Multiple effects of drought on pollination and mating-system traits in
Dalechampia scandens

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16 Abstract

17 *Premise of research.* Selfing rates in mixed-mating plant species are often found to fluctuate
18 greatly across time and space. Environmentally induced changes in floral traits may mediate
19 changes in selfing rates through several mechanisms, including direct effects via changes in
20 traits influencing autofertility rates, and indirect effects via changes in traits affecting the rate
21 of pollinator visitation and/or the efficiency of cross-pollination. In this study we tested how
22 experimentally induced drought affected traits related to these three components of plant
23 mating systems.

24 *Methodology.* We subjected two populations from each of two species in the *Dalechampia*
25 *scandens* species complex to a series of experimental drought events in the greenhouse. We
26 measured drought effects on advertisement (signaling) traits, reward traits, herkogamy,
27 dichogamy, autofertility, and pollination accuracy, and compared these across populations and
28 species.

29 *Pivotal results.* Blossom size (advertisement and reward traits) and dichogamy were
30 consistently reduced under dry conditions. In contrast, the effects of drought on herkogamy
31 and autofertility were population specific. Similarly, despite consistent effects of drought on
32 traits functionally related to pollen transfer, changes in pollination accuracy differed among
33 populations. When plants were returned to a benign moisture environment, phenotypic
34 changes were largely reversed.

35 *Conclusions.* These results show that environmental variation may simultaneously affect
36 multiple traits related to plant mating systems, and thus mediate spatial and temporal variation
37 in selfing rates. However, except for size reductions in advertisement and reward traits, these
38 effects tend to be population-specific and therefore difficult to predict.

39 *Keywords: Dalechampia; dichogamy; herkogamy; mating-system traits; plant-pollinator*
 40 *interactions; pollination accuracy*

41 Introduction

42 Selfing rates in mixed-mating plant species are often found to fluctuate greatly across time
 43 and space, and a current challenge is to understand how environmentally induced variation in
 44 floral traits mediates this variability (Vallejo-Marin and Barrett 2009; Ivey and Carr 2012;
 45 Karron et al. 2012; Spigler and Kalisz 2013). Stressful environmental conditions encountered
 46 when populations experience drought or habitat degradation, for example, are generally
 47 thought to induce phenotypic variants associated with increased self-fertilization (Holtsford
 48 and Ellstrand 1992; Levin 2010). Drought effects on flowers include reduced flower size (e.g.,
 49 Frazee and Marquis 1994; Carroll et al. 2001; Elle and Hare 2002; Caruso 2006), reduced
 50 floral longevity (Jorgensen and Arathi 2013), faster floral development (Ivey and Carr 2012),
 51 and changes in dichogamy or herkogamy (e.g., Vallejo-Marin and Barrett 2009; Kay and
 52 Picklum 2013; Spigler and Kalisz 2013; Van Etten and Brunet 2013). These trait variants are
 53 known to be associated with increased selfing rates (Ornduff 1969), but in order to predict
 54 plant responses to environmental change, we need to understand the different functional
 55 mechanisms by which these responses are mediated.

56 Environmental variation in floral traits can affect selfing rates both directly and
 57 indirectly. As an example of the former, reduced spatial separation of anthers and stigmas
 58 (herkogamy) is expected to directly increase the ability to produce seeds by autonomous
 59 selfing, as demonstrated by the often observed negative association between herkogamy and
 60 autofertility (seedset in the absence of pollinators), and ultimately selfing rate (e.g., Holtsford
 61 and Ellstrand 1992; Moeller 2006; Takebayashi et al. 2006). Similarly, changes in floral
 62 ontogeny may affect the separation of male and female functions in time (dichogamy, Lloyd

and Webb 1986), which may affect selfing rates both directly and indirectly. Greater overlap of sexual functions increases autofertility rates, for example, in some partially protandrous species, where a male phase precedes a bisexual phase (Holtsford and Ellstrand 1992; Brunet and Eckert 1998; Moeller 2006). In other cases, including some partially protogynous species (where a female phase precedes a bisexual phase), reduced dichogamy may not necessarily affect autofertility rates directly, but rather affect the timing and relative importance of selfing by reducing the time available for efficient cross-pollination (Brys and Jacquemyn 2011; Opedal et al. 2015).

Indirect effects of environmental variation in floral traits on selfing rates include reduced opportunities for cross-pollination if pollinator visitation rates are reduced in stressful environments due to reductions in traits involved in floral advertisement (e.g., corolla size) or in rewarding pollinators (e.g., rates of production of nectar or other rewards). Similarly, indirect effects can occur if environmentally induced changes in floral morphology affect the efficiency of cross-pollination. Effective pollination requires that pollen is deposited by the anthers onto a part of the pollinators' body that is likely to contact the stigma during subsequent floral visits. Armbruster and colleagues (2009a, b) defined pollination accuracy as the correspondence between male and female floral organs with regard to the positions where they contact the pollinator. They further partitioned maladaptation into two additive components: the bias corresponding to the mean departure from the optimum, and the imprecision caused by the variance around the mean. Environmentally induced changes in the means and variances of traits involved in pollen transfer to and from pollinators may thus affect both components (Armbruster et al. 2009a). Previous studies of pollination accuracy (Armbruster et al. 2009a, b) have considered patterns of variation across populations and species, but the effect of environmental variation on pollination accuracy has not been assessed previously.

In this study, we investigate how drought affects traits likely to influence the mating system of the neotropical vine *Dalechampia scandens* (Euphorbiaceae). *Dalechampia scandens* occurs in a seasonal environment (tropical dry forest and thorn woodland), and often in disturbed habitats. Flowering starts in the mid to late wet season, and continues into the dry season (Armbruster and Herzig 1984). Thus, we might expect a gradual decrease in soil moisture throughout the flowering period, with potential consequences for the mating system of the species. Field observations made during the transitional period, when periods of rainfall are fewer and farther between, suggest that plants experience repeated episodes of drought stress, as indicated by leaf wilting (Opedal & Albertsen, personal obs.). It is therefore of interest not only to understand the effects of drought on traits related to the pollination and mating system of the species, but also whether these effects are rapidly reversible or not. We specifically distinguish between effects of drought on the autofertility rate, mediated by phenotypic changes in herkogamy, and effects on potential cross-pollination, mediated by changes in dichogamy (length of female phase), pollinator-attraction traits (the opportunity for cross-pollination), and pollination accuracy (the efficiency of cross-pollination).

