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THE EVOLUTION OF MALES: SUPPORT FOR PREDICTIONS FROM SEX ALLOCATION THEORY USING MATING ARRAYS OF *SAGITTARIA LATIFOLIA* (ALISMATACEAE)

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Investment in male function should often yield diminishing fitness returns, subjecting the evolution of male phenotypes to substantial constraints. In plants, the subdivision of male function via the gradual presentation of pollen might minimize these constraints by preventing the saturation of receptive stigmas. Here, we report on an investigation of (1) patterns of investment in male function by plants in hermaphroditic (monoecious) and dioecious populations of *Sagittaria latifolia*, and (2) patterns of siring success by males versus hermaphrodites in experimental mating arrays. We show that in natural populations, males from dioecious populations had greater investment in male function than hermaphrodites in monoecious populations. However, as a proportion of total flower production, males presented substantially fewer flowers at once than hermaphrodites. In comparison with hermaphrodites, therefore, males prolonged the period over which they presented pollen. In mating arrays comprised of females, males, and hermaphrodites, siring success by males increased linearly with flower production. This finding is consistent with the existence of a linear gain curve for male function in *S. latifolia* and supports the idea that the gradual deployment of male function enables plants to avoid diminishing returns on the investment in male function.

KEY WORDS: **Dioecy, floral display, gain curves, gynodioecy, hermaphroditism, pollen presentation theory.**

The evolution of dioecy (separate sexes) from hermaphroditism represents one of the most important evolutionary transitions in reproductive modes in multicellular organisms. For example, without females and males, the evolution of sexual dimorphism, or speciation via sexual selection would not be possible. Sex-allocation theory provides a useful framework for understanding the evolution of dioecy from hermaphroditism (Charnov et al. 1976; Charlesworth and Charlesworth 1978; Charnov 1982; Lloyd 1984; Campbell 1998; Charlesworth 1999). A general expectation of sex-allocation theory is that hermaphroditism is stable when the shapes of the female and male gain curves are decelerating (i.e., there are diminishing returns on investment in each sex function; Charnov et al. 1976). Gain curves describe the relation between investment in each primary sex function and the fitness gains that accrue from that investment. A transition to dioecy is favored if the shapes of the gain curves switch from a decelerating relation between investment and fitness to one that is linear, or even accelerating (Charlesworth 1999). The flowering plants are replete with lineages in which this transition has occurred; dioecy appears to have evolved from hermaphroditism in at least 100 separate angiosperm lineages (Charlesworth 2002). Thus, for lineages in which transitions to dioecy have occurred, there is an expectation that the shapes of gain curves through female and male sex functions should have been rendered more strongly linear.

In the flowering plants, mating success via male function is expected to be influenced by the size and number of flowers produced. The combination of flower size and number influences

total pollen production, and thus the investment in reproduction via male function (e.g., Stanton and Preston 1988). Floral display (i.e., the size and number of flowers that are displayed) regulates attraction to pollinators (Bell 1985; Young and Stanton 1990; Glaettli and Barrett 2008) and the packaging and/or dispensing of pollen can be targets for selection (Lloyd and Yates 1982; Harder and Thomson 1989; Wilson et al. 1994; Castellanos et al. 2006). Because siring opportunities are available for as long as flowers are receptive to pollen, there is a temporal component to mating success. Males can prolong their exposure to pollinators and limit lost mating opportunities by presenting pollen gradually (Harder and Thomson 1989; Wilson et al. 1994). Because the gradual presentation of pollen might linearize the gain curve (Wilson et al. 1994), thereby influencing the evolution of male phenotypes, one might predict differences in the duration of pollen presentation by males versus hermaphrodites. Here, we evaluate differences in attributes of floral display between males and hermaphrodites, and using arrays of plants with known genotypes at microsatellite (SSR) loci, evaluate the consequences of these differences for fitness through the male function.

Previous studies have suggested that resource conditions influence the evolution of separate sexes (e.g., Delph 1990a, b, 2003; Klinkhamer et al. 1997; Ashman 1999; Delph and Carroll 2001; Barr 2004; Dorken and Mitchard 2008). Indeed, there is an association between resource-poor conditions and unisexual frequencies for several gynodioecious species, including *Hebe subalpina* (Delph 1990a), *Nemophila menziesii* (Barr 2004), *Silene acaulis* (Delph and Carroll 2001), and *Fragaria virginiana* (Ashman 1999). However, the same (i.e., resource poor) conditions that might promote the evolution of females could hinder the evolution of males. In particular, resource-poor conditions are often associated with male-biased sex allocations among hermaphrodites (Delph 2003). If so, the difference in the pollen fertilities of males versus hermaphrodites might be small, impeding the spread of male-determining alleles. The idea that phenotypic plasticity of hermaphroditic sex allocation influences frequencies of unisexuals is known as the sex-differential plasticity (SDP) hypothesis (Delph 2003) and there is now substantial evidence that it might play a role in the evolution of females (reviewed in Ashman 2006). A secondary goal of this study was to empirically examine this hypothesis for the evolution of males.

