Notes and Comments
Density-Dependent Regulation of the Sex Ratio in an Annual Plant

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ABSTRACT: Sex ratios are subject to strong frequency-dependent selection regulated by the mating system and the relative male versus female investment. In androdioecious plant populations, where males co-occur with hermaphrodites, the sex ratio depends on the rate of self-fertilization by hermaphrodites and on the relative pollen production of males versus hermaphrodites. Here, we report evolutionary changes in the sex ratio from experimental mating arrays of the androdioecious plant *Mercurialis annua*. We found that the progeny sex ratio depended strongly on density, with fewer males in the progeny of plants grown under low density. This occurred in part because of a plastic adjustment in pollen production by hermaphrodites, which produced more pollen when grown at low density than at high density. Our results provide support for the prediction that environmental conditions govern sex ratios through their effects on the relative fertility of unisexual versus hermaphrodite individuals.

Keywords: androdioecy, gynodioecy, *Mercurialis annua*, sex allocation, sex ratio.

Sex ratios are subject to strong frequency-dependent selection. Because individuals of the minority sex will enjoy the greater reproductive success in a well-mixed population, male : female ratios are predicted to settle at 1 : 1 (Düsing 1884, translated in Edwards 2000; see also Fisher 1930). This important principle applies as much to dioecious populations with males and females as it does to populations of outcrossing hermaphrodites, in which selection should favor an equal allocation of resources to each of the two sexual functions (Charnov 1982). Populations in which males or females co-occur with hermaphrodites (androdioecy and gynodioecy, respectively) are particularly interesting because they illustrate the action of frequency-dependent selection on both the ratio of unisexuals to hermaphrodites and the sex allocation of the hermaphrodites themselves. They also provide revealing study systems because hermaphrodites may often self-fertilize a fraction of their progeny, and inbreeding is known to bias the sex ratio (Hamilton 1967) and the sex allocation (Charlesworth and Charlesworth 1981; Charnov 1982).

Because the selfing rate (e.g., Elle and Hare 2002) and hermaphrodite sex allocation are known to depend on environmental conditions (e.g., Delph 1990b; Ashman 1999; Asikainen and Mutikainen 2003; reviewed in Delph and Wolf 2005), local sex ratios should respond to demographic and environmental stochasticity in a predictable way (Delph 2003). For gynodioecious species with cytoplasmic male sterility, variation in sex ratios can be influenced by stochastic variation in the frequency of nuclear restorers of male fertility (e.g., McCauley 1998; Bailey and McCauley 2005; Bailey and Delph 2007). However, much of the wide variation often observed among populations of gynodioecious and androdioecious species (Pannell 2002), especially for those with nuclear sex determination, is predicted to result from microevolutionary responses to environmental and/or demographic changes to populations (Delph and Wolf 2005). A key variable in this regard is population density.

Population density might affect the local sex ratio for two reasons. First, density can affect the overall availability of resources allocated to reproduction (Snell and Burch 1975; Pannell 1997b). If hermaphrodites vary their allocation to male or female sex functions in response to resource status (e.g., size-dependent sex allocation; Charnov 1982; Delph 1990a; Klinkhamer et al. 1997; Zhang and Jiang 2002) and resource availability is negatively associated with density as a result of competition, density will affect hermaphrodite sex allocation and thus the sex ratio of gynodioecious or androdioecious plants (Delph 2003).
For example, consider an androdioecious population in which the pollen production of hermaphrodites relative to that of males is negatively associated with density (i.e., where hermaphrodites have lower pollen allocation under high densities). Under these conditions we would expect the relative siring success of males to increase with density. Second, for some species, the selfing rate is negatively associated with density (Valdeyron et al. 1977; Burdon et al. 1988; van Treuren et al. 1993; Taylor et al. 1999), especially for wind-pollinated plants (Farris and Mitton 1984; Wolff et al. 1988; Robledo-Arnuncio et al. 2004; Eppley and Pannell 2007). If sex is genetically determined in an androdioecious population, we would expect fewer males in the progeny of parents grown at low density than in the progeny of those grown at high density because as the selfing rate of hermaphrodites increases, the ability of males to transmit genes to the next generation decreases. Similar predictions apply to the frequency of females in gynodioecious populations.

