The association between floral longevity and pollen removal, pollen receipt, and fruit production in flame azalea (*Rhododendron calendulaceum*)

Amy C. Blair and Lorne M. Wolfe

Abstract: Floral longevity is an important feature of a plant's reproductive strategy. The goal of this study was to examine flower life span in the shrub flame azalea (*Rhododendron calendulaceum* (Michx.) Torr.) to determine if it is fixed or plastic and to evaluate its association with pollen removal and receipt. Unmanipulated flowers remained open for ~10 days, while hand-pollinated flowers closed earlier (~7 days), indicating that these flowers are subject to pollination-induced senescence. In 2002, pollen removal was rapid (~70% of pollen was removed in the first 2 h of anthesis), while female function was a slower process (pollen accumulated on stigmas for up to 4 days). Fruit set was pollen limited, as 80% of hand-outcrossed flowers set fruit versus 35% of naturally pollinated flowers. This is likely the result of the pollen-collecting behavior of a common solitary bee, which did not contact stigmas. In sharp contrast, 2003 was a cooler and wetter spring, few flower visitors were observed, and virtually no pollen was removed from or deposited on stigmas after 48 h. These results suggest that the flower life span of flame azalea is a result of selection for increasing the probability of pollen receipt rather than pollen dispersal.

Key words: Ericaceae, flame azalea, floral longevity, *Rhododendron calendulaceum*, pollen limitation, pollen removal.

Résumé : La longévité florale est une caractéristique importante de la stratégie de reproduction des plantes. Les auteurs ont étudié la durée de vie de l'azalée flammée (*Rhodendron calendulaceum*) (Michx.) Torr.), afin de déterminer si elle est fixe ou plastique, et d'en évaluer l'association avec le prélèvement et l'arrivée du pollen. Les fleurs non touchées demeurent ouvertes pendant ~10 jours alors que celles qui sont pollinisées à la main ferment plus tôt (~7 jours), ce qui indique que ces fleurs sont sujettes à une sénescence induite par la pollinisation. En 2002, l'enlèvement du pollen a été rapide (~70 % du pollen enlevé au cours des deux premières heures suivant l'anthèse) alors que la fonction femelle a été un processus plus lent (le pollen s'est accumulé sur les stigmates pendant 4 jours). La mise à fruit est limitée par le pollen, puisque 80 % des fleurs pollinisées à la main ont produit des fruits versus 35 % des fleurs pollinisées naturellement. Ceci résulte probablement du comportement de récolte du pollen par une abeille solitaire, qui ne contactait pas les stigmates. Au contraire, 2003 a connu un printemps plus frais et plus humide et on a observé peu de visiteurs; pratiquement aucun pollen n'a été prélevé ou déposé sur les stigmates dans les 48 heures suivant l'anthèse. Ces résultats suggèrent que la durée de vie de l'azalée flammée résulte d'une sélection favorisant l'augmentation de la probabilité de réception du pollen plutôt que sa dispersion.

Mots-clés : Ericaceae, azalée flammée, longévité florale, *Rhododendron calendulaceum*, limitation par le pollen, enlèvement du pollen.

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Introduction

Flower longevity is an extremely variable phenotypic trait in the angiosperms that ranges from a few hours in certain species of morning glories to as long as months in the case of some orchids (Primack 1985). Individual flower life span is an important feature of a plant's reproductive strategy, as it influences the period of time that pollen grains are available for transport by pollen vectors and, therefore, the probability of reproductive success. We might expect selection to favor flowers that remain open as long as necessary to fulfill the male function of pollen dispersal and the female function of pollen reception. On the other hand, opposing forces may operate to favor minimizing flower longevity. For example, energetic and transpirational maintenance costs (Ashman and Schoen 1994, 1997), the risk of exposure to disease vectors (Alexander and Antonovics 1995; Shykoff et al. 1996), and floral herbivory (Krupnick and Weis 1999; Mothershead and Marquis 2000) all are costs that are likely to increase as flowers remain open for longer periods.

There appear to be two main categories of flowering life spans (van Doorn 1997). Some plant species produce flowers with fixed life spans and senescence occurs regardless

A.C. Blair[1,2] and L.M. Wolfe. Department of Biology, Georgia Southern University, Statesboro, GA 30460, USA.

