

Sexual conflict in protandrous flowers and the evolution of gynodioecy

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Sexual interference between male and female function in hermaphrodite plants is reduced by protandry. In environments with insufficient pollinator service, prolongation of male function owing to limited pollen removal could restrict the duration of female function and lower seed production. We provide evidence that this form of sexual conflict has played a role in the spread of females in gynodioecious populations of *Cyananthus delavayi* in the pollen-limited environments in which this subalpine species occurs. Using field experiments involving artificial pollen removal from the strongly protandrous flowers of hermaphrodites, we demonstrated a trade-off between male- and female-phase duration with no influence on overall floral longevity. Pollen removal at the beginning of anthesis resulted in hermaphrodite seed production matching that of females. In contrast, restricted pollen removal increased the duration of male function at the expense of female function lowering maternal fertility compared to females. This pattern was evident in five populations with females experiencing a twofold average seed fertility advantage compared to hermaphrodites. Gynodioecy often appears to evolve from protandrous ancestors and pollen limitation is widespread in flowering plants suggesting that sexual conflict may play an unappreciated role in the evolution of this form of sexual dimorphism.

KEY WORDS: *Cyananthus*, dichogamy, floral longevity, pollen limitation, sexual dimorphism, sexual interference.

Sexual conflict is likely to be ubiquitous in sexually reproducing taxa and arises when traits enhancing the reproductive success of one sex reduce the fitness of the opposite sex (Parker 1979; Rice and Chippindale 2001; Chapman et al. 2003; Tregenza et al. 2006; Perry and Rowe 2018). Sexual conflict has been identified as an important process in species with diverse evolutionary consequences, including antagonistic coevolutionary arms races between female and male traits (reviewed in Arnqvist and Rowe 2005). Although most research on sexual conflict has involved animals with separate sexes, there is now growing evidence for the importance of this process in shaping sex allo-

cation and trait evolution in hermaphroditic organisms (Charnov 1979; Bedhomme et al. 2009; Abbott 2011; Schärer et al. 2014; Olito et al. 2018). Because most plants are hermaphroditic, sexual conflict between male and female function is also likely to be an important feature of their reproductive ecology and evolution.

Owing to the early reluctance of botanists to extend concepts of sexual selection and sexual conflict to plants (reviewed in Skosmyr and Lankinen 2002; Moore and Pannell 2011), there is a general paucity of studies on these topics compared to the extensive literature for animal species (Prasad and Bedhomme

2006; Lankinen and Green 2015). Although explicit investigations using the concept of sexual conflict are relatively limited in plants (but see Lankinen et al. 2006; Madjidian et al. 2012; Duffy et al. 2013; Lankinen et al. 2016), there is a widespread recognition by plant biologists that conflicts between male and female function occur in hermaphroditic flowers, and that such “pollen-stigma/pistil interference” can potentially exert mating costs by hampering successful maternal and paternal outcrossing (Lloyd and Yates 1982; Bertin and Newman 1993; Barrett 2002a; Dai and Galloway 2011; Fetscher 2015). The extent to which the processes involved in sexual interference in flowering plants can be accommodated within the broader conceptual framework of sexual conflict is at present unresolved.

Fundamental to plant mating in angiosperms are the processes of pollen removal and pollen receipt within and among flowers as they determine the quantity and quality of offspring (Barrett and Harder 2017). But in hermaphrodite flowers, the structures and behaviors that are optimal for one sex function (e.g., pollen dispersal) may not be for the alternate sex function (e.g., pollen receipt by stigmas) resulting in considerable scope for various forms of sexual conflict (reviewed in Barrett 2002a). Several floral adaptations that have evolved repeatedly among angiosperm families, including dichogamy (temporal separation of sex functions), herkogamy (spatial separation of sex functions), and heterostyly (polymorphism involving reciprocal herkogamy), are interpreted as mechanisms that reduce lost mating opportunities and/or fertility arising from sexual interference (Lloyd and Webb 1986; Webb and Lloyd 1986; Bertin and Newman 1993; Barrett 2002b; Armbruster et al. 2014). In such cases, interference may be alleviated through the separation of maternal and paternal function in time and/or space, although empirical testing of this hypothesis has proven challenging. Most empirical research on sexual interference between maternal and paternal function of hermaphroditic plants has considered the costs and benefits of separation of parental functions to mating. But there has been little consideration of how this “self-segregation” of parental functions might be influenced by the ecological conditions in which populations occur.

Sexual conflict between sex functions in hermaphrodites occurs most obviously through trade-offs when increasing energetic investment to one sex occurs at the expense of the other (Charnov 1979). Indeed, theoretical models indicate that when allocation to sex functions in hermaphrodites is sexually antagonistic (Jordan and Connallon 2014), and individuals become partially specialized in one or the other of the two sex functions (Olito and Connallon 2019), the evolution of sexual dimorphism is a possible outcome. And this transition in sexual systems may be promoted by conditions involving pollen limitation (Crowley et al. 2017). Most studies of investment trade-offs have focused on

allocation to morphological structures and gamete production, with less attention given to the maintenance costs of reproductive functions. Where the temporal separation of parental functions in hermaphrodite plants occurs through dichogamy (Lloyd and Webb 1986), floral longevity (the period from flower opening until floral senescence) can play an influential role in securing effective pollen removal and receipt (Ashman and Schoen 1996).

In protandrous plants, conflicts in costs associated with the duration of male and female function may occur because of opposing trait optima for the two sex functions. Intrasexual selection for extending the period of pollen presentation in flowers to increase fitness through male function (Lloyd 1984) has the potential to lead to sexually antagonistic interactions with female function, particularly in animal-pollinated species. For populations occupying environments in which pollinator service is unreliable and seed production is pollen limited, prolonged male-phase duration could potentially result in limited opportunities for pollen receipt. Models predicting the optimal duration for male and female function in protandrous flowers indicate strikingly different outcomes depending on whether populations are pollinator limited or not (Lloyd and Yates 1982). To our knowledge, there has been no consideration of how the resolution of sexual interference by protandry could lead to reduced female fertility under pollen-limited conditions.

In this article, we provide evidence that sexual conflict in the functioning of protandrous flowers may have played an unappreciated role in the evolution of gynodioecy, a dimorphic sexual system in which populations are composed of hermaphroditic and female plants. Sexual interference has been previously proposed as a mechanism favoring the evolution of unisexuality through dioecy (Bawa and Opler 1975; Bawa 1980), but sexual conflict has not been explicitly mentioned as a factor in the evolution of gynodioecy, although this sexual polymorphism is often the first step in the evolution of dioecy in many lineages (Charlesworth 1999; Spigler and Ashman 2012). Theory and empirical evidence from gynodioecious species have primarily focused on the conditions favoring the invasion of male-sterile individuals into ancestral hermaphroditic populations. Generally, these populations are self-compatible and selfing and inbreeding depression in hermaphrodites plays a key role in enabling females to establish and spread owing to their outbreeding advantage (Lloyd 1976; Charlesworth and Charlesworth 1978). Differences in the seed fertility of the sexual morphs (Dufay and Billard 2012; Spigler and Ashman 2012) and the genetic basis of male sterility alleles (Gouyon et al. 1991) are also important factors in the evolution of gynodioecy (reviewed in Charlesworth 1999). A recent comparative study investigated the ecological correlates of gynodioecy among angiosperm lineages (Caruso et al. 2016), and in several gynodioecious species harsh environmental conditions

and/or altered or unreliable pollinator service leading to increased selfing and inbreeding depression have played an important role in promoting the spread of females in populations (Ganders 1978; Delph 1990; Weller and Sakai 1990; Case and Barrett 2004; reviewed in Ashman 2006).