Here, we test the hypothesis of environmentally induced changes to the sex ratio in response to density using the androdioecious herb *Mercurialis annua*, in which wild populations vary widely in their male frequencies, from 0 to about 0.4 (Durand 1963; Pannell 1997b). The species is wind pollinated, so the mating system can be analyzed in terms of a simple process of density-dependent scramble competition among self- versus outcross-pollen grains. Previous work has shown that hermaphrodites produce less pollen than males when grown at high densities (Pannell 1997b). Moreover, using experimental mating arrays, Eppley and Pannell (2007) showed that the hermaphrodite selfing rate decreases with density. As a result, they predicted that the local sex ratio (i.e., the frequency of males in a population) should increase with density. This idea is broadly similar to observations from gynodioecious species that female frequencies are positively associated with environmental conditions that result in lower hermaphrodite seed production (Delph 1990b; Ashman 1999; reviewed in Delph and Wolf 2005). However, the prediction that environmental conditions (including density) should directly affect the sex ratio of the next generation of individuals has not been tested in any gynodioecious or androdioecious plant (although density has been shown to affect the mating system and thus the sex ratio of androdioecious animals; Hollenbeck et al. 2002).

We report the results of an experiment in which we manipulated the density of mating arrays of *M. annua* and compared the frequency of males in the progeny of parents grown at high versus low density. We therefore provide not only a test of the prediction by Eppley and Pannell (2007) that the local frequency of males is determined by the density of plants in the previous generation but also a direct test of the more general prediction made by Delph (1990b, 2003) that the frequency of unisexuals is regulated by environmental conditions that affect the relative fertilities of hermaphrodites and unisexuals. Note that, for an androdioecious population, the critical variable here is the pollen production by males relative to that of hermaphrodites; hermaphrodite allocation to seeds has no direct influence on the sex ratio.

**Material and Methods**

**Natural Populations**

We surveyed naturally occurring variation in the sex ratio in eight androdioecious populations of *Mercurialis annua* in Morocco and Portugal (population locality data is available from the authors upon request). For each population, we sampled a patch of at least 200 plants and recorded its density and its male frequency; these were in the ranges of 30.8–437.5 and 0.06–0.47 plants m$^{-2}$, respectively (table 1). Densities were measured from areas of even density within patches. However, the total area sampled per patch varied across sites because of variation in density. We harvested all plants in a patch and combined all their seeds as a bulk sample for sowing in our experiment.

**Experiment**

We grew plants from each source population under two contrasting densities: 12.25 plants m$^{-2}$ (i.e., 7 × 7 plants in 2-m$^2$ raised beds; hereafter, “low-density plots”) and 250 plants m$^{-2}$ (250 plants in 1-m$^2$ raised beds; hereafter, “high-density plots”), yielding two mating arrays per source population (one for each density). Plots were constructed in open field areas, with plots separated by a minimum of 20 m to avoid pollen flow between plots with contrasting sex ratios. In each plot, we counted the number of males and hermaphrodites. We measured the pollen production of males and hermaphrodites from a random sample of 20 plants per low-density plot (10 of each sex) and 40 plants per high-density plot (30 hermaphrodites and 10 males), following the method described by Pannell (1997b). Specifically, we removed all of the male flowers from these plants and separately dried and weighed the male flowers and the vegetative parts of the plant. We defined the pollen production of males relative to that of hermaphrodites ($\lambda$) as the proportion of aboveground biomass allocated to pollen by males ($\pi_m$) divided by the pollen allocation of hermaphrodites ($\pi_n$). For the low-density plots, we harvested plants individually and dried and threshed them to collect seeds. For the high-density plots, we harvested plants in bulk for their seed. Some recruitment of individuals occurred over the growing season; these plants were easily identified in low-density plots.
Table 1: Effect of density on the relative pollen production of males versus hermaphrodites (λ) and the progeny sex ratio of plants sampled from eight populations in Morocco (M1–M5) and Portugal (P1–P3)

