Sex ratio variation and sex determination in Urtica dioica

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Environmental conditions affect sex expression in monoecious, but not in male and female plants of *Urtica dioica*

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Urtica dioica is a sub-dioecious plant species, *i.e.* males and females coexist with monoecious individuals. Under standard conditions, seed sex ratio (SSR, fraction of males) was found to vary significantly among seed samples collected from female plants originating from the same population (0.05-0.76). As a first step, we investigated the extent to which SSR and sex expression of male, female, and monoecious individuals is influenced by external factors. We performed experiments to analyze: (1) whether the environment of a parental plant affects SR of its offspring, (2) whether SSR can be affected by environmental conditions before flowering, and (3) whether sex expression of male, female and monoecious plants that have already flowered can be modified by environmental conditions or by application of phyto-hormones. Within the range of our experimental design, SSR was not influenced by external factors and gender in male and female plants was stable. However, sex expression in monoecious plants was found to be labile: flower sex ratio (FSR, fraction of male flowers) differed considerably between clones from the same individual within treatments, and increased towards 100% maleness under benign conditions. These results provide strong evidence that monoecious individuals are inconstant males, which alter FSR according to environmental circumstances. In contrast, we consider sex expression in male and female individuals to be solely genetically based. The observed variation in SSR between maternal parents can not be explained by sex-by-environment interactions.

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In dioecious plant populations, both male and female biased sex ratios (SR, fraction of males) among flowering plants have been found. Recent studies indicated that SRs can already be skewed in the seeds (Rychlewski and Zarzycki 1975, Webb 1992, Taylor 1996, Alström-Rapaport 1997). Various mechanisms have been invoked to explain biased SRs in the offspring of plants and animals. These include, for example, pollen competition between X- and Y-bearing pollen in *Silene latifolia* (Correns 1928), sex-chromosome drive in *S. latifolia* (Taylor and Ingvarsson 2003), and cytoplasmic sex-ratio distorters in *Nasonia* wasps (Werren and Beukeboom 1998). To attain an understanding of the mechanisms that maintain biased seed sex ratios (SSR, fraction of males) the sex determination mechanism first has to be analyzed. It is currently generally accepted that determination of sex and the formation of sex organs during growth and development can be determined by both the genetic apparatus (genetic sex determination, GSD) and by environmental factors (environmental sex determination, ESD) (Ainsworth et al. 1998).

Dioecy (separate sexes) has evolved many times from various forms of cosexual ancestors (Vamosi et al. 2003). Hence it is not surprising that sex determination mechanisms in plants are highly diverse, including environmental, physiological and genetic aspects (reviewed by Westergaard 1958, and Ainsworth et al. 1998), so that gender can be determined either by a single factor, or by genotype-environment interactions. Even if genetics lead to a balanced SR in mature seeds however, environmental sensitivity of sex expression may provoke biased SRs in flowering plants. Ignoring this fact can easily lead to erroneous conclusions when estimating SSRs.

Several pre-zygotic and post-zygotic mechanisms may exert influence on sex expression of the offspring, resulting in unequal SRs. Sex ratio theory predicts that, if specific ecological conditions differentially influence the fitness benefits of male and female offspring, parents should adjust number and quality of offspring accordingly to maximize fitness (Charnov 1982). Whereas experimental studies in a large number of animal species reported offspring SRs that vary with parental conditions (e.g., Clutton-Brock et al. 1984, Wiley and Clapham 1993, Nager et al. 1999), investigations associated with the facultative adjustment of SSRs in sexually dimorphic plant species are limited (but see Freeman et al. 1994, Purrington

1993). In *Spinacia oleracea*, Freeman et al. (1994) demonstrated that plants originating from large seeds have a significantly male-biased SR while small seeds yield a female-biased SR. These results are consistent with the prediction of the Trivers-Willard (1973) hypothesis: mothers in good condition produce larger seeds and increase their fitness by producing sons instead of daughters. Also, a large number of dioecious and sub-dioecious plant species are able to alter their sexual state in response to changes in the ambient environment (reviewed by Heslop-Harrison 1957, Freeman et al. 1980, Chailakhyan and Khrianin 1987, and Korpelainen 1998). For example, low soil fertility, dry soils, low temperatures, high stand density and low light intensity all tend to favour male sex expression and therefore incline SR towards males. Several people have suggested that lability of sexual expression might have survival value where a significant proportion of the females must otherwise bear the cost of fruit production under poor conditions (Freeman et al. 1981, Charnov 1982). Likewise, the ratio of male-to-female flowers on monoecious individuals has proven to increase under unfavourable conditions (Heslop-Harrison 1957, Freeman et al. 1981).