Ongoing research has shown that, rather than a single species, *Dalechampia scandens* is actually a species complex, including at least two similar, undescribed species. These differ in several key traits, including blossom size and degree of herkogamy (Pélabon et al. 2005; Armbruster et al. 2009b; Bolstad et al. 2014). We took advantage of this variation to test whether the effects of drought on pollination and mating-system traits were consistent across closely related species that presumably differ in their dependence on pollinators and average pollinator size. We subjected two populations from each of two species of the *D. scandens* complex to a series of experimental drought events in the greenhouse and compared responses in blossom morphology, herkogamy, dichogamy, and autofertility across these four populations. We also measured the effect of drought on leaf growth to assess the degree of

decoupling of phenotypic variation between floral and vegetative traits (cf. Berg 1960; Armbruster et al. 1999; Pélabon et al. 2011; see review in Murren 2012).

Materials and Methods

Study species and populations

Dalechampia scandens L. (Euphorbiaceae) is a species complex of self-compatible vines with mixed mating systems. It is widely distributed in the Neotropics, and populations often have different blossom morphologies (Armbruster 1985; Hansen et al. 2000). Unisexual male and female flowers are aggregated into functionally bisexual blossom inflorescences (pseudanthia), comprising a cluster of three female flowers situated below a cluster of six to ten male flowers (Fig. 1). Each female flower contains three ovules, and each blossom can produce a maximum of nine seeds. A resin-secreting gland is located above the male flowers. The resin is collected by female apid and megachilid bees for use in nest construction (Armbruster 1984). Blossoms with larger resin glands offer more reward (Pélabon et al. 2012), and populations with larger glands tend to be visited by larger bees (Armbruster 1988). The male and female sub-inflorescences are subtended by two involucre bracts that open during the day to allow pollination, and close at night to protect the flowers (Armbruster 1985). The bracts are transformed leaves, and thus bracts and leaves are serially homologous (Webster and Webster 1972; Hansen et al. 2007; Pélabon et al. 2011). Phenotypic-selection studies on several *Dalechampia* species have shown that, depending on the species and population, bees make their foraging decisions based on either the size of the gland (the amount of reward) or the size of the bracts, which honestly advertise the quantity of reward produced (Armbruster et al. 2005; Bolstad et al. 2010; Armbruster et al. 2011; Pérez-Barrales et al. 2013).

The blossoms develop through a series of distinct ontogenetic stages (Webster and Webster 1972; Hansen et al. 2003). In the first phase ('bud', Fig. 1b), bracts remain tightly closed around the young blossom. The second phase starts with the opening of the bracts. During this 'female' phase (Fig. 1c) the stigmas are receptive, while the male flowers stay closed for a few more days. The bisexual phase (Fig. 1d) is defined by the sequential opening of the male flowers over a period of approximately 4-5 days, during which self-pollination can occur (the stigmas remain receptive throughout anthesis). A few days after the opening of the final male flower, the male cymule abscises and the bracts close around the developing fruits. At this time, bracts turn from more or less white to green and increase their rates of photosynthesis (Pélabon et al. 2015).

The experimental populations are second greenhouse generations (except the Tulum population, which is the fifth greenhouse generation), derived from random crosses among plants grown from seeds collected from separate individuals in the field. The seeds were collected by G.H. Bolstad on the Yucatan peninsula in 2007. The populations belong to two undescribed species differing in blossom morphology (Armbruster et al. 2009b; Bolstad et al. 2014). The populations from Tulum (T; 20° 12' N; 87° 27' W) and Puerto Morelos (PM; 20° 51' N; 86° 53' W) belong to the 'large-glanded' species with relatively large bracts and resin-glands, pronounced anther-stigma separation (greater herkogamy), and a mixed mating system (selfing rates range from 0.51 to 0.82; Opedal, unpublished results). The populations from Valladolid (V; 20° 42' N; 88° 15' W) and Cozumel (CO; 20° 22' N; 87° 59' W) belong to the 'small-glanded' species, with smaller bracts and resin glands, and much lower herkogamy (Bolstad et al. 2014). Both species occur in disturbed habitats (roadsides, forest edges, and thorn woodlands).

Experimental design and measurements

In total, 101 plants were used in the experiment (19-28 plants per population; Table 1). Plants were grown between October 2013 and March 2014 and were assigned stratified random positions on four greenhouse tables in a single room, representing either dry or wet experimental environments. Plants in the wet treatment were watered every day by flooding the table to maintain roughly constant soil moisture. Plants in the dry treatment were carefully monitored daily and top-watered individually with 200 mL of water only when they showed signs of drought stress (drooping leaves, indicating loss of turgor pressure). Hence, we imposed, as much as possible, uniform drought conditions on plants in the dry environment by controlling for individual differences in water uptake and use. Effectively, plants in the dry treatment were watered every second to fourth day. Identical conditions of temperature (26°C day/24°C night) and light (13:11 light/dark) were maintained for all plants.

Blossoms take ca. one week from the initiation of the bud to the first day of bract opening (start of the female phase). Therefore, to ensure that the measured blossoms in the dry treatment developed entirely under dry conditions, the treatments were maintained for two weeks before starting measurements and continued throughout the measurements. After the first series of measurements was completed, the plants were fertilized and allowed to recover for three weeks with daily watering. The plants were then swapped between treatments and a second series of measurements was taken after two weeks of acclimation. This design allowed us to assess whether phenotypic responses to the water treatment were rapidly reversible or not, and to remove the possible confounding effect of the position of the tables in the greenhouse.

