# Sex ratio variation and sex determination in Urtica dioica

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# Sex ratio and clonal growth in the stinging nettle (*Urtica dioica*)

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The proportion of female and male flowering shoots of the clonal herb *Urtica dioica* was determined and found to differ considerably among natural populations. Frequently, deviations from the expected 1:1 sex ratio have been attributed to sexual dimorphism in life histories between the sexes. Research has been taken into the behaviour of female and male clones regarding vegetative growth, mortality, plant biomass and height to detect sex-specific differences that might have contributed to the sex ratio bias. A common garden experiment indicated no difference between female and male individuals in the production of flowering and non-flowering shoots during the course of three growing seasons. Also, sex-differential mortality was not observed and mortality rate generally was low. In a laboratory experiment, in which the plants were grown under varying conditions for one season, female and male individuals developed approximately equal numbers of stolons and rhizomes. Other traits such as plant biomass and height support the suggestion that the phenologies of female and male plants differ little. Within the range of our experiments, the results thus indicate that sexual dimorphism in these life history traits is unlikely to have a major effect on the sex ratio. Therefore, alternative arguments to explain the sex ratio bias are discussed.

Submitted manuscript

Natural populations of dioecious plant species often exhibit biased sex ratios among flowering individuals (population sex ratio;

reviewed by Delph 1999). Basically, such biases may arise from sex-based differences (sexual dimorphism) in life history traits, from spatial segregation of the sexes, or from genetic factors leading to skewed sex ratios in the seeds.

Sexual dimorphism in life history traits (e.g., timing of flowering, size at first reproduction, growth, and longevity), which may be a consequence of sex-differential patterns of resource allocation, are likely to influence sex ratios in plants (reviewed by Delph 1999). For example in the clonal shrub *Oemleria cerasiformes*, Allen and Antos (1993) found males to be flowering at an earlier stage than females, leading to a transient flowering bias toward the male morph. At the end of the first flowering season the genet sex ratio (fraction of males) was 1:1 in young mature plants. However, due to higher mortality rates in female plants during the subsequent reproductive years, the genet sex ratios of older plants were predominantly male biased.

Spatial segregation of females and males has been documented in over 20 plant species (reviewed by Bierzychudek and Eckart 1988; see Korpelainen 1991 and Eppley et al. 1998 for recent examples). It may arise, amongst others, in dioecious plants with environmental sex determination or sex-specific differences in germination requirements and seedling mortality.

Whereas there are numerous studies on how sexual dimorphism and spatial segregation may contribute to biased sex ratios, little is known to what extent variation in the primary (seed) sex ratio may account for the preponderance of one sex or the other. Recently Taylor (1999) showed that, indeed, a bias exhibited in several populations of *Silene latifolia* was primarily caused by a bias in the seed sex ratio. Unfortunately for many dioecious species, especially long-lived trees and shrubs, information on the seed sex ratio is scarce (but see de Jong and van der Meijden 2004, Alström-Rapaport 1997), and so primary sex ratios are assumed to approach 1:1. However, it is quite possible that, for some dioecious plant species, the importance of biased seed sex ratios for population sex ratios might be underestimated whilst the impact of sex-differential life histories might be overrated.

The study of population sex ratios in clonal plant species is often complicated due to difficulties identifying genets (genetic individuals). Hence, information on sex ratio is often based on shoot or

ramet (genets that fragmented into independent individuals) rather than genet frequency (e.g., Kitchingham 1979, cited in Kay and Stevens 1986; Lloyd 1981, Doust and Laporte 1991). Such data may lead to erroneous conclusions when females and males differ in the extent of their vegetative propagation. Male-predominant sex ratios in several dioecious species have been repeatedly explained by the fact that female plants invest more in sexual reproduction at the cost of vegetative growth and reduced survival (Delph 1999). Obeso (2002), however, argued in a review that long-lived woody plants confirm to this pattern while herbaceous plants do not and therefore detailed studies on this latter group are worthwhile. Also, sex-differential survival and different timing in flowering alter population sex ratios (e.g., Allen and Antos 1993). These and other factors may introduce biases when recording sex ratios and therefore research into the behaviour of both sexes under controlled conditions as well as long-term studies on experimental and natural populations are needed.

