

# Sexual allocation in the gynodioecious species *Cyananthus macrocalyx* (Campanulaceae) at high elevations in the Sino-Himalaya Mountains

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**Abstract** Sexual allocation pattern is considered highly relevant for the maintenance of females in the process of evolution from hermaphrodites to dioecy. Theoretical and empirical studies predict that gynodioecious plants should invest more resources in male function under harsher environments and/or when female frequency is higher; and that there are trade-offs between male and female function. We studied sexual allocation pattern in the gynodioecious species *Cyananthus macrocalyx* in two populations in SW China. The results showed that although the total flower mass in hermaphrodites was significantly higher than that in females, females and hermaphrodites allocated similar biomass to female function (pistil biomass). As a consequence, females allocated relatively more resource to female function, while hermaphrodites allocated relatively more resource to pollinator attractiveness. There was no difference in total flower mass, pistil mass, and pollen production in hermaphrodites between the two populations. These results suggest that the females compensate for the disadvantage of lacking male function by allocating relatively more resource to female function, while hermaphrodites gain fitness through both male and female functions. This study supports the idea that if females are to be maintained in a gynodioecious population, they must allocate more resource to female functions than is

necessary for hermaphrodites. However, our results were in contrast to those of previous studies, in that trade-offs between male and female functions and male-biased allocation under high female frequency were not observed. This study adds to the body of research on plant sexual allocation, and for that it is, at least at some aspects, contrary to previous statements, it could be helpful for advancing the theoretical predictions in the future.

**Keywords** *Cyananthus macrocalyx* · Female function · Gynodioecy · Pollen production · Sexual allocation

## Introduction

The evolution of plant sexual systems has long fascinated botanists and evolutionary ecologists. Gynodioecy, in which females and hermaphrodites coexist in the same population, is often regarded as a stage in the transition from hermaphroditism to dioecy, or vice versa (Delph 2009; Alonso and Herrera 2011). It is difficult for females to be maintained in a hermaphroditic population, because they have to overcome the disadvantages of not gaining fitness from male function (Charlesworth and Charlesworth 1978). The conditions under which females can become established in populations of hermaphrodites are of considerable interest (Dorken and Mitchard 2008).

Theory predicts that females must gain additional fitness to compensate for the disadvantage of the absence of male function, in order to be maintained in hermaphroditic populations (Charlesworth and Charlesworth 1978). For example, females can completely avoid inbreeding depression due to obligate outcrossing (Sakai et al. 1997; Ramsey et al. 2006). Females can gain additional fitness by allocating the resources saved from male functions to female functions

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(Ashman 1994). Females gain fitness from their ovules and seed production (Charlesworth and Charlesworth 1978; Ashman 1994; Sakai et al. 1997; Williams et al. 2000) and/or indirectly from resources needed for the production of offspring (Bateman 1948), like resources allocated to carpels where the seeds lay and develop. Fitness in hermaphrodites is derived from both ovules and pollen grains and/or indirectly from resources allocated to pollinator-attracting traits (Charlesworth and Morgan 1991). Resource allocation strategies differ between females and hermaphrodites because of the different ways in which they achieve fitness, and this subject has attracted the attention of many ecologists since the time of Darwin (Darwin 1877; Charlesworth and Charlesworth 1978; Goldman and Willson 1986; Ashman and Baker 1992; Sakai et al. 1997; Ashman 1999; Ramula and Mutikainen 2003; Leigh et al. 2006; Delph 2009; Pannell et al. 2014).

Previous studies have suggested that females allocate more resources to female functions than hermaphrodites do (the resource reallocation hypothesis; Darwin 1877; Lloyd 1976; Charlesworth and Charlesworth 1978; Eckhart 1992; Sakai et al. 1997). In addition, females may allocate more resources to fruit and seed production (quantity and/or quality; Charlesworth and Charlesworth 1978; Delph and Lloyd 1991; Ashman 1994; Sakai et al. 1997; Williams et al. 2000) but less to pollinator attractiveness (Ashman and Stanton 1991; Williams et al. 2000; Vaughton and Ramsey 2002), to enhance their female fitness. Theory predicts that individuals will increase pollen production and pollinator attractiveness when they primarily achieve fitness through male function (Charnov 1982; Leigh et al. 2006), while they will increase investment in ovules and seed maturation when individuals achieve fitness primarily through female functions (Bawa 1980).