Blossom morphology - Morphological traits (Fig. 1) were measured on two blossoms haphazardly chosen from each plant. These blossoms were harvested and measured on the first day of the bisexual phase (first male flower open). We measured two traits involved in

pollinator attraction: upper bract area (UBA, the product of the bract length and bract width) and gland area (GA, the product of the gland width and gland height). We also measured gland-stigma distance (GSD, the average of the minimum distances between the gland and the three stigmas), and gland-anther distance (GAD, the minimum distance between the gland and the anthers of the central male flower). These two traits indicate the minimum size of bees that can efficiently transfer pollen to stigmas and export pollen from anthers, respectively (Armbruster 1988, 1990). We measured one trait influencing the rate of self-pollination: anther-stigma distance (ASD, the minimum distance between the anther and the stigmas) (Armbruster 1988; Opedal et al. 2015). The bract blades were carefully removed from the blossom and flattened under an acetate sheet before measurements. All morphological measurements were made by a single observer (JL) using digital callipers (0.01 mm precision) and an optical magnifier ($\times 5$).

Dichogamy - On each plant, an additional blossom still in the bud stage was marked with a short piece of yarn. We recorded each day the ontogenetic stage of these blossoms, scored as either in bud, in female phase, in bisexual phase or post bisexual phase (with the male cymule abscised). The width of the upper bract was measured on the first day of the female phase (the day when the blossom opened for the first time), the first day of the bisexual phase (the day the first male flower opened) and at the end of anthesis (the day the male cymule abscised).

Autofertility - To test whether drought affected the capacity of the blossoms to produce seeds by autonomous selfing, we initiated a third episode of treatments after randomly reallocating the plants to the different tables. After two weeks of acclimation, we marked one to six buds (median = 4) per plant with short pieces of yarn. After two more weeks, we recorded whether each blossom had started to develop fruits, and the blossoms developing fruits were carefully

wrapped with empty tea bags to collect the seeds after explosive capsule dehiscence, ca. 5 weeks after pollination. At this time, plants in the dry treatment were strongly stressed, and the treatment was terminated to allow watering and fertilization of the plants. Hence, seeds from the plants in the dry treatment were initiated in the dry environment, when the traits expected to influence autofertility were manifested, but developed partly under benign (wet) conditions. The estimated autofertilities therefore represent the autofertility rate under dry conditions, but might overestimate actual seed production if increased fruit abortion occurs under prolonged drought.

Leaf growth - During the second treatment period, the growth of a young leaf was measured on each plant. These leaves were marked and measured concurrently with the measurements of the bracts throughout the blossom ontogeny (see above) to allow a direct comparison of the growth of leaf blades and bract blades under wet and dry conditions.

Statistical analyses

All statistical analyses were performed in R, version 3.1.0 (R Core Team 2014). To test whether morphological and developmental traits differed among species, populations and treatments, we fitted three sets of linear mixed-effects models with the lme4 R package (Bates et al. 2014). In the first set, we tested for differences between the two species by including species, treatment, experimental period and their interactions as fixed factors, and population and plant (nested within population) as random factors. To test further for differences between populations within species, we fitted separate models for each species where population, treatment, experimental period and their interactions were treated as fixed factors, and plant as a random factor. We initially tested for differences among the tables in the greenhouse. These were never supported statistically, and therefore we did not include table as a factor in the

main analyses. To avoid the controversies surrounding p-values for mixed-effects models (Bolker et al. 2009), statistical inferences were based on information-theoretic model selection using the Akaike Information Criterion (AIC). AIC values were obtained from models fitted by maximum likelihood, and parameter estimates were obtained from the highest ranked models refitted by restricted maximum likelihood (REML). Bract area and gland area were square-root transformed in order to maintain the same unit dimension (mm) for all traits.

To compare the effect of the treatment on the morphological traits, we partitioned the phenotypic variance into between-treatment, among maternal plant, and among-blossom components. For each population, we fitted separate linear mixed-effects models where treatment was a fixed factor and maternal plant a random factor. The variance explained by the treatment was estimated as the decrease in total random-effects variance when treatment was included in the model. The variance component for each hierarchical level was scaled by the square of the trait mean (the estimated intercept of the model). This method provides scaled components of the variance that can be summed to obtain the CV^2 of the trait as a measure of proportional variation (Pélabon et al. 2011).

To estimate the probability of a seed being self-fertilized, we fitted generalized linear mixed-effect models with binomial errors and logit link-function. Blossom identity, nested within plant, was included as a random factor to account for the non-independence of seeds within a blossom. We tested whether herkogamy affected autofertility at the population level by logistic regression (GLM with binomial errors, with observations weighed by the sample size in each population).

Pollination accuracy

The pollination inaccuracy of a population can be estimated for both male and female reproductive functions (Armbruster et al. 2009a). The inaccuracy for the male function is estimated as:

$$(E[z] - \theta)^2 + \text{Var}[\theta] + \text{Var}[z],$$

where $E[z]$ represents the population-mean position of the anthers relative to the resin gland, the site of reward production (i.e. GAD), θ the optimum for pollen deposition, that is, the population-mean position of the stigmas relative to the gland (GSD), and $\text{Var}[z]$ and $\text{Var}[\theta]$ their respective variances. The expression $(E[z] - \theta)^2$ represents the square deviation of the population mean from the adaptive optimum, that is, a bias, while $\text{Var}[z]$ and $\text{Var}[\theta]$ represent the imprecision in the trait expression and the variance in the optimum, respectively. The pollination inaccuracy for the female function considers the position of the stigma as the focal trait z , and the position of the anthers as the optimum, θ . Therefore, male and female population inaccuracies are identical in *Dalechampia* (Armbruster et al. 2009b), and we treated this value as ‘joint inaccuracy’, estimated as:

$$(\text{Population mean GSD} - \text{population mean GAD})^2 + \text{Variance in GSD} + \text{Variance in GAD}$$

Mean-scaled inaccuracies, which allow comparison between populations and treatments differing in mean values, were obtained by scaling the joint inaccuracy by the product of the male and female trait means in each population and treatment. Ninety-five percent confidence intervals for the estimated inaccuracies were obtained from 1000 non-parametric bootstrap estimates, with individual blossoms as the unit of resampling.