In *U. dioica* sex ratio (fraction of males) variation has been documented to occur in both flowering plants and seeds (Kitchingham 1979, cited in Kay and Stevens 1986; de Jong et al. 2005). De Jong et al. (2005) found that seeds collected from individual maternal plants in the field frequently exhibited biased sex ratios, ranging from extremes such as 0.05 to 0.76. In a subsequent study Glawe and de Jong (2005) indicated that the sex ratio bias was neither a consequence of parental nutrient condition nor environmental sex determination. Sex ratios on flowering *U. dioica* stems from 11 populations in South Britain were found to be either equal or female-biased (up to 89.9% female shoots), and the overall sex ratio being 0.32 (Kitchingham 1979, cited in Kay and Stevens 1986).

The present study was designed to explore to what extent differences in life history traits (growth, mortality, biomass, height) between female and male plants of *U. dioica* contribute to the biased shoot sex ratios observed in natural populations.

## MATERIALS AND METHODS

### *Study organism*

*Urtica dioica* L. (Urticaceae), native to Eurasia, at present occurs all over the world except in tropical regions (reviewed in Šrůtek and

Teckelmann 1998). The sub-dioecious perennial herb is common in habitats rich in nutrients and water. As an 'almost universal follower of man' (Greig-Smith 1948) the plant rapidly colonises ruderal sites due to its great power of spreading vegetatively (in addition to sexual reproduction) by means of above-ground stolons and over-wintering under-ground rhizomes. Patches resulting from rhizome growth often form large compact communities without the intermingling of other species. In dense patches *U. dioica* seedlings usually fail to establish. According to Rosnitschek-Schimmel (1983), seeds are of no importance for the propagation. However, she emphasised the importance of seeds for the colonisation of new sites.

In natural populations, occasionally monoecious plants of *U. dioica* have been found beside pure female and male individuals (Greig-Smith 1948, de Jong et al. 2005). Studies on such monoecious types have suggested them to be inconstant males, i.e. males which occasionally produce seeds (Glawe and de Jong 2005; Glawe and de Jong, Chapter 5). Because there are no secondary sex characteristics identified, the gender of female, male and monoecious plants can be determined at maturity only.

### *Field survey*

The sex ratio of flowering female and male *U. dioica* shoots was determined in 26 natural populations from Leiden area, The Netherlands, inhabiting a range of environmental conditions (sand dunes, roadsides, moist meadows and woodland). The pairwise distances of the populations varied from 0.5 km to 19 km and the size ranged from 175 shoots to 1100 shoots. All populations were studied in July at the time of flowering (June, July) and the peak in biomass production (Al-Mufti et al. 1977). At that time a great many of the female flowering shoots had already initiated seed set. In most of the populations investigated *U. dioica* grew in compact communities, which made it difficult to trace the belowground connections between ramets in order to distinguish between individual genets. Additionally, because clonal species may live for long periods, the interconnected ramets may become disconnected over time. Therefore, for each population the sex of all flowering shoots (female, male and monoecious) was recorded. The flowering shoot sex ratio per population was calculated as the proportion of male flowering

shoots to the number of total flowering shoots (monoecious shoots excluded). In order to perform statistical analyses on the sex ratios, an indirect estimate of clone size was made. In a previous field study (de Jong et al. 2005, in that paper a much smaller sample was investigated), the average shoot number (± SE) of female and male genets was very similar: 20.5 ± 2.3 and 20.7 ± 2.5 for females and males, respectively. Therefore, to obtain a rough estimate for the number of female (male) genets per population, the total number of female (male) flowering shoots was divided by 20. Because de Jong et al. (2005) judged neighbouring shoots to belong to a different genet when morphological (e.g., colouration of stems and leaves), developmental or gender differences were apparent, the estimate should therefore be considered as conservative. Probably, more clones were present than estimated in this way, so the actual shoot number per genet is lower than 20.

