

Size-dependent gender modification in *Lilium apertum* (Liliaceae): does this species exhibit gender diphasy?

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- **Background and Aims** Variation in the relative female and male reproductive success of flowering plants is widespread, despite the fundamental hermaphroditic condition of the majority of species. In many hermaphroditic populations, environmental conditions and their influence on development and size can influence the gender expression of individuals through the formation of hermaphroditic and unisexual flowers. This study investigates the hypothesis that the bulbous, animal-pollinated, perennial *Lilium apertum* (Liliaceae) exhibits a form of size-dependent gender modification known as gender diphasy, in which the sexual expression of individuals depends on their size, with plants often changing sex between seasons.
- **Methods** Variation in floral traits was examined in relation to their size using marked individuals in natural populations, and also under glasshouse conditions. Measurements were taken of the height, flower number, floral sex expression, flower size, flower biomass and pollen production of individuals over consecutive years between 2009 and 2012 in seven populations in south-west China.
- **Key Results** Flowers of *L. apertum* are either perfect (hermaphroditic) or staminate (male) and, in any given season, plants exhibit one of three sex phenotypes: only hermaphrodite flowers, a mixture of hermaphroditic and male flowers, or only male flowers. Transitions between each of these sex phenotypes were observed over consecutive years and were commonly size-dependent, particularly transitions from small plants bearing only male flowers to those that were taller with hermaphroditic flowers. Hermaphroditic flowers were significantly larger, heavier and produced more pollen than male flowers.
- **Conclusions** The results for *L. apertum* are consistent with the ‘size advantage hypothesis’ developed for animal species with sex change. The theory predicts that when individuals are small they should exhibit the sex for which the costs of reproduction are less, and this usually involves the male phase. *L. apertum* provides an example of gender diphasy, a rare sexual system in flowering plants.

Key words: *Lilium apertum*, gender diphasy, plant sexual systems, size-dependent gender modification, sex allocation, sex change, plant mating systems.

INTRODUCTION

Hermaphroditism characterizes populations of the vast majority of flowering plants. In hermaphroditic populations, individuals have the potential to obtain reproductive success as both female and male parents, usually as a result of the production of pollen and ovules in perfect (hermaphroditic) flowers (Horovitz, 1977; Lloyd, 1980a). However, various developmental and genetic mechanisms can cause the abortion of sex organs in perfect flowers, resulting in the origin of pistillate and staminate flowers (reviewed by Diggle *et al.*, 2011), a condition known as dicliny. A major component of the great diversity of angiosperm sexual systems therefore arises from the deployment of hermaphroditic and unisexual flowers in different structural and temporal combinations at the plant and population level (Geber *et al.*, 1999; Barrett, 2002). This diversity results in a range of gender strategies, with individuals varying in their relative contributions to fitness as a result of maternal and paternal

investment (Lloyd, 1979; Lloyd and Bawa, 1984). Whereas sexual systems are most commonly described on the basis of morphological criteria, gender strategies are by definition functionally based and concern the proportion of a plant’s genes that are transmitted to offspring through female versus male function.

Hermaphroditic plants vary in the degree of temporal overlap between female and male function. In simultaneous hermaphrodites, both pollen and ovules are available for mating at the same time and variation in gender usually results from gradual adjustments in sex allocation. This sexual system predominates among flowering plants. A much less common sexual system is gender diphasy in which male and female function comprise discrete ontogenetic phases of the life cycle. In populations with gender diphasy individuals ‘choose’ their sexual condition in any given season according to circumstances (Lloyd and Bawa, 1984; Schlessman, 1988). Gender diphasy is associated with perennial life histories and commonly involves switches in sex expression from male to female, or from male to

hermaphrodite; however, reversals can also occur from female to male function (Bierzychudek, 1984; Schlessman, 1991). Sex expression in gender-diphasic species is controlled by environmental conditions affecting plant vigour (condition), particularly the size and amount of reserves in underground storage organs.

Only a handful of cases of gender diphasy are well documented in flowering plants (e.g. *Arisaema* – Policansky, 1981; Bierzychudek, 1984; Catasetinae – Gregg, 1978; Zimmerman, 1991; *Panax trifolium* – Schlessman, 1988, 1991), probably because evidence for sex change requires demographic observations of marked individuals for several seasons. It is important to recognize that although in any given flowering season populations of gender-diphasic species are composed of individuals with discrete sex phenotypes (e.g. male, female or hermaphroditic), over their lifetime they comprise a single genetically monomorphic class of hermaphrodites. Thus, gender diphasy is not an example of true gender dimorphism in which populations are composed of primarily female- versus male-functioning individuals.

Theory for the evolution of gender diphasy derives from the ‘size advantage hypothesis’ developed for animal species with sex change (Ghiselin, 1969; Leigh *et al.*, 1976; Warner, 1988; Vega-Frutis *et al.*, 2014). It proposes that selection should favour sex change when individuals succeed most efficiently as a member of one sex when small or young, and as a member of the other sex when older or larger. In general, the theory predicts that when individuals are small they should first express the sex for which the costs of reproduction are less, and this usually involves the male phase. Individuals should become female whenever resources increase and the relative returns from maternal investment increase with size. These theories have been refined for plants (e.g. Lloyd and Bawa, 1984; Zhang, 2006) to take account of their particular reproductive traits and life histories, including their sessile nature, quantitative sex expression because of modular growth and the production of repeated reproductive units (e.g. flowers and inflorescences), and the availability of stored resources associated with perennial life histories. Changes in sex expression can occur multiple times over many years associated with both increases and decreases in plant size (Bierzychudek, 1984; Schlessman, 1991). This situation differs from sequential hermaphroditism in animals (see Warner, 1975, 1988), in which sex change is usually not reversible.

The occurrence of size-dependent gender modification is not confined to species with gender diphasy but is a widespread phenomenon in flowering plants. It occurs commonly through gradual adjustments in sex allocation, often by the sterilization of sex organs prior to flowering, or through the cessation of reproductive investment after flowering during early fruit and seed development (Freeman *et al.*, 1980; Lloyd, 1980b; Lloyd and Bawa, 1984; Klinkhamer *et al.*, 1997). Size-dependent gender modification is reported from species with both gender monomorphism (e.g. simultaneous hermaphroditism – Wright and Barrett, 1999; Cao and Kudo, 2008; monoecy – Sarkissian *et al.*, 2001; Dorken and Barrett, 2003) and gender dimorphism (e.g. males in subdioecy – Barrett *et al.*, 1999). The essential difference between gradual gender adjustment, as exemplified in the examples above, is not that gender changes over time, but rather that in the case of diphasy plants ‘choose’ between two more or less discrete phases, according to their condition.

Here, we investigate size-dependent gender modification in the geophytic lily *Lilium apertum* (Liliaceae). Our preliminary observations of this species in south-west China indicated that in addition to plants possessing hermaphroditic flowers, as conventionally described in the literature, a significant number of individuals in populations produced male flowers, in which female sex organs were most often rudimentary and therefore non-functional. These observations motivated us to investigate the relationships between estimates of plant size and the sex expression of flowers. *Lilium apertum* is particularly suitable for this type of investigation for two reasons: (1) it does not form clones and produces a single reproductive shoot with a small number of flowers, thus enabling plant size and flower sex to be measured without difficulty; (2) it possesses a bulb which can be easily excavated and weighed, allowing an assessment of total stored investment at the end of the growing season.

Using both field and glasshouse experiments we investigated seven populations of *L. apertum* in 2009–2012 and addressed the following specific questions: (1) What is the distribution of hermaphroditic and male flowers among individuals of different size in natural populations, and does this distribution result in the occurrence of different sex phenotypes? We predicted that plants with only hermaphroditic flowers would be larger than those with only male flowers. (2) Are there differences in size and total reproductive expenditure in hermaphroditic and male flowers? We predicted that hermaphroditic flowers would be larger, weigh more and produce more pollen than male flowers. (3) Is there evidence for sex change between years for plants belonging to different sex phenotypes? We predicted that plants with only male flowers that grew larger during the growing season would be more likely to produce hermaphroditic flowers the following year. Similarly, we predicted that plants with hermaphroditic flowers that became smaller after reproductive expenditure would be more likely to produce male flowers in the following year. Data on sex change between years in populations of *L. apertum* would provide evidence that the species possesses gender diphasy.