Results

Drought effects on advertisement and reward traits

The sizes of both the advertisement (bract area) and the reward trait (gland area) were reduced in the dry environment (Fig. 2, Table 1). The overall trend was similar across all four populations although the effect size differed between species for both traits, and, for bract area, also between populations (Table 1, see Table S1 for model-selection results). In the dry treatment, the bracts were on average 21.4% and 26.3% smaller in the two large-glanded populations (T and PM, respectively), and 16.7% and 23.7% smaller in the two small-glanded populations (CO and V, respectively). Between 32.7% and 54.2% of the variance in bract area was generated by the moisture treatment (Table 2).

The treatment effect on bract size differed somewhat between the two experimental periods, but this effect was small and inconsistent compared to the effects of the moisture treatment (Table 1, treatment $\times$ period interaction). Indeed, if this difference reflected non-reversible drought effects, we would expect blossoms in the wet treatment to be smaller during the second period compared to the first period, because these plants had previously experienced a drought event. However, the observed difference in bract size between the wet treatment in the first and second period ranged from a 4.6% reduction to an 8.1% increase (Table S4).

The resin gland was affected proportionally less by the treatment than were the bracts, and the response differed between species, but was similar across populations within species (Fig. 2, Table 1). The size of the resin gland was reduced in dry conditions by 16.2% and 11.0% in the large- and small-glanded species, respectively. Compared to bract size, a smaller proportion of the variance in gland size was explained by the treatment (Table 2). Differences in gland size in the wet treatment between the first and second period ranged from a 7.6% reduction to a 0.3% increase (Table S4), and the treatment $\times$ period interaction was marginally significant only for the large-glanded species (Table 1). Again, this suggests that the effects of drought were largely reversible within a few weeks.

Herkogamy

The response of anther-stigma distance (ASD) to the dry treatment differed between species and between populations within species (Fig. 2, Table 1). While ASD was reduced from 0.68 ± 0.12 mm (mean $\pm$ SE) to 0.17 ± 0.05 mm in the dry treatment in the CO population, it was increased from 2.71 ± 0.16 mm to 3.27 ± 0.10 mm in the PM population. In the T and V populations, there was no detectable effect of drought on ASD. For the two populations where ASD was affected by the treatment, the direction of the effect was similar in the two periods, but for the PM population the effect was stronger in the first period (Table S4).

Autofertility

Autofertility was negatively affected by ASD (logistic regression, $\beta_{\text{ASD}} = -1.43 \pm 0.04$, $P < 0.001$) and differed markedly between the two species (Fig. 3). There was a tendency for autofertility to increase with the dry treatment, but this effect was population specific and weakly supported statistically (Table 3, see Table S2 for model-selection results). In the CO population, as could be expected from the reduced herkogamy in the dry treatment, the probability of setting seeds by auto-fertilization increased from 0.52 in the wet environment to 0.60 in the dry environment, although this increase was not statistically supported (Table 3). For the T population, the absolute increase was even weaker, from 0.03 to 0.06. We found no detectable effect of the treatment on autofertility in the PM and V populations.

Dichogamy

Overall, blossom development tended to be more rapid under dry conditions. In the wet treatment, the total receptive period of the blossoms (from the first day of opening to the abscission of the male cymule) lasted for 6.87 ± 0.09 days in the two large-glanded populations and 5.80 ± 0.10 days in the two small-glanded populations (Fig. 4). In the dry treatment, the length of this period was moderately reduced by, on average, 5.4% across all four populations

(Fig. 4, Table 4, see Table S3 for model-selection results). The effect of the treatment on the length of the female phase (dichogamy) was more pronounced, however (Fig. 4, Table 4). For the large-glanded populations, the female phase decreased by 48.5% under dry conditions, from 2.73 ± 0.08 days to 1.40 ± 0.09 days. For the small-glanded populations, the female phase was reduced by 22.3%, from 2.47 ± 0.07 days in the wet treatment to 1.91 ± 0.08 days in the dry treatment.

Positions of stigmas and anthers, and pollination accuracy

In the two large-glanded populations, gland-stigma distances (GSD) were reduced by an average of 26.7% under dry conditions, while gland-anther distances (GAD) were reduced by only 16.4% (Fig. 5, Table 1). Correspondingly, the proportion of phenotypic variance explained by the treatment was more than 40% for GSD, and less than 25% for GAD (Table 2). For the two small-glanded populations, the treatment effects were somewhat weaker; GSD decreased by an average of 15.5%, and GAD by 11.2% in the dry compared to the wet environment. For the two small-glanded populations, GAD tended to be slightly smaller in the wet treatment in the second period compared to the first (8.3% and 6.9% in the CO and V population, respectively, Table S4).

The disproportional changes in GSD and GAD led to changes in the pollination accuracy of the blossoms and the relative importance of bias and imprecision (Table 5). For the two large-glanded populations, the joint inaccuracy increased in the dry treatment. This was caused essentially by an increase in imprecision in the T population, while in the PM population the increased inaccuracy was generated mostly by an increase in the bias. For the two small-glanded populations, there were no significant differences in inaccuracy between treatments (Table 5). Note, however, that the small-glanded populations tended to be more inaccurate in the wet treatment, a pattern opposite to that observed for the large-glanded populations.

Leaf growth

Leaves of all populations responded strongly to the dry treatment (Fig. 2). At the end of the measurement period, leaves were on average 40.9% smaller in the dry treatment, a reduction nearly twice as strong as the reduction observed for the bracts. This effect was highly significant (ANOVA, $F_{1,74} = 55.2$, $P < 0.001$), and similar across all four populations (population effect, $F_{3,74} = 0.62$, $P = 0.60$; interaction effect, $F_{3,74} = 0.46$, $P = 0.71$).