Many ecological factors are considered to be among the selective pressures that drive patterns of sexual allocation. Environmental stress can be an important selective pressure. Ashman (1999) found that females of the gynodioecious species *Fragaria virginiana* are more successful in harsher (lower soil moisture) environments, possibly because females do not need to allocate resource and/or energy to male functions so that the cost of physiological processes is lower. On the other hand, female frequency often has an important role in reproductive success (frequency-dependent theory; McCauley and Brock 1998). Some studies have predicted that the male function of hermaphrodites will be enhanced when the female frequency is high, because hermaphrodites may attain higher fitness by providing pollen than through producing seeds in such populations (Lloyd 1976). For example, Ramula and Mutikainen (2003) showed that in the gynodioecious species *Geranium sylvaticum*, hermaphrodites achieved more fitness through male function in a population with high female frequency. In addition,

pollination environments can determine sex allocation patterns in some plant species (e.g., Arnan et al. 2014). Since the “optimal partitioning theory” predicts that plants should invest more resource in organs/functions which are suffering more stress from resource limiting (Bloom et al. 1985; Gedroc et al. 1996; Poorter et al. 2012), hermaphrodites under conditions where pollinator diversity and activities are low should produce more pollen grains and/or allocate more resources to pollinator attractiveness. According to these predictions, hermaphrodites at higher elevations should produce more pollen grains and/or more or larger flowers than those at lower elevations, due to the increase in environmental stress (Körner 2003) and the decrease in the diversity, abundance, and activities of pollinators that may occur with increasing elevation (Arroyo et al. 1982; Wu et al. 1988; Medan et al. 2002).

Since the total resources available for reproduction are limited, increasing allocation to one organ/function must result in a reduction in allocation to another organ/function (Levins 1968; for review see Obeso 2002), and sex allocation theory predicts that there are trade-offs between male and female allocations (Charlesworth and Charlesworth 1981; Charnov 1982; Burd and Head 1992; Mazer et al. 1999). The problem for plants is how to balance resource allocation among different organs and functions to maximize overall fitness.

Several studies on plants’ reproductive strategies have been carried out on the high mountains in the Qinghai-Tibet Plateau (Duan et al. 2007; see review in Liu et al. 2014). However, few studies focused on sexual allocation pattern in gynodioecious species under particular environment conditions (but see Lu et al. 2012). *Cyananthus macrocalyx* (Campanulaceae) is a gynodioecious perennial, distributed mainly in the Himalayan-Hengduan Mountains, with females coexisting with hermaphrodites in the same population. In a preliminary investigation, we found that the female ratios [females/(females + hermaphrodites)] varies greatly among populations (25–67 %; Niu et al. unpublished data). In this study, we selected two populations with female ratios of c. 50 % ( $n = 128$ ) and c. 28 % ( $n = 141$ ), respectively. Their habitats are similar, i.e., scrub–grasslands, so the abiotic environmental stress gradient is simply represented by elevation (plants at higher elevation may experience lower temperatures and lower levels of pollinator activities). Here, we assumed that individuals of the population at the higher location experience a harsher environment than plants in the lower population. We aimed to determine the pattern of sexual allocation to females and hermaphrodites in the two populations of this gynodioecious species. We explored the following particular hypothesis:

1. According to the resource allocation theory, females should allocate relatively larger proportions of their

resources to female function (pistil biomass) and hermaphrodites should allocate larger proportions to pollinator attractiveness.

2. According to the female frequency-dependent theory, hermaphrodites in the high-elevation population (where there is a higher female frequency) should produce more pollen grains than hermaphrodites in the low-elevation population.
3. According to the trade-off theory, there should be a trade-off between female and male functions.