Lloyd and Yates (1982) introduced the concept of pollen-stigma interference and the segregation of pollen and stigma function by intrasexual selection, using *Wahlenbergia albo-marginata* (Campanulaceae) as its canonical example. In this family, numerous species exhibit a similar floral mechanism in which flowers are strongly protandrous, with little or no overlap in sex function, and pollen is presented to insect visitors on stylar hairs, a phenomenon known as secondary pollen presentation (Yeo 1993; Vranken et al. 2014). The gradual release of pollen down the style and the prolonged duration of the male phase of flowers serve to spread out the presentation of pollen through time. Hence, opportunities for pollinators to remove and disperse pollen are considerably extended compared to flowers in which pollen is presented simultaneously (Lloyd and Yates 1982). Our study investigated the consequences of this floral mechanism on seed fertility in *Cyananthus delavayi*, a gynodioecious member of Campanulaceae that occurs in subalpine habitats in SW China (Chen et al. 2017). Earlier work indicated that populations receive relatively few pollinator visits and permanent floral closure in the female phase of hermaphrodites triggered by fertilization occurred despite low pollen loads (Niu et al. 2011). These observations motivated us to examine whether the sexual strategy of *C. delavayi* might provide a revealing example of how sexual conflict could set up conditions promoting the evolution of gynodioecy and separate sexes in general.

Here, we investigate the reproductive ecology of protandry in five natural populations of *C. delavayi* in SW China. The goal of our studies was to evaluate whether restricted pollen removal, owing to limited pollinator service, extends the male-phase duration of hermaphrodite flowers causing a fertility cost to female function favoring the spread of females. We began by confirming that pollinator service and fruit set in populations was indeed pollen limited and then addressed the following specific questions: (1) What is the influence of experimentally manipulated pollen removal on male- and female-phase duration and is there evidence of a trade-off between sex functions? We predicted that increased amounts of pollen removal from hermaphrodite flowers would shorten male-phase duration and extend female duration. (2) What is the influence of female-phase duration on the maternal seed fertility of hermaphrodites at both the flower and plant level? We predicted that restricted female-phase duration in hermaphrodites would reduce natural seed set. (3) Is there evidence that female plants in natural populations experience a significant fertility advantage compared to hermaphrodites? A

necessary condition for the maintenance of females in gynodioecious populations is a female fertility advantage. This benefit can be as high as twofold in the case of nuclear inheritance of male-sterility alleles, with gains in seed fitness by females offsetting losses in fitness because they produce no pollen (Charlesworth 1999; Shykoff et al. 2003; Dufay and Billard 2012; Spigler and Ashman 2012). In particular, we asked whether restricted female-phase duration in hermaphrodites caused by undispersed pollen may limit their potential fertility in comparison with females, and that any fitness deficit would be sufficient to account for the evolution and maintenance of gynodioecy.

Methods

STUDY SYSTEM AND FIELD SAMPLING

Cyananthus delavayi is a perennial herb endemic to the mountains of SW China occurring in montane grasslands and on rocky slopes between 1900 to 4000 m with a flowering season from mid-August to late September, depending on elevation. Plants of *C. delavayi* do not reproduce clonally and thus the sampling of genets in populations is straightforward. Flowers are showy, violet-blue in color, tubular-campanulate in structure, with five corolla lobes (Fig. 1). The total number of flowers produced per plant in a season ranges from just a few to several hundred, depending on plant size, and is sometimes higher in females than hermaphrodites (Table S1). Hermaphrodite plants produce larger flowers than females (Niu et al. 2011, 2015), but the number of ovules per flowers does not vary significantly between the sexes (Table S2). Plants are self-compatible, but autonomous and facilitated self-pollination within flowers are prevented by complete protandry. The primary pollinators are nectar-feeding bumble bees (*Bombus richardis* and *Bombus festivus*), pollen-collecting halictid bees (*Halictus* sp.), and hoverflies (Niu et al. 2011; Wang et al. 2017). We conducted pollinator observations in three populations to confirm this earlier work suggesting that pollinator activity is generally low in *C. delavayi* populations. We describe these observations and our results are in the Supporting Information (Table S3 and accompanying results and methods).

We conducted our studies during four flowering seasons from 2016 to 2020 in five populations near Shangri-La city (Yunnan province), which were investigated for different amounts of time depending on the questions outlined below. The codes and locations for the five populations and the number of year(s) each was studied were as follows: (1) Shangri-La Botanical Garden (BG), 2016–2019; (2) Napa Lake (NL), 2017; (3) Bigu Woodland (BW), 2017–2019; (4) Chunzong Village (CV), 2017–2020; and (5) roadside site between Geza and Shangri-La city (GS), 2017–2019 (Table 1; Fig. S1).



Figure 1. The hermaphrodite and female flowers of *Cyananthus delavayi*. (A) Dissected views of the male phase (left) and female phase (right) of hermaphrodite flowers from a hermaphroditic plant. (B) Dissected view of a female flower from a female plant.

Table 1. Locality, elevation, population size, and sex ratio (% females) of the five gynodioecious populations of *Cyananthus delavayi* investigated in this study. Population sizes (number of flowering plants) and female frequencies were estimated in 2017 (populations BG and NL) and 2019 (populations BW, CV, and GS).

Population	Latitude (N)	Longitude (E)	Elevation (m)	Population size	Female frequency (%)
BG	27°54'N	99°38'E	3330	669	0.53
BW	27°30'N	99°46'E	3300	704	0.60
CV	27°54'N	99°40'E	3270	1213	0.35
NL	27°53'N	99°38'E	3310	801	0.51
GS	27°59'N	99°42'E	3522	1780	0.21

DOES POLLEN LIMITATION OF FRUIT SET OCCUR AND DO HERMAPHRODITIC AND FEMALE PLANTS DIFFER?

To determine if pollen limitation of maternal fertility occurs in *C. delavayi* populations and, if so, whether hermaphrodites and females differ, we haphazardly chose and marked 100 female and 100 hermaphroditic plants at the beginning of the 2019 flowering season in populations BW and GS. Plants of each sex were randomly assigned to each of two treatments: (1) open pollination, in which all flowers on each individual were unmanipulated and exposed to pollinators, and (2) supplemental hand-pollination, in

which all flowers were cross-pollinated by hand and then exposed to open pollination. The distance between the pollen recipient and pollen donor was always at least 10 m. Thus, a total of 50 hermaphroditic and 50 female plants received supplemental hand-pollination. Every day throughout the flowering period of each population we pollinated all new receptive flowers in the supplemental hand-pollination treatment. We collected fruits at the end of flowering and percent fruit set per plant was determined. Data on the mean number of open-pollinated flowers and those that received supplemental cross pollination per plant in each population and sex type are provided in the Supporting In-

formation (Table S4a). We calculated a pollen limitation index (PL) using the formula, $PL = 1 - (P_o/P_s)$, following Larson and Barrett (2000). P_o is the average percent fruit set of open-pollinated plants of each sex and P_s is the percent fruit set of plants of each sex that received supplemental cross pollen. The PL index ranges from 0 (no pollen limitation) to 1 (complete pollen limitation).