Sex expression in dioecious and sub-dioecious plant species can often be altered by exogenous application of phyto-hormones such as auxins, gibberillic acid, and cytokinins (Chailakhyan and Khrianin 1987, and references therein). The effect is frequently the conversion of one sex to the other (male to female and vice versa), showing that the floral primordia are sexually bipotent and that genetic and physiological systems interact as demonstrated in *Mercurialis annua* (Louis 1989). Analysis of sex determination in *M. annua* was possible because exogenous auxins induced staminate flowers on female plants and cytokinins induced pistillate flowers on male plants, allowing self- and cross-fertilization (Louis 1989, Durand and Durand 1991).

Several species have long been considered to be strictly dioecious, but demonstrate a low frequency of bisexual individuals in natural populations: for instance, *Asparagus officinale* (Rick and Hanna 1943), *Atriplex canescens* (Stutz et al. 1975), *M. annua* (Kuhn 1939), *Ochradenus baccatus* (Wolfe and Shmida 1995), and *Urtica dioica* (Greig-Smith 1948). In sub-dioecious species, occasional bisexual plants occur in both males and females, or in one sex only, and sex expression in such individuals may be strongly dependent on the prevailing condi-

tions (Westergaard 1958). In *A. cansecens*, McArthur (1977) described two groups of plants, one in which sex was fixed as either male or female and the other in which sex varied. Wolfe and Shmida (1995) showed that in *O. baccatus* variability in sex expression differed between males and females: whereas females only reproduced by seed, 65% of males produced pollen and varying amounts of seeds (inconstant males).

In the sub-dioecious *U. dioica*, natural populations often show male- or female-biased SRs (Glawe et al., Chapter 3). Interestingly, SSRs (estimated at standard conditions) of maternal plants coming from the same population are extremely variable (5-76% male offspring, de Jong et al. 2005). Although a multitude of studies has investigated main aspects of biology and ecology in *U. dioica*, little is known about the sex determination mechanism and SSR variation in this species.

The objective of the present work is thus first to examine the extent to which environmental sensitivity may lead to male- and female-biased SSRs. In particular, we ask (1) whether SSR in *U. dioica* can be changed by varying environmental conditions of the parental plants, (2) whether SSR can be influenced by the environmental conditions during vegetative growth, as would be expected if environmental sex determination (ESD) is operating, and (3) whether gender of male, female, and monoecious individuals that have already flowered can be modified by environmental conditions, or by extreme measures such as hormone application (applied only to males and females).

MATERIAL AND METHODS

Study organism

The stinging nettle, *Urtica dioica* L. (Urticaceae), is a sub-dioecious allo-tetraploid perennial plant that propagates sexually through seeds and asexually through rhizomes (biology and ecology reviewed by Šrůtek and Teckelmann 1998). In the Netherlands, natural populations contain monoecious individuals in addition to strictly unisexual individuals (male and females) at frequencies which vary between zero and about 7% (Glawe, unpublished data). Plants established from seed usually initiate vegetative spread as early as in the first year.

Rhizomes are produced in the late summer and over-winter until the next growing period. Preliminary investigations with a few hundred plants showed that measures as cloning, over-wintering, removal of leaves and flowers or crown pruning did not affect gender. After these treatments, pure sexes again consistently produced either 100% male or female flowers, and monoecious individuals remained bisexual, although they varied in the ratio of male and female flowers.

Plant origin and growth procedures

Seeds were collected per individual from open-pollinated females at the dune site Meijendel (near The Hague, The Netherlands) in the autumn of 1999. The plants grown from seed batches (families) were therefore at least half-sibs. Plants were selected along a roadside over a distance of about 800 m. To make sure that plants did not belong to the same genet, female individuals that were sampled were at least 2 m apart, with no *U. dioica* in between. Seeds derived from different maternal plants were stored separately in paper bags. Seeds were germinated under laboratory conditions (at 20°C during 16 h light and 15°C during 8 h dark) on moist filter paper in Petri dishes. After 10 days, seedlings were transplanted to 1.3-L pots containing a mixture of 50/50 dune sand/peat and grown until the flowering stage in a climate chamber. For SSR estimation, plants were grown at standard conditions: 20°C during 16 h light and 15°C during 8 h dark with 70% relative humidity, and with 180-200 μmol m^{-2} s^{-1} PPFD at plant growing level. Plants were watered three times a week with 200 ml water but received no additional nutrients. For experiments using cloned material, cuttings were obtained from the plants, tissue cultured (MS 0 medium) and then transferred to 1.3-L pots containing the sand/soil mixture. Standard conditions as described in all experiments were identical to these described as above. Apart from experiment 2 (5) all experiments were conducted in a growth chamber.