## Materials and methods

### Study species and populations

*Cyananthus macrocalyx* produces flowers ranging from a few to several dozens, depending on the age of the plant. Corollas are yellow, sometimes purple- or red-veined, less frequently purple basally and yellow apically, tubular campanulate, with five corolla lobes. The flowering season is from mid July to late September. The altitudinal range of the species is from 2500 to 5300 m (FOC, <http://foc.eflora.cn>). Its flowers are completely protandrous, so that the female functions are not initiated until the male function has terminated.

This study was conducted in two populations on the Baima snow mountain in the 2014 growing season (from late July to early October for our study sites). One population is located at 4540 m a.s.l. (N28°22'20", E99°00'07"), and the other is at 4320 m a.s.l. (N28°20'05", E99°04'36"). The Baima snow mountain is located in the center of the Hengduan mountains in the south-west of China, which is a global biodiversity hotspot (Mittermeier et al. 1999). The higher population is located ca. 200 m higher than and ca. 10 km away from the lower population. The habitats are scrub-grasslands in both cases.

### Allocation to female functions

To measure resource allocation to female function, at least 25 individuals of each gender morph were randomly selected, and one to three flowers on each individual were collected when they were newly and fully opened but not yet pollinated (this was determined by checking whether pollen was deposited on the stigma, using a handheld  $\times 60$  magnifying glass). Flowers were put in paper bags (one individual per bag). At the same time, we recorded the numbers of flowers as a covariate for the statistical analyses in order to control for the effect of plant size (Ramula and Mutikainen 2003). In the laboratory, flowers were dried at 75 °C for 72 h and then weighed to the nearest 0.1 mg. The

pistils were then separated from the flowers and also weighed to the nearest 0.1 mg. Flower and pistil biomasses from a single individual were averaged and compared between the gender morphs, and between the two populations, i.e., averaged mass from one individual was taken as a replicate in statistical analysis. In this study, total flower mass was taken as a surrogate for pollinator attractiveness, since our preliminary measurements had indicated that total flower mass was positively correlated with flower size ( $r = 0.78$ ,  $P < 0.01$  for hermaphrodites in the high and  $r = 0.44$ ,  $P = 0.02$  in the low population).

We did a supplementary pollination experiment to check whether there is a pollen limiting effect. For this, we randomly marked 30 female individuals and 30 hermaphrodites, at the stage when flowers newly opened and female function had just developed. Pollen grains were collected from individuals at least 20 m apart. One to three flowers per individual per gender morph were manually pollinated. At the same time, we also marked one to three flowers on each of these individuals for natural pollination. After about 4 weeks, when the fruits and seeds were mature, we collected the fruits, storing each in a paper bag. In the laboratory, fruits were air dried for 45 days, and we then measured the seed set in hand- and naturally pollinated fruits. If more than one flower was treated within a single individual, we averaged the values as mentioned above.

To determine whether there is any difference in ovule production between females and hermaphrodites, we randomly collected 30 flowers from at least 20 individuals for each gender morph. The pistils of these flowers were separated and stored in 70 % ethyl alcohol and dissected to count the ovule number under an anatomical lens.

### Pollen production in hermaphrodites

Because the anthers are connate, we could measure only the total pollen production per single flower. To do this, we randomly collected two to three flowers before anthesis from 20 hermaphroditic individuals. The petals were carefully removed, and the anthers with pistils from a single flower were stored in 1.5 ml ethanol in an Eppendorf tube to release the pollen grains. The pollen number per flower was determined using a hemocytometer slide. One 0.03 ml subsample of the pollen suspension was dropped onto the slide, and the number of pollen grains per grid was counted under a microscope. The procedure was repeated ten times for each pollen sample. The mean pollen number in each sub-suspension was then calculated. The total number of pollen grains produced by a single flower was calculated from the mean subsample pollen number multiplied by 50 (the 1.5 ml pollen suspension is equivalent to fifty 0.03 ml sub-suspensions).

## Data analyses

The effects of gender, population, and their interaction on flower mass, pistil mass, and pistil allocation (pistil mass/total flower mass  $\times$  100 %) were analyzed using two-way ANCOVA in the generalized linear model, with the gender and population as fixed factors and the flower number as a covariate to control for the effect of plant size (Ramula and Mutikainen 2003). The flower number was tested using the same model, with flower number as dependent variable and gender and population as fixed factors. Type III sums of squares were used in the ANCOVAs. Normality of the data was checked with Shapiro-Wilks' test, and homogeneity of the variances was checked with Levene's test.

