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The dynamics of pollen removal and deposition, and its effects on sexual phases in a protandrous plant: *Glechoma longituba* (Lamiaceae)

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The duration of sexual phases in dichogamous plants are affected by many factors. Using both experimental and observational studies, we investigated natural patterns of pollen removal and deposition, visiting frequency of pollinators, patterns of nectar secretion, and effects of pollen removal and stigmatic pollen deposition on the duration of sexual phases in a protandrous plant, *Glechoma longituba*. We found that visiting frequency of pollinators correlated with the nectar secretion pattern. The nectar volume during the male phase was higher than during the female phase. In the morning, the main pollinator, the bee *Anthophora plumipes*, mainly foraged for nectar and showed no preference for flowers in male or female phase, despite male phase flowers producing higher amounts of nectar. However, in the afternoon, they changed their behavior and foraged mainly for pollen, and then showed a preference for flowers in male phase. Furthermore, the rates of pollen removal and stigmatic pollen deposition can affect the starting time and the duration of the female phase. When pollen removal and pollination rates are low due to scarcity of pollinator services, the sexual phase can be prolonged, leading to an overlap, and thereby enhance the chance for sexual reproduction through pollinator-facilitated self-pollination. We consider the variation of sexual phases in *Glechoma longituba* an adaptive mechanism prepared for both cross-pollination enhancement and reproductive assurance depending on the available pollination services.

Most hermaphrodite flowers show dichogamy, i.e. temporal segregation of pollen presentation (male phase) and stigma receptivity (female phase) (Lloyd and Yates 1982, Routley and Husband 2003). Dichogamy has previously been interpreted as a mechanism for reducing self-fertilization (Darwin 1862), and most recently as a more general mechanism for reducing the impact of pollen–pistil interference on pollen import and export (Harder and Barrett 1996, Barrett 2002, Evanhoe and Galloway 2002). For example, separation of sexual phases reduces pollen discounting due to losses in self-pollination, enabling more pollen grains to be transported to nearby individuals, and thus, increasing male fitness (Wolfe and Barrett 1989, Harder et al. 2000, Routley and Husband 2003). At the same time, dichogamy reduces the risk of clogging the stigma surface with self pollen, or competition between pollen tubes of self pollen and cross pollen improving siring success (Lloyd and Webb 1986, Spira et al. 1996). Dichogamy is therefore beneficial for both male and female function.

Floral longevity and duration of sexual phases are influenced both by genetic and environmental factors such as temperature, pollinator activeness and mating chances (Primack 1985, Sargent and Roitberg 2000). On the other hand, the plant may be plastic in the longevity of sexual phases in order to compensate for the effects of low pollination rate (Ashman and Schoen 1994, Routley and Husband 2003, Castro et al. 2008). The rate of pollen removal may directly affect the duration of male phase with high efficiency of pollen removal leading to a shorter male phase and possibly decreased floral longevity (Sargent and Roitberg 2000). The variation mechanism of floral sexual phase has been documented in several species such as *Lobelia cardinalis* (Campanulaceae) (Devlin and Stephenson 1984), *Campanula repunculoides* (Richardson and Stephenson 1989) and *Myosotis colensoi* (Borraginaceae) (Robertson and Lloyd 1993). The female phase may also be shortened in response to efficient timely pollination (Richardson and Stephenson 1989, Sargent and Roitberg 2000, but see Proctor and Harder 1995).

Although major advances have been made in dichogamy research in the past decades, only a limited number of studies have paid attention to how the dynamics in pollen removal and deposition affect the variation in sexual phase duration and reproductive success in dichogamous plants (but see Richardson and Stephenson 1989, Preston 1991, Evanhoe and Galloway 2002). The present study on the protandrous *Glechoma longituba* addresses the following issues: 1) variation in sexual phase duration under natural conditions, 2) dynamics of pollen removal and deposition,

and its effects on the duration of sexual phases, and 3) effect of nectar secretion pattern and pollinator behavior on pollen removal and deposition in the species. We also discuss the evolutionary significance of sexual phase duration on the reproductive system in *Glechoma longituba*.