Sagittaria latifolia is a particularly useful species for investigating the relations between investment in reproduction and fitness, and the evolutionary consequences of these relations. First, plants produce unisexual flowers, simplifying the task of evaluating the contribution of floral structures to female versus male function. Second, evaluating patterns of siring by males versus hermaphrodites has been enabled by the development of polymorphic microsatellite markers (Yakimowski et al. 2009). Finally, this plant is unusual among angiosperm species in being comprised of both monoecious populations (i.e., populations of hermaphroditic plants with unisexual flowers) and dioecious populations, enabling inferences about the mechanisms underlying transitions between sexual systems to be made. Previous studies of *S. latifolia* have shown that patterns of floral display (number of open flowers per inflorescence) and rates of pollinator visitation are consistent with diminishing fitness returns on investment in male function (Glaettli and Barrett 2008), Thus, simple increases in the number of open flowers do not appear to be sufficient to promote the evolution of males in *S. latifolia*. Instead, we predict that males in dioecious populations might extend the period of time over which flowers are presented to pollinators, and that this in turn might result in a linearization of the gain curve for male function.

Studies such as the one by Glaettli and Barrett (2008) suggest that, all else being equal, male reproductive success should be substantially higher for those plants that display a small number of flowers over a longer period of time in comparison to plants that display a large number of flowers over a shorter period. Their findings lead to the following expectations for *S. latifolia*: because total reproductive success through male function must be higher for males than hermaphrodites, males should (1) produce more male flowers than hermaphrodites, but these flowers should (2) be presented over a longer period of time. We evaluated these predictions by comparing the number, size, and proportion of open male flowers between plants from monoecious and dioecious populations of *S. latifolia*. If males maintain small daily floral displays over prolonged periods of time, there should be consequences for the fitness of male phenotypes, leading to a third prediction: (3) incremental increases in floral display should result in linear increases in siring success. If so, greater total flower production should lead to (4) greater total siring by males compared to monoecious hermaphrodites. Finally, the SDP hypothesis leads to the prediction that (5) greater siring success by males compared to hermaphrodites will be particularly evident under high-resource conditions, where hermaphrodites are female-biased in their sex allocations. To evaluate predictions (3)–(5), we examined patterns of paternity between males and hermaphrodites in 10 mating arrays subject to high- versus low-resource availability.

Materials and Methods

STUDY SPECIES

Sagittaria latifolia (Alismataceae) is a clonal, emergent aquatic plant that grows in a variety of wetland habitats, including fresh water marshes, shorelines, and roadside ditches. During the flowering season, which in the study region lasts between July and early September, plants produce unisexual flowers borne on vertical racemes. The flowers open for one day, and are visited by a variety of insect pollinators including bees and flies

(Muenchow and Delesalle 1994). Reproductive investment is size dependent, and strongly correlated with the leaf mid-vein length (Sarkissian et al. 2001), a commonly used index of plant size in studies of this species. The sex allocation of hermaphrodites (the proportion of female flowers to male flowers) is also size dependent; female flower production increases with plant size while male flower production remains constant with size (Sarkissian et al. 2001; Dorken and Barrett 2004; Dorken and Mitchard 2008).

FIELD OBSERVATIONS

Sampling

Nine monoecious and nine dioecious populations of *S. latifolia* distributed across southern Ontario, Canada, were visited between August and September 2008 (Table S1). In each population, we recorded the number of open male flowers per inflorescence, the total number of male flowers per inflorescence, the petal width of the largest open male flower, and the mid-vein length (MVL) of the leaf subtending the inflorescence. To avoid measuring multiple ramets of the same clone, plants were sampled along a transect with a minimum distance of 2 m between each shoot. The number of plants sampled per site was determined by sampling at these 2 m intervals until a total of approximately 25 plants were sampled, or all the plants along the transect had been measured, yielding an average sample of 24 plants per site.

Data analyses

Differences in attributes of male floral display between dioecious and monoecious populations were evaluated using mixed-effects models. For our continuously distributed independent variable (flower size, measured as petal width), we evaluated differences between sexual systems using the lme function from the nlme package (Pinheiro et al. 2009) in R (R version 2.11.1; R Development Core Team 2010). We treated sexual system as a fixed effect and site as a random grouping variable and included our measure of plant size (MVL) as a covariate to control for variation in plant size. Petal width was log-transformed to meet assumptions for analysis of variance. Comparisons of floral display between sexual systems that involved count or proportion data (the number of open male flowers per inflorescence, the total number of male flowers per inflorescence, and the proportion of open male flowers per inflorescence) were evaluated using generalized linear mixed models (GLMMs). As for the previous analysis, sexual system was treated as a fixed categorical factor, MVL was a continuous covariate, and site was included as a random grouping variable. The GLMMs were calculated using the lmer function in the lme4 package (Bates and Maechler 2010) in R. Models for count data were fit by specifying Poisson errors (and using a log link function). The model involving the proportion of open male flowers per inflorescence was fit by specifying binomial errors (and a logit link function).