FLORAL LONGEVITY AND THE INFLUENCE OF POLLEN REMOVAL ON SEX FUNCTION

To investigate the floral longevity of sexual morphs and the influence of pollen removal on the duration of male and female sex function, we randomly sampled 37–44 plants at peak flowering in populations BG (2016 and 2017) and CV (2017). On females, two flower buds per individual were marked and randomly assigned to either open-pollinated or bagged treatments. For hermaphrodites, four flower buds per individual were marked, one flower was left intact to be pollinated naturally (open pollination), and each of the three remaining flowers received one of the following treatments assigned randomly: (1) no pollen removed (intact), (2) 50% pollen removed, and (3) 100% pollen removed, and were then bagged. Because removing pollen causes spreading of stigmatic lobes and accelerates entry into female phase (Niu et al. 2015), we predicted that this process may vary depending on the quantity of pollen removed from flowers.

Pollen removal was conducted using a fine paintbrush dampened with distilled water immediately after petal opening when anthers had dehisced (see Niu et al. 2015). In our bagging treatments of hermaphrodite and female flowers, we used nylon nets to exclude pollinators and the possibility of pollination, which can significantly reduce floral longevity in *C. delavayi* (Niu et al. 2011, Niu et al. 2015). All marked flowers were monitored twice daily at 0900h and 1700h until corolla wilting. Total floral longevity was the length of time from the beginning of anthesis until corollas wilted. For marked hermaphroditic flowers, we measured the male phase of each flower as the length of time between the beginning of anthesis and onset of the female phase. We considered the female phase in which stigmas are receptive, the period from stigmatic lobe expansion until corolla wilting. We confirmed that this morphological assessment of stigma receptivity was a reasonable proxy for receptivity using the hydrogen peroxide test in population CV in 2020 (see Fig. S2 and accompanying methods).

THE EFFECT OF FEMALE-PHASE DURATION ON POLLINATION SUCCESS AT FLOWER AND PLANT LEVELS

To investigate whether the female-phase duration of hermaphroditic flowers influenced pollination success, as measured by fruit and seed set, we manipulated the duration

of female function in populations BG in 2016 and 2017 and populations BW, CV, GS and NL in 2017. This was achieved by removing different amounts of pollen from flowers, as described in the preceding section. In each population, we randomly selected ~40 hermaphrodite plants at peak bloom and three flower buds per individual were assigned to each of three treatments: (1) no pollen removed, (2) 50% pollen removed, and (3) 100% pollen removed. Forty flower buds from 40 females were also randomly selected (one bud per individual) in each population at the same time to compare pollination success between hermaphroditic and female flowers under natural conditions. All sampled flowers were then left to be open-pollinated. To limit the influence of display size on pollination success, we maintained a daily display size of four flowers per plant in all populations throughout the experiment by flower removal. When fruits were mature (~30 days after flower wilting), we determined the number of seeds per fruit.

To investigate the influence of female-phase duration in hermaphrodites on pollination success at the plant level, we manipulated female duration of whole plants in three populations (BG, BW, and GS) in 2018. In each population, we randomly selected 120–150 individuals at peak bloom and 40–50 individuals were assigned to each of three treatments as above: (1) open-pollinated flowers left intact, (2) 50% pollen removed from flowers, and (3) 100% pollen removed from flowers. Eight flowers on each plant received one of these treatments and were then left to be open-pollinated. All other flowers on plants were removed to limit the influence of floral display size on pollination success. To compare the pollination success of open-pollinated hermaphrodite and female plants, 40–50 female individuals with eight flowers per plant were also randomly selected. Seed production per plant was then measured, as described above.

DO FEMALE PLANTS EXHIBIT A FERTILITY ADVANTAGE IN NATURAL POPULATIONS?

We determined if female plants experienced a female fertility advantage (FA) in natural populations by comparing the seed production of females and hermaphrodites. This was achieved by haphazardly choosing and individually marking 30–50 individuals of each sex at the beginning of the flowering season in five populations (BG 2016, 2017, 2019; CV, NL 2017; BW, GS 2019). We collected all fruits per plant at the end of the fruiting season before they dehisced and the total number of seeds per plant was determined. To test the prediction that female plants produce more total seeds than hermaphrodite plants, we used one-tailed *t* tests to compare the seed production per individual between the sex types within each population. If, on average, female plants produced more seeds than hermaphrodites, then FA would be evident. We calculated FA by dividing the average total seed

production of open-pollinated females by that of open-pollinated hermaphrodites, following Dufay and Billard (2012).

STATISTICAL ANALYSIS

All statistical analyses were conducted in R (version 3.5.3) and the data are presented as means \pm 1 SE, where applicable. We tested for differences in ovule number per flower between hermaphrodite and female flowers in each population using *t* tests. We investigated how fruit set varied according to sex and treatment using general linear mixed models with sex type (female and hermaphrodite), pollination treatment (open pollination vs. supplementary pollination) and their interaction as fixed effects, flower number per plant as a covariate, and population as a random effect. To examine effects of sex type, pollinator type, and population on the frequency of pollinator visitation to flowers, we used a generalized linear model (GLM) model with a binomial distribution and logit link function.

Differences in total floral longevity of bagged hermaphrodite flowers among pollen removal treatments and populations were examined using two-way ANOVA with “Treatments” and “Population” as main effects, after testing for normality and homogeneity of variances using the Shapiro-Wilks and Bartlett tests, respectively. Data were log transformed to meet assumptions of normality. We determined the male and female duration of open-pollinated (unbagged) hermaphrodite flowers and calculated their relative value by dividing the mean male and female durations by the maximum longevity of bagged hermaphrodite flowers in each population. We tested differences in longevity of bagged and open-pollinated flowers in each population by one-way ANOVA. We compared differences in sex phase duration between bagged and open-pollinated hermaphrodite flowers in each population using *t* tests. We explored the relations between the duration of the male and female phase in bagged hermaphrodite flowers using linear regression, and then tested for differences among the slopes of populations using analysis of covariance (ANCOVA). We tested for differences in female-phase duration among manipulated hermaphrodite flowers (0%, 50% and 100% pollen removal and bagged) and bagged female flowers using one-way ANOVA followed by the Tukey-Kramer HSD, and used GLMs with a Poisson distribution log-link function to examine the effect of female-phase duration on seed production at the flower and plant levels.

Results

POLLEN LIMITATION CHARACTERIZES BOTH HERMAPHRODITES AND FEMALES

In both populations of *C. delavayi* that we investigated, there was clear evidence of significant pollen limitation of fruit set in

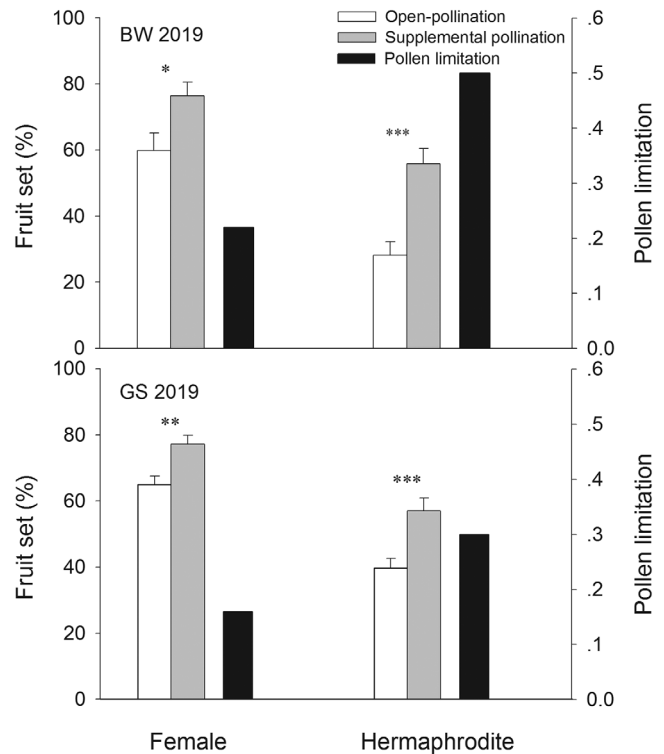


Figure 2. Comparison of mean fruit set following open pollination and supplemental hand-pollination in female and hermaphrodite plants in populations BW and GS of gynodioecious *Cyananthus delavayi*. Fruit set was calculated as the proportion of flowers that produced fruit per individual. Pollen limitation is represented by the pollen limitation (PL) index and ranges from 0 to 1.0; PL index = $1 - P_o/P_s$, where P_o and P_s are shown in white and gray bars, respectively. Asterisks above bars indicate significant differences between pollination treatments in each sex type (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Error bars are 2 SE. See statistical results in Table 2.

