Phenotypic plasticity in Mercurialis annua:

Sex determination and Density detection through 'signalling and response'

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Botany Honours

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I hereby declare that this project is my own work and that any other ideas or input that were not my own were referenced and/or acknowledged. This thesis is towards completing my honours degree at the University of Cape Town and will not be used for any other purposes.

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Phenotypic plasticity in Mercurialis annua:
The role of 'signal detection and response' in sex determination

Abstract

Mercurialis annua has been found to show phenotypic plasticity in terms of their sexual strategies. In androdioecious populations (males coexisting with cosexes), genetically labile males function as cosexes at low densities. At high densities, the frequency of males increases, while cosexes respond by increasing biomass allocation to the female function. Phenotypic plasticity in this species is likely a result of environmental detection. This study investigated possible mechanisms for density detection. Greenhouse experiments showed that *M.annua* responded to an above ground signal by increasing male frequency and male frequency was related to the proportion female allocation in cosexes. Individuals that were isolated from each other below ground and treated with leachate (collected from high density populations) responded by increasing allocation to female function while male frequency decreased. There is most likely an interaction between below and above ground sensing and response to the environment in *M.annua*.

Introduction

Phenotypic plasticity is the ability of a plant to alter its physiology or morphology in response to environmental conditions (Schlighting 1985, Bradshaw 1965). The sessile life styles of plants requires a certain degree of flexibility in terms of resource allocation and sex determination (Schlichting 1985), since plants are constantly faced with the challenges of changes in their environmental circumstances and therefore manipulation of sex ratios for maximum fitness in each sex offer some control over these circumstances (Charnov and Bull 1977). Subsequently the fitness of an individual depends on its ability to respond to its environment, and in a particular environment, selection should favour the phenotype that maximizes fitness relative to that of others under the same conditions (Givnish 2002). A well known example of environmental responsiveness is through light detecting mechanisms relating to competition. Shade-avoidance responses in crowded populations of *Arabidopsis thaliana* demonstrated the existence of phytochrome signalling pathways and the importance of the reliability of the Red to Far Red ratio (R:FRI) cue for phenotypic plasticity (Schmitt *et al.* 2003) in this species. In dense stands these plants manage to out compete near neighbours by stem-elongation, since it ensures a higher lifetime light interception and consequent overall higher fitness. This strategy, to match their phenotype to local conditions ultimately ensures an evolutionary advantage (Weinig and Delph 2001).

Plasticity in terms of reproductive strategies is fundamental for some plants to maximise reproductive success in a given environment. For example, plasticity in sexual strategy has been found in individuals of androdioecious populations, where being a male (unisexual individuals producing only pollen) or a cosex (individuals producing pollen and ovules) determines its ultimate fitness. The success of any particular strategy is based on: a) The efficiency of cross-pollination (the proportion of cosex ovules pollinated by self pollen) (Pannell 2002; b) Inbreeding depression (reduction in fitness of selfed offspring compared to outcrossed offspring) (Schemske and Lande 1985) and/or c) The amount of pollen that a male can produce relative to a cosex (Charlesworth and Charlesworth 1979, Pannell 2005)

In an androdioecious population, a successful male would have to produce at least a threshold amount of pollen in order to have equivalent success as a cosex (Pannell 2002). This depends on the efficiency of outcrossing (1-selfing) and inbreeding depression (fig. A).