EXPERIMENTAL ARRAYS

Sampling

In May 2008 and 2009, 263 *S. latifolia* plants were collected from 14 monoecious and dioecious sites across southern Ontario (Table S1). Plants collected from each site were separated by a minimum of 2 m to avoid sampling multiple ramets of the same genet. The sampled plants were transplanted into 4″ pots and grown in *Sun Gro Horticulture® Universal Mix* (Sun Gro Horticulture, Vancouver, Canada) at the Trent University greenhouse. Pots were kept in horticultural trays and regularly flooded with water. The sex of all plants producing inflorescences was recorded. In September 2008 and May 2009 tissue samples approximately 2 cm in length were excised from the youngest leaf of each sample, dried in air-tight tubes using Sorbead orange silica beads (*eCompressedair*, Oklahoma, USA), and stored at −20°C. Plants collected in 2008 were propagated in 2009 by collecting the corms produced by each plant in the fall and storing them in air-tight containers at 4°C until the following spring.

Genotyping

Genetic profiles were generated from variation at three microsatellite loci for all collected material. Dried leaf tissue was ground into a fine powder using a MM 300 Retsch mixer mill (Haan, Germany). DNA extraction was performed using the *E.Z.N.A.™ Plant DNA Mini Kit Spin Protocol* (Omega Bio-Tek, Inc., GA) for dried specimens according to the manufacturer's instructions, and eluted in a final volume of 100 μl. A total of 263 plants were genotyped. Amplification of nuclear microsatellite loci was performed by adapting polymerase chain reaction (PCR) methods for *S. latifolia* from Yakimowski et al.. (2009). We used 0.2 μM of each forward (F) and reverse (R) primer along with 0.15-mM dNTPs, 1× PCR Buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 2.0 mM $MgCl_2$, 0.3 mM BSA, 0.05 U/μL Taq DNA Polymerase (Invitrogen™). Primers SL06, SL31, and SL65 had the most reliable PCR results, and were used for screening the plants used in this study. PCR was performed using an *Eppendorf Mastercycler™* and amplification conditions were 94°C for 3 min, 30 cycles of 94°C for 30 s, between 60°C and 62°C for 30 s, and 72°C for 45 s, followed by 72°C for 45 min. The annealing temperature varied slightly for each locus, with SL06 at 60°C, and SL31 and SL65 both at 62°C. For each locus, we used the same fluorescent primer labels as Yakimowski et al. (2009). Samples were genotyped using an ABI 3730 (Applied Biosystems™) using 0.75 μl of each PCR product. Genotypes were visualized using *GeneMarker®* software version 1.6 (*Softgenetics®*), and using the ROX 500 (Applied Biosystems™) size standard for reference.

Preparation of vegetative material

In May 2009, the corms collected in the fall of 2008 and plants collected from the field in 2009 were transplanted into 4″ pots, which were placed in horticultural trays and regularly filled with water in the Trent University greenhouse. Plants were transplanted into 5″ pots at the end of May. In early June 2009, plants previously screened for variability at three microsatellite loci (see above) were randomly divided into two groups. One group received a 200-mL dose of a 0.60% solution of 20–20-20 (N-P-K) (*Plant-Prod* ®, Brampton, ON) fertilizer twice weekly, plus a 200-mL dose of 0.20% fertilizer once per week. Plants in this group belonged to the high resource (HR) group. The low resource (LR) group of plants received 200 mL of a 0.20% solution of 20–20-20 (N-P-K) fertilizer twice weekly. Fertilizer applications were repeated until mid-August 2009.

Array setup

Experimental arrays were put outside between 6 July 2009 and 22 August 2009. Each array consisted of five hermaphrodites, five females, and one male growing in 5″pots that were each placed into a 7.5-L water-filled bucket. Arrays were set up on the day that the focal male began to flower and at least one of the hermaphrodites was also starting its male phase. The remaining plants had either already begun to flower (i.e., were in their female phase), or were going to do so before the male finished flowering. We included females and hermaphrodites with staggered dates of flowering so that males would have both mating partners and competition for siring opportunities over the duration of the array. The females and hermaphrodites were randomly assigned to positions around the focal male resulting in a rectangular pattern with at least 30 cm between buckets. Arrays were removed and returned to the greenhouse after the focal male had finished flowering. In total, we set up five LR arrays and five HR arrays.

Males were chosen for inclusion in the arrays if they had alleles at any of the three screened loci that were not shared by the other plants in the array. The hermaphrodites also did not share alleles with the other plants in the array, but in most arrays, they shared alleles with other hermaphrodites. Thus, it was always possible to distinguish whether any offspring produced in an array was sired by the focal male or by a hermaphrodite, but for hermaphroditic sires, it was sometimes not possible to distinguish which hermaphrodite was the father. Therefore, where we explicitly compare differences in siring between males and hermaphrodites in the analyses presented below, we compare siring by focal males versus average siring by hermaphrodites. Hermaphrodites of *S. latifolia* are synchronously protogynous with no overlap between female and male phases within an inflorescence, preventing self pollination by hermaphrodites. To preclude gene flow between arrays, we set out arrays at two locations separated by more than 7 km and at different times such that no two arrays were in the same place at the same time.