hermaphrodite and female plants (Fig. 2; Table 2). In population BW, fruit set was significantly higher when supplemental pollen was added to hermaphrodites (open pollination = 28.1%, supplemental pollination = 55.9%; $\chi^2 = 15.70$, $P < 0.001$) and females (open pollination = 59.8%, supplemental pollination = 76.4%; $\chi^2 = 4.98$, $P < 0.017$). The PL index for hermaphrodites and females was 0.50 and 0.22, respectively. Similar patterns were evident in population GS for hermaphrodites (open pollination = 39.8%, supplemental pollination = 57.1%; $\chi^2 = 10.89$, $P < 0.001$) and females (open pollination = 64.9%, supplemental pollination = 77.2%; $\chi^2 = 8.90$, $P < 0.01$). The PL index for hermaphrodites and females in population GS was 0.30 and 0.16, respectively. The likelihood of open-pollinated fruit set was similar in both populations, but a significant effect of sex type indicated that hermaphrodites exhibited greater pollen limitation than females in both populations (Tables 3 and S4).

Table 2. Fruit set (%) and pollen limitation (PL) index of gynodioecious *Cyananthus delavayi* following open and supplementary pollination in populations BW and GS. The number of plants (*n*) sampled per sex type is provided in parentheses.

Population	Sex type	P_o	P_s	PL index
BW	female	59.8 ± 5.2 (29)	76.4 ± 4.3 (44)	0.217
	hermaphrodite	28.1 ± 42(48)	55.9 ± 4.4(42)	0.497
GS	female	64.9 ± 2.6 (48)	77.2 ± 2.7 (43)	0.159
	hermaphrodite	39.8 ± 3.3(48)	75.1 ± 3.3(47)	0.530

P_o , fruit set of open-pollinated plants; P_s , fruit set of plants that received supplemental cross pollen; PL index = $1 - (P_o/P_s)$.

Table 3. Results of general linear mixed model (GLMM) testing whether fruit set differs significantly between hermaphrodite and female plants of gynodioecious *Cyananthus delavayi* following open and supplementary pollination in populations BW and GS.

Effect	<i>df</i>	<i>F</i>	<i>P</i> -value
Sex type	1	91.6165	<0.001
Treatment	1	49.1737	<0.001
Flowers per plant	1	0.1841	0.6682
Sex type × Treatment	1	2.4672	0.1172

FLORAL LONGEVITY OF BAGGED AND UNBAGGED FLOWERS

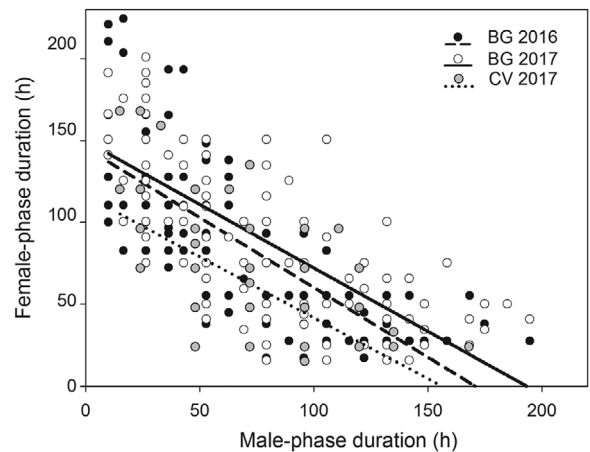
The floral longevity of open-pollinated (unbagged) and bagged flowers in females and hermaphrodites in BG 2016, BG 2017, and CV 2017 revealed similar patterns (Fig. S3). In females, bagged flowers remained open significantly longer than open-pollinated flowers. In contrast, in two of the three populations the floral duration of hermaphrodite flowers did not differ significantly between bagged and unbagged flowers.

SEX-PHASE DURATION OF HERMAPHRODITE FLOWERS

The sex-phase duration of open-pollinated (unbagged) and bagged hermaphrodite flowers in BG 2016, BG 2017, and CV 2017 revealed similar patterns (Fig. S3). Male-phase duration of bagged flowers was significantly longer than open-pollinated flowers. In contrast, female-phase duration of bagged flowers was significantly shorter than that of open-pollinated flowers. On average, the male phase of unbagged hermaphrodite flowers was 45.4% of the maximum longevity of bagged hermaphrodite flowers across the three populations.

POLLEN REMOVAL EXTENDS THE FEMALE FUNCTION OF HERMAPHRODITE FLOWERS

The maximum total longevity of unpollinated (bagged) hermaphroditic flowers of *C. delavayi* varied significantly among populations but was not influenced by pollen removal treatment

**Figure 3.** Relations between male- and female-phase duration for protandrous hermaphroditic flowers in populations: BG (2016), BG (2017), and CV (2017) of gynodioecious *Cyananthus delavayi*. All points are from bagged flowers of the three treatments in the experiment. Lines represent least-squares regressions lines for each population. BG (2016) $y = 126.5 - 0.8x$, $R^2_{120} = 0.53$, $P < 0.001$; BG (2017) $y = 143.1 - 0.8x$, $R^2_{120} = 0.56$, $P < 0.001$; CV (2017) $y = 122.0 - 0.8x$, $R^2_{111} = 0.46$, $P < 0.0001$.

(0%, 50%, and 100% pollen removal), as indicated by the non-significant “treatment” and “treatment × population” interaction (Table S5). Across all treatments, female-phase duration varied negatively with that of male-phase duration (Fig. 3), but the interaction “male-phase duration × population” was not significant ($P = 0.985$) indicating no differences among populations in the slopes of these relations. In each of the three studied populations, female-phase duration of the 100% pollen removal treatment was significantly longer than that of the 50% pollen removal treatment, which in turn was much longer than was observed in intact flowers (Fig. 4). This general pattern indicates that the duration of female function increased with the amount of pollen removed from flowers at the beginning of anthesis. Depending on the amount of pollen removed, hermaphrodite flowers can potentially have female-phase durations matching those of female flowers (Fig. 4).