Material and methods

Study species and site

Glechoma longituba (Nakai) Kupr. (Lamiaceae) is a stoloniferous, gynodioecious perennial herb. It is a widely used medicinal plant in China. Individual ramets develop from over-wintering buds situated at ground level. Flowering commences in early March and continues to the end of April. Corolla mouth width of the hermaphroditic flower is about 5 mm, with a tubular corolla of about 25 mm in length. Nectar is produced at the base of the corolla tube. The hermaphroditic flower has four stamens, all with approximately equal numbers of pollen grains and dehiscing simultaneously (Zhang 2007). The filaments are partially adnate to the corolla. The anthers of the two longer stamens are located at approximately the same level as the stigma. The mature stigma is bifurcated (Li 1977). Plants within the study population had only 2–4 flowers at each node and most flowers opened in the morning. Breeding studies have shown that the plant is self-compatible and protandrous, but insects are necessary for successful pollination and fruiting under natural conditions (Zhang 2007).

The sexual phases in this species were classified as follows: male phase (or pollen presentation) is the period from the time of corolla opening and dehiscence of anthers to the time when the anthers become devoid of pollen; female phase (or stigma receptivity) is the period from the onset of stigma receptivity to floral senescence. Pilot experiments indicated that in this species, anther dehiscence is synchronous with the opening of the corolla and that pollen is viable from the time when it is shed until floral senescence. Stigma receptivity starts when the stigma splits into two lobes. The beginning of the female phase did not always signify the end of the male phase. Flowers in the female phase sometimes carried out male function if they still contained pollen. We defined this period as the sexual overlap phase (or hermaphrodite phase) (Robertson and Lloyd 1993). Unlike the start of the male and female phases and the end of the female phase, the end of the male phase could not be estimated based on visual observation alone. The end of the male phase was estimated by examining the quantity of pollen removed from the anthers. Lack of pollen in the anthers was taken as an indicator of the end of the male phase.

The study was carried out in the Wuhan region of the Hubei Province in central China in March 2006 and 2007. The plants were often subject to consecutive cold (mean daytime temperatures below 15°C) and rainy days in the first half of the flowering season. The study population consisted of several patches (subpopulations) located on the north slope of Luojia hill on Wuhan Univ. campus (150 m a.s.l., 114°21′E, 30°32′N).

Variation in sexual phase duration under natural conditions

To explore the variation in sexual phase duration and the dynamics in the pattern of pollen removal and deposition in *Glechoma longituba* under natural conditions, we used a technique which combined interval-sampling, morphological observation and assessment of the quantity of pollen remaining in the anthers. Fine and warm weather was recorded at the study site during 10–13 Mar 2006. During the experiments, mean daytime air temperature and relative humidity were 14–23°C and 50–65%, respectively. We randomly collected 20 flower buds in the early morning (7 a.m.) before visitor arrival and placed them in micro-tubes in order to count pollen production per flower. In addition, 200 newly opened virgin flowers on different ramets were tagged on the individual flowering nodes. The anthers of these flowers had dehisced and their pollen were viable, indicating the beginning of the male phase. However, no floral visitors were recorded at this time. In the following 2 days, 20 of the marked flowers were picked at 9 a.m., 11 a.m., 1 p.m., 4 p.m. and 7 p.m., and the sexual phase of each flower was recorded before placing them in micro-tubes.

