

# Soil moisture and sex ratio in a plant with nuclear–cytoplasmic sex inheritance

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I investigated whether soil moisture affects relative fitness of females and hermaphrodites and sex ratio in a gynodioecious plant with nuclear–cytoplasmic sex inheritance. I contrast these results with those from species with strictly nuclear sex inheritance. I performed a manipulative watering experiment on seed fitness of the two sexes, and field studies measuring seed fitness and sex ratio as a function of soil moisture. In the dry site, watered hermaphrodites produced approximately twice as many seeds as unwatered hermaphrodites, with little treatment effect on female seed production. Over a natural soil moisture gradient, the ratio of female to hermaphrodite seed production was higher in dry than in wet sites. These data show that the seed fitness advantage of females is a function of soil moisture. Despite this, regression of soil moisture on the sex ratio of 23 populations was not significant. These results indicate a sex-dependent effect of soil moisture on resource allocation to seeds that does not translate into a strong effect on sex ratio. This is consistent with theory based on genomic conflict in which sex ratios are predicted to be only partly determined by fitness differences of the sexes.

**Keywords:** sex ratio; cytoplasmic male sterility; sex allocation

## 1. INTRODUCTION

In hermaphroditic plants, environmental factors can affect plant resource status and sex allocation. In many plants, increasing resource status leads to greater relative allocation to female than to male function (Klinkhamer & de Jong (1997) and references therein).

In species with both female and hermaphroditic individuals, a breeding system called gynodioecy, the relatively greater reduction in seeds versus pollen by hermaphrodites in low-resource environments should result in a higher ratio of female to hermaphrodite seed production in these environments. If the frequency of females is largely a function of the relative seed fitness of females versus hermaphrodites, there should also be a negative relationship between resource quality and female frequency (Delph 1990).

This relationship between relative seed fitness and sex ratio may depend on the genetics of sex inheritance. In some gynodioecious species, sex is determined by nuclear genes (see references below). In species with nuclear sex inheritance, sex ratios are expected to be relatively stable (Charlesworth & Charlesworth 1978), and a relationship between seed fitness and sex ratio is expected.

In many gynodioecious species, however, sex is determined by an interaction between nuclear and cytoplasmic genes (Lewis 1941; Kaul 1988). Cytoplasmic genes cause pollen abortion and male sterility (called cytoplasmic male sterility (CMS)). CMS generally reduces the transmission of nuclear genes, which creates strong selection for nuclear loci that restore male fertility (Frank 1989). Restored populations are then susceptible to invasion by new CMS mutants that are counteracted by different restorer alleles.

The repeated conflict of CMS genes and nuclear restorers has been predicted to result in unstable sex ratios, dependent upon mutation or gene flow events of novel CMS and nuclear restorer genes (Frank 1989; Gouyon *et al.* 1991). If this is the case, the relationship between environmental factors and sex ratio at any given time may not be strong.

In a few gynodioecious species, habitat quality has been shown to affect the relative fitness of females and hermaphrodites (Delph 1990; Delph & Lloyd 1991; Barrett 1992; Wolfe & Shmida 1997; Ashman 1999; Delph & Carroll 2001; Asikainen & Mutikainen 2003; though see Krohne *et al.* 1979). In all of these studies (and also in Alonso & Herrera 2001), environmental factors have also been correlated with sex ratio, with higher frequencies of females found in poorer-quality sites, as predicted by Delph & Lloyd (1991). One exception is that of *Silene acaulis* (Delph & Carroll 2001), in which there was no direct correlation between habitat quality and female frequency.

All of these species with a correlation between environmental factors and sex ratio have either unknown genetics of sex inheritance, or appear to have nuclear sex inheritance (Maletskii *et al.* 1994 (and references therein); Ramsey & Vaughton 2002; L. Wolfe, personal communication), except *S. acaulis*, which appears to have CMS (S. Keller, personal communication).

In the present study, I use a comprehensive approach to test the effect of one environmental factor, soil moisture, on sex-dependent seed allocation and on sex ratio in a plant species with CMS, *Nemophila menziesii*. Because *N. menziesii* is an annual plant, lifetime seed fitness can be measured. I first ran a manipulative experiment to test the effect of water on the relative seed fitness of the two sexes. I then tested whether natural variation in soil moisture of nine populations is correlated with relative seed fitness. Finally, I examined whether soil moisture is a good predictor of sex ratio in 23 populations. The present study

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therefore directly tests the effect of an environmental factor on both female and hermaphrodite lifetime maternal reproductive success.

