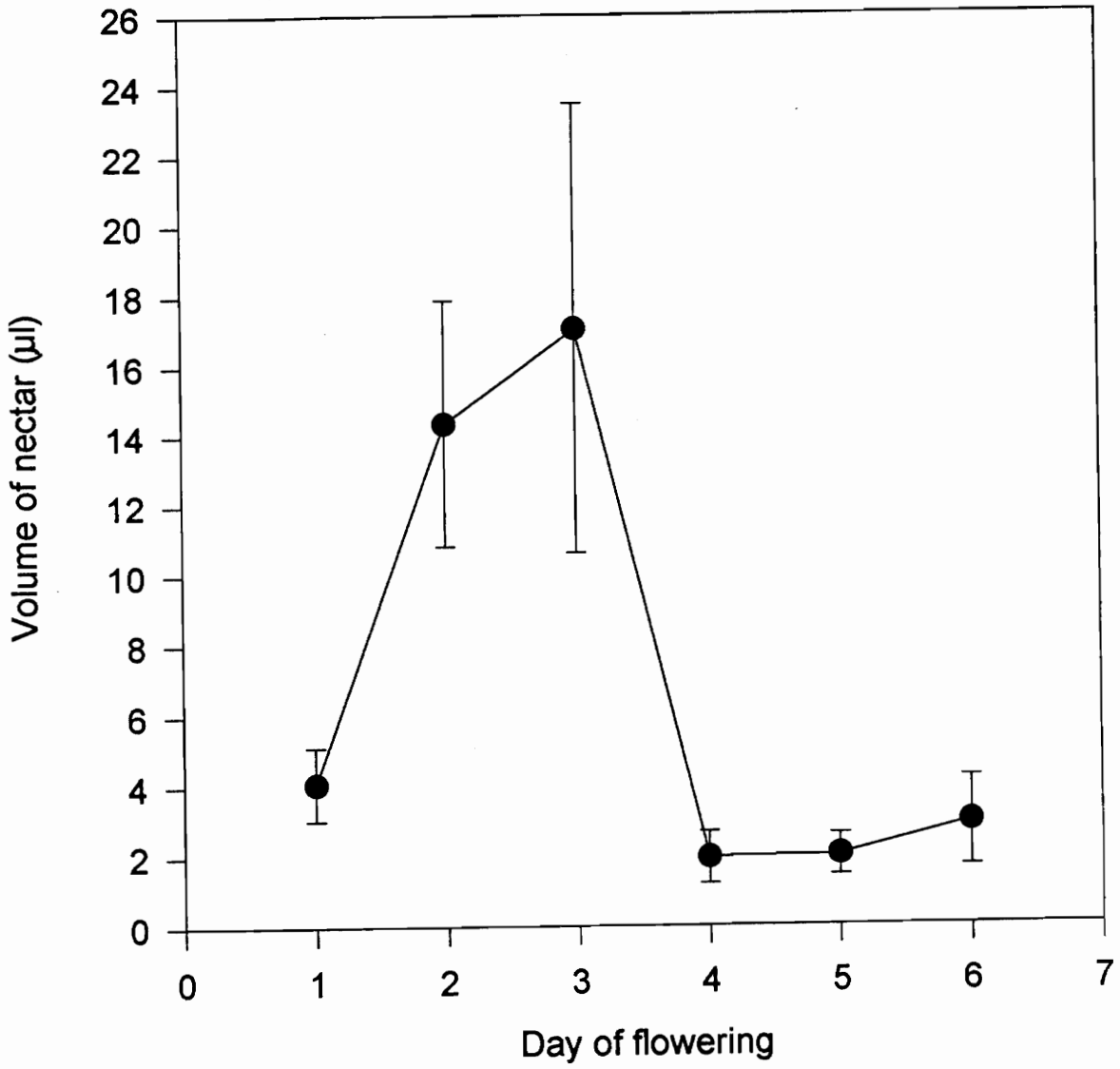


Figure 2.2b. Mean nectar volumes (µl), with standard error bars, secreted by Clematis addisonii flowers 1, 2, 3, 4, 5, and 6 days into anthesis (differences among days not significant; $p > .05$, One Way Repeated Measures ANOVA). August/September, 1994. Site 2.