Pattern of pollen removal and deposition

To assess patterns of pollen removal and deposition, the flowers in male and female phases collected during each observational period were used to determine the amount of pollen remaining in the anthers, as well as the pollen deposited onto the stigma, respectively. Flowers in male phase were placed into small cuvettes, then filled up to the 3 ml scale mark with distilled water. The anthers were then crushed with a dissecting needle and the tube shaken vigorously, an aliquot was removed and placed on a glass slide (3×0.06 ml at a time and repeated twice). Total pollen amount per flower was assessed by applying the same procedure and using flower buds. The proportion of pollen remaining in the flower was calculated by dividing the amount of pollen found in the anthers of the flower by the total amount produced per flower bud. For flowers in female phase, the stigmas were removed with fine forceps and squashed on microscope slides in basic fuchsine gel. The number of pollen grains adhering to the stigma was counted under a compound microscope in the laboratory following the method of Kearns and Inouye (1993). The pollination rate was estimated as the number of pollinated stigmas divided by the total number of observed stigmas.

Pattern of nectar secretion

To explore the effect of the nectar secretion pattern on visitation frequency in *G. longituba*, we measured nectar standing crop at morning (7 a.m.), mid-day (11 a.m.) and afternoon (5 p.m.), respectively. We tagged 20 male phase flowers and 20 female phase flowers in the morning. These flowers were not visited by floral visitors in the period (see pollinator behaviour section). Microcapillaries of 2 µl were used to measure the volume of secreted nectar. Nectar from 5 flowers was measured together because the amount

of nectar in each flower was very low (sensu Corbet 2003). We then covered these flowers with fine bridal netting in order to exclude floral visitors. The same method was used to measure the volume of nectar accumulated at mid-day and afternoon during the same day.

Pollinator behaviour

Pollinator behaviour was recorded during all surveillance periods described in the previous section. Total number of flowers visited per bout, foraged floral reward (nectar and/or pollen), and handling time per flower were recorded for 10 *Anthophora plumipes* individuals (the main pollinator visitor of this plant species). Data were excluded from analysis if we noted that an individual insect which had first left the plant later returned to the observed plant.

In order to investigate pollinator preference for flowers in a specific sexual phase we selected 10 flowers in male and female phase (previously marked). We recorded the number of visiting pollinators and the time spent inside the flower in 15-min observation periods at ca 9 a.m., 11 a.m., 1 p.m. and 4 p.m. Pollinator visiting frequency in *G. longituba* was recorded at ca 7 a.m., 9 a.m., 11 a.m., 1 p.m., 4 p.m., and 7 p.m. We selected three 1×1 m plots in the study population and counted the number of flowers in each plot. Visitation frequency was estimated as the mean number of pollinator visits to each flower, viz. the cumulative number of pollinator visits in a plot during a 15-min observation period divided by the total number of flowers in a plot.

Effect of experimental pollen removal and deposition on duration of female phase

In order to investigate the effects of pollen removal in the early stage of the male phase on the starting and duration of the female phase, we conducted sets of manipulation experiments. In the period 7–9 March 2007, during the full blossom of the plant, the weather was sunny and warm. The air temperature at noon was about 23–25°C, the air humidity 50 cm above ground was about 60–65%. On 7 March, we selected two 1×2 m plots in the middle of the population. All opened flowers in the plots were removed and the plants were covered with a large mosquito net in order to prevent insects from visiting. Before 7 a.m. the next day, 120 newly opened flowers from 120 separate ramets were marked and divided into 4 groups. The groups were subjected to different treatments as follows: 0% pollen removal: untreated flowers; 25% pollen removal: all pollen from one anther removed from each flower; 50% pollen removal: all pollen from two anthers removed from each flower; 100% pollen removal: all pollen removed from each flower; the treated flowers were covered again after treatment. In addition, as control we marked 30 freshly opened flowers and did not cover them (pollen removal under natural conditions).To remove the pollen, we used a piece of paper stained with glue to touch the dehisced anthers. We examined the flowers every 2–3 h in the following 2 days and recorded the starting time of the female phase in each flower.