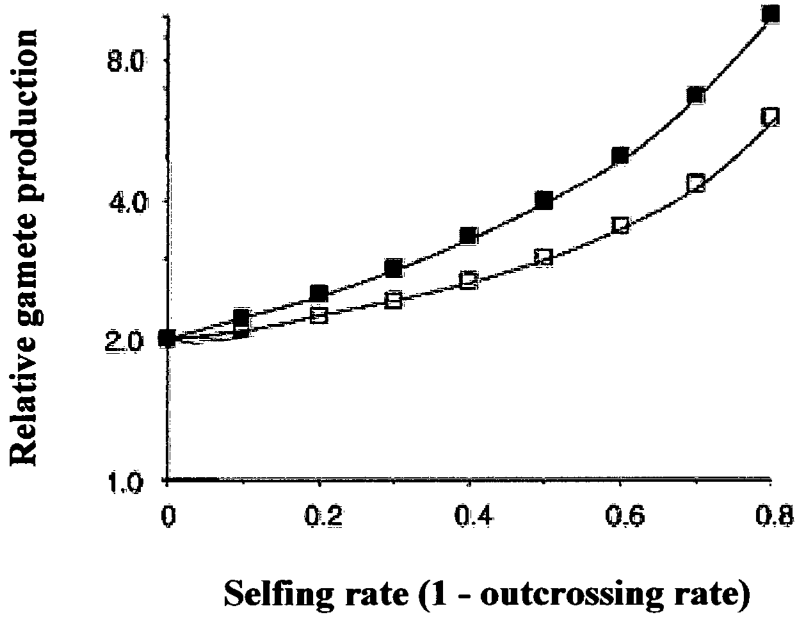

Figure A The minimum relative pollen production of males for their invasion into a population of cosexes as a function of the cosex selfing rate. In the case of inbreeding depression = 0.5 (open squares) males would have to produce relatively less pollen than in the case of no inbreeding depression (closed squares) at the same rate of selfing. (taken from Pannell 2002).

Sex allocation in cosexes also depend on a and b, since outcrossed offspring tend to be more fit than those from self-fertilizing cosexes. In the case of successful outcrossing, the preferred strategy for cosexes is to invest more of their resources into producing ovules than into pollen production (fig. B).

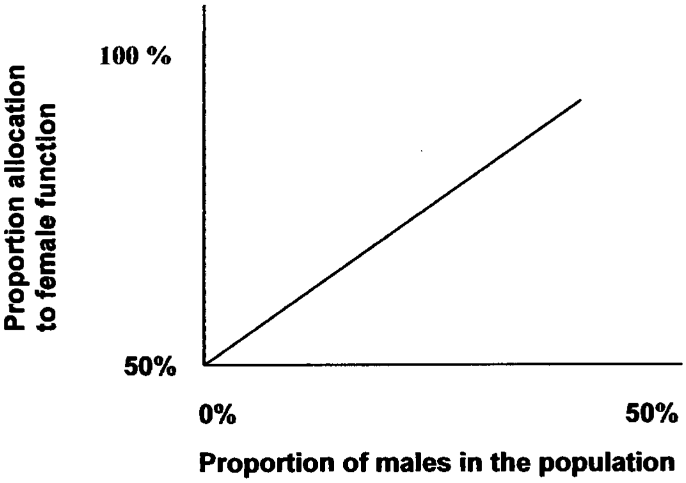

Figure B The relationship between allocation to female function and male frequency in an androdieocious population.

Inbreeding depression and the amount of pollen that a male can produce relative to a cosex are more likely to be evolutionary changes due to the genetic component of sex determination (dominant vs. recessive mutations) and therefore ultimate causes for the mating system of a population. However, the efficiency of cross pollination can change from generation to generation depending on environmental factors such as wind and population density. Consequently these are more likely to affect the optimal reproductive strategy in the short term and therefore affect the structure of an androdieocious population (Pannell 2001, 2006).

The efficiency of cross pollination is largely affected by the density of the population, therefore it would be beneficial for plants to detect density and alter sexual strategies accordingly. In metapopulation dynamics, where sites continually become extinct and recolonized, density is highly variable and individuals with the ability to determine sex in response to the environment would have a fitness advantage. Phenotypic plasticity thus likely plays a major role in the structure of metapopulations and Givnish (2002) pointed out that signal detection and response are fundamental to all aspects of phenotypic plasticity. In order to optimise their sex and strategy, plants must therefore be able to detect the proximity of neighbouring conspecifics.

Density detection and sex determination via chemical cues have been found in the fern *Ceratopteris*. A pheromone (antheridiogen) is excreted by the cosex and promotes the development of males from sexually undetermined gametophytes. This study further showed how the expression of one sex represses the expression of another sex via cues (Banks 1997). Phenotypic plasticity in response to density is thus assisted by communication tools, either via chemical signalling (in the case of *Ceratopteris*) or light detection (in the case of *Arabidopsis thaliana*).