## 2. METHODS

### (a) *Study species and site*

*Nemophila menziesii* (Hydrophyllaceae) is an annual plant with at least two different male-sterility cytotypes and at least two nuclear restorers (Ganders 1978; C. M. Barr, unpublished data). Sex ratios on the Point Reyes peninsula and surrounding coastline vary substantially over very short distances, from 0 to 53% of females (C. M. Barr, unpublished data).

*Nemophila menziesii* is a spring ephemeral, and is insect-pollinated, protandrous and self-compatible (Cruden 1972; C. M. Barr, personal observation). In contrast to many gynodioecious species, hermaphrodites of *N. menziesii* produce substantial numbers of seeds.

Study populations were located on the Point Reyes peninsula, ca. 60 km north of San Francisco, and at Bodega Bay, ca. 30 km north of Point Reyes. *Nemophila menziesii* grows in two types of habitat along the coast, sand dunes and bluffs. Sand dunes have soils with low moisture and organic content, and bluffs have soils with higher moisture and organic content. Dune and bluff sites can be in close proximity.

### (b) *Manipulative water experiment*

In February 2001, 120 plants of each sex at each of two sites, one dune (NB) and one bluff (LH), were used to test the effects of supplemental water on the relative allocation to seed of females and hermaphrodites. Female frequencies in 2001 were 34% and 20% for NB and LH, respectively. Soil moisture was measured (as described below) every week at each site to document site differences. Plants of each sex were selected haphazardly, and the water treatment was assigned randomly to 60 plants of each sex, with 60 unwatered controls. All plants were spaced at least 0.5 m apart (most plants were more than 0.5 m apart), which is enough to prevent overlap of visible roots in test plants, and the diameter of the soil that got wet with treatment was ca. 0.25 m. Watered plants were given 100 ml water every 4–7 days for a total of nine waterings. When plants died, their lifetime total branches, leaves, flowers, capsules and seeds were counted. In a previous greenhouse study, total seed production explained 91% of female and 95% of hermaphrodite variance in total plant seed fitness (measured as seed number  $\times$  fraction germinating  $\times$  fraction surviving) (C. M. Barr, unpublished data); therefore total seed production is used as an estimator of total plant fitness.

The effect of watering on the relative seed production of each sex was analysed by assigning plants into categories based on the numbers of seeds produced. A standard parametric analysis could not be performed because there were many plants with zero seeds, and this non-normality could not be corrected with transformations. Plants were put into five categories at NB (0, 1–5, 6–10, 11–15, and more than 16 seeds per plant) and seven categories at LH (0, 1–5, 6–10, 11–15, 16–20, 21–25, and more than 26 seeds per plant). The effect of watering treatment and sex were analysed with a  $\chi^2$ -test of homogeneity. Because sex-dependent allocation may be a function of plant size, I performed a logistic regression of seeds per plant as a function of plant size measured by branch number and tested for an interaction between the two sexes. Branches were categorized as 1, 2, 3, 4, 5, 6, or more than 7 branches.

To test whether watering might affect seed production by influencing flower number, I tested for the effects of sex and treatment on ln-transformed flower number per plant with an ANOVA.

### (c) *Field correlations*

#### (i) *Study populations and soil moisture*

I measured the soil moisture of 23 populations. Twenty-one populations were measured between 18 and 22 March 2001 at Point Reyes, and two on 19 April at Bodega Bay. Populations at Point Reyes spanned an approximately linear 17 km distance, whereas the Bodega Bay populations were ca. 2 km apart. Soil moisture data were collected either with a 2 cm  $\times$  20 cm soil corer or by digging a 20 cm hole and collecting equal amounts of soil from the bottom to the top of the hole from three locations that spanned each site. Samples were weighed, dried at 110 °C for 48 h, then weighed again, and values at each site were averaged. The percentage of soil moisture was calculated by weight.

#### (ii) *Soil moisture and relative fitnesses*

Seeds from both sexes were collected in 2001 from four dune (all Point Reyes) and five bluff (three Point Reyes and two Bodega Bay) populations. The relative seed production of females and hermaphrodites (F/H seed) was compared between the two types of site. Outliers were tested using Grubbs' test (Sokal & Rohlf 1995) and were removed (0–2 plants per population) from the calculation of population means. I performed a one-tailed *t*-test of F/H seed between dune and bluff populations.