MATERIALS AND METHODS

Study species and sites

Lilium apertum Franch. (Liliaceae) is a perennial geophytic herb that occurs predominantly in mountainous areas of south-west China (i.e. Hengduan Mountains). *L. apertum* is often considered a member of the small genus *Nomocharis* (= *N. aperta*), composed of seven species, all of which are endemic to this region (Liang and Tamura, 2000). However, recent molecular phylogenetic analysis indicates that *Nomocharis* is nested within *Lilium* (Rønsted *et al.*, 2005; Gao *et al.*, 2012, 2013) and therefore in this study we treat our study species as a *Lilium*. Populations of *L. apertum* inhabit shady habitats of broadleaved forests, bamboo scrub and alpine grasslands usually between 3000 and 3900 m a.s.l. Plants are bulbous and in June produce an annual shoot, which is 20–50 cm tall with graceful flower spikes composed of 1–5 large, pink to crimson, nodding actinomorphic flowers (Fig. 1). These can be either perfect (hereafter hermaphroditic) or staminate (hereafter male). Most male flowers have rudimentary female organs and cannot set seed following hand-pollination and are thus functionally male (Z.-Q. Zhang, unpubl. data). Very few male flowers have no obvious rudimentary



FIG. 1. Flowers and inflorescence of *Lilium apertum*. (A) Hermaphroditic flower, (B) male flower, (C) an inflorescence with two hermaphroditic flowers and three buds. Note the protracted period of flowering from basal to distal flower positions within the inflorescence.

female sex organs. The flowering sequence of plants commences with the anthesis of basal flowers and moves upwards over approximately 3 weeks. Flowers of *L. apertum* appear to be nectarless and hence pollen is the primary reward for pollen-collecting bees, which were the major flower visitors to populations we investigated. Hand-pollinations indicate that *L. apertum* is self-incompatible and there was no evidence of pollen limitation of seed set at our study sites (Z.-Q. Zhang, unpubl. data). Fruits of *L. apertum* are capsules, containing more than 100 winged seeds, and these mature about 3 months after pollination.

We conducted our studies during four flowering seasons from 2009 to 2012 and a total of seven populations were investigated for different amounts of time to address the questions outlined below. The codes and locations used for the seven populations and the number of year(s) each was studied are as follows: (1) ABG, Shangri-La Alpine Botanical Garden, Shangri-La, Yunnan province, south-west China (27°55'N, 99°38'E; 3330 m a.s.l.), 2009–2011; (2) SG1 located between Shangri-La City and Geza town, ~17 km from Shangri-La City (27°57'N, 99°42'E; 3400 m a.s.l.), 2009–2010; (3) SG2, located between Shangri-La City and Geza town, ~22 km from Shangri-La City (28°00'N, 99°42'E; 3550 m a.s.l.), 2009–2010; (4) BTF, Bigu Tree Farm (27°36'N, 99°43'E; 3300 m a.s.l.) 2010; (5) BHL, Bigu Heaven Lake (27°37'N, 99°39'E; 3850 m a.s.l.), 2011–2012; (6) XZ1, Xiao Zhongdian (27°30'37"N, 99°46'33"E; 3385 m a.s.l.), 2012; (7) XZ2, Xiao Zhongdian (XZ2, 27°30'15"N, 99°45'55"E; 3669 m a.s.l.), 2012.

Sex expression, gender distribution and sex lability

To investigate patterns of sex expression at the plant and flower level, and to quantify the distribution of gender at the population level, we studied seven populations (ABG, BHL, BTF, SG1,

SG2, XZ1, XZ2) for the durations indicated in the preceding section. In each population, we randomly sampled 71–401 individuals and counted the total number of flowers, their sexual condition (hermaphroditic or male) and their position on an inflorescence (basal flower 1, next 2, etc.).

To quantify the distribution of gender at the population level we employed Lloyd's measure of phenotypic gender (G_i) using data on the sexual condition of flowers (see Lloyd, 1980a; Lloyd and Bawa, 1984). This index depicts the standardized phenotypic femaleness of a plant in a population as:

$$G_i = o_i / (o_i + p_i E),$$

where o_i is the maternal investment, p_i is the paternal investment and E is an equivalence factor based on the ratio of investments in maternal and paternal functions in the population as a whole: $E = \sum o_i / \sum p_i$. We estimated paternal investment as the number of staminate flowers (the sum of hermaphroditic and male flowers) and maternal investment as the number of ovule-bearing flowers (hermaphroditic flowers). G_i ranges from 0 for plants that produce only pollen to 1 for plants that produce only ovules.

To determine whether *L. apertum* exhibits labile sex expression over flowering seasons, we monitored the sexual condition of flowers on marked plants for two or three consecutive years (2009–2011) in populations ABG and SG2, respectively, using plastic tags (sample sizes given in Table 1). Each year, we recorded plant height and the number of male and hermaphroditic flowers. We then determined the direction and frequency of annual transitions between the three sex phenotypes (plants with hermaphroditic flowers only, hereafter H; plants with hermaphrodite and male flowers, hereafter H+M; and plants with male flowers only, hereafter M).

TABLE 1. The changes in gender of marked individuals of *Lilium apertum* between years in two populations (ABG and SG2) from north-west Yunnan, China: number (%) of plants of each of the three sex phenotypes (H, H+M, M) in year t that became members of each sex phenotype in year $t + 1$

Sex in year t	Population ABG sex in year $t + 1$					Population SG2 sex in year $t + 1$				
	n	H+M	H	M	NF	n	H+M	H	M	NF
H+M	54	8 (14.8)	2 (3.7)	23 (42.6)	21 (38.9)	8	1 (12.5)	2 (25.0)	2 (25.0)	3 (37.5)
H	37	0 (0.0)	3 (8.1)	13 (35.1)	21 (56.8)	16	0 (0.0)	6 (37.5)	2 (12.5)	8 (50.0)
M	151	19 (12.6)	14 (9.3)	64 (42.4)	54 (35.8)	31	5 (16.2)	8 (25.8)	9 (29.0)	9 (29.0)

M, plants with male flowers; H, plants with hermaphroditic flowers; H+M, plants with both hermaphroditic and male flowers; NF, non-flowering plants, including plants (9.8 % in ABG and 9.1 % in SG2) that failed to appear in a given year (dead or dormant plants). n , number of plants.

Differences between hermaphrodite and male flowers

To investigate potential size differences between hermaphroditic and male flowers we randomly sampled 73 and 66 flowers, respectively, in population ABG, and 23 and 32 flowers, respectively, in population SG2. To control for position effects, only the basal flower within an inflorescence was sampled from each plant. We used digital calipers to measure the radius of flowers. In populations ABG, BTF and SG2 we randomly collected 20–50 flowers per flower type using the same sampling scheme and these were oven dried at 80 °C for 24 h, and then weighed to the nearest 0.01 mg. In population ABG, we estimated pollen production of hermaphroditic and male flowers following methods detailed by Dafni (1992). To test the prediction that hermaphroditic flowers would be larger, weigh more and produce more pollen than male flowers, one-tailed t -tests were used to compare each parameter (flower radius, biomass and pollen production) between flower types within each population. We also used two-way ANOVA to analyse the first two traits with ‘Flower type’ and ‘Population’ as main effects.