Discussion

Selfing rates in mixed-mating plant species are often found to fluctuate among years within populations (Eckert et al. 2009), and even within a single season (Yin et al. 2016). We have shown that *Dalechampia* floral traits can respond rapidly to reduced soil moisture, and that these responses are largely reversible when a benign, moist environment is restored. It seems likely that such direct, most likely passive, physiological responses to drought contribute to fluctuations in plant mating systems in time and space, the ultimate outcome being variation in the selfing rate and possibly plant fitness (Levin 2010; Peterson and Kay 2015). The observed reductions in advertisement and reward traits, as well as dichogamy, may contribute to reduced cross-pollination in dry conditions due to reduced attractiveness of the blossoms to pollinators and reduced time available for cross-pollination. Changes in herkogamy and autofertility were species and even population specific, making them difficult to predict. Finally, we have demonstrated for the first time that environmental variation can affect the accuracy of pollen transfer, and thus the efficiency of cross-pollination, independently of any effects on pollinator visitation rate.

Reduced expression of advertisement and reward traits under dry conditions

Both the size of the main advertisement trait (bract size) and the amount of reward (gland size) were reduced under dry conditions. Because the bee pollinators of *Dalechampia* base their foraging decisions on the size of one or both of these traits (Armbruster et al. 2005; Bolstad et al. 2010; Armbruster et al. 2011; Pérez-Barrales et al. 2013), these changes could potentially reduce the visitation rate and consequently the opportunity for cross-pollination, and modify patterns of selection on floral traits (Caruso et al. 2003; Caruso 2006). Even so, bract size was reduced proportionally less than leaf size in the dry treatment, suggesting that bracts are more buffered against environmental variation than are vegetative traits (see further discussion below). The response of the bracts to drought was more similar to that of leaves, however, than was observed when nutrients were manipulated (Pélabon et al. 2011). Although we did not measure the water status of the plants directly, the wilted leaves of plants in the dry treatment every second to fourth day (our cue for watering the plants) provided a clear indication that these plants experienced reduced leaf water potential. This might well have driven the reduction in bract and floral organs expansion through a reduced ability of turgor-driven enlargement of cells (Galen 1999; Galen et al. 1999; Carroll et al. 2001; Armbruster et al. 2013; Beauzamy et al. 2014). We further noticed that, after several days without watering, the floral bracts showed much less sign of wilting than did leaves. This could be because: 1) there is a lower density of stomata on bracts than on leaves, as suggested by Pélabon et al. (2015), 2) it is advantageous for bracts not to wilt while flowers are receptive, or 3) the accentuated wilting of leaves is partly an adaptation to reduce solar radiation load at midday (Armbruster, unpubl. obs.).

Because we measured the blossoms at a fixed ontogenetic stage (first day of the bisexual phase), and the timing of ontogenetic events differed between treatments (Fig. 4), it is possible that part of the observed size-reduction in blossom traits in the dry treatment was an artifact of measuring the blossoms at earlier points during their development. However, the

repeated measurements of the bracts throughout late development (Fig. 4) confirmed that the difference in blossom size between the treatments was maintained throughout the entire life of the blossom.

When plants that had previously experienced a drought event were returned to a benign moisture environment, the observed reductions in floral traits were largely reversed, although gland area (GA) and to some extent male-flower elongation (GAD) tended to remain slightly reduced. Although weakly supported statistically, this might indicate that drought effects on these traits are not fully reversible, which might have consequences for the long-term dynamics of plant mating systems during recurrent drought events in natural populations. We do not discuss this issue further here, but note that such ‘lag effects’ warrant further investigation.

Environmental variation in mating-system traits: Dichogamy, herkogamy and autofertility

Environmentally induced variation in herkogamy and dichogamy can influence plant mating systems by affecting the rate or timing of autonomous selfing. In *Dalechampia*, cross-pollination is most likely to take place during the female phase, and the relative positions of male and female flowers suggest that the bisexual phase can be considered to be functionally a male phase (Armbruster et al. 2011). Similar to previous studies on *Clarkia* (Holtsford and Ellstrand 1992; Kay and Picklum 2013), we found a stronger and more consistent environmental effect on dichogamy than on herkogamy (but see Van Etten and Brunet 2013 for an opposite effect). Although dichogamy does not necessarily affect autofertility directly in partially protogynous species such as *D. scandens*, reduced dichogamy will reduce the time available for efficient cross-pollination, and might therefore influence the timing and relative importance of autonomous selfing (Brys and Jacquemyn 2011). All four populations exhibited reduced dichogamy in the dry treatment, although the two large-glanded populations responded more strongly. While the female phase lasted slightly longer in the large-glanded

populations than in the small-glanded populations in the wet environment, the trend was opposite in the dry environment. Hence, the small-glanded species appears to maintain a longer female phase, and hence the opportunity for cross-pollination, in an environment similar to our dry treatment.

Reduced herkogamy increases the probability of self-pollen arriving on the stigmas of bisexual-phase blossoms (Opedal et al. 2015). In the extreme case of contact between the anthers and stigma ($ASD = 0$) the blossom will self-pollinate immediately when the first male flower opens, as happens commonly in the small-glanded *D. scandens* (Opedal, personal observation). Negative associations between herkogamy and autofertility or selfing rate have been demonstrated in many systems (e.g., Armbruster 1988; Holtsford and Ellstrand 1992; Moeller 2006; Takebayashi et al. 2006; Opedal et al. 2015). While population-mean herkogamy was indeed negatively associated with autofertility rate across our study populations, the effect of drought on herkogamy and autofertility was largely unpredictable. Two populations (CO, T) slightly increased their autofertility rate in the dry environment, while there was no detectable effect in the PM and V populations. The small change in autofertility in the CO population might be explained by the reduced herkogamy in the dry treatment. The increase in herkogamy in the PM population in the dry treatment did not reduce autofertility, however, possibly because the autofertility rate was already low (Fig. 3; note the approximately exponential decay of autofertility with increasing herkogamy).