#### (d) *Soil moisture and sex ratios*

I collected sex ratio data from 23 populations at Point Reyes and Bodega Bay between 1999 and 2001. In large populations (generally more than 500 individuals), I ran a 1 m wide transect through the longest dimension of the population and scored all flowering plants for sex within the transect. For smaller populations, all flowering plants in the population were scored. Sample sizes for sex ratios ranged from 92 to 845 individuals with a mean of 363 individuals.

The effect of soil moisture on population female frequency was analysed using a generalized linear model on logit-transformed odds ratio data in S-PLUS (Mathsoft Inc. 1999). This analysis assumes binomially distributed errors and weights the logits from each population by the sample size (Wilson & Hardy 2002).

## 3. RESULTS

### (a) *Manipulative water experiment*

LH soil moisture was ca. 10 times that of NB at the beginning of the flowering season, and dropped to two times at the end of the season. Average soil moisture for LH and NB over the 2001 season was 11.5% and 2.8%, respectively.

At the dune site (NB), there was a significant sex  $\times$  treatment interaction: watering doubled hermaphrodite seed production but slightly decreased female seed production (table 1). At the wetter bluff site (LH), there was no significant treatment effect or interaction.

For both sites, there were no significant effects of sex or treatment or their interaction on total flower number per plant (results from ANOVA, NB: model  $F_{3,200} = 1.30$ ,  $p = 0.27$ ; sex  $F_{1,200} = 2.58$ ,  $p = 0.11$ ; treatment  $F_{1,200} = 1.18$ ,  $p = 0.28$ ; sex  $\times$  treatment  $F_{1,200} = 0.00$ ,  $p = 0.99$ ; LH: model  $F_{3,208} = 0.39$ ,  $p = 0.76$ ; sex  $F_{1,208} = 0.27$ ,  $p = 0.60$ ; treatment  $F_{1,208} = 0.03$ ,  $p = 0.86$ ; sex  $\times$  treatment  $F_{1,208} = 0.75$ ,  $p = 0.39$ ). However, at NB, the two sexes differed, in that watered hermaphrodites converted many more flowers to seeds than control hermaphrodites, whereas female seed production did not respond as strongly

Table 1.  $\chi^2$ -test of heterogeneity results of the effect of watering on females and hermaphrodites of NB and LH. \* $p < 0.05$ .

	d.f.	$\chi^2$	$p$	d.f.	$\chi^2$	$p$
sex $\times$ seed category	4	0.63	0.96	6	11.34	0.08
treatment $\times$ seed category	4	3.87	0.42	6	3.94	0.68
sex $\times$ treatment $\times$ seed category	4	10.61	0.031*	6	4.68	0.59
total	4	15.11	0.004*	6	18.33	0.003*

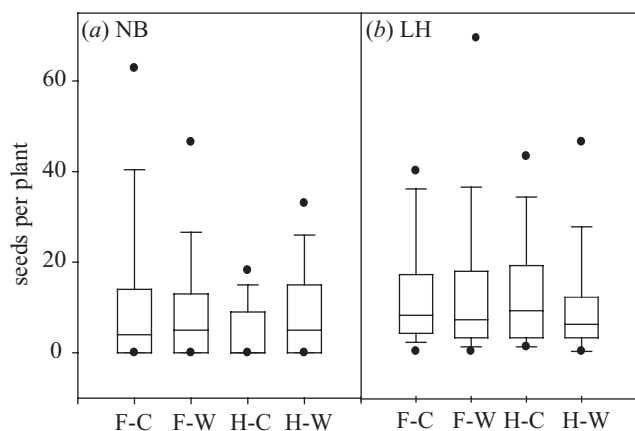


Figure 1. Boxplot of seeds per plant for populations (a) NB and (b) LH. F, female; H, hermaphrodite; C, control; W, watered plants. Shown are the median with tenth, twenty-fifth, seventy-fifth and ninety-fifth percentiles as horizontal bars. Points show fifth and ninety-fifth percentiles.