Gender and size

To determine the relationships among plant height, sex phenotype (H, H+M, M) and biomass, we randomly excavated approx. 20 plants of each sex phenotype in each of three populations (ABG, BTF, SG2) in June 2009. In each population we also included a sample of non-reproductive plants for comparison. For each mature plant we recorded the total number of flowers, the sexual condition of each flower and plant height above the soil. We later dried plants at 80 °C for 24 h, and then weighed their dry biomass to the nearest 0.01 mg. We tested for differences in plant size (total biomass, bulb weight, height) and flower number among sex phenotypes and populations using analysis of covariance (ANCOVA), with total biomass as a covariate.

To examine how gender varies with size in *L. apertum*, we monitored flower production throughout the blooming period for plants maintained in a glasshouse in 2011. The 90 individuals used in this experiment were excavated from three natural populations in 2010 (ABG, SG1 and SG2, $n = 30$ per population) after they had flowered and fruited and were senescing prior to the onset of dormancy. The bulbs were washed, dried and then stored under cool conditions (~ 4 °C) prior to the glasshouse experiment the following year. In 2011, all bulbs were weighed and planted in pots in a glasshouse at the Kunming Institute of Botany. Fifty-one of the bulbs flowered and on the first day

each plant flowered, we measured plant height and subsequently recorded the number of flowers and their sex expression. We tested for differences in size among the sex phenotypes and populations using a two-way ANOVA with ‘Plant height’ and ‘Population’ as main effects. We determined the relationships between plant size and flower traits using linear regression. Because in *L. apertum* plant height correlates strongly with total biomass (see Results; Fig. 6A) we used this measure as a non-destructive surrogate for overall size.

To examine whether plant size was associated with sex phenotype, we measured plant height in six populations during the flowering seasons of 2009, 2010 and 2012: ABG, SG1, SG2 (2009–2010); BTF, (2010); BHL and XZ2 (2012). Differences in plant size between the sex phenotype with only male flowers (M) and the two sex phenotypes with hermaphroditic flowers were tested using ANOVA. To determine if there was an association between size change and switches in sex expression, we marked plants in population ABG and measured plant height for two consecutive years. We then grouped plants based on their change in gender and compared the plant height of individuals between two consecutive years. We used a G -test with William’s correction to determine whether the propensity to become vegetative or smaller was independent of gender, and Fisher’s exact test to evaluate differences between sex phenotypes in their propensity to change gender. For each sex phenotype, we tested for differences in size change between year t and $t + 1$ using paired t -tests.

Throughout, we present data as means ± 1 s.e.m., where possible; G -tests were conducted using the spreadsheet of McDonald (2009), and all of other statistical tests were analysed in JMP (version 6.0.0, SAS Institute, 2005).

RESULTS

Sex expression and gender distributions

Flowers of *L. apertum* are either hermaphroditic or male (Fig. 1A, B); we observed no purely female flowers in our population surveys. The mean proportion of male flowers sampled in our population surveys was 0.56 (range 0.30–0.75), with male flowers the most frequent ($> 50\%$) flower type in all populations except BHL and XZ2 (Fig. 2A). At the plant level, the three sex phenotypes (H, H+M, M) were observed in all populations and years that we sampled (Fig. 2B). The relative proportions varied among populations, and to a lesser extent among years in the four populations (ABG, SG1, SG2 and BHL) in

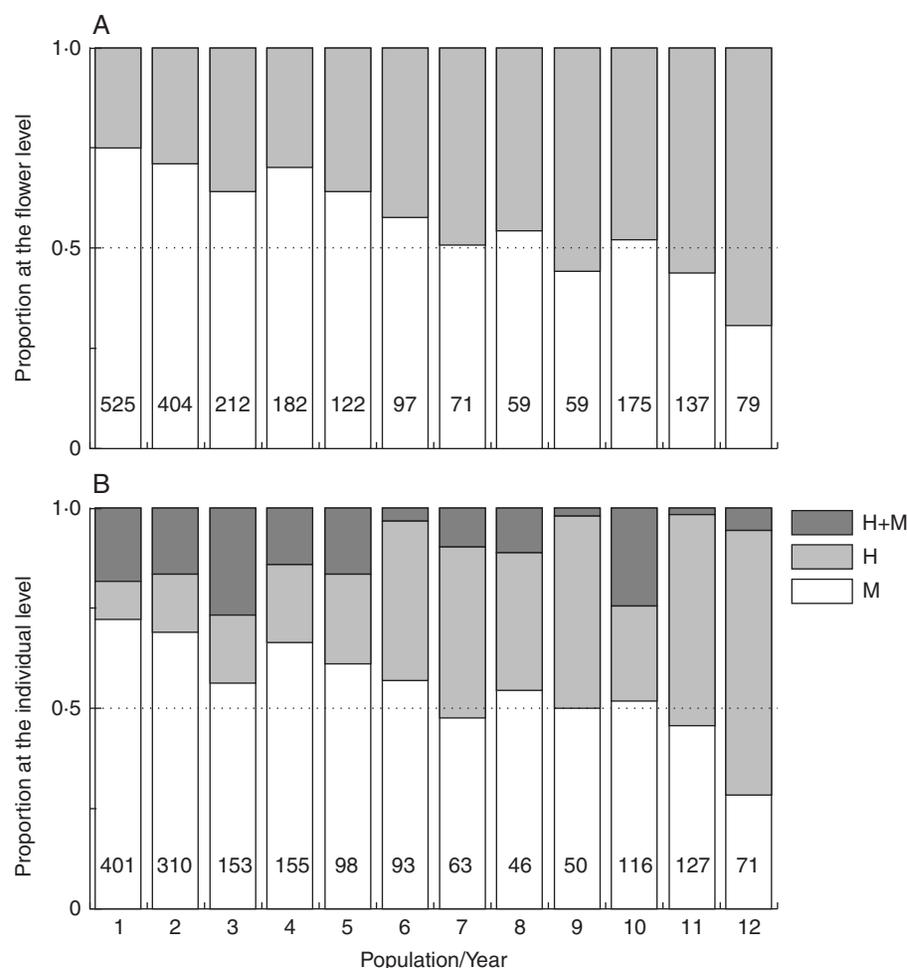


FIG. 2. Variation in sex expression at the flower and population level at 12 sampling intervals in seven populations of *Lilium apertum* (A) flowers, and (B) individuals within each population. M, male flower or plant with only male flowers; H, hermaphroditic flower or plant with only hermaphroditic flowers; H+M, plants with hermaphroditic and male flowers on the same individual. The sample size (n) is given at the base of each bar. 1, ABG (2010); 2, ABG (2011); 3, ABG (2009); 4, SG2 (2010); 5, SG2 (2009); 6, SG1 (2010); 7, SG1 (2009); 8, XZ1 (2012); 9, XZ2 (2012); 10, BTF (2010); 11, BHL (2012); 12, BHL (2011).

which samples were conducted in more than one year. M plants were the most common sex phenotype (mean frequency 0.57, range 0.28–0.72), and plants with this phenotype most commonly produced only a single flower in a flowering season. The next most common sex phenotype was H (mean frequency 0.28, range 0.10–0.66), and the least frequent sex phenotype was H+M (mean frequency 0.15, range 0.03–0.27). In plants with this ‘mixed flower’ phenotype, hermaphroditic flowers were always produced at basal positions within an inflorescence and male flowers were located at distal positions. Among all populations in the survey, the mean number (\pm s.e.m.) of flowers per plant for H, H+M and M sex phenotypes was 1.2 ± 0.03 ($n = 549$), 2.2 ± 0.03 ($n = 209$) and 1.0 ± 0.01 ($n = 925$), respectively. The mean number of hermaphroditic and male flowers for the 209 H+M plants was 1.2 ± 0.02 ($n = 240$) and 1.0 ± 0.01 ($n = 221$), respectively.