Phenotypic plasticity in terms of sexual strategy has also been found in the annual, *Mercurialis annua*, a pan-European species which shows remarkable metapopulation dynamics (Pannell 2006). Plasticity in terms of sexual strategy is particularly important for androdioecious populations of *M.annua*, where colonization dynamics are fundamental for plants with a short growing season (Pannell 1997b). During colonization of a new site, self-fertile cosexes would be favoured (Pannell 2006) since reproductive assurance during population establishment is the primary strategy. Consequently, such populations would consist of partially or wholly inbreeding cosexes which would inhibit the invasion of males (Pannell 2002), which can only gain reproductive success through outcrossing. The difficulty of invasion by males probably explains the raritiy of this sexual system in nature (Charlesworth and Charlesworth 1979). However, certain environmental instances such as high density (following establishment in a colonized environment) and resource availability, allow males to successfully invade because female-biased founding populations are no longer evolutionary stable under these conditions and more ovules are available thus increasing the probability of male fertility advantage over cosexes (Pannell 2001). These dynamics maintain androdioecy (Pannell 2002) and it has been shown that the determination of sex ratios and sex allocation in *M. annua* has a genetic and environmental component (Pannell 1997a). The environmental component is largely density related whereby cosexes increase investment in female function and male frequency increases. Furthermore, it has been pointed out in a review by Pannell (2005) that a plant's gender depends both on its own sex allocation and on the sex allocation of other individuals in the population because there are trade-offs associated with sex allocation therefore cosexes will only increase investment in the relatively more expensive ovules and seeds when resources are readily available or greater outcrossing opportunities arise through increased male frequency (i.e. pollen). Consequently, cosexes will invest less into pollen production if there is a high concentration of males in close vicinity (fig B).

How is density detected by *Mercurialis annua*? This study investigated the possible mechanisms of environmental detection. Density could either be detected by an above ground signal and/or below ground signal. Glasshouse experiments were used to determine an above ground signal which could be chemical signalling or the detection of Red:Far Red ratio, by the isolation of plants below ground. The possibility that *M.annua* communicates via a below ground chemical signal was also investigated in a glasshouse experiment where plants were isolated from each other below ground and watered with leachate collected from plants grown at high density. Density detection through resource availability was investigated in a nutrient experiment where below and above ground density was controlled for.

Methods and materials

Study species

Seeds from hexaploid androdioecious populations from Morocco (collected in 2004 and kindly provided by Marcel Dorken from the Department of Plant Sciences, Oxford) were used in glass house experiments. In natural populations, males were found to occur at frequencies of between 25% and 30%. The inflorescences of males in androdioecious populations have sessile staminate flowers arranged in tight spiral clusters along erect axiallary peduncles. Cosexes are similar in morphology to diploid females, except that their subsessile axillary pistillate flowers are surrounded basally by a tight spiral cluster of staminate flowers (Pannell 1997b). From the age of two weeks to the end of the growing season males and cosexes produce inflorescences and fruits. Sex allocation is easily determined in both males and cosexes by using floral biomass as an index for allocation to pollen and ovule production (biomass of dispersed pollen accounts consistently for about 55% of the staminate floral biomass (Pannell 1997b).

Glass house experiments

To measure sex ratios and sex allocation in androdioecious *M. annua*, varying environmental conditions were mimicked and included high and low nutrient levels, four density treatments and the effect of leachate collected from high density plants. All plants were germinated and grown under glasshouse conditions at Wytham fieldstation (Oxfordshire) from mid July through to the end of August (2006).

Density determination through detection of an above-ground signal

The possibility of an above ground cue was investigated by growing *M.annua* at four different densities and isolating plants from each other below ground. Each density treatment contained 15 trays (fig A and B).

A

X					
		X			
					X

C

X		X		X	
	X		X		X
X		X		X	
	X		X		X

B

X		X		X	
X		X		X	

D

X	X	X	X	X	X
X	X	X	X	X	X
X	X	X	X	X	X
X	X	X	X	X	X

Fig. A. Layout for density treatments: Each treatment consisted of fifteen trays and varied in the number of plants per tray: A) 3 plants per tray, B) 6 plants per tray, C) 12 plants per tray, D) 24 plants per tray.

Each treatment was blocked in order to eliminate edge effects from trays with differing densities, thus treatments were not assorted randomly by intermixing trays within each 15 tray block. The effect of glasshouse position on the blocks was partly compensated for by moving the blocks around in the glasshouse on a regular basis.

Density 6

Density 24

Fig. B. Density treatments at the age of three weeks.

Density determination through detection of a below-ground signal

To test whether *M. annua* respond to below ground cues by, an experiment was conducted using leachate from plants grown at high densities (25 to 50 plants per 35.5 x 21.5 cm tray) to water plants that were isolated from each other below ground (fig Ci)**. These plants were grown in 10 separate trays with 24 compartments (125 cm^3 each). Leachate from soil (containing no plants) was used to water another 10 trays of 'isolated' *M. annua*. Isolation excluded other below ground factors and therefore a response via sex ratio/allocation should be to a signal present in the leachate. The isolated plants as well as the high-density plants were sown and grown in compost (Emerald Range (HTA) Soil based Multi purpose compost). The signal treatment and control treatment were placed on separate benches in the glasshouse.