In order to investigate the effect of the stigmatic pollen load in the early stage of the female phase on female phase duration, we conducted another manipulation experiment. Early in the morning 9 March, before pollinator visitation, 120 open flowers on distinct ramets were selected from the covered plots. The flowers were marked and divided into 4 groups. All flowers entered the female phase, but there was no pollen on their stigmas as the pollinators were excluded by bagging the flowers prior to anthesis. The flower groups were subjected to treatments as follows: putting 0: untreated flowers; putting 1–3: pollinated with 1–3 pollen grains to one of the lobes by touching the stigmatic lobe with a hair strand which has been dipped in xenogamous pollen grain mixture (pilot experiments have shown that 1–3 pollen grains can adhere to the stigmatic lobe by using this method); putting 20–30: pollinated with ca 20–30 pollen grains to one of the two stigmatic lobes by touching the stigmatic lobe only once with a toothpick which had been dipped in xenogamous pollen grain mixture; putting 40–60: pollinated with ca 40–60 pollen grains to both of the stigmatic lobes by touching both stigmatic lobes with a toothpick swab which had been dipped in xenogamous pollen grain mixture. We then covered all treated flowers with a large mosquito net to prevent pollinator visitation. As control, we marked 30 additional flowers which entered the female phase, but were not visited by pollinators and we let these flowers be open pollinated under natural conditions. Corolla and stigmatic lobes withering were assessed every 2 h.

Data analysis

We used a one-way ANOVA multiple comparison test to compare the effects of the removal and deposition of different amounts of pollen grains on the onset and duration of the female phase. A Kruskal–Wallis test was used to examine the variation in visiting frequencies during different observation periods, the time spent inside one flower in the male phase flowers and the female phase flowers, and difference of nectar volume in different sexual phases. A G-test was used to compare differences in visitation frequency between male and female phase (visitor preference) as we expected their value to be 1:1. Data on number and frequency of visitations were processed using square root and arcsine-transformation. All statistical analyses were performed using SPSS (13.0) statistical packages.

Results

Variation in duration of sexual phase under natural conditions

Our investigation showed that the male phase (pollen presentation) started at about 7 a.m. and ended between 4–7 p.m. The duration of male phase was 9.7 h ($\pm$1.6, n = 40, mean $\pm$ SE). The female phase (stigma receptivity) began in the morning of the following day (7–9 a.m.). Stigma lobes remained open and receptive untill the afternoon (4–7 p.m.) after which flower withering occurred. The duration of female phase was 8.9 h ($\pm$1.3, n = 40). In most flowers almost no overlap phase was. There was also 15.2 h ($\pm$1.9, n = 40) of non-sexual phase (mostly at night) when the male phase was already accomplished and female phase

had not yet started. Flowers had significantly longer male phase duration than female phase duration ($F_{1,78} = 4.342$, $p < 0.05$). The floral life-span (male phase + non-sexual phase + female phase) was approximately 33.8 h (±2.3, n = 40).

Pattern of pollen removal and deposition

The pollen removal rates were uneven during different periods of the male phase (Fig. 1). The rate of pollen removal was relatively low between 9 a.m. and 1 p.m. ($\chi^2 = 2.014$, DF = 2, $p > 0.05$), but in afternoon (1–4 p.m.), it increased considerably and about 56.4% of the pollen grains were removed. The difference between the amount of pollen grains that remained in the flowers at 1 p.m. (71.6%) and at 4 p.m. (15.2%) was significant ($\chi^2 = 64.562$, DF = 1, $p < 0.001$). Before the end of the male phase, over 90.8% of pollen in the anthers had been exported. Only 9.2% remained and this did not decrease during the female phase. This pollen was regarded as residual pollen that is never removed from the anther (Bell and Cresswell 1998).

A graph of the pollination rate in the female phase shows that 60.1% of the stigmas had been pollinated 3–4 h after the onset of the female phase (Fig. 1). The remaining flowers, however, still continued to receive pollen, and the total pollination rate was approximately 80.3% (±4.2, n = 99) before the flowers began to wilt. The average number of pollen grains deposited on a stigma was 13.83 (±5.7, n = 99).