Moreover, the height of 20 female and 20 male shoots was measured in 20 populations and the aboveground biomass (dry weight) was estimated for 10 female and 10 male shoots in 5 populations. For both measurements, the female and male shoots were chosen randomly.

### *Clonal growth and mortality*

Sex ratio data based on shoot rather than individual genet frequencies may diverge when the two sexes differ in vegetative propagation. The following experiments were designed to explore to what extent differences in vegetative growth and/or survivorship of females and males contribute to the skewed sex ratios found in natural populations of *U. dioica.*

#### Garden experiment

From 9 populations that were studied in the field survey we collected seeds from 3 maternal plants each, separated by a distance of at least 5 m in order to avoid sampling from the same genet. Per population the seeds from different maternal plants were stored together in paper bags. In February 2003, 55 seeds from each population 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. Next, seedlings were transplanted to 1.3-L pots containing a mixture of 50/50 dune sand/soil and grown in a climate chamber (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). At maturity, all plants were sexed. In May 2003, 8 female and 8 male plants were selected from each population and transplanted into the soil in a common garden near the Gorlaeus laboratory, Leiden. Due to the skewed sex ratios in some of the families, at most 8 females or 8 males could be selected. Per population, female and male plants (=16 plants) were alternately transplanted in a circular design, the interplant distance being 20 cm. This experimental set up was used to allow competition between the individuals during the course of the study. Secondary sex characters, such as sex differential mortality or when one sex outgrows the other, are known to alter population sex ratios (Lloyd and Webb 1977). At the end of July 2003, the number of flowering and non-flowering shoots as well as the height of the longest shoot was recorded for each female and male individual. This allowed us to estimate whether the number of sexually reproductive and vegetative shoots differs between both sexes. All plants were revisited in September to check if any new flowering shoots had emerged. The same procedure was repeated in July 2004 and 2005.

Laboratory experiment

We selected seeds from two maternal plants from the Meijendel population (de Jong et al. 2005) and grew progeny under different environmental conditions. This was done to examine whether varying environmental conditions differentially affect vegetative growth of female and male individuals. For each of the two families, the seeds were germinated in Petri dishes in April 2003 (benign and poor conditions: 25 seeds, garden conditions: 50 seeds). After 10 days, all seedlings were transplanted to pots containing the sand/soil mix that was also used in the previous experiment, and assigned to three environmental regimes.

(1) *Benign conditions*: 25°C/20°C, 1.3-L pots, 150 ml 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 $L^{-1}$) twice a week plus 300 ml water on a third day.

(2) *Semi-natural conditions*: plants were grown in 1.3-L pots in the garden and received only atmospheric water.

(3) *Poor conditions*: 20°C/15°C, small pots (0.5-L), plants were watered only if they showed signs of dehydration.

Plants assigned to regime (1) and (3) were grown in climate chambers for 16 h light and 8 h dark with 70% relative humidity, and with 180-200 μmol $m^{-2}$ $s^{-1}$ PPFD at plant growing level. Beginning July 2003, all plants that had been flowering were sexed. At the same time the number of shoots (stolons) of 8 female and 8 male flowering plants from each of the two families grown under benign and poor conditions was noted. The same trait was measured for both families in 16 female and 16 male flowering plants grown under garden conditions. Thereafter, the selected plants from benign and poor conditions were placed in the garden next to the other selected plants, which had been growing there for the last few months. In the garden, these plants received only atmospheric water. Beginning November, the number of shoots (stolons and rhizomes) was counted.