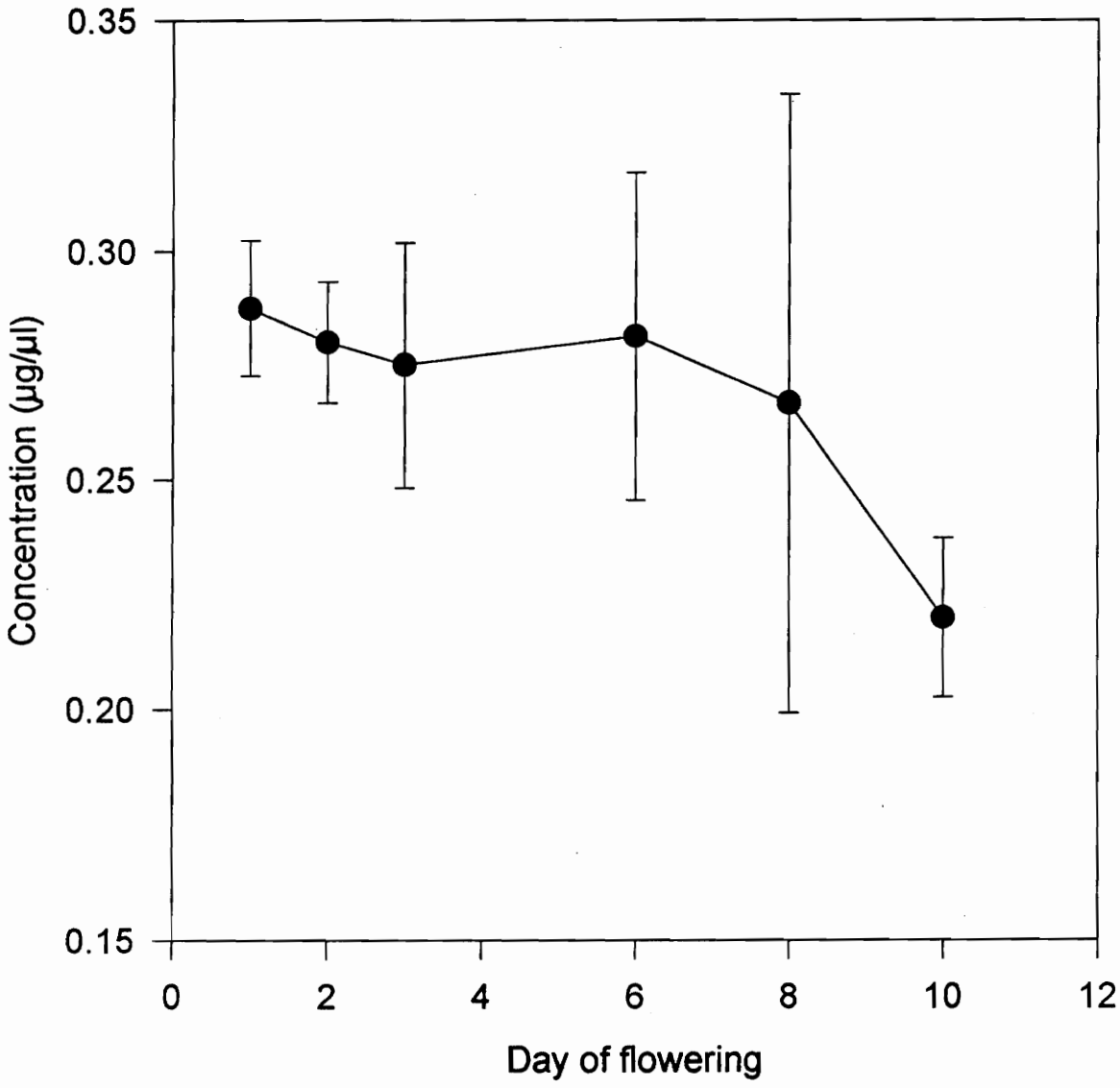


Figure 2.3a. Mean sugar concentrations (µg/µl), with standard error bars, of the nectar of Clematis addisonii 1, 2, 3, 6, 8, and 10 days into anthesis (differences among days not significant; $p > .05$, One Way Repeated Measures ANOVA). May, 1994. Site 1.

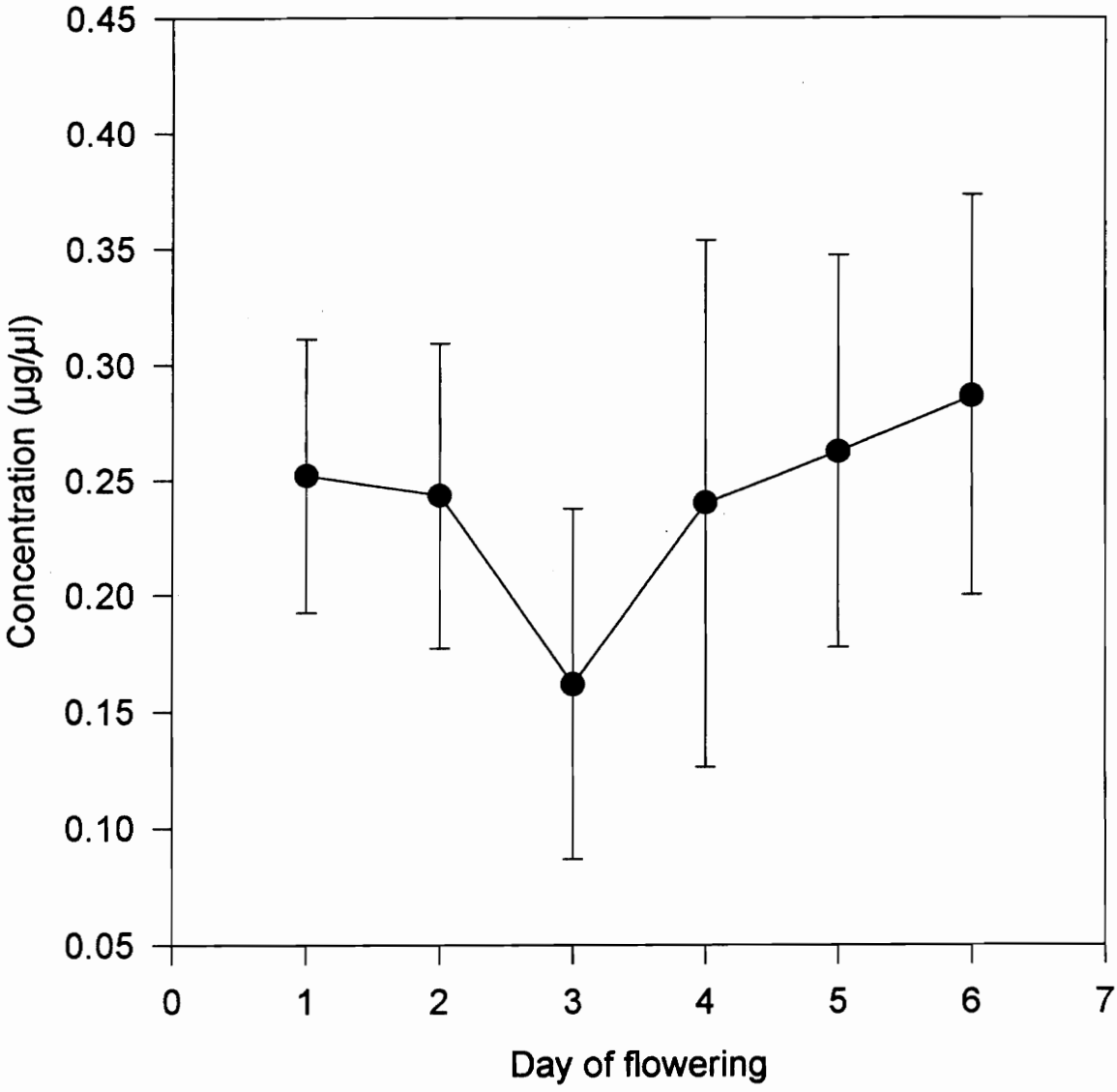


Figure 2.3b. Mean sugar concentrations (µg/µl), with standard error bars, of the nectar of Clematis addisonii 1, 2, 3, 4, 5, and 6 days into anthesis (differences among days not significant; p > .05, One Way Repeated Measures ANOVA). August/September, 1994. Site 2.

Chapter 3

The Breeding System of Clematis addisonii

Literature Review

The potential for genetic recombination in flowering plant populations is influenced by a number of factors. Of these factors, the breeding system is of primary importance. The breeding system is a dynamic property of a plant population, subject to modification by many variables, particularly those of genetical and ecological origin. By regulating the amount of recombination conferred to offspring (Grant 1958), the breeding system has a major impact on the genetic structure of populations and how they respond to selection pressures (Barrett and Eckert 1990).

Breeding systems are often regarded in terms of a dichotomy, with inbreeding on one branch and outbreeding on the other (Lande and Schemske 1985), or a trichotomy, with mixed breeding systems falling on a third branch (Grant 1958; Baker 1959; Cruden and Lyon 1989). Other workers, rather than interpreting breeding systems within the context of distinct classes, view them more as a continuum between inbreeding and outbreeding (Waser 1993). But it is the fitness consequences of inbreeding and outbreeding that ultimately determine the maintenance of breeding patterns or shifts in the breeding patterns of a population.