In addition, we used an independent sample *t* test to measure the differences in pollen production by hermaphrodites between the two populations. Furthermore, to detect whether there were differences in total flower mass and resource allocation patterns between different genders and between different populations, we compared the total flower mass, pistil mass, and pistil fraction using a two-way anova, with gender and population as fixed factors. The seed set data for supplementary and naturally pollinated treatments were not normally distributed, so they were tested non-parametrically using a Mann–Whitney *U* test. All the analyses were performed using SPSS 16.0.

To determine whether there was a trade-off between female allocation and pollinator attractiveness, we did a bivariate correlation analysis of pistil mass and attractive mass (total flower mass—pistil mass; this can be arbitrarily regarded as a component of male function; Schoen 1982; Bazzaz and Reekie 1985). We did this analysis with R and used a Monte Carlo randomization to calculate *p*-values.

## Results

### Total resource allocation

#### Flower number

Gender had no effects on flower number, but population and the interaction between gender and population had

significant effects (Table 1). The two genders produced similar numbers of flowers in both populations (High:  $2.86 \pm 0.25$  female vs  $2.29 \pm 0.17$  hermaphrodite,  $df = 61$ ,  $t = 1.91$ ,  $P = 0.06$ ; Low:  $1.63 \pm 0.14$  female vs  $2.00 \pm 0.21$  hermaphrodite,  $df = 57$ ,  $t = -1.49$ ,  $P = 0.14$ ). Females in the high population produced more flowers than those in the low population ( $df = 57$ ,  $t = 4.31$ ,  $P < 0.001$ ), while flower number in hermaphrodites did not differ between the two populations ( $df = 61$ ,  $t = 1.11$ ,  $P < 0.27$ ).

#### Flower mass

Total flower mass differed significantly between genders, with the flower mass of hermaphrodites being 1.37 times greater than that of females for the high population and 1.21 times greater for the low population (Fig. 1a). When plant size (flower number) was controlled, the total flower mass was not influenced by population, but it was significantly influenced by gender and the interaction between gender and population (Table 1).

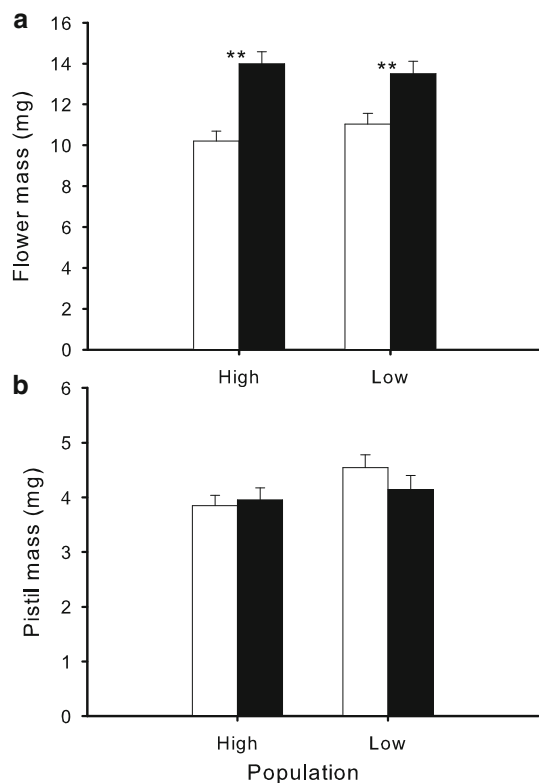
### Female allocation

When plant size (flower number) was controlled, female function, as represented by pistil mass, was not influenced by gender or by gender  $\times$  population interactions, but it was significantly influenced by population (Table 1). Pistil mass did not differ between genders for either the high or the low population (Fig. 1b). Pistil mass of females in the low population was greater than that in the high population ( $4.54 \pm 0.24$  low vs  $3.85 \pm 0.19$  high;  $df = 57$ ,  $t = -2.19$ ,  $P = 0.03$ ), while there was no difference for hermaphrodites ( $4.08 \pm 0.26$  low vs  $3.95 \pm 0.22$  high;  $df = 60$ ,  $t = -0.9$ ,  $P = 0.70$ ).