<table>
<thead>
<tr>
<th>Population</th>
<th>Sex ratio</th>
<th>Density (plants m⁻²)</th>
<th>Density</th>
<th>λ</th>
<th>Sex ratio</th>
<th>Sex ratio</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>.38</td>
<td>Low 438</td>
<td></td>
<td></td>
<td>.39 (49)</td>
<td>.25 (942)</td>
<td>4.4*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High 43.8</td>
<td>.42 (280)</td>
<td></td>
<td>.40 (298)</td>
<td>.2</td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>.25</td>
<td>Low 217</td>
<td></td>
<td></td>
<td>.33 (49)</td>
<td>.29 (735)</td>
<td>.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High 48.9</td>
<td>.32 (266)</td>
<td></td>
<td>.43 (126)</td>
<td>4.1*</td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>.44</td>
<td>Low 13.7</td>
<td>.35 (49)</td>
<td></td>
<td>.38 (1,500)</td>
<td>.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>High 65.7</td>
<td>.42 (290)</td>
<td></td>
<td>.38 (677)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>M4</td>
<td>.40</td>
<td>Low 20.6</td>
<td>.55 (49)</td>
<td></td>
<td>.31 (697)</td>
<td>11.0***</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>High 52.0</td>
<td>.46 (263)</td>
<td></td>
<td>.43 (128)</td>
<td>.3</td>
<td></td>
</tr>
<tr>
<td>M5</td>
<td>.47</td>
<td>Low 13.2</td>
<td>.33 (48)</td>
<td></td>
<td>.26 (987)</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>High 26.5</td>
<td>.39 (269)</td>
<td></td>
<td>.45 (392)</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>.06</td>
<td>Low 3.5</td>
<td>.37 (49)</td>
<td></td>
<td>.31 (612)</td>
<td>.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>High 12.2</td>
<td>.26 (299)</td>
<td></td>
<td>.36 (648)</td>
<td>9.1**</td>
<td></td>
</tr>
<tr>
<td>P2</td>
<td>.16</td>
<td>Low 4.7</td>
<td>.22 (49)</td>
<td></td>
<td>.15 (1,592)</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>High 18.1</td>
<td>.19 (304)</td>
<td></td>
<td>.33 (592)</td>
<td>19.7***</td>
<td></td>
</tr>
<tr>
<td>P3</td>
<td>.15</td>
<td>Low 4.3</td>
<td>.33 (49)</td>
<td></td>
<td>.39 (228)</td>
<td>.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>High 9.3</td>
<td>.46 (314)</td>
<td></td>
<td>.45 (510)</td>
<td>.1</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>Low 9.4 ± 2.1</td>
<td>.36 (391)</td>
<td></td>
<td>.29 (7,239)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>High 34.6 ± 7.4</td>
<td>.37 (2,285)</td>
<td></td>
<td>.40 (3,371)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: The three generations used were the source material collected in the field, the parental generation (i.e., the plants grown in the two density treatments), and the progeny of the parental generation. Sex ratios are expressed as the proportion of males. Averages for each density class are shown at the bottom of the table (±1 SE for λ). Tests evaluating significant effects of the density treatment were conducted using paired t-tests with df = 7. Values in parentheses are sample sizes. G-values are given for tests of independence of male frequencies from the parental versus progeny generation for each plot.

* P < .05.
** P < .005.
*** P < .001.

and were removed. However, they contributed to the final tally of plants in high-density plots, resulting in some variation in the number of plants in these plots (i.e., at the time of harvest, the number of plants per high-density plot ranged between 263 and 314; table 1). The experiment was performed at the Wytham Field Laboratory, University of Oxford, and plants grew under conditions similar to those that they experience in the field in Spain and Morocco. In particular, because *M. annua* is wind pollinated, we do not expect that pollination dynamics in the experiment should be any different from those experienced in the field.