A few recent studies have investigated environmental effects on autofertility. In *Clarkia*, Kay and Picklum (2013) found contrasting responses of two sister species, with one species (*C. concinna*) exhibiting greater autofertility under dry conditions than under more benign conditions, while a second species (*C. breweri*) responded in the opposite direction. In *Collinsia verna*, drought had no overall effect on autonomous seed set, although some families increased their autofertility rate in a stressful environment (Spigler and Kalisz 2013),

while in *Collinsia heterophylla*, autonomous selfing was more likely under wet conditions than under dry conditions (Jorgensen and Arathi 2013). Among these studies, only Kay & Picklum (2013) tested for changes in herkogamy under drought, and found no significant effect. Indeed, most studies testing for environmental effects (including variation in soil moisture, nutrient and light intensity) on herkogamy have found relatively limited or variable effects (Elle and Hare 2002; Mal and Lovett-Doust 2005; Brock and Weinig 2007; Ivey and Carr 2012; Van Etten and Brunet 2013; but see Vallejo-Marin & Barrett 2009). The emerging pattern is that any effect of drought on autofertility mediated by changes in herkogamy will be species and population specific, and more studies are clearly needed to explore further the role of environmental variation in this key feature of plant mating systems.

Changes in pollination accuracy under dry conditions

Specialized pollination systems, like that of *D. scandens*, have been hypothesized to generate canalizing selection on certain floral traits in order to maintain the accuracy of the flower-pollinator fit (Cresswell 1998; Armbruster et al. 2009a, b), and floral traits involved in pollen transfer to and from pollinators are consequently expected to be less sensitive to environmental variation than are vegetative traits (Berg 1960; Armbruster et al. 1999; Hansen et al. 2007; van Kleunen et al. 2008; Pélabon et al. 2011). In *Dalechampia*, the distances between the resin-secreting gland and the anthers (GAD), and between the gland and the stigma (GSD) influence the location of pollen deposition on, and pick-up from, the pollinator, respectively (Armbruster et al. 2009a, b). As expected if these traits have been under canalizing selection, GAD and GSD (in the small-glanded species) were affected proportionally less by the dry treatment than were the bracts, and much less than were the leaves. Interestingly, the observed environmentally induced changes in the means and variances of GAD and GSD led to different effects on pollination accuracy in the two species. While the two large-glanded populations tended to be more inaccurate in the dry environment,

the small-glanded populations tended to be more inaccurate in the wet environment. The interpretation of these changes was complicated, however, because the differences in inaccuracy were caused by changes in imprecision, bias, or both (Table 5). Considering the two populations for which bias differed the most between the two treatments (CO and PM), the change in bias corresponded with the observed changes in ASD (Fig. 2). The CO population had a larger ASD, and a higher bias, in the wet treatment. For the PM population, the trend was opposite. Armbruster and colleagues (Armbruster et al. 2009b; Armbruster et al. 2014) have suggested that because floral morphologies associated with reduced bias will often tend to reduce the physical distance between anthers and stigmas, a functional trade-off (the ‘herkogamy dilemma’) might exist between pollination accuracy and herkogamy. While this trade-off might explain at least part of the variation in bias between the treatments, the changes in trait variances under dry conditions are more difficult to interpret. Overall, these results suggest that environmental variation can affect pollination accuracy, although predicting the direction of the effect is not straightforward and may be population specific.

Conclusions

Despite the tendency of floral traits to be phenotypically decoupled from environmental variation in vegetative traits, stressful environmental conditions can trigger changes to their development that possibly affect pollination and mating systems. This study is, to the best of our knowledge, the first to test simultaneously the effect of drought on autofertility (via changes in herkogamy), the opportunity for cross-pollination (changes in dichogamy and rewarding and advertisement traits), and the efficiency of cross-pollination (changes in pollination accuracy). We found some evidence that all three of these important components of pollination and mating systems could be affected by environmental variation, but that the effect on the mating system might be difficult to predict. These findings illustrate some likely

sources of temporal and spatial variation in selfing rates, and the importance of understanding how they are affected by drought and other environmental stressors.

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Table 1. Effects of taxon (species/population), treatment and experimental period on morphological traits.

Trait	Taxon	Intercept	Taxon effect	Treatment effect	Period effect	Taxon × Treatment	Taxon × Period	Treatment × Period
$\sqrt{\text{UBA}}$ (mm)	Between species	21.08 ± 0.58	-1.30 ± 0.79	-4.47 ± 0.44	0.90 ± 0.44	1.35 ± 0.44	-1.35 ± 0.44	-1.40 ± 0.66
	Large-glanded	21.59 ± 0.54	-1.37 ± 0.65	-4.91 ± 0.65	1.71 ± 0.65	1.52 ± 0.58	-0.83 ± 0.58	-2.13 ± 0.97
	Small-glanded	20.41 ± 0.36	-0.56 ± 0.52	-3.36 ± 0.40	-1.09 ± 0.32	-1.22 ± 0.67		
$\sqrt{\text{GA}}$ (mm)	Between species	4.40 ± 0.10	-1.11 ± 0.14	-0.70 ± 0.06	-0.22 ± 0.04	0.34 ± 0.09		
	Large-glanded	4.22 ± 0.10	0.22 ± 0.11	-0.56 ± 0.11	0.10 ± 0.13		-0.36 ± 0.13	-0.28 ± 0.18
	Small-glanded	3.41 ± 0.05	-0.27 ± 0.05	-0.36 ± 0.05	-0.19 ± 0.05			
GSD (mm)	Between species	5.38 ± 0.17	-1.18 ± 0.24	-1.41 ± 0.11	-0.19 ± 0.08	0.75 ± 0.16		
	Large-glanded	4.99 ± 0.14	0.55 ± 0.16	-1.19 ± 0.16	0.28 ± 0.19		-0.51 ± 0.19	-0.44 ± 0.25
	Small-glanded	4.28 ± 0.10	-0.35 ± 0.14	-0.65 ± 0.13				
GAD (mm)	Between species*	5.32 ± 0.29	-2.14 ± 0.41	-0.65 ± 0.15	0.27 ± 0.15	0.25 ± 0.22	-0.52 ± 0.22	-0.54 ± 0.23
	Large-glanded	5.83 ± 0.12	-0.75 ± 0.14	-0.92 ± 0.11				
	Small-glanded	3.10 ± 0.07	0.07 ± 0.10	-0.34 ± 0.07	-0.28 ± 0.09		0.24 ± 0.15	
ASD (mm)	Between species	2.52 ± 0.20	-2.24 ± 0.27	0.47 ± 0.14	0.26 ± 0.12	-0.64 ± 0.15		-0.33 ± 0.20
	Large-glanded	2.63 ± 0.15	-0.12 ± 0.19	0.53 ± 0.16	0.17 ± 0.11	-0.43 ± 0.22		
	Small-glanded	0.68 ± 0.08	-0.62 ± 0.13	-0.52 ± 0.11		0.54 ± 0.19		