Darwin (1876) was the first to extensively document plant breeding systems and to emphasize the adaptive role of outcrossing. His descriptions of the various mechanisms that facilitate outcrossing and of the reduced fitness of inbred progeny prompted the long-standing view that selfing with its accompanying reduction in genetic variability is inherently disadvantagous. The fitness reduction of inbred progeny, termed inbreeding depression, is one of the most widely documented phenomena of plant evolutionary genetics (Darwin 1876; Schemske 1983; Schoen 1983; Dudash 1990; Holtsford and Ellstrand 1990). Whereas self-fertilization is usually cited in association with inbreeding depression, populational substructuring due to restricted seed and pollen dispersal potentially leads to biparental inbreeding. This type of inbreeding mimics self-fertilization in that it also reduces genetic variation by producing homozygous progeny (Waller 1993). The superior fitness of outbred progeny relative to inbred progeny has classically been attributed to dominance, the masking of deleterious recessive or partially recessive alleles under heterozygosity (Holsinger 1992; Mitton 1993), or over-dominance, the selective favoring of heterozygote individuals over homozygotes (Wright 1977). Both hypotheses are based on the prediction that inbred progeny suffer reduced fitness because of the expression of recessive lethals under homozygosity. Inbreeding depression is believed to be the only selective force strong enough to prevent shifts in the breeding system toward self-fertilization or to maintain outcrossing in self-compatible outbreeding populations (Charlesworth and Charlesworth 1987). The effects of inbreeding depression may vary through the life cycle, expressed in terms of reduced parental fecundity or progeny fitness (Schemske 1983; Schoen 1983; Schaal 1984; Karron 1989; Dudash 1990). Furthermore, the magnitude of its effects may

vary ecologically, being less pronounced under less stressful environmental or competitive conditions (Antonovics 1968; Schemske 1983; Schoen 1983).

It is estimated that approximately one-third of flowering plant species are predominantly self-fertilizing and inbreeding (Allard 1975). Inbreeding in plants, a prerequisite for which is self-compatibility, serves to lower recombination rates and increase homozygosity, and in doing so, can rapidly fix locally adapted genes or gene complexes within the population (Grant 1958; Mather 1973). Although inbreeders gain in terms of local adaptation and the ability to fix rare alleles that might otherwise be lost (Schoen 1982), they sacrifice the variability required to buffer them against changing selection pressures. Inbreeding depression has been observed in inbreeding populations (Barrett and Charlesworth 1991), but the frequency of deleterious alleles is expected to be lower there because homozygosity exposes these alleles to "purifying" selection (Lande and Schemske 1985, but see Karron 1989). Therefore, a history of inbreeding may potentially reduce the negative consequences of inbreeding. Although outbreeding populations are expected to show greater levels of inbreeding depression relative to inbreeders (Wright 1977), similarly low levels of inbreeding depression may be present there also due to historical mating events. Forced inbreeding during past population bottlenecks may have purged the population of deleterious recessives so that in subsequent generations, on return to oubreeding, the population exhibits lower than expected levels of inbreeding depression (Schemske 1983).

Outbreeding plant populations are expected to experience higher rates of recombination, with selection favoring gene combinations that confer fitness on a variety of backgrounds. These populations possess a genetic system capable of

maintaining recessive deleterious alleles in their gene pools because of the sheltering effects of heterozygosity. Therefore, outbreeding is promoted by characters that either prevent self-fertilization altogether or reduce the potential for it. For approximately one-half of flowering plant species, outbreeding is enforced by a self-rejection response that prevents seed set following self-pollination in an otherwise fertile plant (Brewbaker 1959). This phenomenon, referred to as self-incompatibility, is a physiological, genetically controlled mechanism operating at various sites within the pistil which descriminates against self-pollen or self-pollen tubes (de Nettancourt 1977). Although stigmatic and stylar inhibition represent the classic examples of self-incompatibility, ovarian and ovular inhibition or late acting self-incompatibility systems (Seavey and Bawa 1986) are less frequently described (de Nettancourt 1977, but see Seavey and Bawa 1986). While prezygotic ovarian or ovular responses are more easily attributed to self-rejection responses, reactions occurring post-zygotically are not so easily classified. Because of the continuum of response sites, abortion of embryos due to recessive lethals may mimic a late acting self-rejection response, making distinction between the two problematic (Uyenoyama 1993). Although it has been argued that selection should operate so that rejection responses occur at earlier phases to preserve ovules (Lewis 1979), others view delayed reactions as devices for quality control, whereby maternal tissues are allowed a longer period of time to access the genotypes of male gametophytes and their compatibility with the maternal genome (Seavey and Bawa 1986).

Self-incompatibility has been described as the principle device used by flowering plants to control for levels of heterozygosity (and homozygosity) (Arroyo and Squeo 1990) and to avoid the potential problems associated with self-

fertilization and inbreeding depression (Heslop-Harrison 1983a,b). These systems may not be absolute, however, and may vary in their capacity to prevent self seed-set altogether. Larsen (1978) describes incompatibility systems as being dynamic and able to vary along the continuum from self-fertility to self-incompatibility, the strength of the response correlated with the degree of homozygosity present. The basis for the heterosis model proposed by Mulcahy and Mulcahy (1983) follows the same logic. This model assumes that if a style is heterozygous for a deleterious recessive allele and a pollen grain carries that same allele, the tube growth of that pollen grain will be reduced compared to those without the recessive allele. Pollen tube growth rates would then be a function of the heterotic interaction between the style and pollen tube.

Not all outbreeders possess well-defined self-incompatibility systems, however. Despite their capacity for self-fertilization, self-compatible outbreeders typically employ one or several morphological or developmental strategies to promote cross-pollination and fertilization. Dichogamy, one of these devices, was discussed at length in Chapter 1. A physiological mechanism has also been described whereby a weak self-rejection response, called cryptic self-incompatiblity, disfavors self-fertilization in an otherwise self-compatible plant, when self-pollen occurs in combination with cross-pollen on the same stigma (Bateman 1956). The reduced success of self-pollen in plants having such a system results from competitive exclusion rather than from actual self-incompatibility (Clarkia unquiculata, Bowman 1987). Theoretically, this type of response would enforce outcrossing when cross-pollen is available but allow selfing in its absence (Bowman 1987).

Whereas the breeding systems of many plant species

involve a combination of selfing and outcrossing to varying degrees (Jain 1976), occasional selfing in outbreeding populations may be seen as a by-product of bisexuality and self-compatibility (Lande and Schemske 1985). Selection for outcrossing in these populations will be maintained, however, via the selective abortion of selfed seeds or the viability reduction of self-progeny.

The selective pressure to generate optimum levels of genetic recombination has long been viewed as the principle driving force behind the evolution of plant breeding systems. A reduction in fitness following inbreeding is an almost universal response (except in habitual selfers), with a recovery from inbreeding depression expected to result as a response to subsequent outcrossing. However, empirical studies indicate that outcrossing does not always enhance fitness and that for some plant species, an optimal level of inbreeding or outbreeding may exist (Price and Waser 1979; Waser and Price 1991). A reduction in fitness following far-distance outcrossing, referred to as outbreeding depression, is believed to be attributed to the disruption of locally adapted gene complexes that function well together, with the maternal plant exerting an active inhibition against those genotypes that are less related to or less compatible with the maternal genome (Shields 1982; Waser and Price 1991). While it does appear that for some species there is an optimal level of outbreeding beyond which negative gene interaction causes reduced fitness, for others increased interparent distances do not have negative results (Chamaecrista fasciculata, Fenster and Sork 1988; Sabatia angularis, Dudash 1990).

Clematis addisonii Britton (Ranunculaceae) is a geographically restricted species that occurs in approximately twenty scattered populations over a four county region in western

Virginia (Harvill, et al. 1992) (see Figure 3.1). Populations range in size from nine to one-thousand individuals (Van Alstine 1993). The breeding systems of geographically restricted species, particularly those having small, isolated populations are of interest because of the rapid evolutionary changes that often take place in such populations (Kruckeberg and Rabinowitz 1985; Karron 1989). It has been argued that such species are likely to exhibit reduced levels of inbreeding depression as a result of repeated population bottlenecks or pollinator failure and have, as such, undergone selection favoring self-compatibility (Baker 1955; Clegg and Brown 1983; Karron 1987).