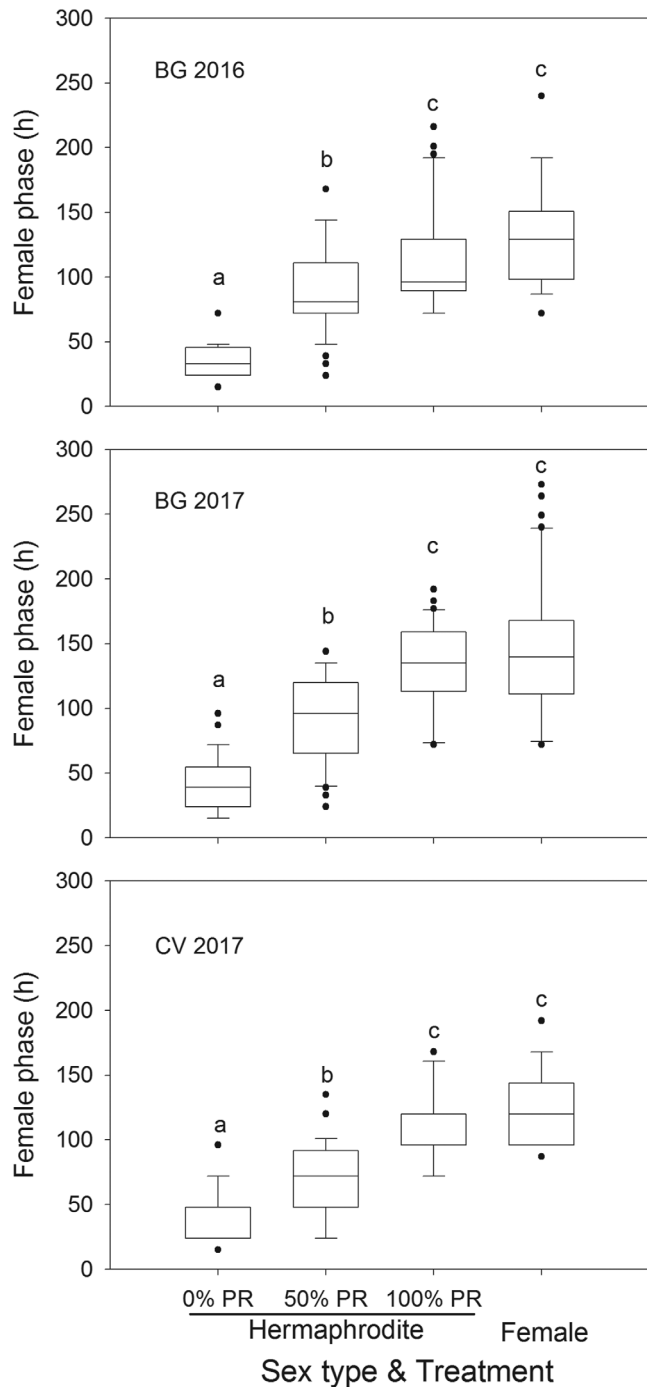


Figure 4. The effects of manipulated pollen removal from flowers on female-phase duration of unpollinated, bagged, hermaphroditic flowers in populations: (A) BG 2016, (B) BG 2017, and (C) CV 2017 of gynodioecious *Cyananthus delavayi*. All panels display box plots based on raw data, indicating the median, interquartile range (hinges showing 25th and 75th percentiles), 1.5 times the interquartile range (whiskers), and outliers. PR = pollen removal. Different letters above the plots indicate significantly different LS means after Tukey-Kramer correction for multiple testing ($P < 0.05$).

GREATER POLLEN REMOVAL RESULTS IN INCREASED SEED PRODUCTION OF HERMAPHRODITE FLOWERS AND PLANTS

In open-pollinated hermaphrodite flowers, fruit and seed production generally increased with the more pollen experimentally removed from flowers at the beginning of anthesis (Fig. 5). This pattern was evident in each of the five studied populations at the flower level. The seed set values obtained for hermaphroditic flowers with 100% pollen removal were not significantly different from flowers on female plants (Fig. 5). No seeds were produced in plants that were bagged before opening in the three populations indicating that autonomous self-pollination is unlikely to have contributed to the variation in seed set of open-pollinated flowers. This result occurred because of complete protandry and the absence of any overlap in male and female function in hermaphroditic flowers.

Similar results demonstrating that greater pollen removal from flowers was associated with increased seed production were also evident in our whole plant manipulations. Seed production of hermaphrodite plants increased with the more pollen that we removed experimentally from flowers (Fig. 6). Also, hermaphrodite plants produced as many seeds as females when all pollen was removed from flowers at the beginning of flowering.

FEMALES EXHIBIT A TWOFOLD FEMALE FERTILITY ADVANTAGE OVER HERMAPHRODITES

We tested the prediction that female plants would produce more seeds than hermaphrodite plants using fruit and seed set data collected from five natural populations of *C. delavayi*. In each population, female plants produced significantly more seed than hermaphrodite plants (Table 4). The average fertility advantage (FA) was 2.1 ± 0.1 ($n = 7$, range 1.6–2.7).

Discussion

Our investigation of gynodioecious *C. delavayi* provides evidence that the combination of sexual conflict between the timing of male and female function in hermaphrodites and pollen-limited conditions favors the spread of females in populations, owing to a twofold advantage in their female fertility. The possibility that the functioning of protandry might be compromised by limited pollinator service resulting in the evolution of gynodioecy has not been considered previously. Below, we discuss our experimental results demonstrating a trade-off between male- and female-phase duration in hermaphrodites and its implications for reproductive function under ecological conditions causing pollen limitation. We also consider alternative hypotheses