Pattern of nectar secretion

Measurements of nectar standing crop at anthesis showed that male phase flowers (first day at anthesis) had significantly more nectar than female phase flowers (second day at anthesis). For example, 5 flowers in male phase did in total secret 1.64 µl (±5.5, n = 12) nectar and in the female phase 1.32 µl nectar (±4.2, n = 12) ($\chi^2 = 8.477$, DF = 1, $p < 0.01$). In addition, the results showed that nectar was secreted mainly in the morning (7–11 a.m.), while in the period from noon to afternoon (11 a.m. to 5 p.m.), in both male and female phase flowers, the amount of secreted nectar was relatively low (Fig. 2).

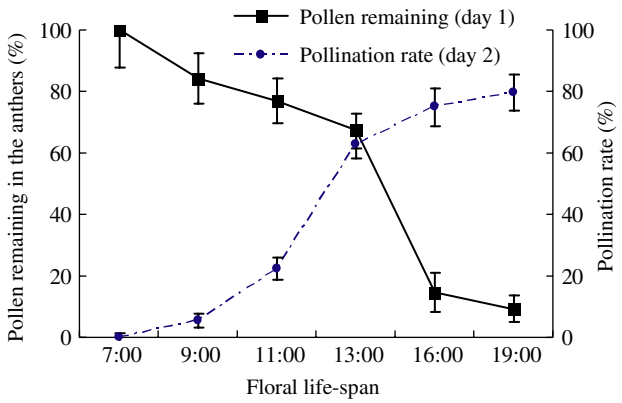

Figure 1. Dynamics of pollen removal and deposition along floral life-span in *Glechoma longituba*. Note that pollen removal occurs during the male phase of anthesis (first day), and that stigmatic pollination occurs in the female phase of anthesis (second day). (bars are mean ± 1 SE).

Pollinator behaviour

Of the 10 pollinator species visiting *G. longituba*, the bees *Anthophora plumipes* and *Apis cerana* had the highest visitation frequency, accounting for 55.1% and 19.4% of total number of visits, respectively (results herein; Zhang et al. 2007). Other visitors including *Habropoda omeiensis*, *Colletes curnicularis*, *Amegilla zonata*, *Mesembrius flaviceps*, *Eeistalinus tarsalis* and *Xylocopa sinensis* were considered of minor importance for pollination due to their low visiting frequencies.

During the study days, floral visitors in the population emerged at approximately 8 a.m. and visitation increased after 9 a.m. The highest visitation frequency was observed between 11 a.m. and 1 p.m., decreasing gradually and ending at about 6.30 p.m. The visitation frequencies varied significantly between the 5 observation periods ($\chi^2 = 21.176$, DF = 4, $p < 0.001$; Fig. 3). Each flower of *G. longituba* received 27.6 (± 3.7, n = 40) visits during its anthesis.

In the morning, *A. plumipes* foraged mainly for nectar. The bee inserted its head into the corolla tube and sucked the nectar using its long proboscis. Grooming involving pollen baskets on the hind legs was rarely observed during this period (23.3%, n = 30). During the period 1–4 p.m. *A. plumipes* actively foraged for pollen and filled pollen baskets were clearly visible on their hind legs (56.6%, n = 30). The difference was significant ($G = 4.67$, $p < 0.01$). The results from marked flowers in male and female phase (focal plants) indicated no significant difference in pollinator visiting frequency in the morning ($G = 3.12$, $p > 0.05$). However, a significant difference was detected in the afternoon during which pollinators preferred male phase flowers ($G = 14.63$, $p < 0.01$). Furthermore, time spent inside flowers in male phase (2.34 ± 1.22 s, n = 10) was significantly longer than time spent inside flowers in female phase (1.26 ± 0.73 s, n = 10) ($\chi^2 = 29.476$, DF = 1, $p < 0.001$).