** **Seeds for the high density trays were sown 2 weeks in advance.**

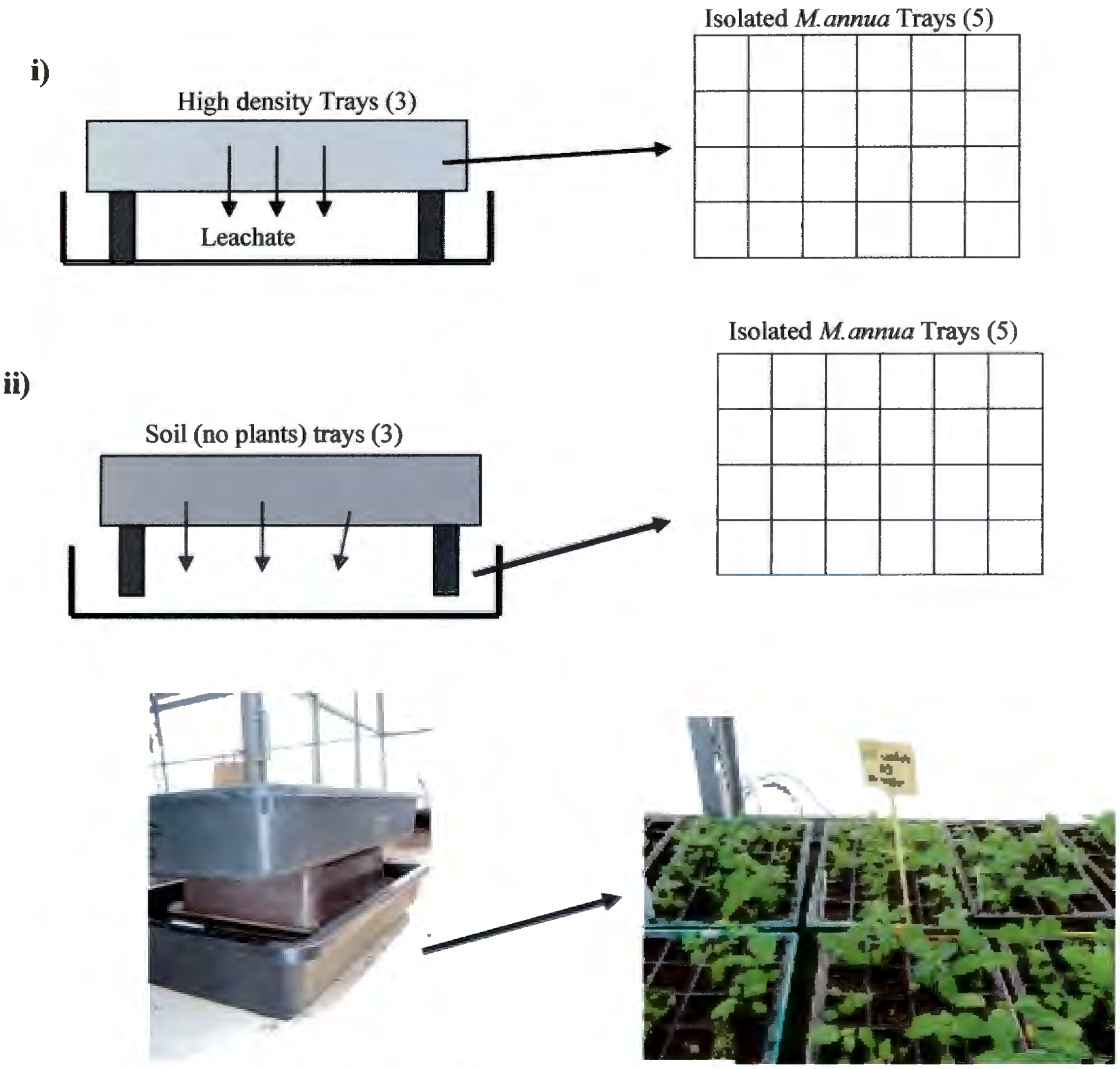

Figure C (i & ii) Signal Experiment no. 1

To control for the possible difference in nutrients between leachate collected from high density plants and leachate collected from soil (containing no plants), dioecious *M. annua* (collected in 2001 from Slapanice in the Czech Republic) was used. Dioecious populations are not expected to show any plasticity in terms of primary sex ratios, therefore potential 'cues' from leachate would have no effect on these plants. However, a difference in biomass between the two treatments would indicate a nutrient effect from the leachate.

The control consisted of 2 trays with dioecious *M. annua*, watered with the leachate collected from high density trays (androdioecious) and 2 trays with dioecious *M. annua* watered with leachate collected from soil (no plants) trays.