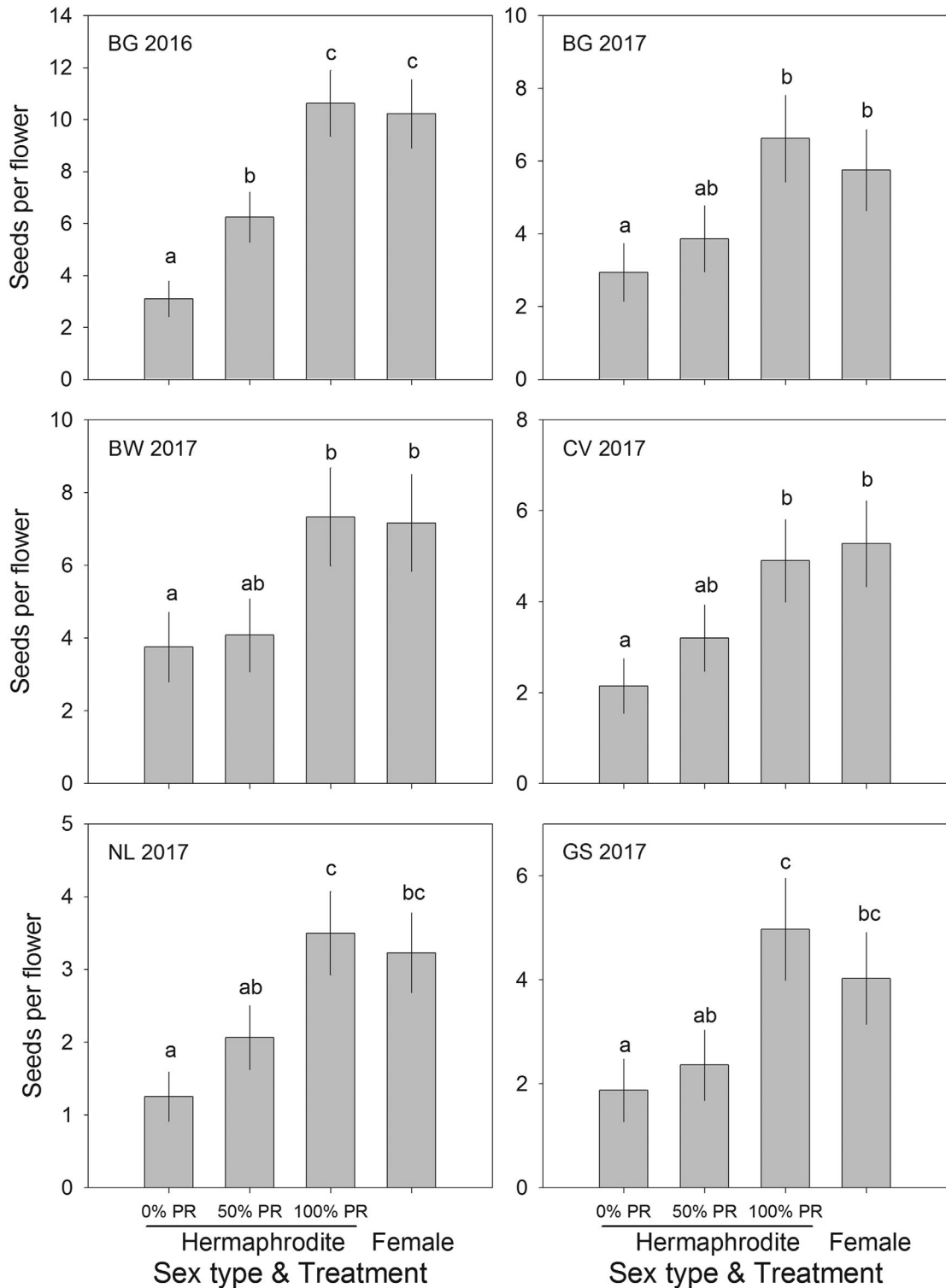


Figure 5. The effects of manipulated pollen removal on mean seed per flower of open-pollinated hermaphroditic flowers in natural populations: (A) BG 2016, (B) BG 2017, (C) BW 2017, (D) CV 2017, (E) NL 2017, and (F) GS 2017 of gynodioecious *Cyananthus delavayi*. Error bars are 2 SE, PR = pollen removal. Different letters indicate significant differences ($P < 0.05$) after Tukey contrasts ($P < 0.05$).

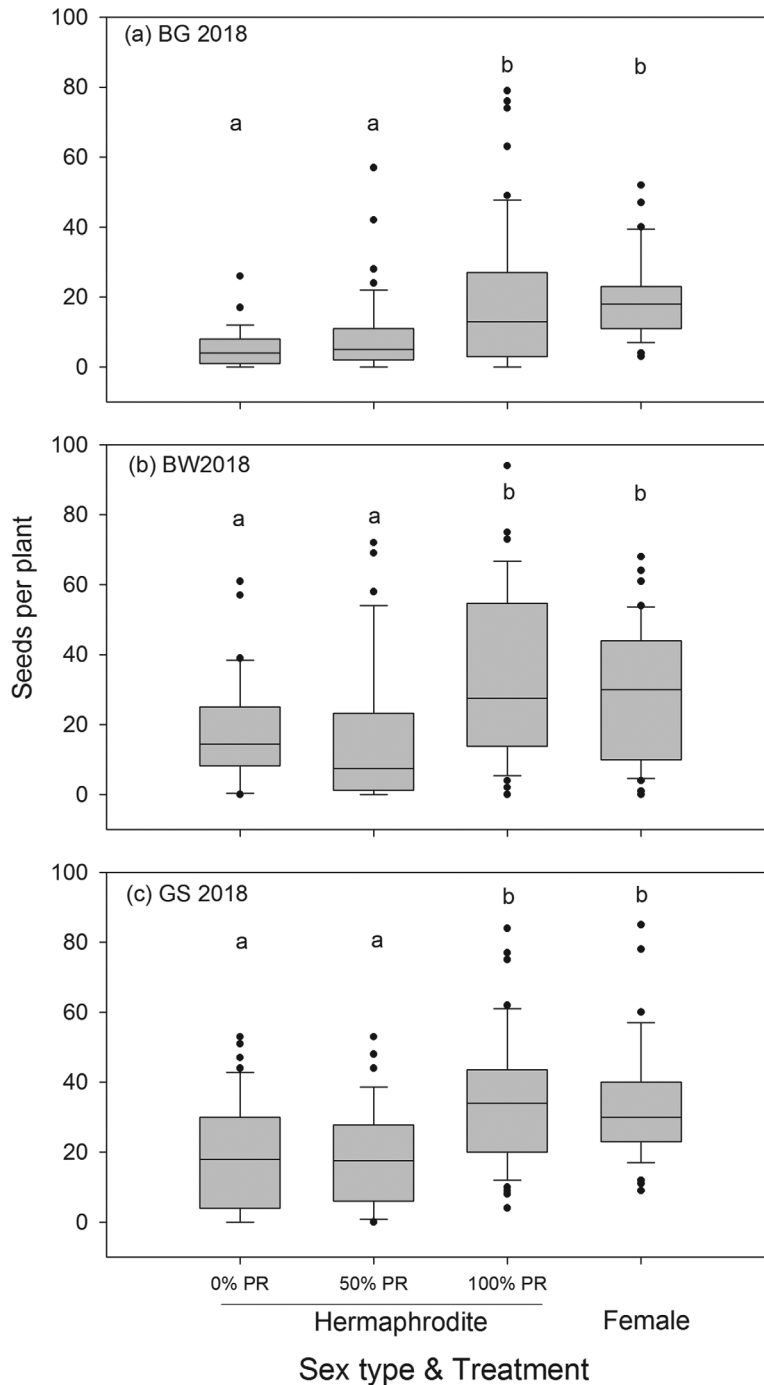


Figure 6. The effect of manipulated pollen removal on mean seeds per plant of open-pollinated hermaphrodite individuals in natural populations: (A) BG 2018, (B) BW, and (C) GS of gynodioecious *Cyananthus delavayi*. All panels display box plots based on raw data, indicating the median, interquartile range (hinges showing 25th and 75th percentiles), 1.5 times the interquartile range (whiskers), and outliers. PR = pollen removal. Different letters indicate significant differences ($P < 0.05$) after Tukey contrasts ($P < 0.05$).

that might contribute to the spread of females in hermaphroditic plant populations and conclude by discussing the relevance of our findings to more general aspects of sexual conflict in plants.

SEXUAL CONFLICT IN PROTANDROUS FLOWERS

Protandry is the commonest form of dichogamy and is most often interpreted as a floral mechanism reducing sexual interference between paternal and maternal sex function in hermaphroditic species (Lloyd and Webb 1986; Bertin and Newman 1993). The

Table 4. Tests of the prediction that female plants should produce more seeds than hermaphrodite plants in five natural populations of gynodioecious species *Cyananthus delavayi*. The number of plants sampled per sex type is provided in parentheses. See Methods section for the calculation of female advantage (FA).

Population (year)	Seeds per individual (Mean \pm 1 SE)			P-value (one-tailed)	Female advantage (FA)
	Female	Hermaphrodite			
BG (2016)	150.1 \pm 21.8 (35)	54.7 \pm 20.6 (39)		0.0011	2.7
BG (2017)	30.1 \pm 5.9 (33)	13.3 \pm 5.9 (33)		0.0175	1.9
BG (2019)	33.4 \pm 4.9 (49)	14.3 \pm 4.5 (58)		0.0024	2.3
BW (2019)	39.4 \pm 6.0 (29)	17.1 \pm 4.7 (48)		0.0023	2.3
CV (2017)	14.1 \pm 2.3 (33)	8.0 \pm 2.3 (33)		0.0317	1.8
NL (2017)	10.6 \pm 1.7 (30)	4.7 \pm 1.7 (30)		0.0088	2.3
GS (2019)	76.1 \pm 8.8 (47)	47.5 \pm 8.7 (48)		0.0117	1.6

life span of protandrous flowers determines the scope for sexual segregation and is also associated with the frequency of floral visits by pollinators and opportunities for pollen export and import (Arroyo et al. 1985; Primack 1985; Bingham and Orthner 1998; Rathcke 2003; Aximoff and Freitas 2010). However, floral lifespan can be constrained by maintenance costs (Ashman and Schoen 1996) and in protandrous species there will necessarily be a trade-off between the allocation of time and resources to the two sex functions (Devlin and Stephenson 1984). Selection experiments on *Chamerion angustifolium* have demonstrated a negative phenotypic correlation between male- and female-phase duration in the protandrous flowers of this species (Routley and Husband 2005).