## RESULTS

### *Field survey*

The overall composition of the 26 sampled populations was 46.9% female, 45.0% male and 2.2% monoecious flowering shoots, and 5.9% non-flowering shoots (Table 3.1). Of these populations, seven contained a high proportion of female flowering shoots (sex ratio < 0.4), another seven comprised a high proportion of male flowering shoots (sex ratio > 0.6), and 12 populations were found to yield female and male flowering shoots at approximately equal numbers (sex ratio 0.4-0.6) (Table 3.1). The ratio of female and male reproductive shoots varied among and between habitats. There was only a slight tendency for the variance in population sex ratio to decrease with sample size and some of the larger populations containing more than 800 shoots exhibited a severe sex ratio bias (Table 3.1). We therefore expect that the effect of sampling bias on the sex ratio was small. The frequency distribution of the sex ratio of reproductive shoots over all 26 populations is shown in Figure 3.1. The average sex ratio of flowering shoots (± SD) was 0.49 ± 0.19. It should be noted that populations with a female-biased sex ratio often were more skewed toward the female morph than it has been observed in male-biased populations for the male morph. By the rough estimate of clone size (see Materials and Methods), the overall sex ratio did not significantly differ from a 1:1 proportion (sex ratio: 0.48; binomial test, $P$=0.2509).

TABLE 3.1 – Number, reproductive status and sex ratios of female and male flowering shoots of *Urtica dioica* populations inhabiting a range of environmental conditions. Monoecious shoots were not included when calculating the flowering sex ratio.

| Habitat | Number of shoots | | Female Shoots | Male Shoots | Monoecious Shoots | Flowering sex ratio (fraction of males) |
|---|---|---|---|---|---|---|
| | Flowering | Non-flowering | | | | |
| Forest 1 | 245 | 14 | 102 | 143 | - | 0.58 |
| Forest 2 | 332 | 24 | 188 | 144 | - | 0.43 |
| Forest 3 | 608 | 27 | 290 | 318 | - | 0.52 |
| Sand dunes 1 | 234 | 4 | 132 | 102 | - | 0.44 |
| Sand dunes 2 | 296 | - | 276 | 20 | - | 0.07 |
| Sand dunes 3 | 798 | 36 | 266 | 532 | - | 0.67 |
| Sand dunes 4 | 1174 | 129 | 456 | 578 | 140 | 0.56 |
| Grassland 1 | 307 | - | 110 | 197 | - | 0.64 |
| Grassland 2 | 336 | 30 | 186 | 150 | - | 0.45 |
| Grassland 3 | 851 | 62 | 589 | 256 | 6 | 0.30 |
| Grassland 4 | 866 | 44 | 158 | 708 | - | 0.82 |
| Grassland 5 | 1097 | 117 | 744 | 343 | 10 | 0.32 |
| Waterfront 1 | 136 | - | 75 | 61 | - | 0.45 |
| Waterfront 2 | 350 | 25 | 171 | 179 | - | 0.51 |
| Waterfront 3 | 365 | 27 | 108 | 251 | 6 | 0.70 |
| Waterfront 4 | 466 | 34 | 403 | 63 | - | 0.14 |
| Waterfront 5 | 476 | 27 | 212 | 264 | - | 0.55 |
| Waterfront 6 | 496 | 33 | 307 | 151 | 38 | 0.33 |
| Waterfront 7 | 852 | 35 | 398 | 454 | - | 0.53 |
| Roadside 1 | 168 | 14 | 63 | 105 | - | 0.63 |
| Roadside 2 | 175 | 8 | 138 | 37 | - | 0.21 |
| Roadside 3 | 274 | 18 | 60 | 191 | 15 | 0.77 |
| Roadside 4 | 416 | 12 | 84 | 265 | 64 | 0.76 |
| Roadside 5 | 507 | 23 | 272 | 235 | - | 0.46 |
| Roadside 6 | 757 | 42 | 336 | 421 | - | 0.56 |
| Roadside 7 | 845 | 54 | 567 | 240 | - | 0.30 |
| Total | 13427 | 839 | 6691 | 6416 | 320 | 0.48 |

Also, of the 26 populations sampled, six had a significantly female-biased sex ratio (binominal test, $P \leq 0.05$), two had a significantly male-biased sex ratio (binominal test, $P < 0.05$), and in 18 populations the sex ratio was non-biased (binomial test, $P > 0.05$).

Monoecious individuals were found in 8 out of 26 populations surveyed, independent from the population size or habitat (Table 3.1). The proportion of monoecious flowering shoots ranged from 0.009 to 0.161.