Studies of various members of the Ranunculaceae have shown that this family includes gametophytic self-incompatibility systems (Anemone canadensis, Douglas and Cruden 1994) and autogamous breeding systems (Hepatica americana, Motten 1982), as well as self-compatible members with outbreeding systems (Delphinium nelsonii, Waser et al. 1987; Aquilegia caerulea, Montalvo 1992). In a study of Clematis subsection Integrifoliae, Keener (1967) describes the group as facultative inbreeders, their breeding system being one of "cyclic autogamy" consisting primarily of inbreeding, with occasional outcrossing. No reference to the breeding systems of members of subsection Viornae, which includes C. addisonii, has been found in the literature. Because of the paucity of information regarding the reproductive biology of C. addisonii, the objective of this study was to obtain information about the breeding system of Clematis addisonii through a series of flower manipulations and controlled hand-pollinations. The purposes of this study were to determine 1) the compatibility status of this species by comparisons of seed set following self- and cross-pollinations; 2) the ability of this species to self-pollinate in the absence of

pollinators; 3) the ability of this species to produce seed via apomixis; 4) if interparent distance, i.e. pollinations between populations, has an effect on fecundity; and 5) if seed set in populations is pollinator or pollen limited.

Methods

1993 Field Season

In 1993, a field site was selected in northern Montgomery county supporting a naturally occurring Clematis addisonii population that contained sufficient numbers of flowering individuals to use for pollination treatments. This site, hereafter referred to as Site 1, consists of a west- to southwest-facing slope supporting a stand of mixed hardwoods and conifers. This population consists of approximately 200 individuals growing beneath a relatively open canopy. Approximately 40 more occur in the opening created by the powerline right-of-way that runs along the northern section of the site.

Sixty plants were selected to use in a pilot study to obtain preliminary information on the reproductive biology of Clematis addisonii. Because these plants were all single-flowered, they were each assigned only one treatment. The treatments were as follows: 1) emasculated; 2) emasculated/bagged; 3) no manipulation/bagged; 4) hand self-pollinated/bagged; 5) hand intra-population cross-pollinated/emasculated/bagged; and 6) hand inter-population cross-pollinated/emasculated/bagged. Ten individuals were tagged and left unmanipulated to serve as controls. Bags were constructed of mosquito netting and measured approximately 10 cm X 12 cm. They were secured at the open end with nylon cording.

The flowers of all plants involved in bagging treatments were bagged while in bud stage. Emasculations were performed

prior to anther dehiscence and carried out by removing the stamens with forceps.

Pollen used for intra-population crosses was taken from freshly dehiscing anthers of at least two donor plants growing a minimum of approximately 10 meters from the recipient plant. Pollinations were conducted once per flower and were accomplished by rubbing collected anthers over the stigmatic surfaces. Inter-population crosses were conducted in the same manner, but pollen was taken from donors growing in a population several kilometers from the study site. Pollen was transported as quickly as possible between sites to reduce potential for viability loss.

Self-pollinations were also conducted once per flower in the early portion of the staminate phase of the flower. These were accomplished by brushing self-pollen over the stigmatic area with a small paint-brush. A different brush was used for each flower.

Following hand-pollinations, bags were replaced and left until early signs of fruit development. Bags were then removed so as not to interfere with normal fruit development. Bag replacement was necessitated roughly 3 weeks later when severe predation by the nymphal stage of the Hemipteran Euschistus tristigmus was observed on the majority of fruits. Bags remained over the fruits until ripening. Ripened fruits were collected in September for counting. Because aborted or unfertilized ovules had dropped from the plants during the unbagged interval, percent seed set for these treatments could not be calculated. All seeds that were fully developed, regardless of insect damage, were scored as viable.

1994 Field Season

During May of 1994, a field study similar to the 1993 study was performed using Site 1 and a second site (Site 2) located

approximately .5 kilometer from Site 1. Site 2, also on a southwest facing slope, has experienced relatively recent disturbance as indicated by an old home-site at the southern section of the slope. Site 2 supports a smaller population of approximately 60 flowering individuals growing under a dense canopy of Ailanthus altissima (Miller) Swingle.

Utilizing the information obtained from the previous year's field study, the number of treatments was reduced to three: 1) hand self-pollinated/bagged; 2) hand intra-population cross-pollinated/emasculated/bagged; 3) hand inter-population cross-pollinated/emasculated/bagged. Because of substantial differences in population sizes between the two sites, replicate numbers were smaller for Site 2.

Sixty plants at Site 1 and forty plants at Site 2 were each assigned one pollination treatment or not manipulated and used as a control. The flowers of all treatment plants were bagged prior to anthesis. Emasculations for hand cross-pollination treatments were performed prior to anther dehiscence. Pollinations were conducted once per plant. Pollen for hand crosses was taken from freshly deh isced anthers, with the exception of one occasion which was noted accordingly. Pollen for intra-population crosses was taken from two plants growing at least 10 meters from the recipient plant at Site 1 and 5 meters from the recipient plant at Site 2. Pollen for inter-population crosses was taken from two donor plants and transported as quickly as possible between the two sites. To determine if flower age at the time of pollination influenced seed set, the day of anthesis when hand pollinations were made was recorded for each flower.

Bags remained on the treatment plants for the duration of the study to prevent fruit predation. Fruits of control plants were bagged at the first sign of fruit development. In September, all fruits and undeveloped ovaries were collected,

counted, and percent fruit set calculated. All fruits that were filled and fully developed were scored as viable. Undeveloped ovules were not differentiated as unfertilized or aborted. Because each ovary contains only one ovule, fruit set and seed set are equal entities. In the following discussion, data will be stated in terms of percent seed set, although actual counts were made of fruits.

Percent seed set among the treatment and control plants was statistically compared using One Way ANOVA.

Results 1993

Percent seed set could not be calculated in 1993 because insect damaged ovaries had senesced and dropped from plants during the unbagged interval. Because ovary number is so variable among individual plants (from 3 to 20 in the 1994 study), comparisons of seed numbers rather than percent seed set can lead be misleading. Since percent seed set could not be calculated in 1993 as a result of senesced ovaries, numbers of seed producing individuals per treatment were examined and then utilized as a basis for designing the 1994 field study. No statistical comparison of the results from the 1993 study was conducted.

Seeds were produced in all groups, although the open-pollinated and hand-pollinated treatment groups had greater numbers of seed producers than those not hand-pollinated. The highest number of seed producers was found in the emasculated open-pollinated, intra-populational hand-crossed, and control plants. Nine of the ten plants in each of these groups produced seeds. Seven of the ten hand-selfed plants produced seeds and five of the ten inter-populational crosses produced seeds.

The emasculated/bagged and bagged/unmanipulated treatments resulted in the lowest numbers, with 3 of the 10

plants in each group producing seeds.