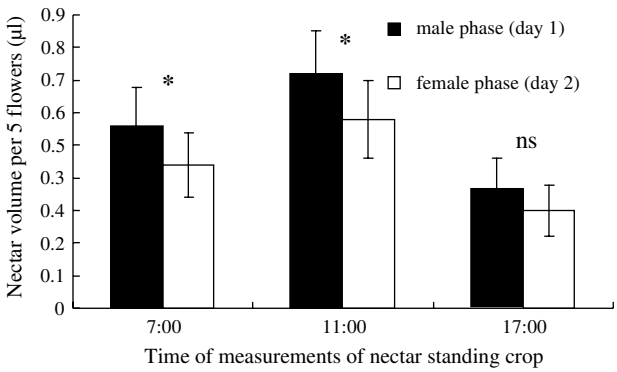

Figure 2. Nectar accumulation in the flowers of *Glechoma longituba* during different sexual phases and different periods when excluded from flower visitors. Values are means (±1 SE). * = $p < 0.05$, ns = $p > 0.05$.

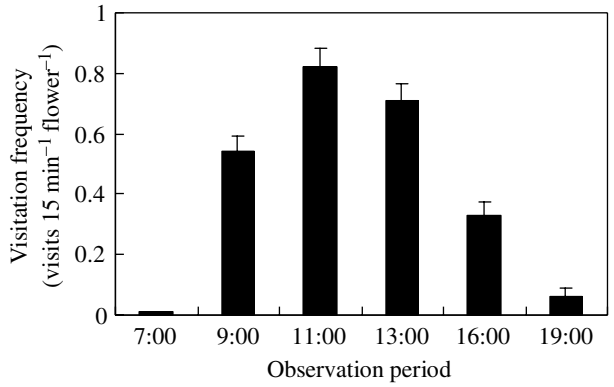

Figure 3. Visitation frequency of pollinators for *Glechoma longituba* in different observation periods in the study population on a fine and warm day (bars are mean ± 1 SE).

Effect of pollen removal and deposition on female phase duration in artificial conditions

As observed in the first experiment, on warm days with fine weather, the stigma lobes opened at about 7–8 a.m. on the second day of flower anthesis under natural conditions, i.e. the female phase started 24.9 h after flower opening. When pollinators were excluded, the female phase started at noon the second day, 27.5 h (±2.8, n = 30) after flower opening. When 25% of the pollen grains were removed allready at the onset of flowering, female phase started earlier: 25.8 h (±2.4, n = 30) after flower opening. When 50% of the pollen grains were removed, the onset of the female phase occurred at dusk of the first day, 10.7 h (±1.7, n = 30) after flower opening. When all pollen grains were removed, female phase started in the afternoon of the first day, 7.4 h (±1.6, n = 30) after flower opening. Thus, the starting time of the female phase was significantly affected by the rate of pollen removal ($F_{4,\ 145} = 26.257$, $p < 0.001$; Fig. 4).

When pollinators were excluded after male phase, female phase was prolonged from about 8.9 h (under natural conditions) to 28.1h (±2.7, n = 30). The duration of female phase was significantly different in the 4 treatments ($F_{4,145} = 19.486$, $p < 0.001$). To conclude, controlled pollinations can significantly shorten the duration of female phase in flowers of *G. longituba* (Fig. 5).

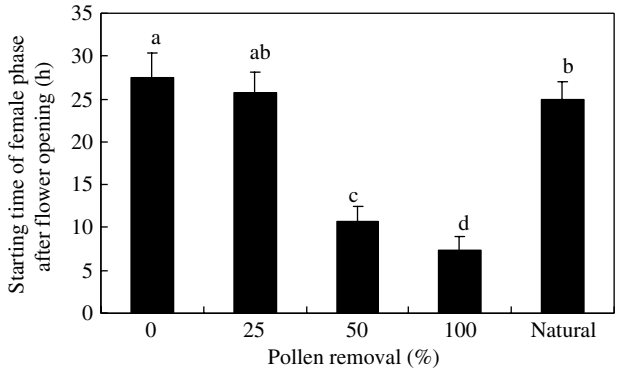

Figure 4. Effect of experimental pollen removal in an early stage of floral male phase on female onset. '0' = no pollen removal; '25' = all pollen in 1 anther removed; '50' = all pollen in 2 anthers removed; '100' = all pollen removed; 'Natural' = pollen removal under natural conditions. Bars displaying the same letters do not differ significantly at $p < 0.05$. (bars are mean ± 1 SE).