When plant size (flower number) was controlled, gender, population, and their interaction all had significant influences on pistil allocation (pistil mass/total flower mass  $\times$  100 %; Table 1). At the individual level, pistil allocation in females was higher than those in hermaphrodites for both high and low populations (Fig. 2a). At the population level, pistil allocation of females in the low

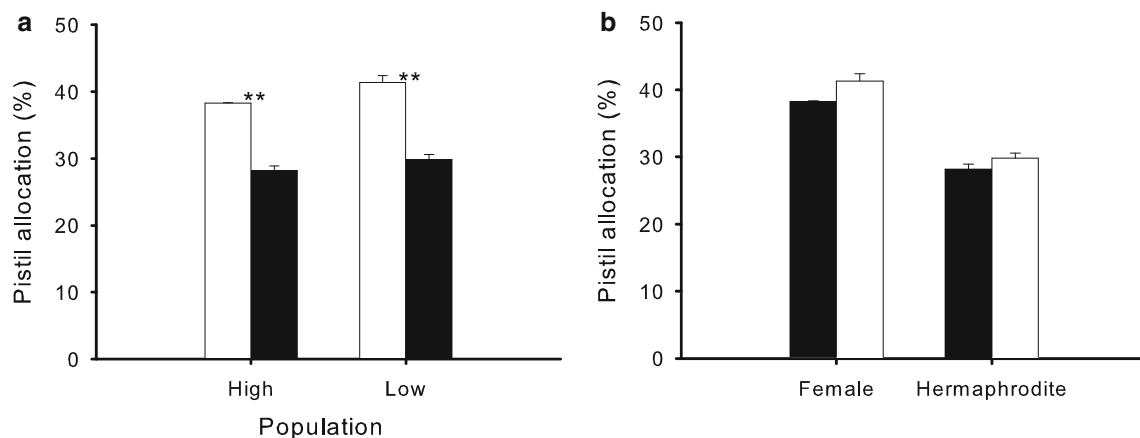
**Table 1** Results of generalized linear models of resource allocation patterns

Source	Flower number			Total flower mass			Pistil mass			Pistil allocation (%)		
	<i>df</i>	$\chi^2$	<i>P</i>	<i>df</i>	$\chi^2$	<i>P</i>	<i>df</i>	$\chi^2$	<i>P</i>	<i>df</i>	$\chi^2$	<i>P</i>
Gender	1	0.31	0.58	1	297.94	<0.001	1	0.67	0.41	1	3525.6	<0.001
Population	1	17.61	<0.001	1	0.84	0.36	1	5.8	0.02	1	168.36	<0.001
Gender $\times$ population	1	6.63	0.01	1	13.18	<0.001	1	1.85	0.17	1	16.52	<0.001



**Fig. 1** **a** Total flower mass in female and hermaphroditic individuals from two populations at different elevation. **b** Total pistil mass in female and hermaphroditic individuals from two populations at different elevation. Female *white bars*, Hermaphrodites *black bars*. \*\*Indicates significant difference between the two genders at the  $P = 0.001$

population was a little higher than that in the high population ( $P = 0.05$ ), while that of hermaphrodites did not differ between the two populations (Fig. 2b).