Families and SSR

We selected four families from the larger seed collection sampled in 1999 and used by de Jong et al. (2005), and estimated SSRs at standard conditions: M01(SSR=0.52), M16(SSR=0.39), M18(SSR=0.69), and M31(SSR=0.62). SSR per family was calculated as the proportion of males and monoecious plants to total progeny. SSRs in the families

M16, M18 and M31 differed significantly from 0.5 (binominal test, Siegel and Castellan 1988, M16 {P=0.0495}, M18 {P=0.0018}, and M31 {P=0.0367}). Because germination rate in the four families averaged 96% and mortality was negligible, variation in SSR was not due to sex differential germination or mortality. When determining the gender of n seeds from a large sample size, the standard error (SE) of the estimated SSR equals $\sqrt{[p(1-p)/(n-1)]}$, where p is the fraction of male individuals. If, for instance, n=100 seeds, and SSR is 0.5, the SE of the SSR is 0.05 and confidence limits are 0.05 x 1.96 = +/-0.098.

Experiment 1: Parental effects on SSR

One male and one female plant from family M01 were cloned, and crosses were performed. The same procedure was followed for single male and female sibs from family M31. The parental plants were grown at varying nutrient regimes during growth and pollination period to determine whether parental environment can affect SR of offspring. Six male [M01/31(1-6)] and female [M01/31(1-6)] clones of both families were transferred to 1.3-L pots containing the sand/soil mix and placed in a climate chamber. Three times a week every plant received 200 ml water (when nutrients were applied, the volume of water was substituted by the nutrient solution). Nutrients were given as Steiner nutrient solution (Steiner 1968, macro-nutrients: N 167 mg L^{-1}, P31 mg L^{-1}, K 282 mg L^{-1}, S 111 mg L^{-1}, Ca 180 mg L^{-1}, Mg 49 mg L^{-1}). From one week after planting up to the flowering stage, the following nutrient regimes were applied to plants of both families: (1) both maternal and paternal parent received 150 ml Steiner twice a week, (2) either maternal or paternal parent received 150 ml Steiner twice a week, and (3) neither maternal nor paternal parent received additional nutrients. With the exceptions above, plants were grown at standard conditions as described above. As soon as flowering started, male and female individuals were placed together in pollination chambers (Table 4.1, first column). To test whether pollen viability was affected by the different nutrient regimes, pollen grains from each male (a pollen mixture of 15 flowers) of both families were analyzed using the cotton blue in lactophenol procedure outlined in Dickison (1974). After pollination and seed ripening, seeds were collected. Sixty-five seeds of each parental combination were sown in Petri dishes and grown at standard conditions until maturity, at which point all individuals were sexed.

Experiment 2: Environmental effects on SSR

For this experiment, we selected seeds from two maternal plants from the Meijendel population that were found to produce biased SSRs at standard conditions: M16 (SSR=0.39) and M18 (SSR=0.69). Offspring from M16 and M18 were grown from seedling to flowering stage under different environmental conditions. For each of the two families, 5 x 65 seeds were sown in Petri dishes. After germination, all seedlings were transplanted to pots containing the sand/soil mixture and assigned to five environmental regimes. With the exceptions below, plants were grown at standard conditions as described above. (1) Poor conditions: small (0.5 L) pots, plants were only watered if they showed dehydration. (2) Benign conditions: 25°C/20°C, 150 ml Steiner twice a week. Altogether plants were watered three times a week (2 x 150 ml Steiner plus 1 x 300 ml water). (3) High density: 1.3-L pots (5 plants/pot). (4) Low light intensity: 70% light intensity. (5) Semi-natural conditions: plants were grown in 1.3-L pots in the garden and only received atmospheric water. Gender of the flowering plants was recorded.