On average, female shoots were higher compared to male shoots (102.5 cm ± 2.2 SE and 98.3 cm ± 2.1 SE for females and males, respectively), however, plant height did not significantly differ

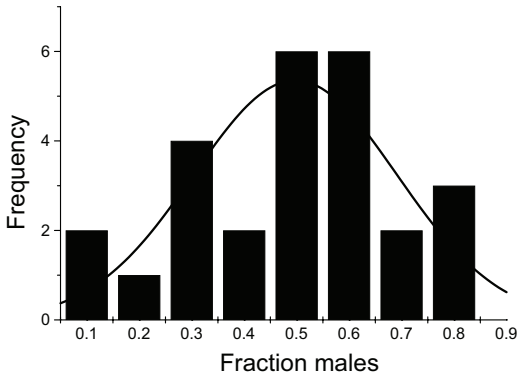


FIGURE 3.1 – Frequency distribution of flowering shoot sex ratio (fraction of male shoots) of *Urtica dioica* from 26 natural populations from The Netherlands. The distribution of the data is approximately normal (Kolomogorov-Smirnov test, $P>>0.05$).

between both sexes. Also, shoot biomass (g) was not significantly different between female and male shoots (11.01 ± 0.84 SE and 8.90 ±1.26 SE for females and males, respectively).

### *Garden experiment*

During the course of the experiment, females and males did not differ significantly from each other in terms of overall shoot production (repeated-measures ANOVA on flowering and non-flowering shoots, gender effect $F_{1,113}=0.882$, $P=0.3498$; population effect $F_{8,113}=0.912$, $P=0.5089$) (Figure 3.2). Likewise, there was no significant difference in the production of flowering shoots between both sexes (repeated-measures ANOVA on flowering shoots, gender effect $F_{1,113}=0.687$, $P=0.4089$; population effect $F_{8,113}=0.606$, $P=0.7712$). In the third growing season the overall composition was 48.8% female, 44.0% male flowering shoots and 7.2% non-flowering shoots. Furthermore, there was no significant difference with respect to shoot height between female and male individuals (ANOVA, gender effect $F_{1,128}=1.327$, $P=0.2515$; population effect $F_{8,128}=1.389$, $P=0.2407$). During the course of the study, twice as many males (12.5%) as females (5.6%) died. The difference in mortality rate however, was not significant (binomial test, $P=0.1655$). None of the individuals

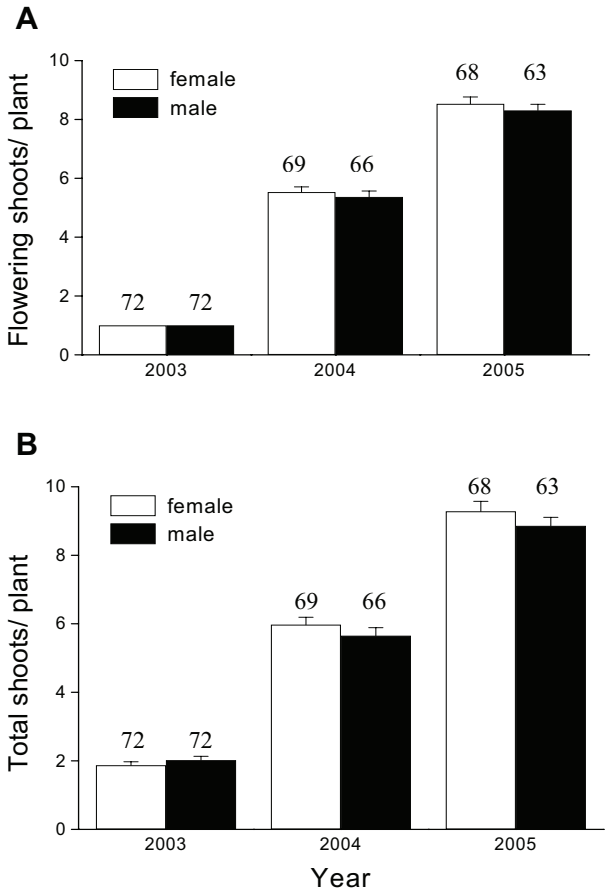


FIGURE 3.2 – Vegetative growth of female and male *Urtica dioica* plants from a common garden experiment measured as mean (+ 1 SE) (A) flowering shoots and (B) non-flowering shoots per plant for three consecutive years. Numbers above the bars represent the number of replicates; during the course of the experiment several female and male plants died.

checked at flowering time in the subsequent years changed the sexual phenotype.