We estimated the distribution of phenotypic gender at 12 intervals (years) in seven populations of *L. apertum*. We present only six representative samples in four populations to illustrate overall patterns (Fig. 3). All populations showed variation in gender, including plants with only male function to plants that have the potential to gain fitness through both

maternal and paternal function. The patterns of gender distribution over a 3-year period (2009–2011) in ABG (Fig. 3A–C) provide evidence that gender expression at the population level remains relatively stable, despite transitions between sex phenotype classes at the plant level (see below). The remaining gender plots (Fig. 3D–F), from populations BHL–2011, BTF–2010 and XZ2–2012, illustrate variation among populations in the relative frequencies of the three sex phenotypes. Although the distributions give the appearance of discontinuity, because of the occurrence of varying proportions of plants with only male function, in reality the lifetime gender of plants is more likely to exhibit continuous variation, owing to transitions to hermaphroditism with size and age (see below).

Comparisons of hermaphroditic and male flowers

Hermaphroditic flowers were significantly larger than male flowers, based on measurements of flower radius (Fig. 4A: ABG: hermaphroditic 43.5 ± 0.5 mm, $n = 73$; male 41.6 ± 0.5 mm, $n = 66$; t ratio = 2.518, $P = 0.0065 < 0.05$; SG2: hermaphroditic 38.5 ± 0.8 mm, $n = 23$; male 34.4 ± 0.5 mm,

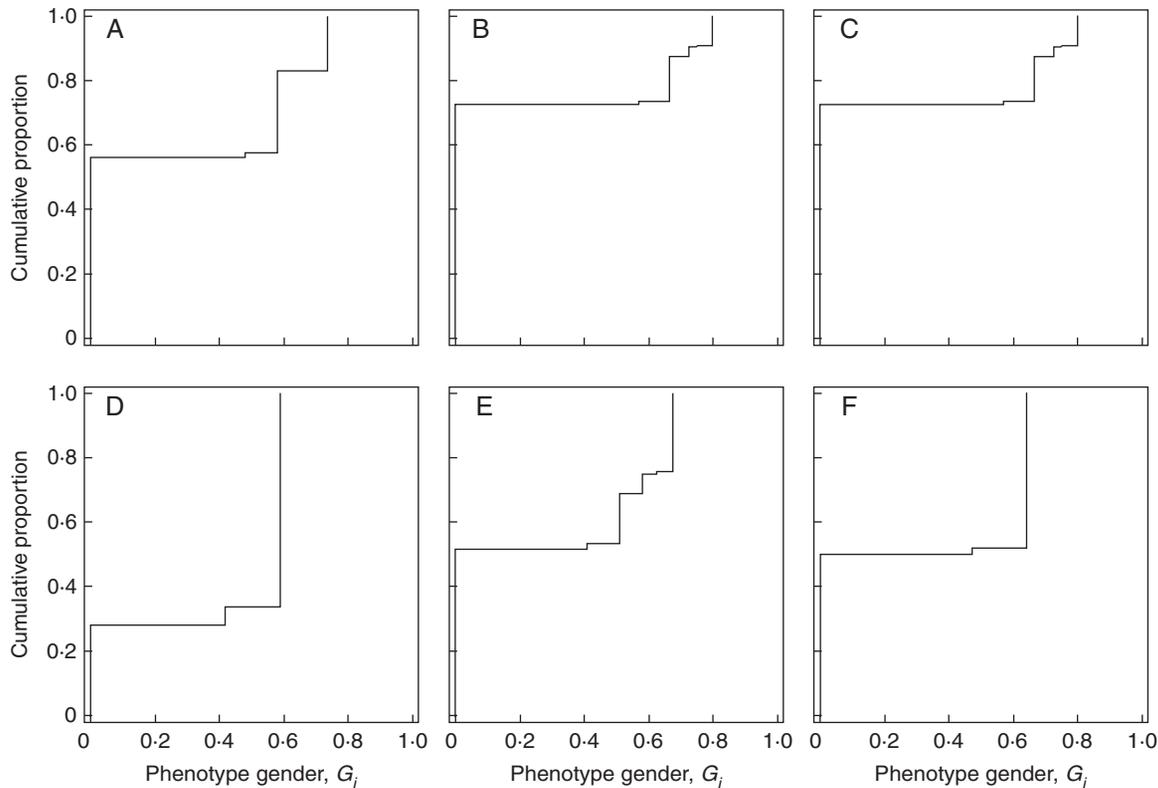


FIG. 3. The distribution of phenotypic gender in four populations of *Lilium apertum*. The lines represent the cumulative frequency distribution of standardized phenotypic femaleness (G_i) for plants sampled from each population. (A–C) |ABG, 2009–2011; (D) |BHL, 2011; (E) |BTF, 2010; (F) |XZ2, 2012. Gender can potentially vary from purely male (= 0) to purely female (= 1); however, in populations of *L. apertum* no plants were observed with only female gender expression.

$n = 32$; t ratio = 3.913, $P = 0.0001$). The effects of flower type and population on flower size were significant, but the interaction ‘population \times flower type’ was not ($P = 0.092 > 0.05$). Not unexpectedly, the dry biomass of hermaphroditic flowers was significantly greater than that of male flowers (Fig. 4B: ABG: hermaphroditic 86.1 ± 2.8 mg, $n = 39$; male 64.2 ± 2.5 mg, $n = 48$; $t = 5.801$, $P < 0.0001$; BTF: hermaphroditic 90.7 ± 4.1 mg, $n = 24$; male 55.3 ± 4.4 mg, $n = 21$; $t = 5.835$, $P < 0.0001$; SG2: hermaphroditic 83.9 ± 4.3 mg; $n = 27$; male 54.5 ± 3.7 mg; $n = 36$; $t = 5.200$, $P < 0.0001$). Flower type had a significant effect on floral biomass, but the interaction ‘population \times flower type’ was not significant ($P = 0.163 > 0.05$). In population ABG, hermaphroditic flowers produced significantly more pollen (32.5 %) than male flowers (hermaphroditic $329\,400 \pm 17\,398$, $n = 19$ flowers; male $248\,522 \pm 14\,873$, $n = 26$ flowers; $t = 3.534$, $P = 0.0005$).

Dynamics of gender expression

By investigating marked individuals in populations ABG and SG2 for consecutive years, we found that individuals are able to switch sex from one year to the next (Table 1). Of the six possible annual transitions between sex phenotype classes, all except the transition from H plants to H+M plants were observed. For example, in ABG 35.1 % of H plants in year 1 produced only male flowers (M) the following year, and 42.6 % of H+M plants became M plants in the second year of observation.

Similarly, among M plants in year 1, 12.6 and 9.3 % of plants were H+M or H, respectively, the following year. Changes in gender between years were also evident in population SG2. For example, among M plants observed in the first year of observation, 16.2 and 25.8 % of the plants in the following year were either H+M or H, respectively.

In both populations a significant proportion (39.7 % in ABG, 36.4 % in SG2) of flowering plants surveyed in the first year did not flower in the second year. The three sex phenotypes differed with respect to their likelihood of not flowering in the second year of observation. H plants were the least likely to flower, followed by H+M plants, and M plants were the most likely to continue flowering in the second year. Interestingly, with one exception among the sex phenotype (H plants in SG2) the proportion of plants switching gender was lowest among M plants with 42.4 and 29.0 % of plants in ABG and SG2, respectively, remaining M in the second year of observation. Of the 70 and 16 plants that were observed for three consecutive years in ABG and SG2, 17.1 and 18.8 %, respectively, changed sex twice.