Density determination through resource availability

Seeds were sown in 10 seed trays (35.5 X 21.5 cm) containing compost and watered (every other day) with fertilizer in solution (1.1 g *Phostrogen/ 1L water) imitating high resource (nutrient) availability. Ten trays with compost lacking fertiliser represented low resource (nutrient) availability. Each tray consisted of 24 compartments (with a total volume of 125 cm^3) therefore plants were isolated from one another (thus excluding any below ground density effects on sex determination and sex allocation). Plants were watered with equal amounts of water every day or every other day depending on the weather conditions. *

Phostrogen NPK fertiliser 14-4.4-22.4 (Mg 1.5 – S 3.0 %)

Plant harvesting

M.annua produced inflorescences after three weeks of growing under the various treatments and males and cosexes were distinguishable at the age of five weeks when sex ratios were determined at the various densities, leachate treatments and nutrient levels. Sex allocation was also measured by harvesting the plants (at the age of seven weeks) and weighing dried inflorescences of male and female inflorescences of the cosexes.

Statistical analyses

The density effect on male frequency was determined via a chi-square test for trend (Armitage 1971) since the density treatment groups contained natural order of increasing densities including 3, 6, 12 and 24. In all of the analyses, sex ratios were transformed using an angular transformation to achieve normality of the residuals and homogeneity of error. To determine female allocation, we considered the method used by Lloyd (1980) whereby an equivalence factor (E) is introduced to compensate for a possible difference in biomass allocation to fruits vs. pollen. However, since this was an androdioecious population, we could disregard this assumption. However, for my purposes, the equivalence factor was used to normalize the error distribution due to the large differences between male and female reproductive (dry) weights. Since this factor made no difference to the results, E was excluded for the analyses. As an alternative, female sex allocation was determined as a proportion of the total biomass allocated to male and female reproductive function. Differences in female allocation at the various density levels were detected via ANOVA.

The relationship between sex allocation in cosexes and male frequency was determined via a linear regression. A Student's t-test was used to determine significant differences in sex ratios and differences in sex allocation between the signal treatment and control treatment. Differences due to resource availability were tested via a Student's t-test.

Results

Density determination through detection of an above-ground signal

Androdioecious *Mercurialis annua* showed significant differences in male frequency (sex ratio male: total) at the four different density treatments ($X^2_{df3} = 11.410$, $P = 0.010$, Chi-square test for trend) (fig. 1). Male frequency was lowest at the lowest density and increased at density level 6. The highest male frequency (*c.a.* 50%) was found at density treatment '24 plants per tray'.

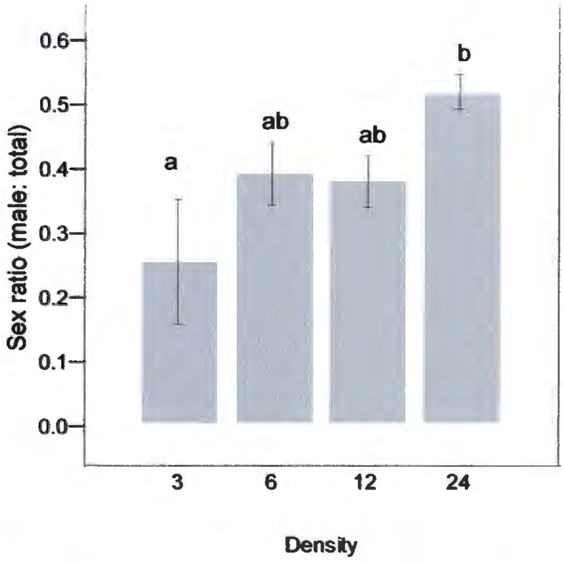

Fig. 1. The effect of density on male frequency (ratio of males to cosexes) in androdieoecious *M. annua*. Letters above error bars indicate significant differences (One-way ANOVA with post hoc Neuman Keuls multiple range test. Values are means of 15 replicates +/- stderrors

There was no significant density effect ($R^2 = 0.009$, $p = 0.4841$) on the sex allocation of cosexes. However the frequency of males and the sex allocation in cosexes were significantly correlated ($R^2 = 0.07$, $p = 0.0468$) (fig. 2).