In each array, we measured the MVL of the leaf subtending the inflorescence for all individuals. On each day, we recorded the number and sex of open flowers on each plant. The fruits from all female and hermaphrodite plants were allowed to mature in the Trent University greenhouse, collected individually in labeled envelopes, and dried for three weeks at room temperature. The dried seeds were then stored at 4°C for three months. In December 2009, seeds were soaked in distilled water for three weeks and germinated in flooded 3″ pots containing *Pro-Mix 'PGX' Plug and Germination Growing Medium* (*Premier Tech Horticulture*, Rivière-du-Loup, Canada). In February 2010, one plant was randomly selected from among the germinated seeds of each fruit produced in each of the 10 arrays using destructive sampling, yielding a sample of 427 seedlings from 427 fruits. The seedlings were genotyped using the methods described above.

Statistical analysis

Differences in plant size (MVL) and male flower production between males and hermaphrodites, and between plants in HR and LR arrays were evaluated using mixed-effects models. Differences in plant size were evaluated using a linear mixed-effects model using the lme function in R, and differences in the number of flowers produced were evaluated using a GLMM with Poisson errors using the lmer function in R. For each of these tests, sex, fertilizer treatment, and the year plants had been sampled were included in the model as fixed effects, and array was considered a random effect. We evaluated the relationship between the production of flowers by the focal male versus the number of offspring sired by that male in each array using a GLMM with Poisson errors. For this analysis, the cumulative number of seeds sired by the focal male was the dependent variable, and fertilizer treatment, the cumulative number of flowers produced by the focal male, and their interaction were included as fixed effects, with array included as a random grouping variable. The association between the proportion of male flowers produced in each array by the male versus hermaphrodites and the proportion of seeds sired by the focal male was evaluated using a generalized linear model with binomial errors using the glm function in R. For this analysis, resource treatment and the proportion of flowers produced by the focal male plus their interaction were included as predictor variables. The value for the slope of the line describing the relation between the proportion of male flowers in each array that were produced by focal male and the proportion of offspring sired by that male was tested against a value of 1 using the slope.test function in R (in the smatr library; Warton and Ormerod 2007). Total siring by males versus hermaphrodites was compared with a linear mixed-effects model using the lme function in R. The number of seeds sired by males versus the average number of

seeds sired per hermaphrodite in each array was used as the dependent variable, and these values were square-root transformed to meet assumptions of analysis of variance. Treatment, the sex of the plants that sired seeds, plus their interaction were included as fixed independent variables. We also included the total number of receptive female flowers available in each array as a covariate to control for differences in the availability of ovules across arrays, and as with our previous analyses, we included array as a random grouping variable. To examine whether there was a bias in male siring success due to a potential difference in siring ability between males and hermaphrodites for seeds produced by females or hermaphrodites, we performed an analysis of deviance between the identity of the father (male vs. hermaphrodite) and identity of the mother (female vs. hermaphrodite) using the contingency table approach outlined by Crawley (2007) using the glm function in R.

Results

FIELD OBSERVATIONS

Attributes of male floral display differed significantly between the two sexual systems. Male flowers in dioecious populations were 36.8% larger (average = 1.56 cm ± 0.05 SE) than those in monoecious populations (1.14 cm ± 0.05; linear mixed-effects model, sexual system effect: $F_{1,16} = 20.1$, $P < 0.001$). In dioecious populations, males also had larger floral displays with approximately 50% more open male flowers per inflorescence than plants in monoecious populations (average for dioecious populations = 2.88 flowers ± 0.29 SE, compared to 1.93 ± 0.23 in monoecious populations; GLMM: Wald's $Z = -2.75$, $P < 0.01$). Overall, males in dioecious populations produced more than twice as many male flowers per inflorescence (average = 12.0 flowers ± 1.0 SE) compared to plants in monoecious populations (5.7 ± 0.6; GLMM: Wald's $Z = -5.85$, $P < 0.001$). In comparison with hermaphrodites, males opened a smaller proportion of their male flowers per day than hermaphrodites; males opened less than one third of their flowers per inflorescence per day (proportion of open flowers = 0.28 ± 0.03 SE) compared to more than one-third for hermaphrodites (0.40 ± 0.06 SE; GLMM: Wald's $Z = 3.12$, $P < 0.01$).