Gender and size among the sex phenotypes

For all components of size, female-functioning plants (H+M and H) tended to be larger than plants that produced exclusively staminate flowers (M), but H and H+M plants were similar in size (Fig. 5; Table 2). In each population there were significant

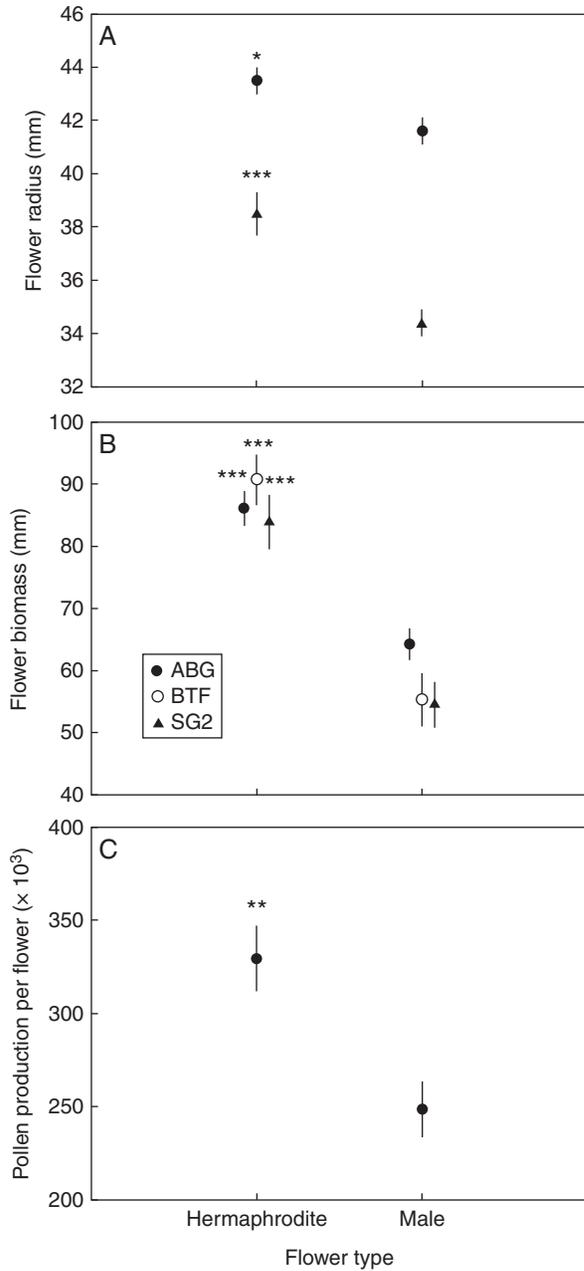


FIG. 4. Differences in floral traits between hermaphroditic and male flowers of *Lilium apertum*. (A) Flower radius, (B) flower biomass and (C) pollen production per flower. * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$. Population codes are indicated.

differences among the sex phenotypes in total biomass and plant height (Fig. 5A, C) and total biomass was positively related to bulb biomass. For bulb biomass, differences between H and M phenotypes were only significant in SG2 (Fig. 5B). H+M plants produced significantly more flowers than the other two sex phenotypes (Fig. 5D). The effects of sex phenotype and population on total biomass, bulb biomass and height were significant, but the interaction ‘population \times sex’ was not (Table 2). In each population, plant height, total flower production and the number of hermaphroditic flowers per plant varied

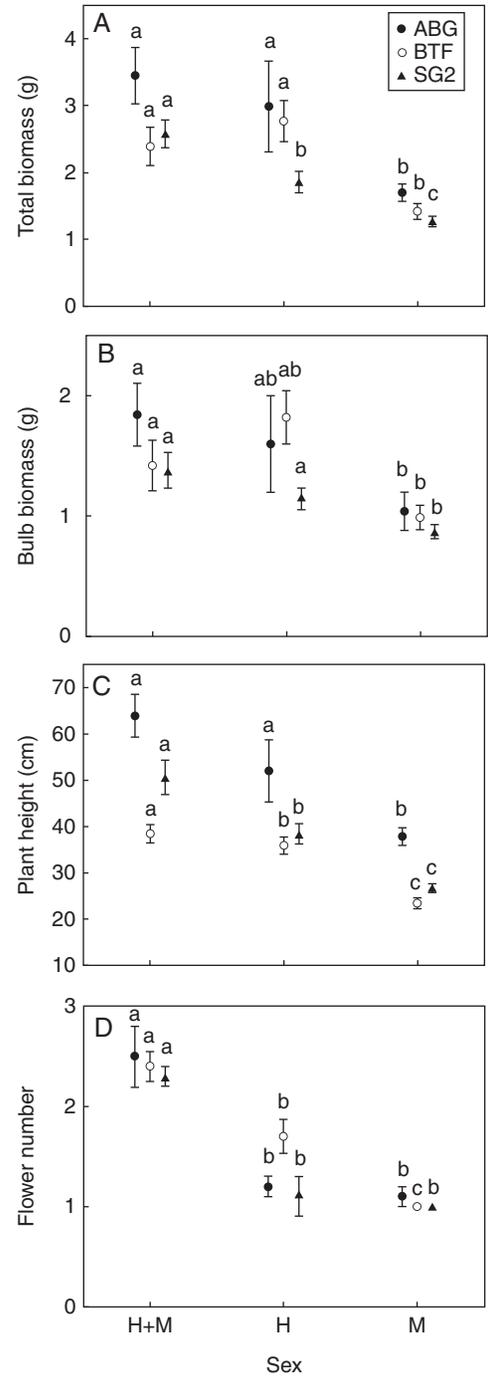


FIG. 5. (A) Total plant biomass, (B) bulb biomass, (C) plant height and (D) flower number among three sex phenotypes (M, H, H+M) in three populations (ABG, BTF, SG2) of *Lilium apertum* in the flowering season of 2009. Lower-case letters indicate means that are significantly different from one another in each population (Tukey–Kramer comparisons, $P < 0.05$). M, plants with only male flowers; H, plants with only hermaphroditic flowers; H+M, plants with hermaphrodite and male flowers. The statistical results are reported in Table 2.

positively with total biomass (Fig. 6). In contrast, there was no significant relationship between size and the number of male flowers per plant, largely because all M plants but one produced a single flower.

TABLE 2. ANCOVA of total biomass, bulb biomass, height and flower number among sex phenotypes (H, M, H+M) of *Lilium apertum* in three populations (ABG, BTF and SG2) from NW Yunnan, China

Source (d.f.)	Total biomass	Bulb biomass	Height	Flower number
Population (2)	7.37***	3.59*	41.53****	3.03
Sex (2)	30.81****	14.42****	61.23****	114.51****
Population × sex (4)	2.16	3.81	2.07	3.83**
Total biomass (1)	–	1076.99****	128.61****	54.29****
Total biomass × sex (2)	–	3.91*	–	4.60*
Total biomass × population (2)	–	6.18**	8.71***	–
Total biomass × population × sex (4)	–	–	–	6.33**

Total biomass was used as a covariate in each analysis. Interactions between covariates and main effects were tested and removed using backwards elimination ($\alpha = 0.05$). *F*-values are shown for each response variable. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Considering plant height as a surrogate of total biomass (or size) in the glasshouse experiment, we found that ovary length, ovary width, style length and flower diameter all varied positively with height (Fig. 7, Table 3: ovary length: $r^2 = 0.19$, $F_{1, 50} = 11.55$, $P = 0.0014$; ovary width: $r^2 = 0.15$, $F_{1, 50} = 8.97$, $P = 0.0043$; style length: $r^2 = 0.15$, $F_{1, 50} = 8.78$, $P = 0.0047$; flower diameter: $r^2 = 0.34$, $F_{1, 50} = 24.81$, $P < 0.0001$). In contrast, floral traits directly associated with male function (e.g. anther length and filament length) were not associated with plant height.

Surveys of plant height and total flower number in five populations of *L. apertum* revealed consistent patterns between the sex phenotypes. Female-functioning sex phenotypes (H and H+M) were significantly taller (Fig. 8A) and produced more flowers (Fig. 8B) in all but one comparison (plant height in SX1) compared with plants that produced exclusively male flowers (M). However, the lack of a difference in height between the sex phenotypes in SX1 did not occur in this population the following year and female-functioning phenotypes were taller than purely male-functioning phenotypes (Fig. 8A).

We tested several predictions concerning changes in the size and gender of sex phenotypes between consecutive flowering seasons using data collected from population ABG. First, we predicted that M plants would be more likely to increase in size between years, whereas H and H+M sex phenotypes would be more likely to decrease owing to the greater costs associated with female function. We found evidence to support this prediction (Table 4A). Second, we predicted that M plants that grew larger would be more likely to become female functioning than those that became smaller, and that female functioning plants that became smaller would be more likely to become exclusively male functioning compared with females that became larger. These two predictions were also supported by our data (Table 4B).