Results 1994

Because undeveloped ovaries/ovules were not examined microscopically to ascertain the reasons behind their failure to develop, it is uncertain whether they represent aborted embryos or unfertilized ovules. It is possible that a percentage of these were actually unfertilized ovules. A likely explanation for the 0% seed set following intra-populational crossing in one of the plants is that inviable pollen was used for the pollination. Difficulty in finding flowers with freshly dehiscing anthers on that date necessitated the use of pollen from older flowers. Reduced seed set in three plants may be attributable to the short style lengths of those pistils failing to produce fruit. It is possible that the stigmas of these pistils were not contacted during hand-pollinations and failure to set seed resulted from lack of pollination rather than as a response to the pollination treatment itself.

Prior to statistical analysis of treatment effects on seed yield, a regression was performed for each treatment at both sites to determine if the day of pollination exerted any influence on seed set. No relationship was found between the two parameters at either site for any of the three treatments.

Percent seed set between treatment and control plants was statistically indistinguishable (see Appendix III for individual seed yield data). Statistical comparisons did not show significant differences between treatment and control plant groups at Site 1 or Site 2 ($p > .05$, One Way ANOVA). A significant difference was found, however, in overall seed yield between sites ($p < .05$, One Way ANOVA). The significantly lower seed production at Site 2 may have resulted from the lower light regimes there which may not

allow plants to accumulate sufficient resources to support developing embryos (see May 1963). The lower seed yield of control plants at this site may also reflect reduced levels of pollinator activity under shadier conditions.

Discussion

Although the small sample sizes for both years offer limited statistical power for a conclusive assessment of the breeding system of Clematis addisonii, the 1993 and 1994 field studies revealed some important aspects of the reproductive biology of C. addisonii.

The number of individuals producing seeds following the emasculation treatment demonstrates that seed set is not limited by pollinator activity or availability of cross-pollen. Flowers are highly attractive to insects because of the abundant nectar they secrete from the first day of anthesis (see Chapter 2). Seed set, therefore, is not likely to be restricted by lack of insect activity. The first individuals to initiate anthesis may experience a degree of pollen limitation, however, as a result of protogyny. Because the sex ratio in protogynous populations is expected to be female biased early in the season, pollen may be of limited availability then since sufficient time may not have elapsed for flowers to progress into the staminate phase. Given the amount of time flowers remain functionally pistillate (approximately 8 days; see Chapter 1), it is likely that the earliest flowering individuals will receive pollen during the time interval that stigmas are receptive.

The low number of seed producing individuals from the emasculated, bagged treatment indicates that Clematis addisonii is not apomictic. The seed production in three of the individuals may be the result of pollen contamination because of incomplete emasculations rather than apomixis. No

reference to apomixis in Clematis has been found in the literature.

The equally low number of seed producers from the bagged, unmanipulated treatment indicates that Clematis addisonii has limited capacity for self-pollination in the absence of insects. Plants, therefore, depend on the activity of insects to facilitate pollination, which is achieved primarily by bumblebees and, to a lesser extent, butterflies. Observations of individuals not included in the study indicate that flowers are also frequented by ants which are attracted to the abundant nectar found inside the flower. It is possible that during the course of their nectar thievery, the movement of ants over the flower may effect self-pollination. Since the bodies of ants are unfavorable for the actual transport of pollen (Faegri and van der Pijl 1979), it is unlikely that they have the capacity to effect cross-pollination between plants. However, for isolated plants occurring at the margins of populations where visitation by primary pollinators may be infrequent, ant visitation may serve as a mechanism to facilitate self-pollination, in the absence of visitation by "legitimate" pollinators.

The results from both 1993 (although less conclusive) and 1994 indicate that inter-parent distance has no significant effect on parental fecundity. Although Price and Waser (1979) and Waser and Price (1983, 1991) have found a correlation between interparent distance and reduced fitness in Delphinium nelsonii and Ipomopsis aggregata, no correlation has been found in certain other taxa (Chamaecrista fasciculata, Fenster and Sork 1988; Sabatia angularis, Dudash 1990, Lobelia cardinalis, Schlicting and Devlin 1992). Without knowledge of pollinator flight distances, it is difficult to predict whether pollen transfer between populations of Clematis addisonii is even possible in nature.

Results from both seasons indicate that Clematis addisonii is self-compatible, with some variability in response among individuals following self-pollinations. Although regressions did not demonstrate a relationship between day of pollination and seed set, the low seed yield of some individuals may be the result of diminished stigma receptivity at the time of self-pollinations. Four individuals from Site 1 having 0% seed set following selfing were pollinated when flowers were 9 or 10 days into anthesis. Likewise, two individuals from Site 2 that failed to produce seed following selfing were pollinated when flowers were 9 days into anthesis. However, some individuals self-pollinated 7 and 4 days into anthesis also failed to produce seeds at Site 2, indicating that factors, in addition to inbreeding depression may be influencing seed set there.

If genetic load is responsible for reduced seed set, then plants will, indeed, vary in their ability to produce seed following self-pollination (Seavey and Bawa 1986). Seed production in individuals harboring few detrimental recessives should approach normal levels whereas those harboring many will suffer reduced fecundity or produce low viability offspring following selfing. Although inbreeding depression is usually found to be most pronounced during seed development (Levin 1984, Schlicting and Devlin 1992), absence of significant fecundity reductions following selfing in Clematis addisonii may indicate that inbreeding depression is not present because past inbreeding or frequent bi-parental inbreeding has reduced the genetic load or because the effects of inbreeding depression are too small to detect early in the life cycle (see Schoen 1983; Karron 1989). Inbreeding depression is sometimes not detectable until later in the life cycle when the performance of selfed-progeny proves to be inferior to that of outcross-progeny, particularly under

competitive conditions or environmental harshness and variability (Schoen 1983). A full assessment of inbreeding depression should, therefore, be based on several life-history stages from zygote to adult.

While the controlled self-pollinations of this study do demonstrate self-compatibility in Clematis addisonii they fail to reveal the frequency of successful self-pollinations that may actually occur in nature. That C. addisonii is protogynous and produces nectar throughout much of anthesis suggests that it is adapted for outcrossing. But because stigmas remain receptive into the staminate phase, self-fertilization is a possibility for those stigmas which were not cross-pollinated during the morphological pistillate phase. A cryptic self-incompatibility system may be in operation, however, so that self-fertilization is prevented if self- and cross-pollen occur in combination on the same stigma (see Bateman 1956; Bowman 1987). This would enforce cross-fertilization when cross-pollen is available, yet allow for selfing in its abscence.

Interestingly, the majority of plants in both study populations produced only a single flower over the entire season, precluding the occurrence of geitonogamy in these plants. Multiple-flowered individuals do occur, however, in the more open areas along road and powerline cuts, indicating that the single flowered habit may be a response to lower light regimes or to different light quality. Whether geitonogamous pollinations occur in multiple-flowered individuals is unclear because the degree of overlap in anthesis of the flowers on a single plant is not known.

Although this study has demonstrated that Clematis addisonii is self-compatible, it does not indicate the levels of recombination that occur under natural conditions. Outcrossing in this species will be influenced by a number of

factors including plant density and the foraging behavior of pollinators, the availability of outcross pollen during female biased portions of the flowering season, and variation in the degree of protogyny among individuals. Occasional self-fertilization in C. addisonii may confer the advantages of increased probability and efficiency of seed set and provide for adaptation to local conditions (Jain 1976). However, any fecundity advantage that occasional selfing may have will depend on the magnitude of reduced fitness of selfed progeny.