Discussion

Effects of pollen removal and/or pollen receipt on the duration of sexual phases

Our results show that pollen removal rate in the male phase affects the onset of the female phase. In addition, pollen deposition at an early stage of the female phase affects female phase longevity. However, after a certain period without pollen removal, the onset of the female phase would occur and be accomplished independently. We also found that artificial removal of most pollen allready at flower opening led to an earlier onset of the female phase, which then started in the afternoon of flower anthesis (during which time pollination rate was low owing to few pollinators). The results support the hypothesis that floral longevity may be changed in response to pollen removal or pollen receipt (Devlin and Stephenson 1984, Proctor and Harder 1995, Ashman and Schoen 1996, Evanhoe and Galloway 2002, Ashman 2004, Giblin 2005, Castro et al. 2008). Changes in floral longevity have been considered as strategies to optimize the balance between resource allocation and reproductive achievements (Schoen and Ashman 1995, Ashman and Schoen 1996, Ishii and Sakai 2001). Total floral longevity, especially in dichogamous species, depends on the longevity of sexual phases (Evanhoe and Galloway 2002, Giblin 2005). When male function (i.e. pollen exportation) is not achieved, onset of female phase is delayed leading to longer floral longevities and greater resource investments in the maintenance of reproductive structures, which may constitute a major energetic cost to the plant (Schoen and Ashman 1995, Ashman and Schoen 1996, 1997). In this context, mechanisms involving specific patterns of pollen presentation may also have

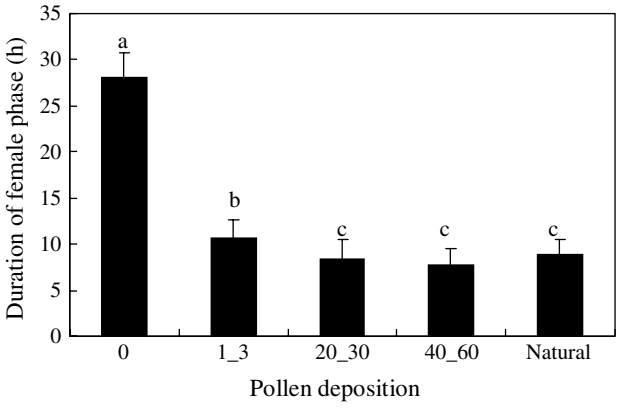

Figure 5. Effect of flowers receiving experimental controlled pollen deposition in the early stage of the female phase on female phase duration. '0' = no pollen put on the stigma; '1_3' = 1–3 pollen grains were on one of the two lobs of the stigma. '20_30' = 20–30 pollen grains put on one of the two lobs; '40_60' = 40–60 pollen grains put on two lobs of the stigma. Bars displayed with the same letters do not differ significantly at $p < 0.05$ (bars are mean ± 1 SE).

evolved to improve male function in response to resource limitation (Dafni 1984, Bertin and Newman 1993). Furthermore, pollen reception significantly diminished the length of female phase leading to flower withering. This occurred even when a low amount of pollen grains was deposited and may be correlated with the low number of ovules in *G. longituba* flowers.