**Fig. 2** Pistil allocation in females was significantly higher than that in hermaphrodites (**a** Female *white bars*, Hermaphrodites *black bars*), but there were almost no differences between the two populations at different elevation (**b** High *black bars*, Low *white bars*)

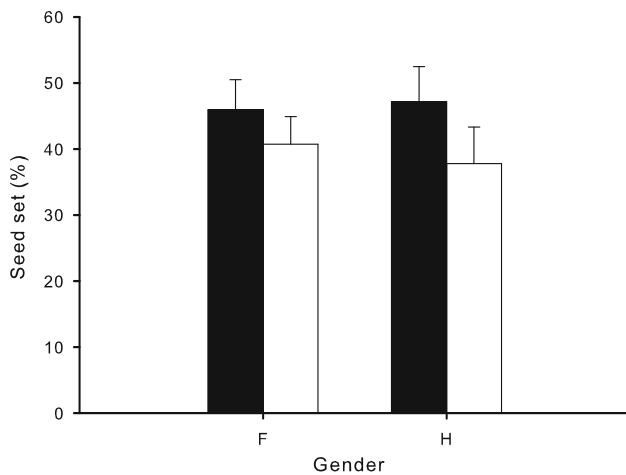
The two gender morphs produced similar numbers of ovules in both high ( $32.39 \pm 3.10$  female vs  $31.56 \pm 3.28$  hermaphrodite;  $df = 34$ ,  $t = 0.19$ ,  $P = 0.86$ ) and low ( $27.83 \pm 1.71$  female vs  $31.24 \pm 1.43$  hermaphrodite;  $df = 62$ ,  $t = -1.54$ ,  $P = 0.13$ ) populations, which may indicate that they allocate similar resources to potential seed production. In the high population, for both morphs, seed set in supplementary pollinated individuals was similar to those in naturally pollinated individuals (Fig. 3), which indicates that there was no pollen limitation in this population. In the low population, more than 70 % of the supplementary treatments were destroyed by grazing, so we excluded the data from these plants from our results.

### Pollen production

Hermaphrodites from high and low populations produced similar numbers of pollen grains per flower ( $19182 \pm 853.46$  high vs  $20816 \pm 1128.78$  low;  $df = 37$ ,  $t = -1.15$ ,  $P = 0.26$ ). Moreover, the pollen to ovule ratios did not differ between the two populations ( $633.46 \pm 36.02$  high vs  $717.29 \pm 38.79$  low;  $df = 37$ ,  $t = -1.58$ ,  $P = 0.12$ ).

### Correlations between female function and attractive structure

Pistil mass was positively correlated with attractive mass, indicating that *C. macrocalyx* allocates consistent proportions of its resources to female function and to pollinator attractiveness. That is to say, there was no trade-off between resources allocated to female function and those allocated to pollinator attractiveness (treated as a component of male function, Schoen 1982; Bazzaz and Reekie 1985; Fig. 4).