[1]Corresponding author (e-mail: amyblair@lamar.colostate.edu).
[2]Present address: Department of Bioagricultural Sciences and Pest Management, Colorado State University, Fort Collins, CO 80523, USA.

of pollination success (e.g., Commelinaceae and Pontederiaceae; Primack 1985). Other plant species have plastic schedules in which flowers cease functioning as a result of pollination-induced senescence (Primack 1985; Arathi et al. 2002). In these species, the cessation of floral function may be triggered by either pollen removal or pollen receipt (Devlin and Stephenson 1984; Ackerman 1989; Proctor and Harder 1995). For this second group, floral longevity will ultimately be dependent on the availability and efficacy of pollinators in transporting pollen among conspecifics in a population. The fixed life span strategy may be riskier than the plastic strategy owing to the dependence on pollinators appearing sometime in the time period before senescence. This would be particularly true in species that flower in habitats at a time when pollinator service is unpredictable.

A large literature has developed around the hypothesis that selection on hermaphroditic floral characters is driven largely by male reproduction (Willson and Burley 1983). The basic premise is that animal-pollinated hermaphroditic flowers require only one or very few insect visits to fulfill female fitness by fertilizing almost every ovule, while male fitness increases with numerous visits, each resulting in additional pollen dispersal (Bell 1985). While this hypothesis holds in some cases (Emms et al. 1997; Queller 1997; Gehring et al. 2004; but see Broyles and Wyatt 1990; Conner et al. 1996; Delph et al. 1996), selection on floral traits is expected to be driven by female function when seed production is limited by pollen deposition instead of resources (Burd 1995). Limited pollen deposition may occur through discrepancies between the services that a flower visitor provides as both a pollen remover and depositor. For example, certain bees harvest pollen, but in effect they act as pollen thieves by not coming in contact with the stigma during flower visits. Wilson and Thomson (1991) found that in *Impatiens capensis* Meerb., a group of pollen collectors removed almost twice as much pollen as the pollen collector *Bombus*, but the latter delivered nearly an order of magnitude more pollen to stigmas of virgin female phase flowers.

The overall goal of this study was to evaluate the relationship between floral longevity and sex-specific pollination dynamics in flame azalea (*Rhododendron calendulaceum* (Michx.) Torr.) (Ericaceae). This understory shrub is among the first species to flower in spring in eastern North American deciduous forests. Owing to the unpredictabilty of the weather and presence of pollinators at this time of the year (personal observations, 2002, 2003; Schemske et al. 1978; Primack 1985; Motten 1986; Bell and Cresswell 1998), we might expect fruit and seed production to be pollen limited. This is precisely the sort of situation that we hypothesize will favor a plastic, pollinator-induced senescence schedule for flowers. We specifically addressed the following questions. (*i*) How variable is individual flower life span within a population and is floral longevity fixed or subject to pollinator-induced senescence? (*ii*) What is the time course of pollen removal and deposition and does this vary from year to year? (*iii*) Is fruit production pollen limited?

Materials and methods

Study species and site information

Flame azalea is a common nonclonal understory shrub of eastern deciduous forests from Pennsylvania and southern Ohio to Georgia and Alabama (Gleason and Cronquist 1963). During May and June, the 1–3 m shrub produces inflorescences containing 5–15 orange to yellow flowers (Wolfe and Rissler 1999). Each flower has five poricidal anthers, and viscin threads hold pollen grains together. Flowers are not dichogamous, and individual fruits contain 100–200 seeds (D.W. Hyatt, personal communication).

Our study was conducted in June 2002 and June 2003 at the University of Virginia's Mountain Lake Biological Station in the Appalachian Mountains of southwestern Virginia (Giles County; 37°22′N, 80°32′W; elevation ~1160 m). We studied 25 flame azaleas within a 2 km area of the Mountain Lake Biological Station. The density of flame azalea in the study area was about 80 plants/ha.

Floral longevity

To quantify the life span of individual flowers, in 2002, we enclosed inflorescences of unopened buds on six trees with ziplock bags. Ziplock bags were used because we found that pollen grains stuck to other materials (e.g., bridal veil) but not to the plastic. The next day, we removed the bags, tagged all freshly opened flowers, and then inspected them daily. We considered senescence to occur when the corolla fell off the flower.