EXPERIMENTAL ARRAYS

The resource treatment had a significant effect on the size of plants in the arrays (measured as MVL). Plants growing in HR arrays were 28% larger than plants in LR arrays (average MVL across HR arrays: 9.1 cm ± 0.4 SE; LR arrays: 7.1 cm ± 0.3 SE; linear mixed-effects model, treatment effect: $F_{1,8} = 8.79$, $P < 0.05$). There was no evidence that the size (MVL) of males and hermaphrodites differed in our arrays (average MVL of males: 8.68 cm ± 0.73 SE; hermaphrodites: 7.99 cm ± 0.30 SE; linear mixed-effects model, sex effect: $F_{1,47} = 1.39$, $P > 0.20$). There was also no evidence that plants sampled in 2008 versus 2009 differed in size (average MVL for plants sampled in 2008: 7.7 cm ± 0.4 SE; 2009: 8.9 cm ± 0.9 SE; linear mixed-effects model year effect: $F_{1,47} = 1.25$, $P > 0.25$). As has been found in other studies of *S. latifolia*, resource availability affected total reproductive investment by plants from monoecious and dioecious populations and patterns of sex allocation by hermaphrodites from monoecious populations. Males from high resource arrays produced almost twice as many flowers as they did in low resource arrays (average for HR arrays: 19.0 ± 3.6 SE; LR arrays: 11.0 ± 1.5 SE). However, hermaphrodites in HR and LR arrays produced similar numbers of male flowers (average for HR arrays: 3.6 ± 1.0 SE; in LR arrays: 4.7 ± 0.6 SE), resulting in a significant sex by treatment interaction in the GLMM (Wald's $Z = -3.27$, $P < 0.01$). Instead of increasing the number of male flowers, hermaphrodites produced approximately 40% more female flowers when grown in HR arrays than when grown in LR arrays (average for HR arrays: 5.1 ± 0.4 SE; LR arrays: 3.6 ± 0.3 SE). Averaged across arrays, males produced more than three times as many male flowers as hermaphrodites (average number of flowers per male: 14.7 ± 2.3 SE; hermaphrodite: 4.3 ± 0.4 SE; GLMM sex effect: Wald's $Z = 8.48$, $P < 0.001$).

Two observations from the array experiment indicate that siring by males in the experimental arrays was positively related to their production of flowers. First, we found a positive association between the cumulative production of flowers by the focal male and cumulative siring success (Fig. 1; Wald's GLMM: $Z = 8.52$, $P < 0.001$). This same analysis failed to reveal a difference between treatment types in the relation between siring and flower production (Wald's $Z = -1.73$, $P > 0.05$) and there was no interaction between cumulative flower production and treatment on cumulative siring success (Wald's $Z = 1.34$, $P > 0.15$), indicating that the association between flower production and siring success was similar across treatments. Second, we found a positive relation between the proportion of flowers produced by the focal male and the proportion of seeds sired by that male (Fig. 2, GLM: Wald's $Z = 2.87$, $P < 0.01$). A linear model indicated that the slope of this relation was not significantly different from one (estimated slope = 1.03; test that the obtained slope was equal to one: $r = 0.04$, $P = 0.91$, 95% C.I. for the estimated slope = 0.42 – 1.64). Thus, as the focal male produced additional flowers there appeared to be directly proportional increase in siring success by the male that was scaled by the production of male flowers by competing hermaphrodites in each array.

For total seed siring per array, there was a significant interaction between treatment and the sex of the plants siring seeds in the arrays ($F_{1,8} = 9.6$, $P < 0.05$). This interaction was consistent with SDP, for which the sex allocation of hermaphrodites regulates the fitness of males. However, the direction of the response was opposite to that proposed to favor the evolution of females under