Experiment 3a: Environmental effects on sex expression of male, female and monoecious individuals

Two male, two female and four monoecious plants that also originated from the Meijendel population were cloned and grown to the flowering stage under different environmental conditions. Prior to cloning, sex expression of the plants was observed for two flowering seasons, and was found to be constant under standard conditions. FSR (FSR, fraction of male flowers) of the monoecious individuals which only was recorded for the second season was female biased in two of the plants ($FSR_{\#1}$=0.19, $FSR_{\#2}$= 0.23). In the other two, FSR was found to be male biased ($FSR_{\#3}$=0.78, $FSR_{\#4}$=0.64). Individual plants were cloned into two times seven cuttings each, and were transferred to 1.3-L pots, which were subsequently assigned to two environmental regimes: (1) standard conditions as described above, and (2) benign conditions: plants received 150 ml Steiner twice a week and an additional 300 ml of water on a third day; other see standard conditions. Plants were grown to the flowering stage and FSR was estimated for each individual.

Experiment 3b: Effect of hormones on male and female individuals

Two male and two female plants each were selected from Meijendel families M01, M16, M18 and M31 in attempt to modify flower sex expression by treating the plants with several growth-regulating substances. Experimenters (e.g., Heslop-Harrison 1956, Louis 1989) have successfully accomplished sex conversion either by applying the hormone in lanolin paste medium or by spraying in aqueous solution. Since the action of particular hormones in feminizing or masculinising flowers is species-dependent (Chailakhyan and Khrianin 1987 and references therein), each hormone was applied to both male and female plants, and different concentrations were used. Two males and females per family were thus cloned and then transferred to 1.3-L pots. One clone (clones were numbered according to treatment) per sex and family was placed in treatment groups as following: (1) 0.5% auxin (IAA,) in lanolin paste, (2) 0.8% gibberellic acid (GA_3) in lanolin paste, (3) auxin (IAA, NAA) sprayed at concentrations of 15, 20, 40, 55 mg/L, (4) gibberellic acid (GA_3) at conc. of 20, 35, 50, 70 mg/L, (5) cytokinin (kinetin) at conc. of 0.5, 1.5, 2.5 mg/L, and (6) cytokinin (BAP) at conc. of 0.5, 1.5, 2.5, 5 mg/L. All substances were obtained from Sigma-Aldrich (Steinheim, Germany). For the substances in lanolin paste about 0.25 g was applied to the lower leaf surface of the 5th and 6th node (after which on the 6th node the floral meristems emerged). In all other treatment groups, plants were sprayed two times a day for 12 consecutive days, just before floral meristems appeared. In other experiments (Chailakhyan and Khrianin 1987 and references therein) flowers of the opposite sex appeared as soon as plants started reproductive growth.

RESULTS

Experiment 1: Parental effects on SSR

Neither nutrient availability to maternal nor paternal parent significantly affected SSR of the parental combinations in both families: there was no significant heterogeneity in SSR among crosses between parents from the M01 family (heterogeneity G-test, G_5=0.547, P=0.990) or among parents from the M31 family (G_4=0.418, P=0.981) (Table 4.1). Nutrient supply resulted in noticeably more

TABLE 4.1 – Seed sex ratio (SSR), germination percentage (G%) and mortality rate (M%) among progeny of *U. dioica* resulting from different nutrient treatments of maternal and paternal parents.

Family and	Nutrient supply		Progeny					
clone no.[a]	Mother	Father	Females	Males	Monoecious	SSR[b]	G%	M%
M01 (1)	yes	yes	27	34	1	0.56	98	0
M01 (2)	no	no	26	33	1	0.57	98	3
M01 (3)	yes	no	25	36	0	0.59	97	0
M01 (4)	yes	yes	26	33	3	0.56	100	2
M01 (5)	no	no	28	31	1	0.53	95	0
M01 (6)	no	yes	26	31	0	0.54	95	5
M31 (1)	yes	no	22	30	4	0.61	94	3
M31 (2)	no	no	25	31	3	0.58	95	2
M31 (3)	yes	yes	27	31	3	0.56	98	0
M31 (5)	no	yes	24	32	4	0.60	95	0
M31 (6)	yes	yes	23	33	5	0.62	97	0

[a]One male and one female plant from family M01 were cloned and crosses were performed, the same procedure was followed for family M31. [b]SSR is calculated as the proportion of males and monoecious plants to total progeny.

vigorous and bigger plants with higher seed set (up to twofold) than the control treatment. In females that did not receive nutrients, seed set was limited and from M31(4) we could not collect any seeds. Pollen grain viability did not significantly differ between males of the different treatment groups in both families. Overall, 88% to 96% of the grains stained darkly blue and were considered as viable. Whit the exception of cross M31(3) and cross M31(6) not only male and female but also monoecious individuals were produced.