We manually pollinated some plants to determine whether floral longevity is fixed or sensitive to pollen deposition. Inflorescences on 13 trees were enclosed with ziplock bags, and the following day, freshly opened flowers were either selfed (*n* = 9), outcrossed with pollen from a neighboring tree (*n* = 20), or left unmanipulated as a control (*n* = 20). All of the pollen from one anther was used for each hand-pollination. When sufficient flowers were available, we assigned each of the three treatments to a single tree. Flowers were tagged and inspected daily, as above.
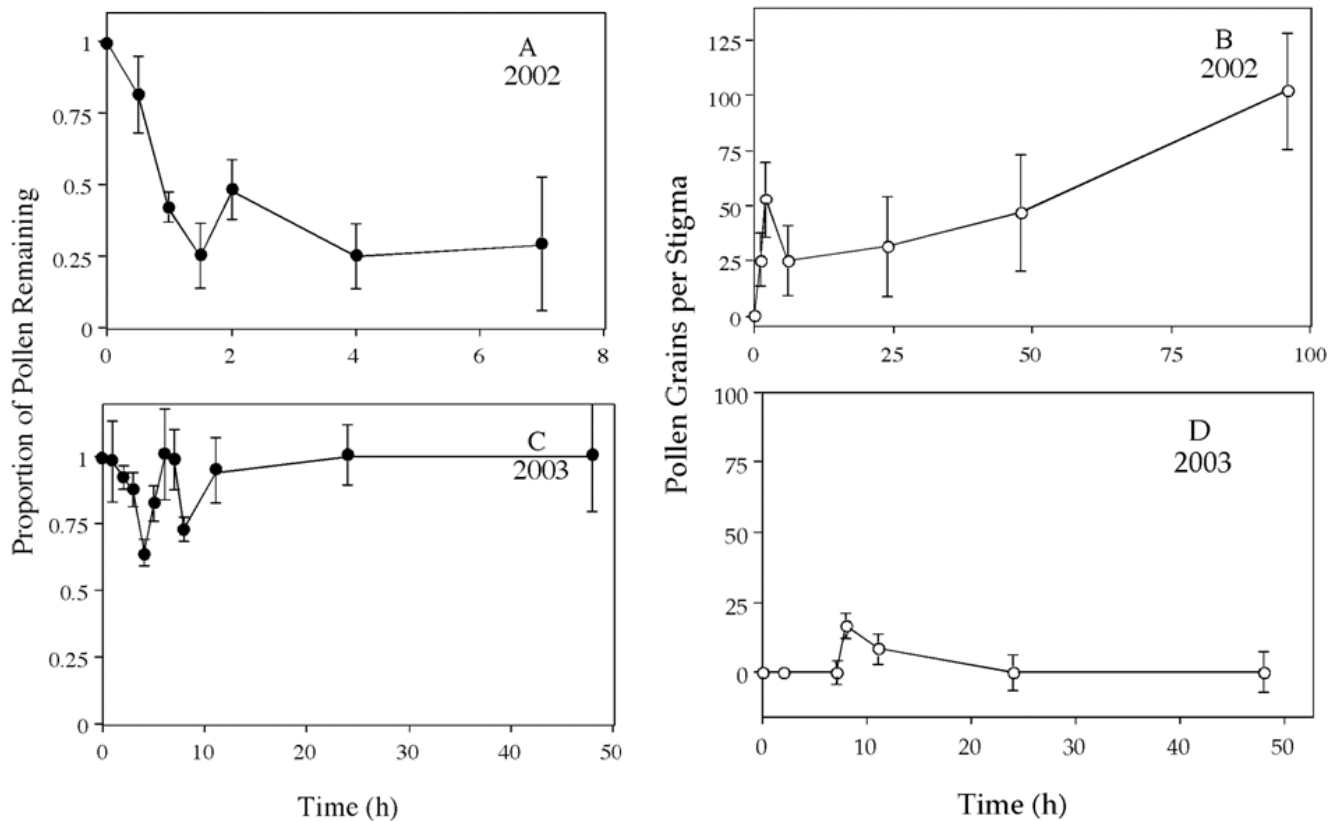
Pollen removal