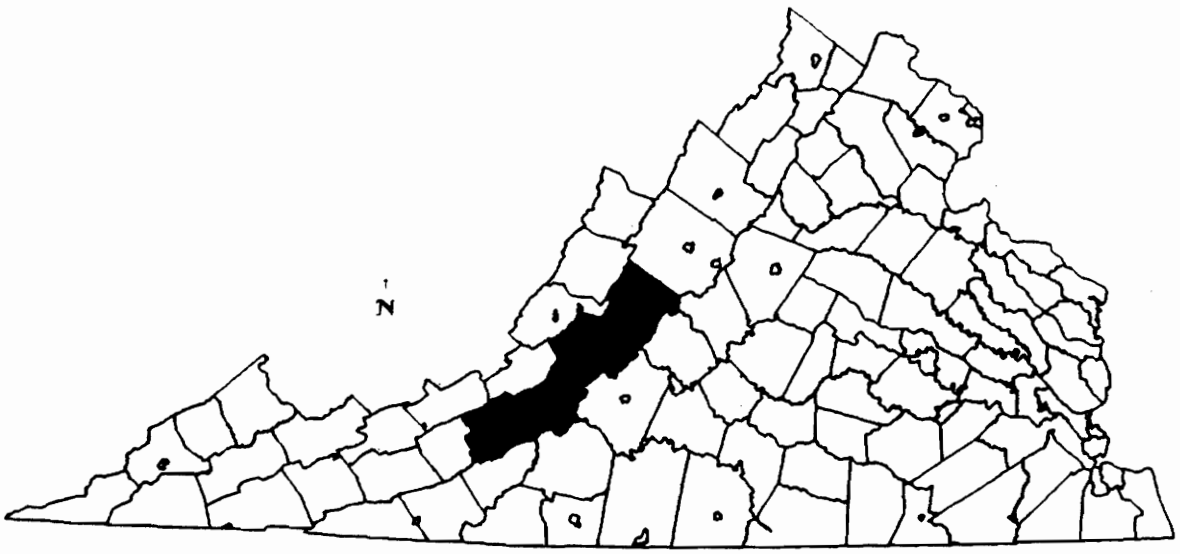


Figure 3.1. Geographical distribution of the Virginia endemic Clematis addisonii. Highlighted areas represent counties in which C. addisonii populations have been reported; Montgomery, Roanoke, Botetourt, and Rockbridge.

Conclusions

This investigation has revealed several important aspects of the reproductive biology of Clematis addisonii. First, hand-pollinations indicate that the plants are self-compatible, showing no statistically significant reduction in fecundity following self-pollination.

The ability of Clematis addisonii to produce seed following self-pollinations may not be indicative of the level of selfing that occurs under natural conditions. Field observations indicate that this species is intraflorally protogynous, with the pistillate phase lasting a sufficient amount of time for some level of outcrossing to occur prior to the shedding of self-pollen in the flower. In vivo pollen tube growth supports the morphological observations. But because stigma receptivity extends into the staminate phase of anthesis, as indicated by self-seed production as well as pollen tube growth in eight day old pistils, there is the potential for self-fertilization in pistils not receiving cross-pollen during the morphological pistillate phase.

Given that the majority of plants in both study sites are single-flowered and geitonogamous self-pollination is, therefore, precluded, protogyny functions as a highly effective outcrossing mechanism in these plants. It is not known if protogyny effectively promotes outcrossing by

preventing geitonogamous pollinations in multiple-flowered plants because they were not included in this study.

The low seed yield of flowers that were bagged and not manipulated suggests that flowers require visitation by insects for cross-pollination as well as self-pollination. The high seed yields of both control and emasculated plants indicate that seed set is not pollinator limited and that the flowers are highly attractive to pollinators. This study demonstrates that nectar secreted by Clematis addisonii flowers encourages visitation by insects. The nectar of C. addisonii is sucrose rich, a nectar type associated with flowers that are pollinated by long-tongued bees and butterflies. Field observations indicate that bumblebees are the primary pollinators, although butterflies are also frequently seen foraging on them.

Nectar is produced throughout the pistillate and staminate phases of anthesis, its secretion from the onset of anthesis increasing the probability of insect visitation to pistillate phase flowers and, thus, cross-pollination prior to the staminate phase. Secretion of nectar throughout the staminate phase continues to encourage insect visitation for pollen removal and dispersal.

In conclusion, Clematis addisonii is a self-compatible hermaphrodite that is highly adapted for outcrossing by virtue of protogyny and the production of nectar from the onset of anthesis.

Appendix I

Duration of sexual phases and anthesis

and

Pollen tube data by individual pistil

Table 1.1a. Total number of days in pistillate and staminate phase for each plant (n=15) and number of days to senescence (n=10). May 1994.

Plant number	Number of days pistillate	Number of days staminate	Number of days to senescence
1	7	5	15
2	5	5	11
3	6	3	12
4	5	4	
5	5	5	
6	6	4	13
7	5	4	
8	5	3	
9	6	5	13
10	4	4	13
11	6	3	
12	6	4	18
13	5	4	14
14	6	4	14
15	6	4	19

Table 1.2a. Approximate number of pollen grains and pollen tubes and mean tube lengths (μm) for each age class (days); one hour after pollination.

Day of anthesis	Hours after pollination	Number pollen grains	Number pollen tubes	Mean tube length
1	1	44	44	80.5
1	1	18	8	73.1
1	1	57	45	76.4
2	1	16	16	23.7
2	1	1	1	10
2	1	20	18	73.1
2	1	65	65	161.6
3	1	80	77	51.3
3	1	10	10	154.4
4	1	17	17	30
4	1	13	7	84.3
5	1	0	0	0
5	1	10	4	14.7
5	1	45	27	33.5
5	1	20	3	18.3
8	1	25	20	33

Table 1.2b. Approximate number of pollen grains and pollen tubes and mean tube lengths (μm) for each age class (days); 3 hours after pollination.

Day of anthesis	Hours after pollination	Number pollen grains	Number pollen tubes	Mean tube length
1	3	80	80	3000
1	3	40	40	1800
1	3	135	135	2050
2	3	100+	100+	2000
2	3	40	40	900
2	3	50	50	1050
2	3	60	50	135
3	3	100+	100+	1100
4	3	40	40	150
4	3	27	27	600
5	3	25	25	1000
8	3	29	16	40

Table 1.2c. Approximate number of pollen grains and pollen tubes and mean tube lengths (μm) for each age class (days); six hours after pollination.

Day of anthesis	Hours after pollination	Number pollen grains	Number pollen tubes	Mean tube length
2	6	50	40	113
2	6	85	85	2300
2	6	40	35	1150
5	6	0	0	0
5	6	23	23	114
8	6	12	12	114

Table 1.2d. Approximate number of pollen grains and pollen tubes and mean tube lengths (µm) for each age class (days); ten hours after pollination.

Day of anthesis	Hours after pollination	Number pollen grains	Number pollen tubes	Mean tube lengths
2	10	135+	135+	4900
2	10	50	50	2100
2	10	35	35	3100
5	10	30	20	306
5	10	24	24	900
5	10	75+	75+	1700
8	10	27	18	51

Table 1.2e. Approximate number of pollen grains and pollen tubes and mean tube lengths (μm) for each age class (days): fourteen hours after pollination.