Experiment 2: Environmental effects on SSR

In both families M16 and M18, SR of the flowering plants did not change significantly under a wide range of conditions: there was no significant heterogeneity in SSR among different environments in the M16 family (heterogeneity G-test, G_5=2.678, P=0.749) or in the M18 family (G_5=3.447, P=0.631) (Table 4.2). Also, SSRs under most unfavourable conditions (poor environment) did not differ significantly from SSRs produced under most favourable conditions (benign environment) in both families (M16: df=1, P=0.219; M18: df=1, P=0.131). Together, SSRs produced in all other environments were not significantly different from SSRs produced under standard conditions. At standard as well as at varying conditions, SSR was female-

TABLE 4.2 – Seed sex ratio (SSR) and mortality rate (M%) among progeny of maternal plants of *U. dioica* grown at different environmental regimes.

Family[a]	Environment	Progeny				
		Females	Males	Monoecious	SSR[b]	M%
M16	Standard	38	20	4	0.39	5
	Poor	32	29	1	0.48	5
	Benign	40	24	0	0.38	2
	High density	38	22	3	0.40	3
	Low light int.	39	24	2	0.40	0
	Garden	37	21	2	0.38	8
M18	Standard	19	38	5	0.69	5
	Poor	29	34	1	0.55	2
	Benign	21	43	1	0.68	0
	High density	26	38	0	0.59	2
	Low light int.	22	42	0	0.67	2
	Garden	21	35	2	0.64	11

[a]In both families, maternal plants were found to produce biased SSRs at standard conditions (M16: female-biased, M18: male-biased). [b]SSR is calculated as the proportion of males and monoecious plants to total progeny.

biased in family M16 and male-biased in family M18. Plants that were grown at poor conditions and high stand density showed reduced stalk length, delayed flowering and produced less biomass generally compared to plants grown at benign and garden conditions. Seed set at favourable conditions was up to two times higher than at unfavourable conditions.

Experiment 3a: Environmental effects on sex expression of male, female and monoecious individuals

Gender expression in male and female plants was found to be stable. In both treatment groups, male and female clones produced consistently 100% male and 100% female flowers, respectively. In contrast, sex expression in monoecious individuals was labile. Mean FSR for all four plants was significantly different between clones grown at standard conditions and clones grown at benign conditions (ANOVA, $F_{1,46}=13.127$, $P=0.0007$). The fraction of male flowers increased when clones were grown under favourable conditions (Figure 4.1), 43% of the clones even increased FSR towards 100% male flowers, particularly clones that derived from plants in which the phenotype already showed a male biased FSR at standard conditions (plant no. 3 and 4, Figure 4.1). Moreover, FSR significantly differed between plants of the different genotypes (ANOVA, $F_{3,46}=35.865$, $P<0.0001$). There was

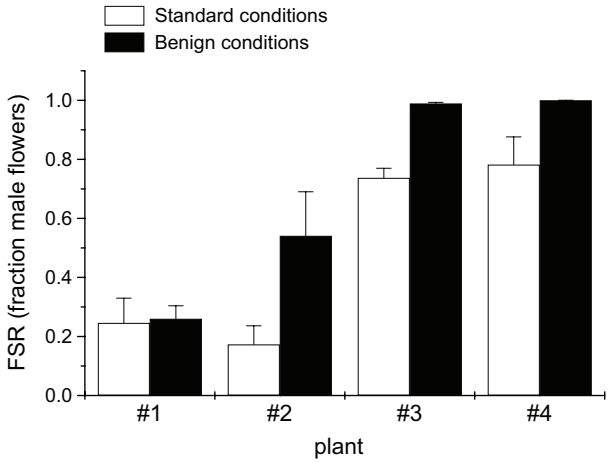

FIGURE 4.1 – Flower sex ratios (FSR, fraction male flowers) of clones of four monoecious individuals of *U. dioica* grown at standard and benign (nutrient-rich soil) conditions. Values are the mean (+1 SE) of 7 clonal replicates for each monoecious plant and environment, for plant #1 mean FSR of clones grown at standard conditions was calculated from 5 replicates. The following FSRs were observed before at standard conditions: $FSR_{\#1}$=0.19, $FSR_{\#2}$= 0.23, $FSR_{\#3}$=0.78, and $FSR_{\#4}$=0.64.

no significant environment-plant interaction (ANOVA, $F_{3,46}$= 1.963, P=0.133) although additional nutrient supply had little effect on the phenotype of clones taken from plant #1. Plants grown at favourable conditions produced on average twice as many flowers as those grown at standard conditions.