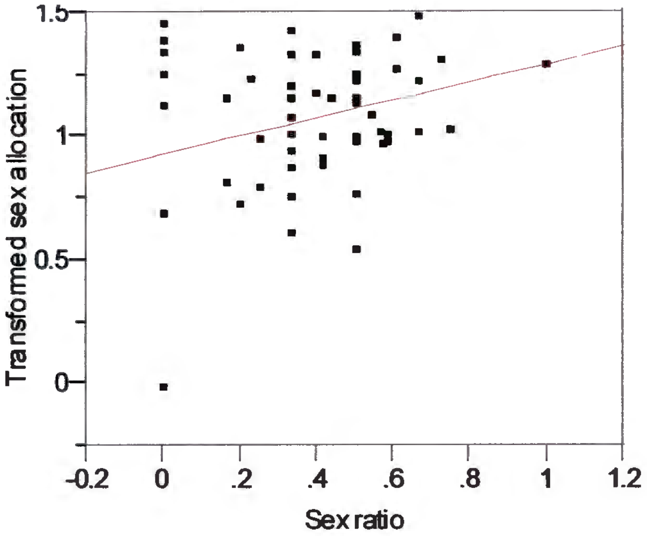

Fig. 2. The relationship between sex ratio (male: total) and female sex allocation (in cosexes).

Density determination through detection of below-ground signal

There were significant differences in sex ratio (male :cosex) between plants treated with leachate collected from high density trays and control plants. Male frequency was significantly lower ($t_{df18} = 3.809$, $p = 0.0013$, Students t-test) in plants treated with the 'signal' leachate (fig. 3 a) than control plants.

Mercurialis annua showed a further response to a belowground cue by increasing allocation to female function (in cosexes). This was evident in the significant difference ($t_{df18} = -2.61$, p

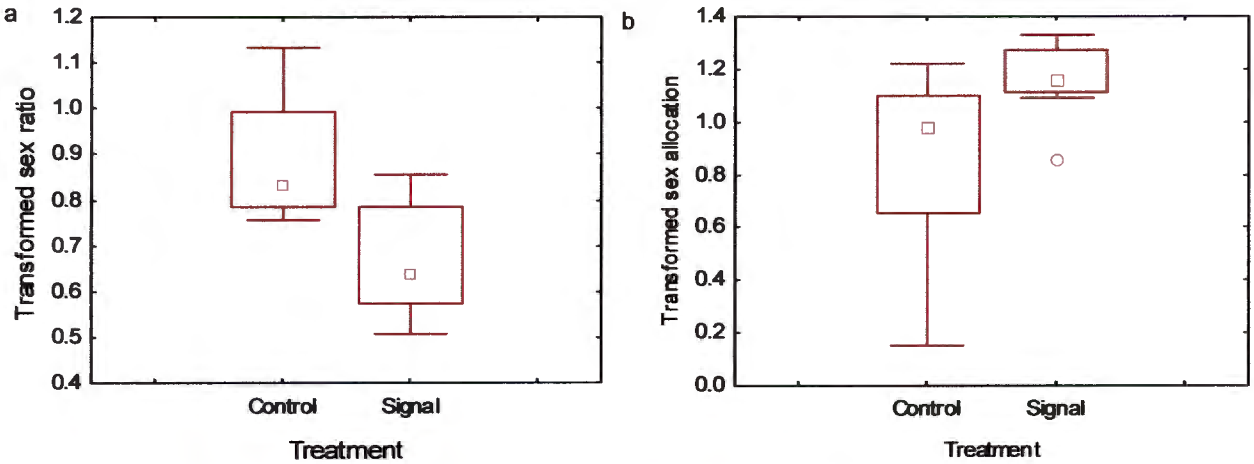

Fig. 3 a) Male frequency and b) Allocation to female function in control plants and plants treated with 'signal' leachate. Values are means of 10 replicates +/- stdev.

= 0.0177, Student's t-test) between female sex allocation in control plants and 'signal' plants (Fig. 3b).

Density determination through resource availability

There was no significant difference in the frequency of males or allocation to female function between the high and low nutrient treatments.

Discussion

Density determination through detection of an above-ground signal

Male frequency in androdioecious *Mercurialis annua* was found to increase with increasing density (Fig 1). This conforms to the general notion that maleness in *M. annua* is genetically determined and that some males have the ability to function as either cosexes or males depending on an environmental factor such as density and therefore are phenotypically plastic (Pannell 1997a). Since plants were isolated from each other below ground in this experiment, density is most likely to be detected via an above ground signal. I propose two possible mechanisms for detecting density above ground. Firstly, individuals may respond to density through light detection whereby neighbour proximity is expressed as a reduction in the Red to Far Red ratio (Schmitt *et al.* 2003). A second mechanism involves chemical signaling, in which case either males or cosexes produce a signal. It is then interpreted as the presence of the particular sex. In high densities, these cues are likely to be produced in high concentrations and interpreted as high density. Reproduction via outcrossing ensures viable offspring which increases with density.