Day of anthesis	Hours after pollination	Number pollen grains	Number pollen tubes	Mean tube length
2	14	50+	50+	4900
2	14	30	26	3600
5	14	23	21	1350
8	14	15	10	63
8	14	8	8	75

Table 1.2f. Approximate number of pollen grains and pollen tubes and mean tube lengths (µm) for each age class (days); eighteen and twenty four hours after pollination.

Day of anthesis	Hours after pollination	Number pollen grains	Number pollen tubes	Mean tube lengths
2	18	70	55	7600
2	18	66	62	1200
5	18	150+	150+	4400
5	18	75+	75+	6300
5	24	150+	150+	4600
5	24	50	50	6250
5	24	24	10	3400
8	24	16	10	64

Appendix II

Nectar data by individual plant

Table 2.1a. Nectar volumes (µl), nectar concentrations (µg/µl), total sucrose, glucose, fructose (mg) and total sugars (mg) in the nectar of Clematis addisonii 1, 2, 6, 8, and 10 days into anthesis. May, 1994. Site 1.

Day of anthesis	Volume collected	Concentration	Total sucrose	Total glucose	Total fructose	Total sugars
1	.6	.25	.15	0	0	.15
2	3.7	.28	.99	0	.04	1.03
6	2.8	.33	.70	0	.17	.87
8	2.2	.16	.12	0	.13	.25
10	3.0	.19	.05	0	.52	.57

Table 2.1b. Nectar volumes (µl), nectar concentrations (µg/µl), total sucrose, glucose, fructose (mg) and total sugars (mg) in the nectar of Clematis addisonii 1, 2, 3, 6, and 8 days into antheis. May, 1994. Site 1.

Day of anthesis	Volume collected	Concentration	Total sucrose	Total glucose	Total fructose	Total sugars
1	1.1	.28	.30	0	0	.30
2	2.4	.23	.55	0	0	.55
3	5.6	.17	.96	0	0	.96
6	4.5	.23	.39	0	.67	1.06
8	3.2	.08	0	0	.26	.26

Table 2.1c. Nectar volumes (µl), nectar concentrations (µg/µl), total sucrose, glucose, fructose (mg) and total sugars (mg) in the nectar of Clematis addisonii 2, 3, 6, 8, 10, and 11 days into anthesis. May, 1994. Site 1.

Day of anthesis	Volume collected	Concentration	Total sucrose	Total glucose	Total fructose	Total sugars
2	1.9	.32	.62	0	0	.62
3	3.0	.34	1.03	0	0	1.03
6	3.5	.16	.58	0	0	.58
8	3.2	.18	.42	0	.14	.56
10	1.5	.21	.06	0	.25	.31
11	1.5	.12	0	0	.19	.19

Table 2.1d. Nectar volumes (µl), nectar concentrations (µg/µl), total sucrose, glucose, fructose (mg) and total sugars (mg) in the nectar of Clematis addisonii 3, 6, 8, and 10 days into anthesis. May, 1994. Site 1.

Day of anthesis	Volume collected	Concentration	Total sucrose	Total glucose	Total fructose	Total sugars
3	1.8	.33	.60	0	0	.60
6	1.0	.29	.29	0	0	.29
8	1.5	.26	.32	.07	0	.39
10	1.0	.25	.22	0	.04	.26

Table 2.1e. Nectar volumes (µl), nectar concentrations (µg/µl), total sucrose, glucose, fructose (mg) and total sugars (mg) in the nectar of Clematis addisonii 1, 2, 3, and 6 days into anthesis. May, 1994. Site 1.

Day of anthesis	Volume collected	Concentration	Total sucrose	Total glucose	Total fructose	Total sugars
1	.75	.32	.24	0	0	.24
2	1.8	.26	.47	0	0	.47
3	6.0	.23	1.40	0	0	1.40
6	3.2	.30	.54	0	.69	1.23

Table 2.1f. Nectar volumes (µl), nectar concentrations (µg/µl), total sucrose, glucose, fructose (mg) and total sugars (mg) in the nectar of Clematis addisonii 2, 3, 6, 8, 10, and 11 days into anthesis. May, 1994. Site 1.

Day of anthesis	Volume collected	Concentration	Total sucrose	Total glucose	Total fructose	Total sugars
2	1.4	.31	.44	0	0	.44
3	5.2	.31	1.62	0	0	1.62
6	4.5	.45	2.03	0	0	2.03
8	5.4	.52	2.05	0	.78	2.83
10	3.5	.18	.37	0	.25	.62
11	1.4	.06	0	0	.10	.10

Table 2.1g. Nectar volumes (µl), nectar concentrations (µg/µl), total sucrose, glucose, fructose (mg) and total sugars (mg) in the nectar of Clematis addisonii 1, 2, 3, 6, 8, and 10 days into anthesis. May, 1994. Site 1.

Day of anthesis	Volume collected	Concentration	Total sucrose	total glucose	Total fructose	Total sugars
1	.75	.30	.22	0	0	.22
2	1.8	.28	.51	0	0	.51
3	2.8	.27	.75	0	0	.75
6	3.3	.21	.46	0	.24	.70
8	1.2	.40	.20	0	.28	.48
10	1.0	.27	0	0	.27	.27

Table 2.2a. Nectar volumes (µl), nectar concentrations (µg/µl), total sucrose, glucose, fructose (mg) and total sugars (mg) in the nectar of Clematis addisonii 1, 2, 3, 5, and 6 days into anthesis. August and September, 1994. Site 2.

Day of anthesis	Volume collected	Concentration	Total sucrose	Total glucose	Total fructose	Total sugars
1	1.5	.10	.15	0	0	.15
2	19.75	.07	1.47	0	0	1.47
3	18.75	.11	1.74	0	.31	2.05
5	2.1	.17	.14	.05	.17	.36
6	.4	.12	0	0	.05	.05

Table 2.2b. Nectar volumes (µl), nectar concentrations (µg/µl), total sucrose, glucose, fructose (mg) and total sugars (mg) in the nectar of Clematis addisonii 1, 2, and 3 days into anthesis. August and September, 1994. Site 2.

Day of anthesis	Volume collected	Concentration	Total sucrose	Total glucose	Total fructose	Total sugars
1	6.5	.22	1.46	0	0	1.46
2	9.0	.10	.91	0	0	.91
3	9.75	.05	.52	0	0	.52

Table 2.2c. Nectar volumes (µl), nectar concentrations (µg/µl), total sucrose, glucose, fructose (mg) and total sugars (mg) in the nectar of Clematis addisonii 2, 3, 4, and 5 days into anthesis. August and September, 1994. Site 2.

Day of anthesis	Volume collected	Concentration	Total sucrose	Total glucose	Total fructose	Total sugars
2	26	.20	5.12	0	0	5.12
3	11.75	.11	1.13	0	.21	1.34
4	1.75	.08	.04	0	.09	.13
5	.5	.07	.03	0	0	.03

Table 2.2d. Nectar volumes (µl), nectar concentrations (µg/µl), total sucrose, glucose, fructose (mg) and total sugars (mg) in the nectar of Clematis addisonii 1, 2, 3, 5, 6, and 7 days into anthesis. August and September, 1994. Site 2.