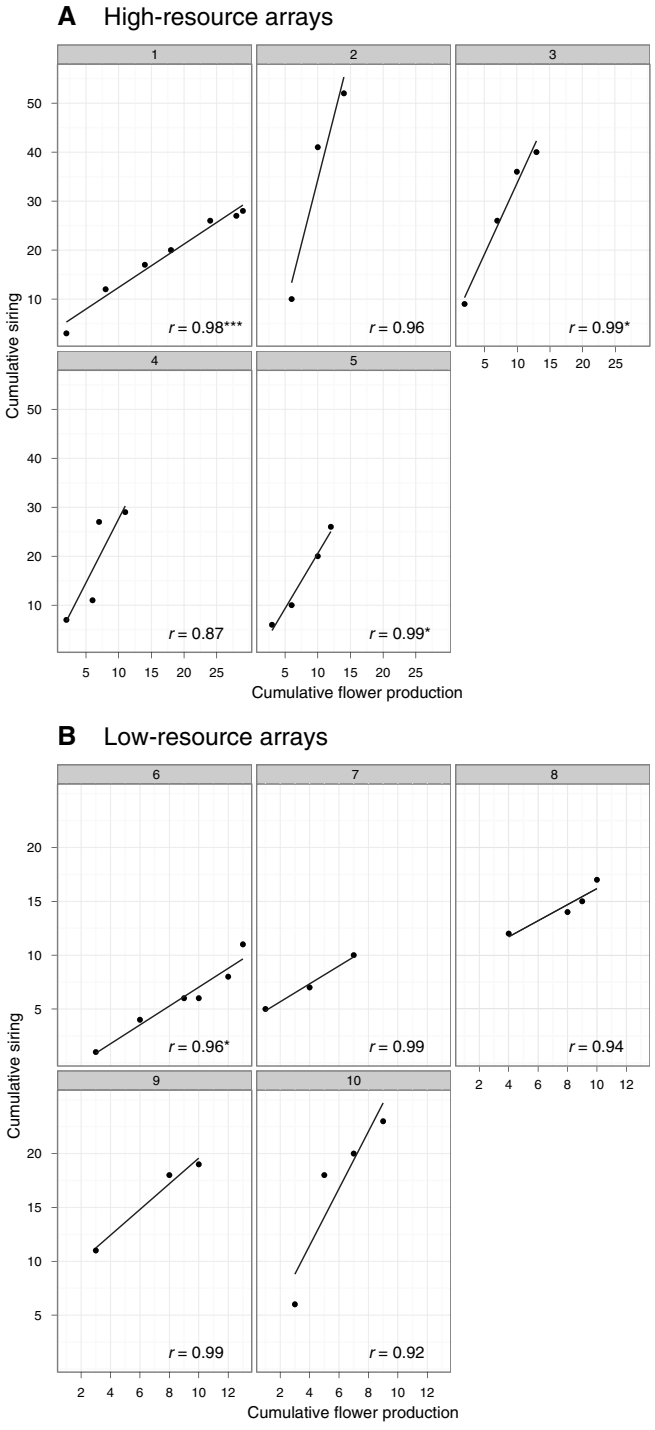

Figure 1. Correlations between the cumulative production of flowers and the cumulative number of offspring sired by the focal male in each of the five high-resource arrays (A) and the five low-resource arrays (B). Correlation coefficients are shown for each panel (*$P < 0.0001$; **$P < 0.005$; *$P < 0.05$).**

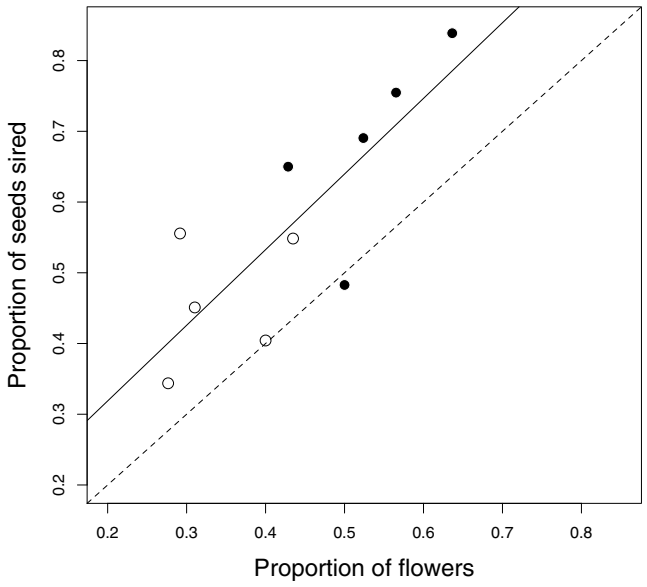

Figure 2. Relation between the proportion of male flowers in each array that were produced by the focal male and the proportion of offspring sired by that male. High-resource arrays are indicated with filled circles, low-resource arrays with open circles. The least-squares regression line is indicated with a solid line. The one-to-one line is shown for comparison (dashed line).

the SDP, which has been invoked to explain female fertility advantages under low-resource conditions. Instead, we found enhanced siring by males under high-resource conditions. In HR arrays, the average proportion of seeds sired by males was 48% higher than in LR arrays, whereas the reverse was true for hermaphrodites, which had 41% higher seed siring in LR than in HR arrays. There was also a significant effect of plant sex on patterns of siring ($F_{1,8} = 96.1$, $P < 0.001$) that was driven by higher overall rates of siring by males compared to hermaphrodites across treatment levels. Males sired on average 7.1 × and 4.0 × more offspring than hermaphrodites in the HR and LR arrays, respectively. On average, each focal male sired 35.0 ($\pm$ 4.9 SE) seeds per HR array, and 16.0 ($\pm$ 2.4 SE) seeds per LR array. In contrast, each hermaphrodite sired an average of 4.9 ($\pm$ 2.2 SE) seeds per HR array and 4.0 ($\pm$ 0.7 SE) seeds per LR array. There was a significant treatment effect ($F_{1,7} = 11.4$, $P < 0.05$) that was driven by greater fruit production in HR arrays, and therefore a higher number of seeds in HR versus LR arrays (number of fruits produced in HR arrays: 26.6 fruits $\pm$ 4.4 SE; LR arrays: 17.9 $\pm$ 2.3 SE). Finally, there was no evidence that males were any more likely to sire seeds produced by females than by hermaphrodites (analysis of deviance: deviance = 1.44, df = 1, $P > 0.20$).

Discussion

This study reports three key findings: (1) males of *S. latifolia* open only a fraction of their flowers at once and thereby extend

the duration of their floral displays compared to hermaphrodites; (2) in the mating arrays, siring success by males increased linearly with flower production; and (3) males always sired substantially more offspring than hermaphrodites, however the magnitude of this difference varied between resource levels, with males siring substantially more seeds under high-resource conditions. Patterns of siring by males in each of the 10 arrays did not indicate that males might be subject to diminishing fitness returns on investment in reproduction. Instead, two lines of evidence are consistent with the existence of a linear gain curve for *S. latifolia* males. Below, we discuss each of these three main findings, and conclude by considering their implications for understanding evolutionary transitions to dioecy.