Males respond to density by producing female inflorescences at reduced density (Pannell 2005). Furthermore, density is likely to affect the rate of selfing in cosexes to such a degree that males can invade the population at higher densities, resulting in this increase of male frequency. At low densities, the male frequency was found to be closer to the expected ratio for natural Moroccan populations (25 – 30%) (Pannell 1997*b*). These densities are likely a simulation of environments with high colonization rates where reproductive assurance is fundamental. Low male frequencies are therefore due to individuals' assessment of the environment as being 'crowded' and therefore show an increase in the expression of cosexual function rather than being male.

Cosexes invested increasingly into female function at higher densities. The possibility that cosexes in androdioecious popoulations respond to shading by reducing allocation to the female function (Pannell 1997b) has been contradicted in this experiment since there was no evidence for a relationship between sex allocation and density and female allocation increased at higher densities.

It would seem that cosexes respond to the increase in male frequency rather than density as such (Fig. 2). The increase in male frequency is most likely the result of individuals responding to the 'light cue'. The cue probably equates to competition and therefore pollen production possibly increases. Cosexes in return sense the increased pollen production via increased fertilization from non-self pollen. The higher probability for out crossing results in an alteration of biomass allocation to female function evident in the significant relationship between male frequency and allocation to female function. Chemical signaling as an above ground mechanism for density detection is largely overridden by this relationship between cosexes and male pollen production.

Density determination through detection of below-ground signal

Since sexually non-plastic plants (dioecious *M. annua*) and androdioecious *M.annua* showed no significant difference in biomass between plants treated with leachate collected from soil and plants treated with leachate collected from high density plants, it could be assumed that resource availability had no effect on the 'signal' results.

Male frequency in the control plants were higher than in plants watered with 'high density' leachate (Fig. 3a). The frequency in control plants was close to 50%, which is higher than the expected frequency for natural populations, but similar to the frequency at the same density (24 plants per tray). Plants treated with the 'signal' leachate and grown at this same density had a male frequency of close to 30%. The significant difference between the two treatments leaves little doubt that there is a below ground signal, however the nature of this cue remains tentative since the effect was found to be in the opposite direction than was anticipated. This leads us to consider various possible scenarios (illustrated in Fig 5).

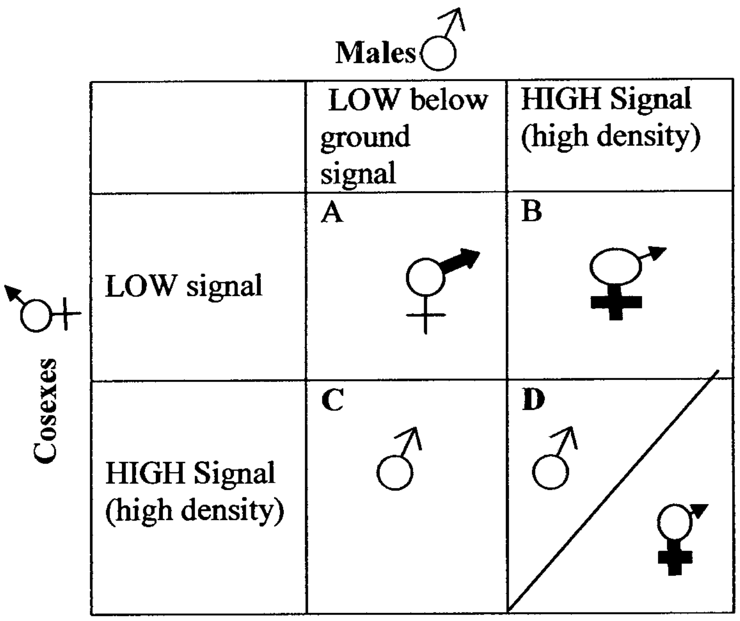

Fig. 5. Possible scenarios for the optimum strategy that any individual could 'choose'. Individuals can respond by becoming male (♂) or cosexual. ⚥ is the symbol for cosexes with higher allocation to male function relative to female function. ⚥ symbolises cosexes with higher allocation to female function relative to male function.

In the case of low/no below ground signal the most likely outcome is for cosexes to respond in the same fashion as in sparse sites, i.e. increasing allocation to male function relative to female function thereby ensuring pollination through selfing (Fig. 5 A).
On the other hand, if there is a chemical cue and exclusively from males, high concentrations would be detected below ground. Assuming reproductive biomass is a proxy for inheritance of genes in the next generation, an individual would benefit more from being a female- biased cosexual than from being a competing male (Fig. 5 B). Consequently, sex allocation would be positively affected as was found in the leachate experiment.