To quantify pollen removal, we enclosed unopened inflorescences with ziplock bags to exclude flower visitors. We removed the bags the following morning at approximately 0800, which was the time that flower visitors became active (personal observations). In 2002, we removed one flower per inflorescence over 7 h beginning at time = 0 (0800), followed by removals at 0.5, 1, 1.5, 2, 4, and 7 h (*n* = 90 flowers). Because of the different insect dynamics in 2003 (see Results), we instead sampled flowers over 48 h beginning at time = 0 (0800), 1, 2, 3, 4, 5, 6, 7, 8, 11, 24, and 48 h (*n* = 180 flowers). To include the potential variation across the flowering season, we sampled 10 days throughout the month-long flowering period. Flowers were transported to the laboratory in egg cartons to prevent pollen removal from the anthers en route. Pollen removal was quantified by estimating the amount of pollen remaining in anthers. The poricidal anthers of flame azalea are transparent and the pollen mass can easily be seen under a dissecting microscope. We examined all five anthers per flower and the amount remaining was recorded as 0% (i.e., all pollen removed), 25%, 50%, 75%, or 100% (i.e., no pollen removed).

Pollen deposition

We used the same bagging technique as above to sample

Fig. 1. Temporal pattern of (A and C) pollen removal from anthers and (B and D) pollen deposition on stigmas in 2002 and 2003 in *Rhododendron calendulaceum*. Values are means ± SE.

stigmas for pollen deposition. Because our preliminary observations in 2002 indicated that this occurred over a longer time period than pollen removal, flowers were sampled over 96 h beginning at time = 0 (0800), followed by sampling at 1, 2, 6, 24, 48, and 96 h (n = 49 flowers). In 2003, flowers were sampled over 48 h beginning at time = 0 (0800), followed by sampling at 2, 7, 8, 11, 24, and 48 h (n = 148 flowers). Upon inspection with a dissecting microscope, the pollen load on the stigma was recorded as being "none", "trace", "few", "some", or "heavy". These qualitative groupings were quantified by making five slides from stigmas that corresponded to each of these groupings and then counting the individual pollen grains with a compound microscope (mean ± SE: none = 0, trace = 5.3 ± 2.8, few = 46.6 ± 10.45, some = 134.3 ± 22.7, and heavy = 329.0 ± 41.0).

Fruit production in naturally pollinated versus artificially outcrossed flowers

In 2002, we conducted hand-pollinations on an additional set of flowers to determine whether fruit production was limited by pollen. Twenty pairs of freshly opened flowers on inflorescences were haphazardly chosen from 13 trees and marked with small tags. One flower was pollinated with pollen taken from another tree in the population, while the second flower was unmanipulated and served as a control for natural level of fruit production. As inflorescences contain up to 15 flowers, it is highly unlikely that hand-pollination of one flower influenced fruit production of the control. All flowers were revisited in July 2002 to record presence or absence of fruit set. Maturing but still unripe fruit were har-

vested in October 2002. We measured the length and width of each fruit, and area was calculated with the equation for the area of an ellipse (π(1/2 width)(1/2 length)).

Flower visitor behavior

In 2002, we observed and documented the foraging behavior of the visitors to flame azalea's flowers. We monitored each insect during its visit to a single tree, recorded the event with a digital video camera, and noted whether or not the insect contacted the stigma and (or) anthers and (or) entered the flower to probe for nectar.
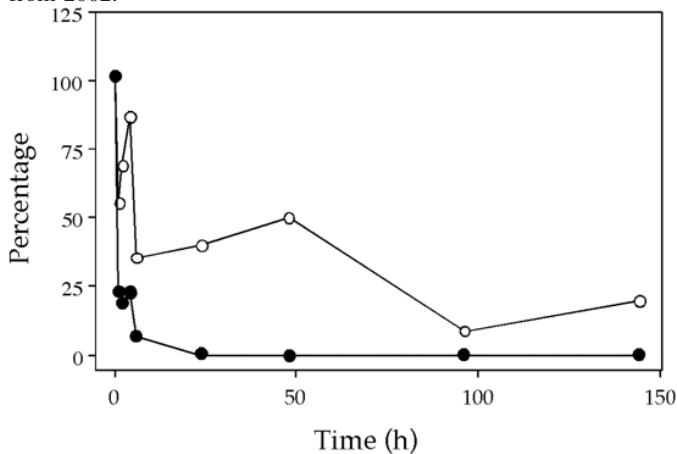
Data analysis