Our results support the findings of Richardson and Stephenson (1989) working on *Campanula rapunculoides*, that timely pollination shorten the duration of the female phase and the floral life-span. Lack of pollinator visitation (either artificially caused by pollinator exclusion or natural due to bad weather) can prolong the floral life-span and the stigma presentation period. In *G. longituba* this situation leads to sexual overlap (e.g. under overcast days; Zhang 2007). In the flower, the anthers of the longer stamens are located at approximately the same level as the stigma, and visiting pollinators are likely to mediate self-pollination during the period of sexual overlap as they forage the flowers for nectar or pollen. It has been reported that self-pollination occurs easily in the sexual overlap phase in other species (Holtsford 1985, Lloyd and Webb 1986, Robertson and Lloyd 1993). In a previous study using pollen staining technique, we demonstrated that almost all pollen on the stigmas of *G. longituba* during the sexual overlap period is self-pollen (Zhang 2007). This results mainly from the low accrual rates of the male and female components which lead to extended periods of male and female phase, and finally, to longer floral longevities and sexual overlap with high levels of self-pollination being observed under natural conditions. These patterns may have several implications. Extended longevities have been shown to compromise reproduction due to trade-offs between sexual accrual rates and resource allocation (Ashman and Schoen 1997, Castro et al. 2008, but see Holtsford 1985) or due to sexual interference. However, under unpredictable conditions and/or scarce pollination services, sexual overlap may constitute a mean of sexual assurance (Robertson and Lloyd 1993, Navarro 1997).

Pollinator behaviour, pattern of nectar secretion and dynamics of pollen removal and deposition

In *G. longituba*, both pollen removal and deposition rates show temporal variation. Pollen was gradually and slowly removed during the morning, with a peak after 1 p.m., while pollen deposition slowly started in the first hour of the morning, reaching its peak at noon. Many studies suggest that the dynamics of pollen removal and deposition are affected by the behavior and number of pollinators, which in turn, are affected by floral rewards (e.g. patterns of nectar secretion; Richardson and Stephenson 1989, Castro et al. 2008). Two possible explanations are proposed for the observed patterns. The first explanation proposes that the main visitors to the flowers of *G. longituba* (the bee *A. plumipes*) forage mainly for nectar from the morning to noon, because there is more nectar during that period, independent of male or female phase flowers (Fig. 2). Despite the differences in nectar secretion by flowers in male and female phase, foraging bees visited flowers indiscriminately during the morning and mid-day, mainly collecting nectar as nectar was more attractive than pollen. In this process, bees remove very few pollen grains but still efficiently pollinate the flowers in the female phase (Fig. 1). This pattern is not yet clearly understood, but several other species present similar nectar secretion patterns and they have been proposed to promote male fitness (Devlin and Stephenson 1985, Mitchell 1993, Aizen and Basilio 1998). In the afternoon only minimal amounts of nectar remained in most of the flowers and *A. plumipes* began to switch for pollen collection. Because most flowers with pollen were in their male phase, *A. plumipes* displayed a preference for male phase flowers during this period, which caused the peak of pollen removal to coincide with this period. A second interpretation is that the species being a low herb, relative humidity around the flowers close to ground is higher in the morning (ca 80–90%) making it difficult for floral visitors to collect pollen, while drier air (ca 40–50%) in the afternoon makes it more conducive for visitors to forage for pollen (Wells and Lloyd 1991, Proctor and Harder 1995). It is likely that the latter situation is more probable. The dynamics of pollen removal and deposition is an intriguing area that is still not well understood and further research is required.

Although in most cases cross-pollination is advantageous to plants, plants may switch to self-pollination for higher reproductive assurance when the environment is unfavorable (Holtsford and Ellstrand 1992, Barrett 1998, 2002). Our investigations show that the sexual phases in *G. longituba* are separated to enhance cross-pollination and avoid self-interference when the weather is fine and warm, and pollinators are abundant. When the weather is cold and overcast, and pollinators are scarce, a significant sexual overlap phase occurs, which is caused by a prolongation of the male phase. The overlap phase may enhance pollinator-facilitated self-pollination and thus serve as a mechanism to ensure reproductive success (Lloyd and Yates 1982, Mallick 2001). This feature of the reproductive system could play an especially important role in species like *G. longituba* that flower in early spring and are commonly exposed to cold, rainy days during blooming. The results of the present study provide support for the hypothesis that dichogamy is a mechanism involving an avoidance of sexual interference and a promotion of cross-pollination (Lloyd and Webb 1986, Evanhoe and Galloway 2002), and provides new insights on mechanisms for sexual assurance in dichogamous species.

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