DISCUSSION

Our study of size-dependent gender modification in *L. apertum* revealed three major findings. (1) A significant proportion of plants in populations (mean frequency 0.57, range 0.28–0.72) produced only staminate flowers and thus functioned exclusively as male parents (Fig. 1). All of these plants except one produced a single flower, and were shorter than plants with female function, which generally produced more flowers (Figs 5 and 8). (2) Hermaphroditic flowers were larger, weighed more and

produced more pollen than male flowers (Fig. 4). (3) Censuses of marked plants among years indicated sex change among all three sex phenotypes (M, H, H+M) associated with changes in their plant size (Table 1). Specifically, plants producing only male flowers were more likely to produce hermaphroditic flowers the following year if they grew larger, whereas plants with hermaphroditic flowers that became smaller were more likely to switch to producing male flowers the following season (Table 4). We begin by first considering the occurrence of unisexual flowers in *L. apertum* and related taxa and the implications that dicliny has for the sexual systems and mating biology of populations. We then examine the evidence for gender diphasy in *L. apertum* and consider the developmental and ecological mechanisms that cause size-dependent gender modification.

The origin, distribution and consequences of dicliny for sexual systems

The origin of unisexual flowers from hermaphroditic flowers broadens the spectrum of sexual systems available to flowering plants, and increases the opportunity for variation in sex allocation and gender strategies. Our study indicates that rather than being of infrequent occurrence, as is the case in most hermaphroditic species, unisexual flowers are a common feature of *L. apertum* populations. Indeed, the mean proportion of male flowers sampled in our survey was 0.56, with male flowers the most frequent flower type in the majority of populations.

Loss of organ function causing floral unisexuality can occur at all stages of development, ranging from the absence of initiation to the failure of organ maturation, but within a species the stage of organ abortion tends to be similar for both pistillate and staminate flowers (Diggle *et al.*, 2011; Fig. 2). Virtually all male flowers of *L. apertum* displayed conspicuous non-functional pistils (pistilodes), indicating that the processes responsible for the termination of female function were often late in development, perhaps precluding significant opportunities for reproductive compensation. Indeed, there was no evidence of increased pollen production in male flowers, which were in fact smaller and produced less pollen than hermaphroditic flowers. Significantly, pistillate flowers were never observed in populations of *L. apertum*, implying that male flower production is an adaptive feature of the size-dependent sex allocation strategy of populations.

The occurrence of male flowers in members of the Liliales is not uncommon and a significant number of taxa are dioecious

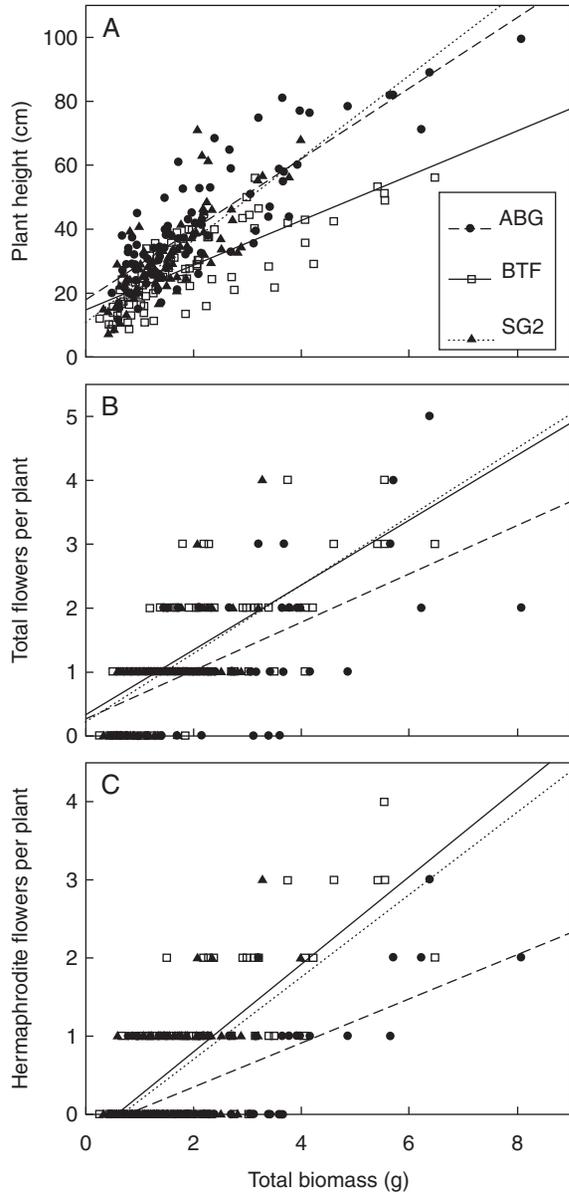


FIG. 6. Relationships between total biomass and (A) height, (B) total flowers and (C) number of hermaphroditic flowers per plant in populations ABG, BTF and SG2 of *Lilium apertum*. Lines represent least-squares regressions lines for each population. (A) ABG: $y = 18.1 + 11.0x$, $r^2 = 0.74$, $P < 0.0001$; BTF: $y = 14.8 + 7.0x$, $r^2 = 0.56$, $P < 0.0001$; SG2: $y = 11.2 + 12.9x$, $r^2 = 0.60$, $P < 0.0001$. (B) ABG: $y = 0.3 + 0.4x$, $r^2 = 0.38$, $P < 0.0001$; BTF: $y = 0.3 + 0.5x$, $r^2 = 0.50$, $P < 0.0001$; SG2: $y = 0.2 + 0.5x$, $r^2 = 0.41$, $P < 0.0001$. (C) ABG: $y = -0.2 + 0.3x$, $r^2 = 0.44$, $P < 0.0001$; BTF: $y = -0.3 + 0.6x$, $r^2 = 0.60$, $P < 0.0001$; SG2: $y = -0.3 + 0.5x$, $r^2 = 0.41$, $P < 0.0001$.

(e.g. *Chamaelirium* – Meagher, 1981; *Smilax* – Sawyer and Anderson, 1998; *Wurmbea* – Barrett and Case, 1996) or andromonoecious (e.g. *Gagea* – Wolfe, 1998; *Veratrum* – Liao and Zhang, 2008; *Zigadenus* – Emms, 1993). Surveys of male flowers in *Fritillaria*, *Lilium* and *Tulipa* have revealed frequencies ranging from 11 to 38 % (Peruzzi, 2012), indicating that female sterility is also a feature of other normally hermaphroditic taxa of lilies (and see Mancuso and Peruzzi, 2010). A detailed study of flower sex expression in seven populations of