By bagging hermaphrodite flowers of *C. delavayi* and preventing pollination, we were able to measure the maximum duration of male and female sex phases and floral longevity (Figs. S3 and S4). We found a significant difference in duration of the sex functions, with the male phase two to three times longer than the female phase, a pattern consistent with sexual selection to increase mating opportunities through male function (Lloyd and Yates 1982). However, by experimentally manipulating the quantity of pollen removed from flowers at the beginning of anthesis we demonstrated considerable plasticity in sex-phase duration (Fig. 4), but not in overall floral longevity (Table S5), which in some species can also be plastic (Webb and Littleton 1987; Ashman and Schoen 1997; Spigler 2017).

When all pollen was removed from flowers, male function rapidly transitioned to female function, whereas restricting pollen removal induced a prolongation of the male phase, with the duration depending on the amount of pollen removed. As long as some pollen remained, the stigmatic lobes failed to spread open, which is a signal of receptivity. Plotting the duration of male and female phases of bagged flowers among these experimental treatments in each of three populations clearly revealed a negative correlation between sex functions (Fig. 3). We have not identified the proximate physiological mechanism enabling female function to respond plastically to the quantity of pollen present on styles; however, this flexibility in sexual behavior may be adaptive and related to variable pollination environments. Plants with secondary pollen presentation (Yeo 1993) may be more likely to possess mechanisms for recognizing the presence of undispersed pollen and adjusting female function, and this possibility would be worth investigating.

Significantly, if all pollen was removed from flowers at the start of anthesis the female phase was very similar to the total duration in which flowers on female plants were functional (Fig. 4). This equivalence in duration of female function was associated with similar levels of seed set between the sexual morphs at both flower and plant levels (Figs. 5 and 6). The implication of this result is that under ecological conditions in which pollinator

service to *C. delavayi* populations is reliable and pollen is effectively dispersed among plants, females would experience no seed fertility advantage over hermaphrodites and gynodioecy is unlikely to evolve. Limited or no pollen removal from hermaphroditic flowers resulted in a significantly longer male-phase duration at the expense of female function. The shortened female duration provided reduced opportunities for pollen import and this was associated with a significantly reduced seed set of hermaphrodites in comparison with females. Importantly, these experimental results were quite general occurring in all five populations that we investigated at the flower level (Fig. 5), and all three populations at the plant level (Fig. 6). Thus, we have demonstrated using experimentally manipulated flowers that a shortened duration of female function results in a significant fertility cost to hermaphrodite plants, and that this establishes conditions favoring an evolutionary transition from hermaphroditism to gynodioecy.

The hermaphrodite disadvantage in seed fertility is only likely to be realized in environments with unreliable pollinator service. Our measurements of pollinator visitation (Table S3) support earlier work on *C. delavayi* indicating low levels of pollinator activity (Niu et al. 2011). Consistent with these observations is our finding, based on comparisons between open- and hand-pollinated flowers, that fruit set in both sexual morphs was significantly pollen limited (Fig. 2). This finding was also reported by Chen et al. (2017) in three populations of *C. delavayi*. Montane regions commonly experience harsh environmental conditions that limit pollinator activity (Arroyo et al. 1985; Körner 2003) causing pollen limitation (García-Camacho and Totland 2009). Indeed, there is evidence that pollen-limited conditions in the subalpine habitats occupied by *C. delavayi* can promote other transitions in reproductive systems, although this more often involves shifts from outcrossing to selfing (e.g., Richards 1997; de Vos et al. 2012; Yuan et al. 2017) than the spread of unisexuals. A phylogenetic reconstruction of the evolutionary history of sexual systems in a clade of five species of *Cyananthus* inferred a single origin of gynodioecy associated with a shift from wet meadows to alpine habitats (Zhou et al. 2013). The transition from sexual monomorphism to dimorphism may be associated with contrasting pollinator environments and deterioration in reliable insect visitation at higher altitudes.

EVOLUTION OF GYNODIOECY AND CAUSES OF THE FEMALE ADVANTAGE

Gynodioecy is reported from less than 1% of flowering plants but is distributed across numerous plant families, especially those that are herbaceous and temperate in distribution (Godin and Demyanova 2013; Caruso et al. 2016). Bertin and Newman (1993) drew attention to the prevalence of protandry in hermaphrodites

of gynodioecious species, although a rigorous comparative analysis would be valuable to confirm this association and determine how frequent protandry is as an ancestral state to gynodioecy. These authors suggested that intrasexual (male) competition because of the availability of female flowers might explain this apparent association. An alternative perspective is that protandry under unreliable pollination environments might also favor the spread of females and promote the evolution of gynodioecy, the hypothesis we propose here.

Theoretical work indicates that a necessary condition for the evolution and maintenance of females in gynodioecious populations is a “female advantage” in seed fertility (Lewis 1941; Lloyd 1976; Charlesworth 1999; Dufay and Billard 2012). The threshold advantage allowing females to spread can vary in magnitude from high to low, depending on whether the genes causing male sterility are nuclear or cytoplasmic, respectively. In theory, this could range from a single additional seed under cytoplasmic male sterility, to a twofold differences in seed production with nuclear inheritance. Key fitness components influencing the seed fertility of the sexual morphs include flower number, ovule number, fruit and seed production, and seed quality (reviewed in Shykoff et al. 2003; Dufay and Billard 2012). In *C. delavayi*, there was no evidence that ovule number per flower differed between sexual morphs (Table S2) and female flower number was higher in some populations but not others (Table S1). In common with many other gynodioecious species (see Fig. 2 in Spigler and Ashman 2012), we found that females on average produced twice as much seed compared to hermaphrodites (Table 4). This seed fertility advantage was evident in each of the seven samples we obtained from five populations. The magnitude of this difference should allow females to be maintained in *C. delavayi* populations, assuming that their seed quality is equivalent or higher than hermaphrodites, as seems probable given they are obligately outbreeding. Although the genetic basis of male sterility in *C. delavayi* is unknown, the magnitude of the female advantage is clearly sufficient for the maintenance of females under nuclear inheritance.

An unresolved question concerns the proximate cause of the female fertility advantage in *C. delavayi*. As there is no evidence in this species for strong reproductive compensation involving ovule or flower number, the answer likely involves differences between the sexual morphs in pollination and postpollination processes. Our investigation of pollen limitation provided some insight to this question. Females in gynodioecious populations might be predicted to suffer greater pollen limitation than hermaphrodites because they cannot self-pollinate. However, we found the reverse with significantly higher pollen limitation of fruit set in hermaphrodites (Fig. 2). Although a meta-analysis of gynodioecious species reported no consistent differences in pollen limitation between sexual morphs, female fruit set was

generally higher than that of hermaphrodites (Shykoff et al. 2003; Spigler and Ashman 2012), a pattern in accord with our findings. However, in *C. delavayi* differences in pollen limitation cannot fully explain the observed female advantage because although hand-pollinated flowers of both sexual morphs had significantly higher fruit set than open-pollinated flowers, the female fertility advantage was still evident (Fig. 2).