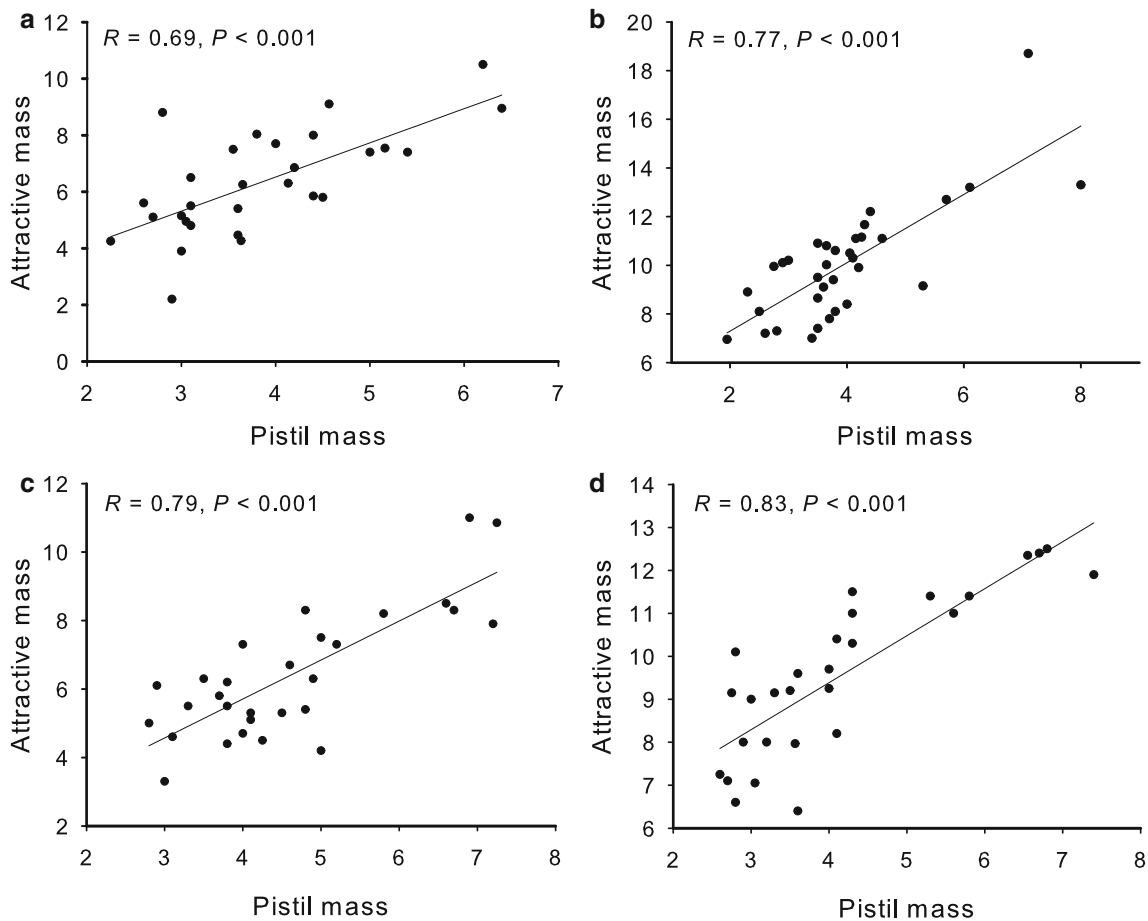


**Fig. 3** Seed sets resulting from supplementary (black bars) and natural (white bars) pollination treatments in the population at higher elevation

## Discussion

### Allocation to female function

Previous studies have shown that in a sexually dimorphic species, the pattern of resource allocation is, at least at certain stages, female-biased (Ehlers and Thompson 2004; Leigh et al. 2006; Zhang et al. 2008), in order to increase female reproductive success. In addition, male and/or hermaphroditic plants frequently produce more and/or larger flowers than female plants (Ashman and Stanton 1991; Williams et al. 2000; Vaughton and Ramsey 2002). Our findings support these observations. In this study, the total individual flower mass in hermaphrodites was significantly larger than that in females, though they allocated similar absolute mass to pistils as did females, and they produced similar number of flowers with those of females; this



**Fig. 4** Correlations between pistil mass and pollinator attractive mass. High population: **a** female; **b** hermaphrodite. Low population: **c** female; **d** hermaphrodite

indicated that hermaphrodites allocated relatively more resources to pollinator attractiveness. Two hypotheses could explain this. Firstly, according to the “male-competition” hypothesis, hermaphrodites may adopt a “flower enlargement” strategy, which requires more resources allocated to flowers, to attract enough pollinators for the transport of their pollen (Charlesworth and Morgan 1991). Secondly, the “enclosing-function” hypothesis proposes that flowers may develop large petals in order to protect larger sex organs (Bawa and Opler 1975; Delph 1996). High UV radiation and unpredictable rainfall in alpine regions could reduce the viability of pollen grains (Chen et al. 2013; Song et al. 2013). Hence, in our study species, in order to ensure the viability of pollen, it is possible that hermaphrodites develop larger petals to protect pollen grains from damage caused by adverse environmental conditions.

It has been stated that female individuals should have some properties that compensate for the disadvantage of not transmitting their genes through pollen; otherwise they could not be maintained in gynodioecious populations (Charlesworth and Charlesworth 1978). Although the total individual flower mass in females was lower than that in hermaphrodites, females and hermaphrodites allocated similar mass to pistils (Fig. 1). Thus, females allocated a relatively greater proportion of their resources to pistils than did hermaphrodites (Fig. 2). It has long been proposed that the cost of male function which is avoided by females may be reallocated to female function, thus enhancing female fitness, and that this could compensate (completely or partially) for the genetic disadvantage of not producing offspring through pollen (Darwin 1877; Charlesworth and Charlesworth 1978). Our results are consistent with this sex allocation theory.