Day of anthesis	Volume collected	Concentration	Total sucrose	Total glucose	Total fructose	Total sugars
1	2.2	.38	.84	0	0	.84
2	5.4	.43	2.04	0	.28	2.32
3	4.0	.46	1.47	0	.36	1.83
5	2.0	.41	.56	0	.27	.83
6	4.0	.41	.94	0	.70	1.64
7	2.0	.39	.25	0	.54	.79

Table 2.2e. Nectar volumes (µl), nectar concentrations (µg/µl), total sucrose, glucose, fructose (mg) and total sugars (mg) in the nectar of Clematis addisonii 1, 2, 4, 5, and 6 days into anthesis. August and September, 1994. Site 2.

Day of anthesis	Volume collected	Concentration	Total sucrose	Total glucose	Total fructose	Total sugars
1	3.6	.40	1.43	0	0	1.43
2	6.0	.45	2.34	0	.33	2.67
4	3.3	.49	1.22	.10	.29	1.61
5	3.4	.41	1.03	.03	.34	1.40
6	4.4	.34	.96	0	.52	1.48

Table 2.2f. Nectar volumes (µl), nectar concentrations (µg/µl), total sucrose, glucose, fructose (mg) and total sugars (mg) in the nectar of Clematis addisonii 1, 2, 3, and 4 days into anthesis. August and September, 1994. Site 2.

Day of anthesis	Volume collected	Concentration	Total sucrose	Total glucose	Total fructose	Total sugars
1	6.5	.16	1.02	0	0	1.02
2	19.9	.21	4.16	0	0	4.16
3	41.0	.08	3.41	0	0	3.41
4	.75	.19	.06	0	.08	.14

Appendix III

Seed yield data

by individual plant

Table 3.1a. Total number of ovules and seeds and percent seed set of each Clematis addisonii individual. Control group. Site 1, 1994.

Plant	Number of ovules	Number of seeds	Percent seed set
1	14	8	57
2	14	11	79
3	10	6	60
4	6	5	83
5	9	8	89
6	8	7	87
7	7	2	29
8	7	7	100
9	10	3	30
10	10	-	-
11	9	-	-
12	3	1	33
13	16	12	75
14	14	13	93
15	7	4	57

- indicates seeds that were removed by predators

Table 3.2a. Total number of ovules and seeds and percent seed set of each individual of Clematis addisonii following self-pollinations. Site 1, 1994.

Recipient flower	Day of anthesis when pollinated	Number of ovules	Number of seeds	Percent seed set
1	7	14	10	71
2	6	6	3	50
3	7	7	4	57
4	10	8	0	0
5	8	-	-	-
6	10	12	0	0
7	6	-	-	-
8	4	5	3	60
9	9	5	0	0
10	7	-	-	-
11	6	10	5	50
12	3	6	2	33
13	10	6	0	0
14*	6	7	5	71
15	8	9	8	89

* indicates flowers with pistils of shorter style lengths that may not have been contacted during hand-pollination

- indicates seeds that were removed by predators

Table 3.3a. Total number of ovules and seeds and percent seed set of each individual of Clematis addisonii following intra-populational cross-pollinations. Site 1, 1994.

Recipient flower	Day of anthesis when pollinated	Number of ovules	Number of seeds	Percent seed set
1	4	14	12	86
2	4	9	7	78
3	3	11	10	91
4	2	6	4	67
5	7	9	8	89
6	4	-	-	-
7	6	6	0	0
8	5	7	3	43
9	6	14	10	71
10**	2	3	0	0
11	1	6	4	67
12	4	13	7	54
13*	5	6	3	50
14	5	7	5	71
15	6	20	16	18

* indicates flowers with pistils of shorter style lengths that may not have been contacted during hand-pollinations

** indicates flower pollinated with old pollen

- indicates seeds that were removed by predators

Table 3.4a. Total number of ovules and seeds and percent seed set of each Clematis addisonii individual following inter-populational cross pollinations. Site 1, 1994.

Recipient flower	Day of anthesis when pollinated	Number of ovules	Number of seeds	Percent seed set
1	2	3	3	100
2	4	6	5	83
3	4	11	6	54
4	2	8	2	25
5	3	-	-	-
6	2	7	0	0
7	5	13	6	46
8	3	8	1	12
9	5	7	0	0
10	3	9	7	78
11	3	5	2	40
12	4	4	2	50
13*	6	7	2	29
14	2	8	2	25
15	3	9	6	67

* indicates flower with pistils of shorter style lengths that may not have been contacted during hand-pollination

- indicates seeds that were removed by predators

Table 3.1b. Total number of ovules and seeds and percent seed set of each Clematis addisonii individual. Control group. Site 2, 1994.

Plant	Number of ovules	Number of seeds	Percent seed set
1	19	16	84
2	7	0	0
3	-	-	-
4	11	0	0
5	13	10	77
6	13	9	69
7	10	0	0
8	13	13	100
9	12	12	100
10	9	8	89

- indicates seeds that were removed by predators

Table 3.2b. Total number of ovules and seeds and percent seed set of each Clematis addisonii individual following self-pollinations. Site 2, 1994.

Recipient flower	Day of anthesis when pollinated	Number of ovules	Number of seeds	Percent seed set
1	7	11	0	0
2	7	7	1	14
3	9	14	0	0
4	9	6	0	0
5	5	12	5	42
6	4	7	0	0
7	4	12	0	0
8	4	4	2	50
9	4	12	7	58
10	4	7	0	0

Table 3.3b. Total number of ovules and seeds and percent seed set of each Clematis addisonii individual following intra-populational cross-pollination. Site 2, 1994.

Recipient flower	Day of anthesis when pollinated	Number of ovules	Number of seeds	Percent seed set
1	4	9	3	33
2	6	9	3	33
3	3	11	4	36
4	4	8	0	0
5	3	11	3	27
6	4	7	0	0
7	2	8	7	87
8	3	6	2	33
9	4	9	0	0
10	3	9	0	0

Table 3.4b. Total number of ovules and seeds and percent seed set of each Clematis addisonii individual following inter-populational cross-pollinations. Site 2, 1994.

Recipient flower	Day of anthesis when pollinated	Number of ovules	Number of seeds	Percent seed set
1	6	9	4	44
2	6	7	0	0
3	3	6	0	0
4	2	6	4	67
5	6	5	0	0
6	3	9	9	100
7	4	11	0	0
8	4	9	6	67
9	4	7	0	0
10	3	6	0	0

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Vita

Rhonda Edwards was born August 15, 1960 at Radford, Virginia. She lived in Hillsville, Virginia with her parents, three brothers, and grandmother until she moved to Blacksburg in 1980 to attend Virginia Tech. In 1983 she received a Bachelor of Science Degree in Horticulture. During the time interval between receiving her undergraduate degree and beginning her graduate work at Virginia Tech in 1991, she worked primarily as a "professional" gardener. While pursuing her Masters Degree in Biology she restored and now lives in the house that her grandfather helped to build and her father grew up in. She is quite content there with her dog, Chelsea, and her flower and vegetable gardens.