Parameter estimates ± SE were obtained from the highest ranked models following model selection (see Table S1 for details). For the between species comparison, the intercept represents the mean value for the large-glanded species in the wet treatment in the first period. The taxon effect represents the contrast between the large- and small-glanded species, the treatment effect, the contrast between the wet and dry treatment and the period effect, the contrast between the first and second period. For the within species comparisons, the intercept represents the mean trait value for the PM population (in the large-glanded species) and the CO population (in the small-glanded species) in the wet treatment in the first period. *Three-way interaction not shown (estimate ± SE = 0.66 ± 0.34).

Table 2. Components of phenotypic variance in morphological traits

Pop	Trait	Mean $\pm$ SE	Treatment	Among-plant	Within-plant	CV ²
<i>Large-glanded species</i>						
T	$\sqrt{\text{UBA}}$	18.5 $\pm$ 0.37	1.44	0.88	0.76	3.08
			46.8 %	28.5 %	24.7 %	
	$\sqrt{\text{GA}}$	3.96 $\pm$ 0.07	0.64	0.24	1.90	2.78
			23.1 %	8.7 %	68.3 %	
	GSD	4.73 $\pm$ 0.11	2.21	0.82	2.11	5.14
			43.0 %	16.0 %	41.0 %	
GAD	4.62 $\pm$ 0.10	0.93	0.61	2.39	3.93	
		23.8 %	15.4 %	60.8 %		
ASD	2.64 $\pm$ 0.08	≈ 0	0.05	9.09	9.14	
		0.0 %	0.1 %	99.9 %		
PM	$\sqrt{\text{UBA}}$	19.29 $\pm$ 0.40	2.33	0.42	1.61	4.36
			53.4 %	9.6 %	37.0 %	
	$\sqrt{\text{GA}}$	3.90 $\pm$ 0.07	0.91	0.40	1.25	2.55
			35.6 %	15.6 %	48.8 %	
	GSD	4.39 $\pm$ 0.10	2.50	0.00	2.56	5.07
			49.4 %	0.0 %	50.6 %	
GAD	5.35 $\pm$ 0.10	0.69	0.32	2.60	3.61	
		19.2 %	8.8 %	71.9 %		
ASD	3.00 $\pm$ 0.14	0.83	4.30	5.76	10.9	
		7.7 %	39.5 %	52.8 %		
<i>Small-glanded species</i>						
CO	$\sqrt{\text{UBA}}$	18.35 $\pm$ 0.29	0.79	0.25	1.38	2.43
			32.7 %	10.4 %	56.9 %	
	$\sqrt{\text{GA}}$	3.15 $\pm$ 0.04	0.32	0.04	1.08	1.44
			22.5 %	2.5 %	75.0 %	
	GSD	3.98 $\pm$ 0.10	0.93	0.00	5.29	6.21
			14.9 %	0.0 %	85.1 %	
GAD	2.81 $\pm$ 0.05	0.48	0.00	2.18	2.66	
		30.9 %	6.0 %	63.0 %		
ASD	0.45 $\pm$ 0.08	31.16	13.59	235.52	280.28	
		11.1 %	4.8 %	84.0 %		
V	$\sqrt{\text{UBA}}$	17.36 $\pm$ 0.40	1.72	0.15	1.30	3.17
			54.2 %	4.9 %	41.0 %	
	$\sqrt{\text{GA}}$	2.89 $\pm$ 0.04	0.35	0.11	0.85	1.31
			26.8 %	8.6 %	64.6 %	
	GSD	3.63 $\pm$ 0.11	0.30	1.12	2.05	3.47
			8.7 %	32.3 %	59.0 %	
GAD	3.01 $\pm$ 0.07	0.09	0.02	3.34	3.44	
		2.6 %	0.4 %	97.0 %		
ASD	0.07 $\pm$ 0.03	≈ 0	0.00	1073.35	1073.35	
		0.0 %	0.0 %	100.0 %		

Variance components were obtained from linear mixed-effect models fitted with plant as a random factor, and treatment as a fixed factor. Traits are upper bract area (UBA), gland area (GA), gland-stigma distance (GSD), gland-anther distance (GAD) and anther-stigma distance (ASD). The reported means ($\pm$ SE) are estimates of the intercepts in the models. Variance components are scaled by the square of the trait mean, and given as percentages. The variance components sum to the reported CV². Percentages are the amount of the total trait variance explained by each component.

Table 3. Effects of taxon (species/population) and treatment on autofertility rate.

Species	Intercept	Taxon effect	Treatment effect
Between species	-2.77 ± 0.69	4.17 ± 0.97	0.35 ± 0.21
Large-glanded	-2.28 ± 0.23	-0.98 ± 0.27	0.40 ± 0.27
Small-glanded	0.26 ± 0.21	2.76 ± 0.38	

Parameter estimates $\pm$ SE (on logit scale) were obtained from the highest ranked models following model selection (see Table S2 for details). For the between species comparison, the intercept represents the mean autofertility for the large-glanded species in the wet treatment. The taxon effect represents the contrast between the large- and small-glanded species, and the treatment effect, the contrast between the wet and dry treatment. For the within species comparisons, the intercept represents the mean for the PM population (in the large-glanded species) and the CO population (in the small-glanded species) in the wet treatment.