Experiment 3b: Effect of hormones on male and female individuals

External hormone application, regardless of treatment or concentration, had no effect on sex expression in *U. dioica*; males produced consistently 100% male flowers and females produced consistently 100% female flowers. We observed plant responses typical for the different growth regulators (e.g., induced root growth and induced stem elongation for IAA/NAA and GA_3 treatments, respectively), which shows that hormones were successfully absorbed by the plant tissue. Moreover, we found that IAA/NAA and GA_3 applied in aqueous solutions at 55 mg/L and 70 mg/L, respectively, totally suppressed flowering.

DISCUSSION

Our experiments demonstrated that the observed SR bias in the seeds of *U. dioica* (de Jong et al. 2005) is not due to environmental sensitivity in the pre- or post-zygotic stage. In experiment 1, SSR did not differ between nutrient-supplemented parents and unsupplemented parents. The data show that maternal parents always produced approximately the same SSR regardless of the prevailing conditions at which maternal and paternal parents were grown. A similar result was obtained in *S. latifolia* by Purrington (1993) who hypothesized that selection against Y-carrying pollen would be increased if parents were nutrient–stressed, causing a female-biased SSR. However, he found no evidence for progeny SRs being influenced by parental nutrient regime.

Fisher (1930) predicted that SR bias may persist if there is a difference in the cost of rearing the two sexes. Assuming that seed mass is an estimate of parental expenditure in an offspring (Freeman et al. 1994, Taylor 1996) we conclude that there is no difference in the cost of rearing males versus females in *U. dioica* since there was no relationship between sex of a seed and its seed mass (Glawe, unpublished data). Shaw and Mohler (1953) found that for a large panmictic population, individual families with male- and female-biased SRs in their progeny can coexist provided that over the population as a whole SSR is at equilibrium. However, in smaller populations that are less well mixed one would expect that a single SSR-type would (de Jong et al. 2002). The problem of the maintenance of large genetic variation in SSR in *U. dioica* is therefore not yet solved.

SR from flowering plants in experiment 2 did not differ significantly from SSR produced at standard conditions when offspring were grown at different environmental conditions. Whereas there was evidence for a considerable effect of the environment on seed production there was no ESD operating in males and females. These findings were confirmed by experiment 3a, in which we found that gender expression in clones of male and female plants, when grown at different conditions, was fixed as all-male and all-female floral phenotypes. In clones of monoecious plants however, floral phenotype varied considerably within a treatment and between treatments. Similar findings have been reported for sub-dioecious *A. canescens* (McArthur et al. 1992). Because the ability to vary floral phenotype was confined to

monoecious genotypes, the authors suggested that the ability to regulate sex expression and the ability to change sex are determined by different genetic mechanisms. Interestingly, at favourable conditions, the ratio of male-to-female flowers on clones of all monoecious *U. dioica* plants increased and 43% of the clones produced exclusively male flowers. This is in contrast to findings of other experimenters (e.g., Dorken and Barrett 2003) who report a decrease of the ratio of male-to-female flowers in monoecious individuals under favourable conditions. Typically, such increases in resource allocation to the female function (seeds) under favourable conditions are thought to be due to higher costs of female reproduction as compared to male reproduction. The reverse conclusion in *U. dioica* is unlikely because plants produce enormous quantities of pollen. However by varying the amount of pollen that is produced, a plant could easily set a limit to these costs. Furthermore, reproductive assurance is sacrificed when a plant switches to become 100% male. At present we can only explain the phenomenon in *U. dioica* in physiological terms. Possibly, additional nutrient supplies exert an influence on the endogenous hormone levels that, in turn, affect sex expression. Self-fertilization of such plants and crosses with pure males and females provided genetic evidence that the monoecious individuals are inconstant males and heterozygous at the sex determining locus (Glawe and de Jong, Chapter 5). In the Meijendel population we detected sex inconstancy only in male plants (=inconstant males) of *U. dioica*, but not in female plants (Glawe and de Jong, Chapter 5) which is similar to findings reported in other sub-dioecious species (e.g., Galli et al. 1993, Testolin et al. 1995, Dorken and Barrett 2003). Generally, monoecious plants in natural populations occur in low numbers together with pure male and female plants, and never have been observed to dominate populations or to occur on their own (Glawe et al., Chapter 3). In crosses between males and females, inconstant males appeared in the progeny in proportions of 0-4.7% (Glawe and de Jong, Chapter 5). To estimate SRs of progenies and flowering plants in the field we include the monoecious individuals (inconstant males) to the fraction of males and calculate the SR. Therefore, sexual lability of inconstant males neither affects the outcome of SSRs nor population SRs.