We germinated a standardized sample of seeds from each plant (low-density plots) or from each plot (high-density plots) by sowing equal volumes of seeds evenly in pots (corresponding to 250 seeds per 11-cm pot). Germination and plant growth occurred under uniform greenhouse conditions, and all plants that germinated survived to flowering. We tallied the sex of each plant as soon as its sex could be determined, after which we removed each plant from its pot. Note that total sample sizes differed between the high- and low-density treatments because of this standardization. Specifically, because we grew progeny from each hermaphroditic plant from the low-density plots, to maintain standard densities, many more progeny than were required to accurately estimate the sex ratio had to be grown for this treatment.

**Analysis**

To evaluate differences between density treatments in the relative pollen production of males versus hermaphrodites and in the frequency of males in the parental and progeny generations, we used paired t-tests, with each source population serving as a replicate in the analysis. To test the prediction that the progeny sex ratio should increase at high density and decrease at low density, we conducted a paired t-test of the difference in the sex ratio between the parental and progeny generations (i.e., the sex ratio among the progeny minus the sex ratio among the parents). Our results were robust to changes in scale (e.g., arcsine transformations of the sex ratio) and to whether tests were parametric or nonparametric (Wilcoxon signed-rank test). We therefore present the results only for parametric tests.
of untransformed data. Finally, we evaluated changes in the frequencies of males from the parental to progeny generations using G-tests of independence (Sokal and Rohlf 1995). Because this procedure involved multiple tests of the same hypothesis, levels of significance were adjusted using the sequential Bonferroni method (Sokal and Rohlf 1995).

Results

We detected a significant change in sex ratio among the progeny of plants grown under high versus low densities (i.e., the difference in the sex ratio between the parental and progeny generations depended on whether plants were grown under high or low densities; paired t-test: $t = 2.6$, $df = 7$, $P < .05$; fig. 1). On average, the frequency of males among the progeny of parents in low-density plots was 28% lower than among those in high-density plots (table 1). This difference in male frequency was associated with a difference in the relative pollen fertility of males versus hermaphrodites ($\lambda$) between density treatments, which was more than three times as high in high-density as in low-density plots (table 1). The difference in the relative pollen fertility of males versus hermaphrodites was caused by a change in the pollen production of hermaphrodites across density treatments. Specifically, hermaphrodites produced 3.6 times as much pollen per unit biomass under low densities (mean $\pm SE$: $\pi_n = 31.2 \pm 3.9$ mg pollen g$^{-1}$ aboveground biomass) as under high densities ($\pi_n = 8.7 \pm 3.9$ mg g$^{-1}$; paired $t$-test: $t = 5.3$, $df = 7$, $P < .005$). Finally, at the level of the individual plots, G-tests of independence revealed significant changes in male frequencies across generations for three plots (table 1). In each case, the change occurred in the expected direction.

Discussion

Sex-Ratio Evolution in Mercurialis annua

The direction of adjustments in the sex ratio between generations depended on parental density, with higher male frequencies occurring in the progeny of parents that grew at higher densities. Our results also indicate that density governs the ability of males to sire seeds through its effect on the relative pollen fertilities of hermaphrodites and males. Previous studies have demonstrated that the local sex ratio is associated with environmental variables such as site quality (e.g., Delph 1990b; Barrett 1992; Ashman 1999; Asikainen and Mutikainen 2003; Case and Barrett 2004; Vaughton and Ramsey 2005; see also Barr 2004) and resource availability (e.g., Pannell et al. 2008). These studies inferred that sex-ratio variation arose through changes in hermaphrodite sex allocation among environmental gradients (see also Dorken and Barrett 2004; Delph and Wolf 2005). Our study confirms that such environmental effects on hermaphrodite sex allocation occur in Mercurialis annua and that they influence the sex ratio of the next generation of adults.