POLLEN PRESENTATION AND THE SHAPE OF THE GAIN CURVE

Larger floral displays generally receive greater visitation by pollinators, but the proportion of flowers visited by pollinators often declines with display size (Harder and Barrett 1996; Mitchell et al. 2004; Huang et al. 2006; Glaettli and Barrett 2008). Decelerating relations between floral display and per-flower visitation rates, however, do not necessarily mean that the shape of the gain curve is decelerating. For plants with protracted periods of flower production, such as *S. latifolia*, pollinator responses to daily floral display size might be largely uncoupled from total pollinator visitation over the duration of an entire flowering season. For example, our field observations indicate that males display up to nine flowers at a time, and this is the maximum number of flowers used by Glaettli and Barrett (2008) to evaluate the association between floral display and pollinator visitation for *S. latifolia*. However, our field observations also indicate that males usually only display approximately three flowers at once. This value corresponds both with the lowest number of flowers used in the experiment by Glaettli and Barrett (2008) and with the highest per-flower rates of pollinator visitation in that study. Indeed, even though males in natural populations produced about twice as many flowers per inflorescence as hermaphrodites, they displayed a similar number of flowers at any given time (three flowers vs. two for hermaphrodites). Male plants of *S. latifolia* would therefore appear to be avoiding the most saturating portion of the fitness gain curve by displaying only a small proportion of their flowers.

The gradual presentation of pollen has been predicted to linearize the gain curve for male function by maximizing opportunities for the transfer of pollen to stigmas on other plants (Wilson et al. 1994). Our results were consistent with this expectation, showing that siring by males increased with the production of additional flowers. However, our data do not enable direct evaluation of the shape of the gain curve for male function. Directly measuring the shape of the gain curve requires measures of lifetime fitness. *Sagittaria latifolia* is a clonal perennial, making measures of lifetime fitness exceedingly difficult to obtain. Moreover, siring success is a component of fitness through the male function, but realized fitness will also be influenced by the germination, survival, and reproduction of the progeny produced by males versus hermaphrodites. The shape of the gain curve can also be influenced by the level of competition for siring events (Yund 1998). Indeed, taking these three issues together, no studies have directly evaluated the shape of the female or male gain curves in plants. Perhaps the best example of a study examining the shapes of gain curves for plants was done in a natural population of an annual plant in which patterns of siring were evaluated using neutral genetic markers (Campbell 1998). However, even here, the realized fitness of plants and the effect of competition among plants for siring on the shape of the gain curve were not evaluated.

The approach taken in this study provides indirect evidence that the shape of the gain curve for male function in *S. latifolia* is linear. The strongest evidence supporting this inference comes from the observation that the relationship between cumulative flower production and siring success for male plants was linear (i.e., as males produced additional flowers in the mating arrays, they sired additional seeds). Thus, in terms of the first issue involved with measuring the shape of the gain curve mentioned above, longevity on its own should not affect the inference of a linear gain curve; the production of inflorescences in other years would be expected to additively affect siring in the same manner found here. That the gain curve for male function might be linear is, to a lesser extent, also supported by the data presented in Figure 2. Note that, using a linear model, a linear relationship between the proportion of flowers produced by males and their proportional siring success would have been inferred whether the underlying gain curve is linear or not. The interpretation of a linear male gain curve is based on the observation that the slope for the relation between relative flower production and relative siring success was close to unity, consistent with a one-to-one relationship between flower production and siring. However, the 95% confidence interval for the slope of the line shown in Figure 2 includes values substantially above and below one, and so these data on their own do not provide unequivocal evidence for a linear gain curve for male function.

For clonal plants, lifetime fitness will also be influenced by the fertilities of the ramets comprising the clone, and for two reasons, clonality might strongly affect male fitness gains. First, increases in clone size increase the likelihood that pollen is dispersed geitonogamously within clones, leading to pollen discounting (Handel 1985; Charpentier 2002; Routley et al. 2004; Vallejo-Marín et al. 2010). For hermaphrodites, this would tend to yield diminishing fitness gains for male function via increased local mate competition (de Jong et al. 1999). Alternatively, it is

possible that clonal expansion could yield increased fitness gains per unit investment in male function if the production of functionally independent fertile ramets results in a subdivision of male reproduction effort (Dorken and Van Drunen 2010). This idea is analogous to the one proposed by Wilson et al. (1994), with the subdivision of male reproductive effort among spatially separated ramets substituting for the subdivision of reproductive effort among temporally separated flowers or inflorescences. Both of these impacts of clonal growth should primarily affect the male fertility of hermaphrodites, not males. Specifically, the subdivision of reproductive effort via clonal growth is not likely to enhance the pollen fertility of males (i.e., the effects described by Dorken and Van Drunen require decelerating male gain curves). Moreover, males avoid pollen discounting via geitonogamous pollen transfer, making per ramet mating opportunities more strongly independent of genet size. Indeed, the reduction in pollen discounting associated with the loss of female function by hermaphrodites may be one of the factors favoring the evolution of males (Barrett 2003). For these two reasons, a linear gain curve for male ramets should approximately translate to a linear gain curve for male genets.