Numerous studies have demonstrated a role of phyto-hormones in modifying sex expression in dioecious plant species such as

Cannabis sativa, *Humulus lupulus*, *M. annua*, and *S. oleracea* (reviewed by Chailakhyan and Khrianin 1987). Sex reversal by hormone application in these plants indicates that the genes required for the development of male and female sex organs are functional but suppressed (e.g., in *M. annua*; Louis 1989, Durand and Durand 1991). In *U. dioica*, hormones that have been applied had no effect on the sexuality of flowers of male and female plants. However, we cannot exclude the possibility of hormonal influences on sex expression in dioecious individuals because there are far more gibberellins, and combinations of different kinds of hormones that still may affect gender. Also, an effect of phyto-hormones on sex expression may be dependent on the developmental stage at which the hormone is applied (reviewed by Chailakhyan and Khrianin 1987). A similar result to ours, however, was found for *S. latifolia*, which was insensitive to any hormone treatment (Dellaporta and Calderon-Urrea 1993). Individual plants of this dioecious species, which exhibit strict genetic control of sex expression, are either male (XY) or female (XX). *Silene* has an active-Y system of sex determination, with dominant male factors and female suppressing factors (Westergaard 1958, van Nigtevecht 1966).

The sex determination mechanism in *U. dioica* has, to our knowledge, not been analyzed. In our study, experiments were carried out with few genotypes that all derived from one (the Meijendel) population. Although the results did not differ between individual plants, we acknowledge that our findings may not necessarily be generalized. Yet, our findings provide strong evidence that environmental conditions do not affect gender of the male and female plants used in our study. Sex expression in these dioecious individuals seems to be solely determined by the genetic apparatus. However, labile sex expression in monoecious plants of *U. dioica* may occur as a gene-environment condition. Sexual lability as it was reported for several male and female plants by Strasburger (1910) could not be confirmed in any of our experiments. The results on monoecious individuals in experiment 3a clearly show that phenotype and genotype can be different and possibly, the plants that Strasburger (1910) regarded as pure sexes in fact were monoecious. Cytological investigations failed to provide evidence for the existence of heteromorphic sex chromosomes in *U. dioica* (Meurman 1925, Parker 1990). The possible absence of heteromorphic chromosomes and the existence of inconstant males suggest that dioe-

cy in *U. dioica* may have been derived relatively recently. The same was assumed for the sub-dioecious plant *A. officinalis* in which sex is determined by homomorphic sex chromosomes and the males are the heterogametic sex (reviewed by Bracale et al. 1991). In natural populations of *A. officinalis*, male individuals with a few bisexual flowers are occasionally found (Rick and Hanna 1943).

Finally, we have demonstrated that the observed variation in SSR in *U. dioica* cannot be explained by sex-by-environment interaction in male and female plants at different stages (zygotic, vegetative and flowering stage) in the life cycle. Also, no sex change, from male to female or the converse, has been observed since investigations on *U. dioica* were initiated 5 years ago. Moreover, germination and survival frequencies in this study were high, and no trend in favour of one sex was observed. Our findings in *U. dioica* are in line with the results obtained for *S. latifolia* in which SSRs differed also significantly among families (Taylor 1996). Likewise, Taylor (1996) found no evidence that life cycle stage affected SSRs: in families that exhibited a biased SSR, SR estimates from different life cycle stages showed that SRs in the mature seeds were nearly identical to the SRs in the adult plants. As in *S. latifolia*, our results strongly imply that the enormous variation in SSRs between maternal plants of *U. dioica* may be entirely genetically based. Genetic analysis can show if this variation in SSR is a consequence of a complex genetic mechanism of sex determination and/or if differences in SSRs are due to other genes that act within the parent to modify SR among its progeny.

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