The largest changes in sex ratio were observed in those populations most likely to be furthest from sex-ratio equilibrium in the parental generation. For example, a significant decrease in sex ratio was observed in the low-density plot with the highest male frequency in the parental generation. Similarly, significant increases in sex ratio were observed in the high-density plots with the two lowest male frequencies in the parental generation. In addition to these expected changes in sex ratio, some male frequencies did not display a shift between parents and their progeny. These apparent discrepancies were, in fact, not unexpected. For example, those high-density plots in which the sex ratio remained unchanged had a high male frequency in the parental generation. Because we expect parents at high density to produce progeny with a high proportion of males, these populations might already have been at their sex-ratio equilibrium for high density in the parental generation.

Density had a strong effect on the sex allocation of hermaphrodites of M. annua. At high density, hermaphrodites produced substantially less pollen than they did at low density, confirming the earlier findings of Pannell (1997b). The shift in allocation toward reduced pollen
production by hermaphrodites at high density favored the siring success of males and thus led to an increase in their frequency from one generation to the next. Sex-allocation plasticity has been inferred for a number of gender-dimorphic species (reviewed in Delph and Wolf 2005). Because hermaphrodite sex allocation affects sex ratios in androecious and gynodioecious populations (Lloyd 1975, 1976), we should expect phenotypic plasticity for sex allocation to lead to variation in sex ratios across environmental gradients. Indeed, Delph (1990b, 2003) has argued that plasticity in hermaphrodite sex allocation can promote the spread of unisexual plants by increasing the relative fertility of unisexuals under a subset of environmental conditions. Our results provide direct experimental support for this hypothesis.

Our results make sense to the extent that sex is genetically determined. In a simple crossing experiment, Pannell (1997a) found that males segregated among the progeny of hermaphrodites grown in the presence, but not the absence, of males. While that experiment was rather crude, the result was consistent with sex determination by a dominant male-determining allele in *M. annua*, with males heterozygous for this allele. (This mode of sex determination has also been found in the androecious plant *Datisca glomerata*; Wolf et al. 2001.) Pannell’s (1997a) results also pointed to an environmental contribution to sex determination, with an elevated expression of maleness at high densities. To prevent the influence of any such plasticity on our results, we grew progeny from our experiment under standardized, high-density conditions for all parental treatments. Although we cannot exclude the possibility that some of the variation among arrays in the male frequency of the parents was due to environmental effects, such effects could not account for differences in the sex ratio of progeny sets grown under the same conditions.

Assuming dominant male determination, it follows that the expected frequency of males in the progeny of an androecious population will be given by

\[
m' = \frac{m\lambda\alpha}{2(m\lambda\alpha + (1 - m))(1 - s - \delta)}, \tag{1}
\]

where *m* and *m’* are the respective proportions of males in the parental and progeny generations, *s* is the proportion of selfed progeny, *λ* is the relative pollen production of males versus hermaphrodites (i.e., *πm/πs*), *δ* is the magnitude of inbreeding depression, and *α* is the success in reaching a stigma of a pollen grain dispersed by a male relative to that of reaching a pollen grain dispersed by a hermaphrodite (see Buggs and Pannell 2006). In general, we expect *α* > 1, because males disperse pollen from erect inflorescence stalks, whereas hermaphrodites release their pollen from flowers in the leaf axils. Note that the product \(λα(1 - s - δ)\) denotes the expected outcross siring success of male individuals relative to that of hermaphrodites. In other words, increases in the relative pollen production, the relative success of male versus hermaphrodite pollen reaching stigmas, decreases in the selfing rate, and increases in the magnitude of inbreeding depression will all cause an increase in the relative siring success of males over hermaphrodites.