#### Allocation to male functions in the two populations

Our results showed that for both females and hermaphrodites, there were no differences in biomass allocation (total flower and pistil biomass) between the high and the low population. Pollen production by hermaphrodites in the high population (where female frequency was high) did not differ from that in the low population (lower female frequency). In addition, our data showed that hermaphrodites produce a similar number of ovules in the high and the low population, thereby indicating that it was not necessary for hermaphrodites to reduce allocation to female functions under harsher environment conditions; in other words, hermaphrodites achieved fitness through both male and female functions. The fixed female and male allocations under two different environmental conditions were in contrast with other studies which have predicted that hermaphrodites would increase allocation to male function and reduce allocation to female function under high environmental stress and when female

frequency is high (Charlesworth and Charlesworth 1978; Ramula and Mutikainen 2003; reviewed in Delph and Wolf 2005; Reekie and Bazzaz 2005). There are two possibilities for these discrepancies. Firstly, the total resources that a hermaphroditic individual can gain from its surrounding environments may not be limited. The conditions that plants experience at high elevation maybe less extreme than was previously imagined (Körner 2003). If this is true, hermaphrodites of higher population may have sufficient resources for allocation to both vegetative and reproductive functions, and they have to balance these allocations to optimize the performance of these functions. Secondly, pollination efficiency may be particularly high in the population at higher elevation. On the one hand, our results showed no difference in seed set between supplementary pollination and natural pollination, what indicates that there was no pollen limiting effect for *C. macrocalyx* in either population. On the other hand, plant species diversity decreases with elevation (Zhang et al. 2009). It is possible that in populations at higher elevations, the number of other species blooming simultaneously is smaller than that at lower elevations, and this might result in a lower level of pollinator competition and higher pollination efficiency. In fact, pollen is generally produced in just the right quantities to ensure ovule fertilization (Charnov 1982). In any case, if pollen transfer is efficient enough, even low pollen to ovule ratio is able to guarantee maximum seed set (Cruden 1977, 2000).

#### Trade-off between female and male functions

Finally, our results showed that there were no trade-offs between female allocation and pollinator attractiveness (which can be arbitrarily regarded as a component of male function; Schoen 1982; Bazzaz and Reekie 1985). This is contrary to the sex allocation theory which assumes that genotypes investing more resources in male production have less resource available for female reproduction (Koelewijn and Hunscheid 2000). Goldman and Willson (1986) argued that trade-offs between male and female functions are not required, because these functions may be limited by different resources. Furthermore, female costs may be met chiefly from resources that are accrued after male function is over (Goldman and Willson 1986; Baker et al. 2005). If this is true, trade-offs between male and female function within one reproductive season or episode would not be expected (Goldman and Willson 1986). Making this assumption and taking into account the fact that *C. macrocalyx* is protandrous, and the corolla withers immediately once pollination is completed (Niu et al. 2011), we argue that the costs of seed maturation might be met after all male costs have been met, so that no trade-off between female and male functions is necessary.

## Conclusion

In summary, our results support only in part our hypothesis “1” and completely contradicted our hypothesis “2.” In addition, we found no trade-off between male and female functions in contrary to hypothesis “3.” Our study added new data to the body of research on plant sexual allocation, but its findings are mostly contrary to those of previous studies (Charlesworth and Charlesworth 1978; Charnov 1982; Ramula and Mutikainen 2003). However, we are cautious in drawing conclusions, since we acknowledge that our study has some limitations. Firstly, we compared only two populations, so it is not possible to extrapolate our findings to a larger gradient of environmental conditions. Secondly, we did this investigation only in a single growing season, and whether sex allocation might be influenced by different flowering phenology among years (Baker et al. 2005; Zhang et al. 2008; Ishii and Harder 2012) remains unknown. Thirdly, due to grazing damage, we lost information about seed production, and as a result, we do not know whether females can gain more fitness than hermaphrodites from seed performance (individual seed mass and seed viability). In short, this study found some new findings about sexual allocation pattern of gynodioecious species under different environmental stress, which could, at some extent, advance the existing theoretical predictions.

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