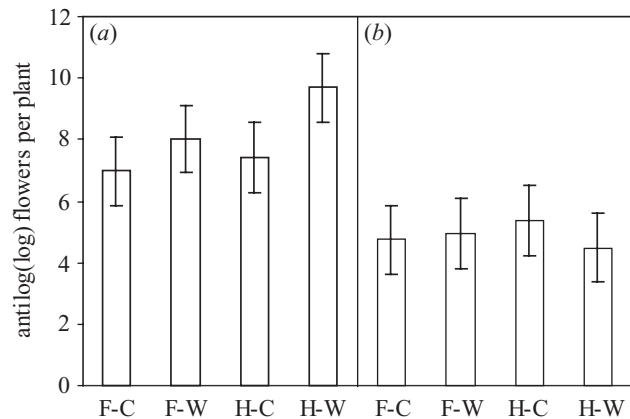


Figure 2. Average flower numbers per plant  $\pm$  s.e. (back-transformed log values) for populations (a) NB and (b) LH. F, female; H, hermaphrodite; C, control; W, watered plants.

to watering conditions (figures 1 and 2). This difference in response to water results in a higher ratio of female to hermaphrodite seeds in the dry control conditions than in wetter conditions. Both control and watered LH (bluff) females and hermaphrodites made approximately the same number of flowers and seeds (figures 1 and 2).

At NB, there was no significant effect of the watering treatment on branch number, nor a significantly different response between the sexes (ANOVA results of ln-transformed data: model  $F_{3,203} 1.28$ ,  $p = 0.28$ ). There was a significant effect of branch number on seeds per plant, but this effect did not differ significantly between the sexes

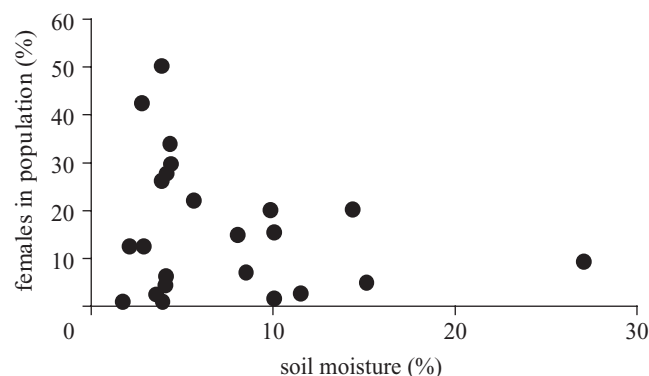


Figure 3. Percentage of females per population as a function of percentage of soil moisture.

for either population (results from logistic regression, NB: model  $\chi^2_9 = 25.75$ ,  $p = 0.003$ ; branch  $\chi^2_1 = 22.86$ ,  $p = 0.0001$ ; sex  $\chi^2_1 = 0.85$ ,  $p = 0.36$ ; branch  $\times$  sex  $\chi^2_1 = 1.69$ ,  $p = 0.19$ ; LH: model  $\chi^2_{15} = 29.73$ ,  $p = 0.01$ ; branch  $\chi^2_1 = 87.73$ ,  $p = 0.0001$ ; sex  $\chi^2_1 = 3.31$ ,  $p = 0.07$ ; branch  $\times$  sex  $\chi^2_1 = 3.76$ ,  $p = 0.053$ ). Results using flowers and leaves per plant as measures of plant size were qualitatively the same as those for branch number.

#### (b) Field correlations

##### (i) Soil moisture and relative seed fitness

Average soil moisture for the four dune and five bluff populations was 4.06% and 10.68%, respectively. Average F/H seed was significantly higher for the dune than for the bluff populations (F/H seed mean  $\pm$  s.e., dune:  $2.03 \pm 0.34$ , bluff:  $1.31 \pm 0.20$ ) ( $t_7 = 2.21$ ,  $p < 0.05$ ).

##### (ii) Soil moisture and sex ratio

The effect of soil moisture on logit-transformed female frequencies was not significant ( $F_{1,22} = 0.78$ ,  $p = 0.39$ ). Both maximum female frequencies and variance in female frequency decreased with increasing soil moisture (figure 3).

## 4. DISCUSSION

### (a) Manipulative water experiment

There was a significant effect of watering on the relative seed fitness of females and hermaphrodites at the dry site NB, but not at the wet site LH (table 1). Control NB hermaphrodites made fewer than half the seeds of control females, while there was little difference between watered females and hermaphrodites (figure 1). Hermaphrodites and females allocated similarly to flowers, but in control conditions hermaphrodites converted relatively fewer flowers to capsules and seeds (see figures 1 and 2).

This sex-dependent effect of an environmental factor on fitness has not been directly shown in other gynodioecious

(or subdioecious) species, although five studies showed that fruit production of hermaphrodites decreased with qualitative decreases in soil moisture (Delph & Lloyd 1991; Sakai & Weller 1991; Barrett 1992; Wolfe & Shmida 1997; Asikainen & Mutikainen 2003), and Delph & Carroll (2001) showed a borderline significant sex-dependent correlation between fitness measures and site quality for *S. acaulis*. Greater relative allocation to female function on more fertile or wet soils has also been shown for dioecious, monoecious (Costich 1995) and hermaphroditic (Klinkhamer & de Jong 1993) species.