The product of the selfing rate and the relative fitness of selfed progeny, \(s(1 - δ)\), can be inferred through rearrangement of equation (1) as

\[
s(1 - δ) = 1 - \frac{2(m\lambda\alpha + 1 - m)}{m\lambda\alpha}. \tag{2}
\]

The parameters *δ* and *α* were not measured in our experiment. Fitness comparisons of experimentally selfed versus outcrossed progeny suggest that *δ* is close to 0 for androecious populations of *M. annua* (S. M. Eppley and J. R. Pannell, unpublished data). There is also little evidence from this study that inbreeding depression affected our results. For example, because we predicted higher selfing rates in the low-density plots, inbreeding depression would have obscured our finding of decreased male frequencies among the progeny of plants grown under these conditions. Specifically, if selfed seed had had lower germination rates or survival to flowering than outcrossed seeds (many of which should have been sired by males), it would have led to an increase in the male frequency of low-density compared to high-density plots, all else being equal, which is contrary to what was found. The parameter *α* was estimated by Eppley and Pannell (2007) to equal 1.7. In their experiment, *α* was estimated from mixed arrays of diploid males and hexaploid hermaphrodites, and Buggs and Pannell (2006) showed that mating success was somewhat lower between ploidy levels than within ploidy levels in *M. annua*. Our use of Eppley and Pannell’s (2007) estimate of *α* is therefore conservative; that is, our inferred selfing rate may be positively biased.

If we assume these parameter values (\(δ = 0.0, α = 1.7\)), the inferred *s* across low-density arrays (\(s = 0.31 ± 0.06\)) was twice the inferred *s* across high-density arrays (\(s = 0.15 ± 0.03\)). Indeed, this doubling in the inferred selfing rate from high to low densities holds across a wide range of values of *δ*. It would therefore seem that in addition to the differences in the relative pollen production of males versus hermaphrodites described above, selfing also contributed to differences in male siring success across density treatments and thus to shifts in sex ratios observed between generations. Our results thus not only confirm Delph’s (2003) hypothesis that the spread of unisexuals will be mediated by environmentally induced
plasticity in hermaphrodite sex allocation but also point to the importance of environmentally induced variation in hermaphrodite selfing rates (Epplcy and Pannell 2007).

Are Natural Populations of Mercurialis annua at Sex-Ratio Equilibrium?

Male frequencies vary dramatically across populations of M. annua, from 0% to more than 40% (Durand 1963; Pannell 1997b). Moreover, densities typically fluctuate over two orders of magnitude among populations (M. E. Dorken, R. P. Freckleton, and J. R. Pannell, unpublished data). Our study provides a link between these measures of variability by showing that sex ratios respond to population densities. However, it is unknown whether the variation in male frequency reflects variation in equilibrium conditions across populations with different densities or whether densities fluctuate over time, perturbing local sex ratios from their sex allocation and inbreeding equilibria. Moreover, other environmental factors not considered in our experiment might also contribute to variation in sex ratios across populations. For example, hermaphrodite sex allocation in M. annua is known to respond plastically to variation in the resource environment (Pannell et al. 2008), which is likely to vary between populations. Such variation might contribute to differences in male frequencies among populations by affecting the relative siring success of males by altering the contribution of male versus hermaphrodite pollen to the pollen pool. In general, sex-ratio variation in natural populations is likely to reflect a variety of deterministic (e.g., the hermaphrodite sex allocation) and stochastic factors (e.g., variation in population density, resource availability, and the frequency of males among the population founders) that characterize a population’s recent history.

By experimentally demonstrating that the sex ratio changes across generations in response to environmental gradients, our results build on previous studies that have inferred similar fluctuations in the populations of gynodioecious species. For example, Delph (1990b) showed that hermaphrodite vigor is associated with female frequency in Hebe strictissima and argued that the sex ratio is thus modulated across environmental gradients. Similarly, Ashman (1999) posited that variation in site quality affects the sex ratio of Fragaria virginiana through its effects on hermaphrodite sex allocation. In these cases, we would expect evolutionary adjustments of sex ratios if populations were exposed to varying environmental conditions, as shown here for M. annua.

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Literature Cited