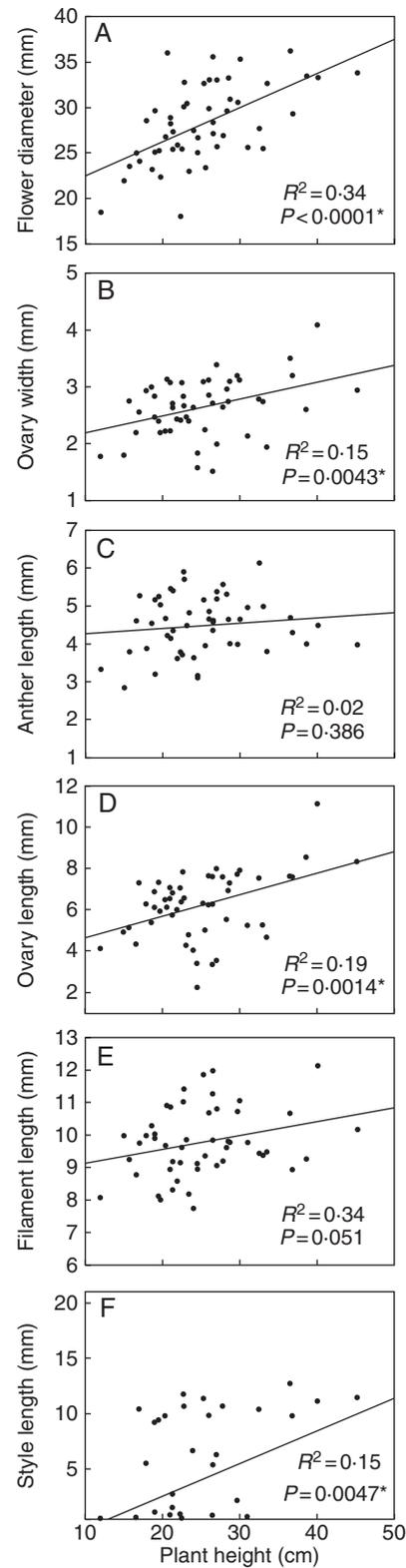


FIG. 7. Relationships between plant height and (A) flower diameter, (B) ovary width, (C) ovary length, (D) style length, (E) filament length and (F) anther length in *Lilium apertum* plants excavated from three populations (ABG, SG1 and SG2) and grown in a glasshouse for one flowering season. The points are data pooled from three populations (ABG, SG1 and SG2) and lines represent least squares regressions lines for each floral trait. Asterisks indicate a significant relationship. Two-way ANOVA results are reported in Table 3.

TABLE 3. F-values for two-way ANOVAs of the effect of plant height and population origin on flower diameter, anther length, filament length, ovary width, ovary length and style length in *Lilium apertum* plants excavated from three populations (ABG, SG1 and SG2) from north-west Yunnan, China, and grown in a glasshouse for one flowering season

Source	Flower diameter	Male function		Female function		
		Anther length	Filament length	Ovary width	Ovary length	Style length
Plant height	20.41 (1, 46)***	0.87 (1, 46)	6.40 (1, 46)*	14.45 (1, 46)***	8.84 (1, 46)**	9.49 (1, 46)**
Population	0.73 (2, 46)	1.72 (2, 46)	1.69 (2, 46)	2.93 (2, 46)	0.13 (2, 46)	1.29 (2, 46)
Plant height × population	1.17 (2, 46)	1.99 (2, 46)	0.68 (2, 46)	0.42 (2, 46)	0.20 (2, 46)	0.57 (2, 46)

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

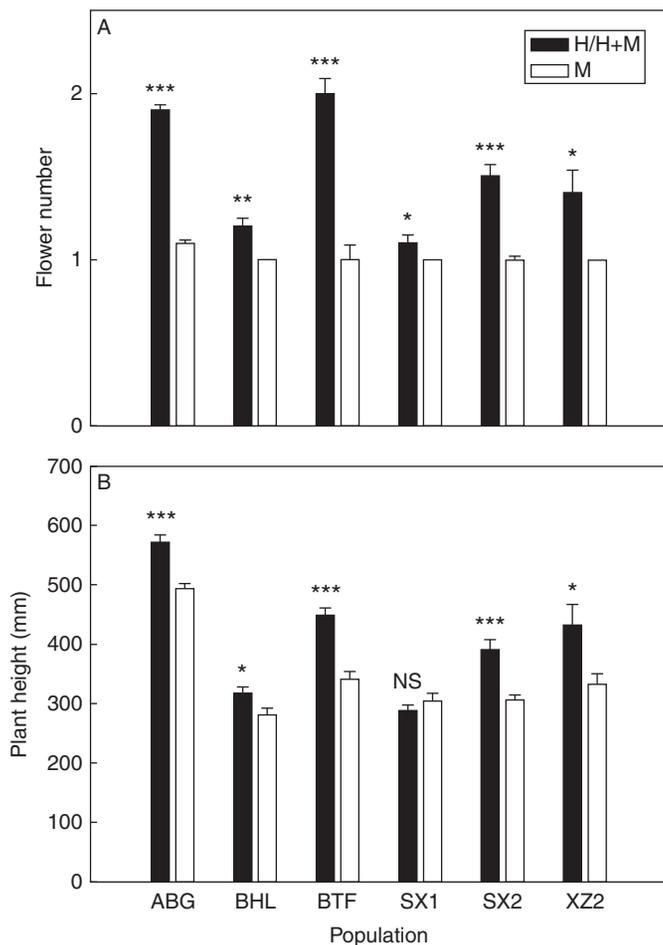


FIG. 8. The differences in (A) flower number and (B) plant height between sex phenotypes that produce only male flowers (M) and the two sex phenotypes that produce hermaphroditic flowers (H/H+M) in *Lilium apertum*. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0001$. NS, not significant.

F. montana revealed that male flowers represented 14–48 % of the flowers sampled, with the remainder being hermaphroditic (Peruzzi *et al.*, 2012). Similarly, in a survey of 11 populations of hermaphroditic *Lloydia serotina*, 48.5 % of the flowers were male (Manicacci and Després, 2001). In both these species the vast majority of plants produce a single flower during the flowering season and in both studies plants with male flowers were on average smaller for various traits than those with hermaphroditic

flowers. Observations between years in *Lloydia serotina* provided evidence of switches in gender (Manicacci and Després, 2001; and see Jones and Gliddon, 1999), and these changes were similar to those observed in the present study. Size-dependent gender modification may be especially important in geophytic species with underground storage organs, such as members of the Liliaceae. Indeed, there is evidence for changes in sex allocation with size in *Veratrum nigrum* (Liao and Zhang, 2008) and *Cardiocrinum cordatum* (Cao and Kudo, 2008). In these cases gender adjustment is gradual, rather than involving discrete changes in sex phase between seasons, and this may, in part, be because of the larger floral displays that these species possess.

The significant number of plants producing only male flowers in members of the Liliaceae has resulted in some confusion concerning the sexual systems of populations. For example, based on studies of *Fritillaria*, *Lilium* and *Tulipa*, Peruzzi (2012) and Peruzzi *et al.* (2012) concluded that species in these genera were androdioecious (and see Mancuso and Peruzzi, 2010). In contrast, based on very similar patterns of sexual variation, Manicacci and Després (2001) concluded that *Lloydia serotina* was not androdioecious, as had been previously suggested, but was andromonoecious. This inference was based on the assumption that all plants within the populations if large enough over their lifetimes would produce hermaphroditic flowers. Given the overall rarity of androdioecy in angiosperms, and the severe constraints on the spread and maintenance of female sterile plants in predominantly hermaphroditic populations (reviewed by Pannell, 2002a), this interpretation seems more probable. The occurrence of plants with only male function in populations of Liliaceae genera (including *L. apertum*), although superficially resembling androdioecious gender dimorphism, is more likely to reflect the influence of resource status on hermaphroditic species with size-dependent gender modification (and see Pannell, 2002b). However, long-term demographic studies would be required to completely rule out the presence of female sterile plants. Notwithstanding the difficulties in establishing the sexual system of these populations, in any given season the strong bias towards ‘maleness’ in populations will have important implications for mating biology, particularly the intensity of intrasexual (male–male) competition for mates.

Does *L. apertum* exhibit gender diphasy?