All analyses were conducted with SAS version 9.1 (SAS Institute Inc., Chicago, Ill. 2002). Residuals were inspected for normality and heteroscedasticity and log transformed when required for parametric analyses. As multiple flowers were studied from individual trees, we used Proc Mixed repeated-measures ANOVA. For the floral longevity data, we used a model with the following main effects: tree (random) and treatment (fixed: selfed, outcrossed, or control). For the fruit production data, we used a similar model, but the treatments were either outcrossed or control. Values are presented as means±SE throughout.

Results

Floral longevity

Naturally pollinated flowers remained open 4–14 days. Floral longevity differed significantly for outcrossed, selfed,

Fig. 2. Percentage of flowers that have anthers with no pollen removed (solid circles) and stigmas with no pollen deposition (open circles) through time in *Rhododendron calendulaceum*. Data are from 2002.

and control flowers ($F_{[2,34]}$ = 13.73, P < 0.0001). A Tukey–Kramer post-hoc test revealed that flowers that received supplemental pollen senesced earlier than naturally pollinated control flowers (outcrossed = 7.1 ± 0.4 days, selfed = 7.0 ± 0.5 days, control = 9.7 ± 0.5 days).

Pollen removal and deposition

Full anthers contained ~1300 pollen grains. In 2002, pollen removal from fresh flowers was rapid, with ~70% being removed within the first 2 h (Fig. 1A). In contrast, pollen deposition was a slower process, with pollen accumulating on stigmas for days rather than minutes. While some pollen was deposited almost immediately, it continued to accumulate for 100 h (Fig. 1B). The temporal disparity between male and female function is further evidenced by the fact that only ~20% of the anthers were still full of pollen after 1 h. On the other hand, 50% of the stigmas had not yet received any pollen after 2 days (Fig. 2).

The pattern of pollen removal and deposition was completely different in 2003 compared with 2002. After 2 days, virtually no pollen had been removed from anthers or deposited on stigmas (Figs. 1C and 1D).

Fruit production in naturally pollinated versus artificially outcrossed flowers

Fruit production in flame azalea was pollen limited, as 79.7% of the flowers that were manually outcrossed set fruit, while only 34.8% of the naturally pollinated flowers were successful (G = 27.15, P = 0.0001). Cross type significantly affected fruit size ($F_{[1,51]}$ = 39.47, P < 0.0001); flowers that were artificially outcrossed had larger fruits than those left to be naturally pollinated (133.2 ± 7.3 versus 61.7 ± 10.3 mm^2).

Flower visitor behavior

Flame azalea flowers were visited by bumble bees, honey bees, solitary bees, and swallowtail butterflies. Solitary bees predominated the flower visitor pool. For example, when we made several digital video recordings of each insect group to monitor their behavior, we saw 25 solitary bees in comparison with only three bumble bees, one honey bee, and six swallowtail butterflies. Few floral visitors were seen in 2003 because of inclement weather. While mean June temperature differed slightly between years (17.6 °C in 2002 versus 15.2 °C in 2003), there was a large difference in the amount of rainfall. In June 2002, 89 mm of rain fell over 8 days compared with 253 mm over 18 days in 2003.

Based on the video recordings made in 2002, the bumble bees tended to crawl into the corolla to collect nectar. The solitary bees and the one observed honey bee typically climbed up the filament and gathered pollen from the anthers and occasionally also collected nectar. We never observed any bee type contacting the stigma; thus, these floral visitors acted as pollen thieves. The swallowtail butterflies also collected nectar but while hovering their wings often contacted both the anthers and stigma within a flower.