Selfing and inbreeding depression could potentially contribute to the lower hermaphrodite fruit and seed set evident in our study. Opportunities for self-pollination are, however, restricted in *C. delavayi* because intrafloral self-pollination is prevented because of strong protandry. Moreover, daily floral display sizes are relatively small and geitonogamous self-pollination is therefore unlikely to be as pervasive as in many other species with larger floral displays. Our observations of pollinators indicated that they visited very few flowers per plant during foraging bouts (Table S6). Nevertheless, some limited degree of geitonogamy is certainly possible, especially as *C. delavayi* is visited by generalist pollinators, including halictid bees and hoverflies (Niu et al. 2011; Wang et al. 2017; Table S6), that often forage within a plant. Early-acting embryo abortion resulting from selfing and inbreeding depression could therefore play some role in reducing female fertility in hermaphrodites. Also, differences in resource allocation may also contribute to the lower fertility of hermaphrodites because they invest in pollen production and have larger flowers.

A significant component of female advantage in *C. delavayi* resulted from percent fruit set differences between the sexual morphs (Fig. 2). Empirical evidence demonstrating inbreeding depression for fruit set is surprisingly sparse and much less common than for seed set (see Raduski et al. 2012, p. 1277), thus alternative hypotheses for female advantage are worth considering. One possibility is that the reduced fruit set of hermaphrodites is caused by female sterility alleles that are commonly associated with increased “maleness” of hermaphrodites in the gynodioecious pathway (Lloyd 1974, 1976). Another potential explanation is that under pollen-limited conditions and a brief duration of hermaphrodite female function, low pollen loads may result in fruit abortion because of few developing embryos within fruits (Stephenson 1981). Experimental studies in *C. delavayi* have demonstrated permanent floral closure with low pollen loads insufficient to produce fruit despite fertilization of some ovules (Niu et al. 2011). Because of the fitness cost to female function in hermaphrodites of permanent floral closure, Niu et al. (2011) proposed that this behavior may represent an example of sexual conflict in which early pollen donors secure their own paternity at a cost to female fitness. Similar arguments invoking sexual conflict have also been proposed by Lankinen and colleagues concerning the timing of stigma receptivity in *Collinsia* (Lankinen and Kiboi 2007; Madjidian et al. 2012; Lankinen and Strandh 2016).

GENETICS AND ECOLOGY OF PLANT SEXUAL CONFLICT

Incorporating elements of the conceptual framework of sexual conflict theory to hermaphroditic plants has proven challenging. Currently, there are limited data on sexually antagonistic genetic variation and selection in plant populations (but see Campbell 1989; Delph and Ashman 2006; Delph and Herlihy 2012). Unlike dioecious species in which sexually antagonistic selection operates on the total fitness of individuals, studies of selection in hermaphrodites involve identifying several fitness components. Nevertheless, as first pointed out by Morgan (1994), because reproductive success through both male and female function cannot evolve separate fitness peaks, as occurs in dioecious species, the potential for sexual conflict may in fact be larger in hermaphrodites.

Motivated by their studies of intrasexual selection and the avoidance of pollen-stigma interference in *W. albomarginata* (Campanulaceae), a species with a similar floral biology to *C. delavayi*, Lloyd and Yates (1982) investigated theoretically the optimal duration of male and female sex phases in a population with strongly protandrous flowers. They found that the evolutionary stable strategy was for flowers to have equal male and female sex phases under pollinator-limited conditions. In contrast, under conditions in which pollinator service was reliable, with maternal fitness limited by resources and paternal fitness limited by competition for ovules, male duration should exceed female duration, a pattern in accord with our data from open-pollinated and bagged flowers of *C. delavayi* (Fig. S4). However, we have also shown that populations of *C. delavayi* receive low pollinator service and experience pollen-limited conditions (and see Niu et al. 2011; Chen et al. 2017). How can the apparent mismatch between Lloyd and Yates' (1982) theoretical predictions and the pollination environments in which *C. delavayi* occurs be reconciled?

A possible explanation is that developmental and/or genetic constraints prevent selection for increased female duration in hermaphrodite flowers. If true and changes to sex-phase duration are constrained in hermaphrodites, the spread of females in populations may be a consequence of the conflict created by pollen-limited conditions. Unsatisfactory pollinator service has most often been invoked to explain the evolution of selfing as a result of the benefits of reproductive assurance under chronic pollinator limitation (Eckert et al. 2006). However, pollinator limitation can promote diverse reproductive outcomes, including the selection of alternative means of achieving outcrossing (Harder and Aizen 2010), including the evolution of sexual dimorphism (Crowley et al. 2017). Thus, as long as pollinator service is not chronic and females maintain a fertility advantage over hermaphrodites, the evolution of gynodioecy may be an unexpected outcome of sexual conflict in protandrous plant populations.

AUTHOR CONTRIBUTIONS

ZQZ, SCHB, and QJL conceived and designed the study. HW, XYL, and YN performed the experiments. HW, ZQZ, and YWD analyzed the data. ZQZ and SCHB wrote the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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DATA ARCHIVING

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. (a) Total flower number per plant (Mean \pm 1SE) between hermaphrodites and females in natural populations of *Cyananthus delavayi*.

Table S1. (b) Results of generalized linear model (GLM) with a Poisson distribution and link-logit function to test whether total flower number per plant differs significantly between hermaphrodite and female plants in six populations of gynodioecious *Cyananthus delavayi*.

Table S2. Ovule number per flower of hermaphrodite and female plants in four populations of gynodioecious *Cyananthus delavayi*.

Table S3. (a) Evidence for the low frequency of insect visits to hermaphrodite (H) and female (F) flowers of gynodioecious *Cyananthus delavayi* in three natural populations.

Table S3. (b) GLM examining the effects of sex type, population and pollinator type on insect visitation in three populations of gynodioecious *Cyananthus delavayi*.

Table S4. (a) The number (mean \pm 1SE) of open-pollinated and supplemental-pollinated flowers per female and hermaphrodite plants in two populations of gynodioecious *Cyananthus delavayi*.

Table S4. (b) GLM examining the effects of sex type and population on pollen limitation index in two populations of gynodioecious *Cyananthus delavayi*.

Table S5. Two-way ANOVA examining the effects of experimental treatment (0%, 50%, 100% pollen removal) on the total floral longevity of unpollinated and bagged, hermaphrodite flowers in three populations of gynodioecious *Cyananthus delavayi*.

Table S6. The number of hermaphrodite flowers that were visited on each plant (Mean \pm 1SE) per foraging bout by pollinators in three natural populations of *Cyananthus delavayi*.

Figure S1. The location of the five populations of gynodioecious *Cyananthus delavayi* in the vicinity of Shangri-La city, Hengduan Mountains, SW China that were investigated in this study.

Figure S2. Estimate of stigma receptivity based on the percent of stigmas each day that produced bubbles after hydrogen peroxide treatment for female and hermaphrodite flowers in populations CV (2020) of gynodioecious *Cyananthus delavayi*.

Figure S3. Floral duration of open-pollinated and bagged flowers in populations BG (2016 and 2017) and CV (2017) of gynodioecious *Cyananthus delavayi*.

Figure S4. Male-phase (top) and female-phase (bottom) duration of hermaphroditic flowers in populations BG (2016 and 2017) and CV (2017) of gynodioecious *Cyananthus delavayi*.