Gender diphasy may also be difficult to distinguish from more gradual forms of size-dependent gender adjustment in hermaphroditic species with gender monomorphism. The essential

TABLE 4. Tests of predictions about plant size and gender change based on yearly observations of marked plants of *Lilium apertum* in population ABG, north-west Yunnan, China

(A) Tests of the prediction that plants with only male flowers would be more likely to increase in size than decrease, whereas plants with hermaphroditic flowers would be more likely to decrease than increase in size				
Gender in year t	Size in year $t + 1$		G_{adj}	P (one-tailed)
	Larger	Smaller		
Male	63	44	3.376	0.033*
Hermaphroditic	18	59	22.853	< 0.0001*

(B) Tests of the prediction that plants with only male flowers that grew larger would produce hermaphroditic flowers, whereas those that became smaller would not, and plants with hermaphroditic flowers that became smaller would change to produce only male flowers whereas those that grew larger would not				
Gender in year t	Size in year $t + 1$	Gender in year $t + 1$		P (one-tailed)
		Male	Hermaphroditic	
Male	Larger	32	29	0.0001*
	Smaller	21	1	
Hermaphroditic	Larger	10	8	0.0265*
	Smaller	28	5	

*Significant difference.

feature of gender diphasy is that individuals in a population belong to a single genetic class in which their sex expression in any particular season is controlled by circumstances, particularly plant size. Thus, within a flowering season populations should be composed of two primary gender classes: male-functioning and female-functioning plants. Although female-functioning plants can also produce pollen, the majority of their fitness is likely to be obtained through their ovules because of the strongly male-biased sex allocation at the population level, as we observed in *L. apertum*. Thus, the sex phenotypes H and H+M in our study are often referred to as ‘female’ (see Lloyd and Bawa, 1984; Schlessman, 1988), even though they are not strictly unisexual. Despite the occurrence of two primary gender classes in diphasic populations, from the perspective of between-season, lifetime fitness all plants on average should contribute genes to the next generation as both male and female parents. As a result, the lifetime functional gender of populations with gender diphasy will be unimodal, rather than bimodal as occurs in species with gender dimorphisms such as androdioecy.

As discussed above, although we cannot completely rule out the possibility that some *L. apertum* plants are genetically determined males, we believe this is unlikely for two reasons. First, given the various constraints on the evolution and maintenance of androdioecy it would be unexpected that male plants in androdioecious populations would produce fewer and smaller flowers with less pollen than coexisting female-functioning plants, as occurs in *L. apertum*. Both theoretical studies and empirical evidence indicate that for males to be maintained in androdioecious populations it is necessary for them to compensate for their lack of female function (Lloyd, 1975; Charlesworth, 1984; Pannell, 2002a, b). This is usually achieved by a significant increase in pollen production compared with hermaphrodites, the reverse pattern to what we observed in *L. apertum*. Second, we directly observed between-year switches in gender from male to female function and from female to male function associated with increases and decreases in plant size, respectively. These patterns have not been reported in androdioecious species and are more consistent with the seasonal switches in gender that are the hallmark of gender diphasy.

The lifetime functional gender of populations of gender-diphasic species should be monomorphic rather than dimorphic. However, in any given season the distribution of gender variation in a population will be discontinuous, as we have shown (Fig. 3). This pattern is the direct result of the ‘choice’ that plants make between two contrasting modes of pre-fertilization investment in maleness versus femaleness. This discontinuity differs from what would be observed in most hermaphroditic species that exhibit gradual gender adjustment. In these species phenotypic gender is regulated by modifications to the sex allocation of flowers, and by post-fertilization phenomena associated with fruit and seed abortion (Lloyd and Bawa, 1984). Gender diphasy thus represents an extreme case of size-dependent sex allocation in which populations in any given season are made up of two distinct categories or phases corresponding to male- versus female-functioning plants, despite populations being fundamentally hermaphroditic.

Developmental and ecological mechanism causing size-dependent gender modification

Gender in natural populations of *L. apertum* is governed primarily by size, with increased size resulting in greater investment in female function. These effects were manifested at both the flower and the plant level. For example, in the glasshouse ovary length, ovary width and style length all varied positively with plant height, whereas floral traits directly associated with male function (e.g. anther length and filament length) were unrelated to size (Fig. 6). Similarly, in natural populations both the number of hermaphroditic and the total number of flowers per plant were positively correlated with plant height, indicating that larger plants have more resources to invest in female function as a result of the ‘budget effect’ of plant size (see Klinkhamer et al., 1997). In contrast, male flower production was unrelated to plant height and large plants produced more flowers capable of female function and small plants either produced only male flowers or were non-flowering (Figs 5 and 7). These results are consistent with size-dependent sex allocation theory, which

predicts that plants should invest in female function only when absolute investment in survival and male function exceeds a certain threshold, and that small individuals should express the gender that incurs the lower cost (Lloyd and Bawa, 1984; Zhang, 2006). The smaller size of staminate flowers and shorter stature of plants with only male flowers is consistent with the hypothesis that in *L. apertum* male function is less costly than female function.

The size-advantage hypothesis predicts that changes in sex expression are controlled by size. In animals, these changes are most often unidirectional from male to female and thus occur just once over a lifetime (Warner, 1975, 1988). However, in plants the amount of resources stored in underground organs may change from year to year depending on annual climatic conditions, levels of herbivory, pollinator activity, and the amount of fruits and seeds produced. As a result, both increases and decreases in below-ground reserves can occur, resulting in reversals in gender (Bierzychudek, 1984; Schlessman, 1991). Our results from *L. apertum* confirm these expectations: we not only observed changes from male to female function with increased size, but also cases where primarily female-functioning plants reverted to male function in the second year associated with a decrease in their size. Although we did not explicitly investigate the extent to which flowers of *L. apertum* plants were pollen-limited in this study, our preliminary observations indicated that all marked flowers set fruit, which produced as many seeds as fruits from flowers that received supplemental hand-cross pollination (Z.-Q. Zhang, unpubl. data). If *L. apertum* is most often resource- rather than pollen-limited, annual reproductive expenditure associated with female function may play an important role in determining changes in plant size and gender.

Our studies of size-dependent gender modification in *L. apertum* raise several important questions concerning the proximate mechanism governing sex expression. At this stage we do not know the specific developmental stages at which the gender of individuals is controlled. Given the geophytic life history of *L. apertum*, flower number in any given season may be determined at the end of the previous growing season, based on the amount of stored reserves in the bulb and the number of floral primordia that are initiated. Preformed flowers could contain rudimentary female and sex organs and the decision whether to maintain or abort female function may occur in the subsequent spring prior to flowering based on an ‘assessment’ of current resources and future expenditures. For small plants with only a single male flower this decision may occur early in the growing season; however, for large plants producing both hermaphroditic and male flowers the developmental decision to abort female function in distal flowers may depend on whether basal flowers are pollinated and initiate fruit set. Because of the extended period over which flowers and buds are present on plants (e.g. see Fig. 1C) this seems quite likely. Experimental pollination studies (e.g. see Diggle, 1997) could be used to test this hypothesis.

L. apertum produces only a small number of large flowers (e.g. 1–5) and allocation ‘decisions’ affecting the sex expression of flowers may be best understood in terms of theories for the fractional allocation of resources among a small number of units (see Ebert, 1994; Charnov *et al.*, 1995). If the optimal investment for hermaphroditic flowers falls between the necessary integer values male functioning flowers may be the only option. In

populations of *L. apertum* the largest plants with the most flowers produced both hermaphroditic and male flowers (Fig. 5). This suggests that the resources available to these plants were not sufficient to produce additional hermaphroditic flowers, but rather were invested in less costly male flowers. Future studies might usefully measure variation in flower size and number in *L. apertum* and consider ways to determine the threshold allocation to flowers below which female function is aborted.

The selective factors responsible for the evolution of size-dependent gender modification differ depending on whether gender diphasy or gradual gender adjustment are considered. Although in both cases an individual’s environment and reproductive status must differentially affect paternal and maternal fitness, the particular shape of the fitness gain curve for each sexual function is of critical importance in determining the form of gender modification that can evolve (Lloyd and Bawa, 1984; Zhang, 2006). A range of other factors including the scale of environmental heterogeneity in a population and its influence on local gamete ratios are also likely to be influential. Given the rarity of gender diphasy in angiosperms compared with other forms of size-dependent gender modification it would appear that the environmental circumstances and particular plant traits that promote the evolution of this unusual sexual system are restrictive. Comparative studies of the ecology and reproductive biology of related species of *Lilium* may aid in identifying the mechanisms responsible for the evolution of gender diphasy from simultaneous hermaphroditism.

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