The degree of competition among individuals for siring opportunities can change the shape of the gain curve, with reduced competition yielding diminishing returns on investment in male function (Yund 1998). We indirectly manipulated the intensity of competition in our arrays by altering the resource budgets for the investment in sexual reproduction by hermaphrodites (via the fertilizer treatment). This treatment affected the production of male flowers by males, but to a much lesser extent by hermaphrodites (i.e., male flower production is largely independent of ramet size for monoecious hermaphrodites of *S. latifolia*, but not males; Sarkissian et al. 2001). As a result, focal males experienced varying degrees of competition for siring events across arrays, with males producing the majority of male flowers in some arrays (up to 67%), and a much smaller proportion of male flowers in other arrays (as little as 29%). However, in spite of this variation, siring by the focal male appeared to remain directly proportional to the production of male flowers by the male versus its hermaphrodite competitors.

EVOLUTION OF DIOECY

The evolution of dioecy occurs via specific pathways that are defined by the order in which unisexual phenotypes evolve. With a few exceptions (e.g., Lloyd 1980; Rosas and Domínguez 2009; Li et al. 2010), the available evidence indicates that dioecy has most commonly evolved via the gynodioecy pathway (reviewed in Webb 1999; gynodioecy refers to the co-occurrence of females and hermaphrodites). If so, the first step toward the evolution of separate sexes would generally involve the origin and spread of female phenotypes, followed by the evolution of males. The various factors affecting the evolution of females have received considerable attention (reviewed in Ashman 2006); much less attention has been paid to the evolution of males.

One of the factors that might be important in the first step of the gynodioecy pathway, the evolution of females, is the resource environment and the response of hermaphrodites to variation in the availability of resources (Delph 1990b). Specifically, under resource-poor conditions, hermaphrodites are often male biased in their sex allocations, favoring the evolution of female phenotypes (i.e., the SDP hypothesis; Delph 2003). However, if hermaphrodites are largely functioning as males under resource-poor conditions, this might prevent the evolution of males by reducing the siring advantage of males over hermaphrodites. Therefore, the environmental conditions that promote the evolution of females might interfere with the evolution of males. On one hand, our results are consistent with this expectation; we found that the siring success of males relative to hermaphrodites was substantially lower in low-resource conditions than in high-resource conditions. Thus, although low-resource conditions might promote the evolution of females, our results suggest they might constrain the evolution of males. On the other hand, across the 10 arrays males always had at least 2.0 times greater siring than hermaphrodites, and on average, approximately 5.5 times the siring of hermaphrodites. Therefore, at least for the conditions in which our experiment was conducted, the resource environment would not appear to be a major impediment for the evolution of males.

The magnitude of the difference in siring between males and hermaphrodites might have been influenced by the layout of our arrays. To standardize arrays, males were placed in the centre of the array. This enabled comparisons of the relative fitness of males across arrays, a major goal of our experiment. However, the central position of males in each array meant that they were often closer to plants in female phase than any given hermaphrodite. The degree to which this might have biased our inference regarding the relative siring ability of males versus hermaphrodites is not clear, however, for two reasons it is unlikely to have qualitatively affected our inference that males outcompeted hermaphrodites for siring. First, as discussed above, our data are consistent with the existence of a linear gain curve for male function. Thus, considering the data presented in Figure 2, the placement of males in the centre of arrays might have increased the intercept, but not the slope of the line. Second, in the presence of females, only a small siring advantage is required by males to enable an increase in their frequency over hermaphrodites. In a related study using mating arrays of *S. latifolia* involving females and hermaphrodites, Dorken and Mitchard (2008) calculated that males would only need a 15–35% increase in pollen fertility over hermaphrodites to increase in frequency in a gynodioecious population. Because, on average, males produce roughly twice as many male flowers

as hermaphrodites, this threshold should usually be exceeded by *S. latifolia* males.

Males can displace hermaphrodites in a gynodioecious population if their inability to produce seeds is more than compensated for by enhanced siring success. Although a general expectation for the evolution of separate sexes is that unisexual phenotypes have at least double the fertility of hermaphrodites through one sex function (e.g., males must have at least double the pollen fertility of hermaphrodites to be maintained in a population of hermaphrodites; Lewis 1941; Lloyd 1976), the presence of the alternate unisexual phenotype alters this dynamic. For example, the presence of females in gynodioecious populations facilitates the evolution of males (Charlesworth and Charlesworth 1978; Charlesworth 1999); the increased availability of ovules in populations with females provides greater fitness rewards for plants with increased pollen production. In particular, the presence of females determines whether male evolution is enabled by a linear versus accelerating gain curve for male function. In the absence of females, an accelerating gain curve for the male function is necessary for male invasion (Charnov 1982; Charlesworth 1999). However, a linear gain curve is sufficient for males invading a gynodioecious population (Charlesworth 1999). Consistent with this scenario, our data suggest that siring by *S. latifolia* males increases linearly with their investment in male function and that this linearity is achieved via the gradual presentation of male flowers.

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Supporting Information

The following supporting information is available for this article:

Table S1. Locality information for the 12 dioecious and 10 monoecious populations of *Sagittaria latifolia* from which data and material were collected.

Supporting Information may be found in the online version of this article.

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