There are two possible mechanisms for this sex-dependent allocation effect as a result of resources: plant size effects or greater hermaphrodite seed plasticity. As plant size and flower number increase, allocation to maternal relative to paternal function has been shown to increase (e.g. Delph 1990; Klinkhamer *et al.* 1997; Ashman 1999) because of local mate competition (de Jong *et al.* 1999) and/or the effects of inbreeding depression with pollen discounting because of geitonogamy (Lloyd 1987). If soil moisture is correlated with plant size, then larger hermaphrodites in wetter sites should allocate relatively more to seeds than to pollen.

Plant size is not the cause of the sex-dependent allocation found in this study. There was no effect of the watering treatment on plant size for either sex, and the effect of size on seeds per plant does not differ significantly between the sexes for either population. Outcrossing rates have been shown to be high in *N. menziesii* (72–99%; Schick 1999), and the number of open flowers on a plant on any given day is generally small (one to three flowers; C. M. Barr, unpublished data); thus there is little opportunity for geitonogamy.

A second mechanism for the sex-dependent effect of resources is greater plasticity in hermaphrodite than female seed production (Delph 1990, 2003; Delph & Lloyd 1991). Hermaphrodites in populations with females gain more fitness through pollen than through seeds (e.g. Ramula & Mutikainen 2003), therefore selection may favour the maintenance of pollen production in all environments with seed allocation dependent on resources. I did not measure pollen production in this study; however, if there is a trade-off between pollen and seed production, this mechanism could explain the present results.

### (b) *Field correlations*

In addition to the manipulative experiment, I tested whether a natural gradient in soil moisture affects sex-dependent seed fitness, and thus whether natural environmental effects may be contributing to sex ratio variation seen in the field. F/H seed was significantly higher in dry dune than in wetter bluff sites. This indicates that the seed advantage of females over hermaphrodites is greater in drier sites, consistent with the results from the manipulative experiment.

There was no significant effect of soil moisture on female frequency (figure 3). This result is in contrast to other published reports of gynodioecious (or subdioecious) species (Barrett 1992; Wolfe & Shmida 1997; Ashman 1999; Asikainen & Mutikainen 2003), all of which appear to have nuclear sex inheritance (*Geranium sylvaticum* studied by Asikainen & Mutikainen (2003) may have nuclear–cytoplasmic sex inheritance (P. Mutikainen and E. Asikainen,

personal communication), but sex ratios appear to be fairly stable over time in this species), but similar to that of Delph & Carroll (2001) with *S. acaulis*, with apparent nuclear–cytoplasmic sex inheritance. This difference may be explained by the relative stability of the sex ratio predicted for species with nuclear sex inheritance versus the unstable dynamics predicted for those with nuclear–cytoplasmic sex inheritance.

If sex ratios are unstable and in part determined by gene flow, then environmental effects on the female seed fitness advantage (F/H seed) will affect the rate at which the cytoplasmic equilibrium is reached and thus the maximum female frequency, but will not be a predictor of the female frequency at any given time. The present results with *N. menziesii* are consistent with this idea, suggesting that the maximum female frequency decreases with soil moisture, but that individual population sex ratios can be anywhere below this maximum (figure 3). Alternative explanations to these data, however, include a positive association between gene flow (and introduction of nuclear restorers) and soil moisture (e.g. through pollinator movements), and thus a negative association between female frequency and soil moisture; or, a shift from resource to pollinator limitation as soil moisture increases, resulting in a large resource-induced difference in female and hermaphrodite seeds at low soil moistures, but a limited difference between the sexes at higher soil moisture as a result of equal pollen limitation. While large-scale differences in soil moisture among sites are likely to be fairly constant, one caveat to an interpretation of an observed correlation between soil moisture and sex ratio is that soil moisture and selection for changes in the sex ratio may change at different rates; therefore any one-year measure of soil moisture may not be predictive of current sex ratios.

In conclusion, it appears that soil moisture can affect the relative fitness of females and hermaphrodites in *N. menziesii*; however, environmental effects are not the sole determinant of the sex ratio. A plausible explanation of natural sex ratio variation is the combination of environmentally controlled fitness effects and gene flow dynamics driven by conflict.

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