Table 4. Effects of taxon (species/population), treatment and experimental period on blossom development.

Trait	Species	Intercept	Taxon effect	Treatment effect	Period effect	Taxon × Treatment	Taxon × Period	Treatment × Period
Total (days)	Between species	7.08 ± 0.43	-1.12 ± 0.59	-0.35 ± 0.12	-0.42 ± 0.12			
	Large-glanded	6.46 ± 0.19	1.09 ± 0.20	-0.37 ± 0.18	-0.27 ± 0.18			
	Small-glanded	6.13 ± 0.18	-0.47 ± 0.19	-0.04 ± 0.24	-0.33 ± 0.23			-0.60 ± 0.37
Female (days)	Between species	2.42 ± 0.26	0.08 ± 0.38	-1.32 ± 0.14	0.60 ± 0.14	0.79 ± 0.22	-0.80 ± 0.22	
	Large-glanded	2.26 ± 0.16	0.32 ± 0.16	-1.32 ± 0.16	0.60 ± 0.16			
	Small-glanded	2.80 ± 0.13	-0.62 ± 0.14	-0.53 ± 0.14	-0.20 ± 0.14			

Parameter estimates ± SE were obtained from the highest ranked models following model selection (see Table S3 for details). For the between species comparison, the intercept represents the mean value for the large-glanded species in the wet treatment in the first period. The taxon effect represents the contrast between the large- and small-glanded species, the treatment effect, the contrast between the wet and dry treatment and the period effect, the contrast between the first and second period. For the within species comparisons, the intercept represents the mean value for the PM population (in the large-glanded species) and the CO population (in the small-glanded species) in the wet treatment in the first period.

Table 5. Inaccuracy statistics under wet and dry experimental conditions

Pop	Treatment	Bias ²	Male variance	Female variance	Joint inaccuracy (95% CI)	Mean-scaled inaccuracy (95% CI)
<i>Large-glanded species</i>						
T	Wet	0.14	0.25	0.48	0.87	0.03
		16.1 %	28.8 %	55.1 %	(0.62, 1.17)	(0.02, 0.04)
	Dry	0.05	1.00	0.80	1.85	0.11
		3.0 %	53.8 %	43.2 %	(1.42, 2.33)	(0.08, 0.14)
PM	Wet	0.53	0.94	0.58	2.05	0.07
		25.8 %	46.0 %	28.2 %	(1.56, 2.66)	(0.05, 0.09)
	Dry	1.42	0.69	0.40	2.51	0.14
		56.7 %	27.4 %	15.9 %	(1.94, 3.09)	(0.11, 0.17)
<i>Small-glanded species</i>						
CO	Wet	1.83	0.15	0.9	2.87	0.22
		63.7 %	5.1 %	31.3 %	(2.10, 3.71)	(0.17, 0.28)
	Dry	0.93	0.19	0.73	1.85	0.20
		50.2 %	10.5 %	39.4 %	(1.21, 2.57)	(0.14, 0.27)
V	Wet	0.55	0.33	0.53	1.41	0.12
		38.8 %	23.5 %	37.7 %	(0.82, 2.20)	(0.07, 0.19)
	Dry	0.29	0.24	0.24	0.77	0.08
		37.7 %	30.9 %	31.4 %	(0.43, 1.23)	(0.04, 0.13)

Bias² is the mean squared deviation from the hypothesized adaptive optimum. The reported percentages are the proportion of the joint inaccuracy explained by each component. To obtain the mean-scaled inaccuracy, the joint inaccuracy was scaled by the product of the male and female trait means. Ninety-five percent confidence intervals (95% CIs) were obtained from 1000 non-parametric bootstrap estimates of the joint and mean-scaled inaccuracies, respectively.

Figure legends

Fig. 1. *Dalechampia* blossom with illustration of functional traits (a), and ontogenetic development. Blossoms are shown in bud (b), female phase (c), and early bisexual phase with the first male flower open (d). The resin-secreting gland can be seen above the male flowers. Trait abbreviations are GSD: gland-stigma distance, GAD: gland-anther distance and ASD: anther-stigma distance. Drawing by M. Carlson, photos by Ø. H. Opedal.

Fig. 2. Reaction norms to soil moisture for upper bract area, gland area, leaf length and anther-stigma distance (ASD) in two populations from each of two species of the *D. scandens* complex. Mean trait values ($\pm$ SE) are shown for each population and trait, across experimental periods. All blossom traits were measured on the first day of the bisexual phase. Note the difference in y-axis scale for leaf length and ASD.

Fig. 3. Relationship between population-mean anther-stigma distance (ASD, herkogamy) and autofertility, expressed as the probability of an ovule being self-fertilized, across two moisture treatments.

Fig. 4. Blossom ontogeny in two populations from each of two species of the *D. scandens* complex under wet (closed symbols, solid lines) and dry (open symbols, dashed lines) experimental treatments. Mean bract width ($\pm$ SE) is shown for three ontogenetic stages, namely the day the bracts open for the first time, the day the first male flower opens, and the day the male cymule abscises.

Fig. 5. Reaction norms to soil moisture for gland-stigma distance (GSD) and gland-anther distance (GAD) in two populations from each of two species of the *D. scandens* complex. Mean trait values ($\pm$ SE) are shown for each population and trait, across experimental periods. Both traits were measured on the first day of the bisexual phase.

667 **Supplementary materials**

668 Table S1. Model selection results for morphological traits

669 Table S2. Model selection results for autofertility

670 Table S3. Model selection results for blossom ontogeny

671 Table S4. Trait means $\pm$ SE in the two experimental periods