Discussion

Flower life span is a reproductive trait that represents a trade-off between the benefits of pollen dispersal and receipt and the costs associated with remaining open. It is likely that flame azalea flowers are energetically costly to maintain, since they are relatively large, are regularly damaged by floral herbivory, and are susceptible to fungal infection (Wolfe and Rissler 1999). Given these costs, it is curious that the flowers can remain open for 14 days. Because flame azalea initiates flowering in the spring before most other species have begun to flower, interspecific competition for pollinators is not likely to be driving the duration of floral longevity. Instead, highly stochastic pollinator activity in eastern deciduous forests in the spring (Schemske et al. 1978), resulting from the dependence of emergence and activity pattern of insects on local temperature and precipitation (Danks 1994; Alonso 2004), could be favoring longer periods of floral longevity. In this study, the dramatic difference in pollen removal and stigmatic pollen loads between 2002 and 2003 can likely be explained by weather patterns. Nearly three times as much rain fell in June 2003 as in June 2002 and very few flower visitors were present in 2003 (personal observations). Thus, the year-to-year variation in climate is associated not only with flower visitor abundance but also with the movement of pollen grains from flower to flower, which is the true measure of pollination. Variation in weather across seasons, therefore, may play an important role in flame azalea floral longevity.

To offset the costs of increased floral longevity, plants that experience unpredictable pollination appear to have pollination-induced senescence (Primack 1985; van Doorn 1997; Arathi et al. 2002). Keeping flowers open for a long, fixed time could be unnecessarily costly if in some flowering seasons, climate patterns were favorable and pollinators abundant. In essence, these plants are hedging their bets in stochastic pollinator environments. Indeed, flame azalea exhibits pollination-induced senescence. Naturally pollinated flowers stayed open on average 10 days, while flowers that received supplemental pollen senesced after 7 days.

Within a flowering season, plant reproductive success can also be affected by the behavior and efficiency of the flower visitor fauna (Schemske and Horvitz 1984; Brunet and Sweet 2006). For example, Conner et al. (1995) found that nectar-feeding butterflies had higher pollination efficiency

than nectar- and pollen-feeding honey bees in wild radish. Similarly, the flower visitors in the flame azalea system appeared to differ in their ability to transport pollen from flower to flower. The common solitary bees typically landed on the filament, climbed up to the anther, and then gathered pollen with their front legs and mouthparts. Because of the vertical and horizontal separation between the stigmas and anthers in a flame azalea flower, these visitors were able to remove pollen without contacting the stigma.

Alternatively, only the swallowtail butterflies contacted both reproductive parts as they landed on flowers. Because of the viscin threads connecting pollen grains, the wings of the swallowtail butterfly could extract long threads of pollen while also contacting the stigma (personal observation). The pattern of rapid pollen removal (on the order of hours) and more gradual pollen deposition (on the order of days) that we observed in 2002 may have resulted from swallowtail butterflies being much less common than bees at our study site. While it is not uncommon for pollen to be removed rapidly in other species, contrary to our findings, most studies find similarly rapid rates of pollen deposition (Wolfe and Barrett 1989; Ashman and Schoen 1994; Rush et al. 1995). For example, Wolfe and Barrett (1987) found both pollen removal and deposition to occur within the first few hours of flower opening in pickerelweed (*Pontederia cordata* L.).

It is of interest to ask whether the discrepancy between pollen removal and deposition affected flame azalea reproductive success in 2002. Indeed, it appears that flame azalea was pollen limited. Only 35% of the naturally pollinated flowers set fruit compared with 80% of the artificially outcrossed flowers. Seed set, a more direct measure of fitness, is likely to be associated with fruit set; in a meta-analysis of 63 data records in which both fruit set and seed set were reported, Knight et al. (2005) found a strong correlation between the magnitude of pollen limitation reported for fruit set and seed set per plant. Thus, it is probable that the rapid pollen removal that we observed did not translate into male and female reproductive success (i.e., seed production). This is likely to be a direct result of the foraging behavior of the common solitary bee. Although these insects removed pollen, they appear to have very low pollen transfer efficiency. This creates a strong disjunct between apparent pollen removal and deposition. With regard to floral longevity, contrary to the general assumption that floral reproductive traits are driven by male fitness (Bell 1985), it appears that pollen limitation in this system may, in fact, result in selection for increased floral longevity to enhance female fitness.

Natural selection should favor evolution of life history strategies that maximize lifetime fitness. Floral longevity is a trait likely to be influenced by a suite of external factors. In this study, we have demonstrated that both unpredictable pollinator service across seasons because of varying weather and inefficient pollen deposition rates within a season should favor plants that maintain open flowers for longer periods. We further posit that fitness will be enhanced by flower life spans having a plastic component, therefore reducing the associated costs of maintenance.

Acknowledgements

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