In the case of the below ground cue being produced by cosexes only, leachate collected from high density populations would imply the presence of ovules and low density/the absence of functional males, in which case an individual would benefit more from being an above average male rather than a cosexual (Fig. 5 C).

In dense natural populations, the most 'sensible' evolutionary strategy would be for males to increase in frequency and cosexes to invest extensively in their female function, since this would maximize outcrossing rates. For this to be possible, a below ground chemical cue has to be produced by both males and cosexes (Fig. 5 D). However, the contrary was found and some individuals responded by reducing the expression of maleness and cosexes increased allocation to the female function. This is likely the effect of an above ground cue operating in combination with a below ground signal. Such complex relationships among trait means, plasticities and underlying mechanisms have been found to be a caution against generalizing about the genetic architecture of plastic traits in *Arabidopsis thaliana* (Callahan and Picliucci 2005). Given this, individuals in a *M. annua* population might be responding to one stimulus while another independent stimulus might affect another aspect of the plasticity. In this case the above ground stimulus is interfering with the below ground cue so that male expression is possibly repressed by near neighbour proximity. While below ground, the signal possibly originates from males, inferring increased outcrossing rates.

The differences in sex allocation between the two treatments were not due to differences in biomass, since the effect of biomass on sex allocation was not significant. Plasticity in terms of gametic allocation is related to fitness, and the proportion of fitness gained through male function for each genotype (male or cosex) will constrain the selection for males. Males at high frequency pose a greater fitness prospect for cosexes than for genetically labile males and if the signal for male presence is concentrated, an individual's optimal strategy would be to ensure cross fertilization; for cosexes this requires increased investment in female gametes.

Having considered these 'if-then' scenarios, the best possible explanation can only be found in a follow-up series of experiments, where the origin and nature of the signal could be clarified. Leachate collected from males only and from cosexes only is a good starting point, with possible extensions into exploring the effect of these on various densities of the receiving individuals. Furthermore, there should be a close relationship between the strength (density) of the signal and the response. To test this, chemical analysis of the leachate should be carried out. Alternatively, sex ratios from the leachate plants should be recorded. For this experiment, leachate was collected from a variety of densities; therefore no inferences could be made regarding this relationship. Further experimentation should be done where leachate is collected from males only, and from cosexes only to establish where the signal originates from and how it is received. Furthermore, in future experiments, the effect of the glasshouse position should be completely eliminated, even though it is unlikely that this played a major role in these significantly large differences between control and signal plants (there was no significant difference in biomass between control and signal plants).

Density determination through resource availability

The lack of differentiation in male frequency and allocation to female function in cosexes between low and high nutrient treatments is most likely an artefact of experimental flaw in that the nutrient addition to compost might not have been adequate to create a significant difference in nutrient availability. However, density detection through resource availability would most likely result in a higher male frequency at low nutrient availability, since allocation to female function is more costly and an individual would have a greater fitness advantage by being an above average male rather than a below average female, therefore sexually labile males would not function as cosexes under these conditions (Charnov and Bull 1977, Pannell 1997a, Charlesworth and Charlesworth 1979).

Concluding remarks

Plasticity in *M. annua* has evolved under conditions of highly variable environments, therefore despite heterozygosity in males, sufficient genetic variation in this species allowed for optimal strategies to evolve. It is therefore fair, as suggested by Pigliucci (2005), to explore a series of 'if-then' hypothetical scenarios about natural selection rather than to make quantitative predictions about actual evolutionary pathways. In high density, the optimal strategy for males is to reduce the production of female inflorescences and for cosexes to increase investment in the female function. Independently of the underlying genetic mechanism for these responses, we can say that males are plastic in response to an above ground assessment of the environment, which in the absence of a below ground signal would infer a situation where being male and a female biased cosexual ensures maximal outcrossing. This would therefore result in the most fit offspring ensuring the transferral of both male and cosexual genes to the next generation. However, where above ground and below ground stimuli interact, the outcome is such that functional male expression is constrained and female allocation in cosexes is promoted. Even though this experiment can not define the cue, *M.annua* indeed shows a response to the environment, which is most likely the product of an interaction of below and above ground density cues.

Acknowledgements

My utmost thanks to Dr John Pannell and Mark Harris, for their input and advice experimental design and analyses. Thanks to Marcel Dorken for providing the seeds collected in Morocco. Thanks to Phil Smith at Wytham Fieldstation for assistance in plant care and watering. Thanks to Benoit Pujol for his valuable input and transporting plants. Thanks to Christine for watering the plants.

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