### *Laboratory experiment*

While we found no significant difference in the number of shoots produced between the sexes from both families (ANOVA, $F_{1,122}=0.110$, $P=0.7411$), varying environmental conditions significantly affected

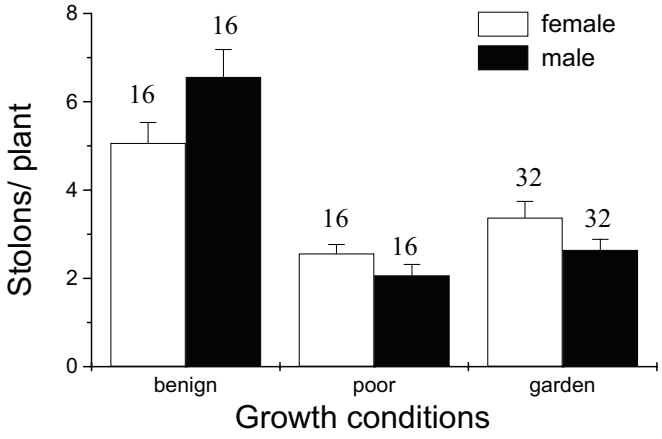


FIGURE 3.3 – Vegetative growth of female and male *Urtica dioica* plants measured as mean (+ 1 SE) number of stolons per plant after individuals were grown under different environmental regimes from seedling to flowering stage. Individuals reared under benign and poor conditions were kept under laboratory conditions whereas plants grown in pots under garden conditions were placed outside. Numbers above the bars represent the number of replicates.

shoot production in female and male plants (ANOVA, $F_{2,122}$=40.316, $P$<0.0001) (Figure 3.3). Here, a significant environment-plant interaction (ANOVA, $F_{2,122}$=4.940, $P$=0.0086) was found. However, after growing all plants under equal conditions for another four months, the environmental effect decreased and the significant interaction had disappeared (ANOVA, environmental effect $F_{2,119}$=4.017, $P$=0.0205; environment x gender $F_{2,119}$=1.589, $P$=0.2085).

## DISCUSSION

Because of the difficulties to identify genetic individuals, the genet sex ratio of *U. dioica* is not yet known. However, the sex ratio of flowering shoots has been observed to differ enormously among natural populations. According to the indirect estimate of clone size, both female- and male-biased sex ratios were found to significantly differ from 1:1. The sex ratio as expressed by reproductive shoots varied among and between habitats. Populations which showed a female-biased sex ratio of flowering shoots often were more skewed toward the female morph than it was observed in male-biased populations for

the male morph. For several *U. dioica* populations in South Britain, Kitchingham (1979) reported on predominantly female-biased shoot sex ratios.

In dioecious plants, deviations from the expected 1:1 sex ratio have usually been attributed to sexual dimorphism in life histories between the sexes. We have taken a variety of approaches to detect such sex-specific differences. Experimental surveys investigating vegetative growth of female and male clones revealed no significant difference. Nevertheless, if the vegetative propagation of female and male plants is approximately equivalent, then gender-related differences in reproductive investment (generally females incur a higher cost of reproduction) can result in reduced growth and/or survival in one of the sexes. We find in *U. dioica* that height and biomass did not significantly differ. Also, there was no significant difference in mortality between female and male individuals and mortality rate in both sexes generally was low. Similar findings for some life history traits in *U. dioica* have been reported by Koskela (2002): shoot and root biomass as well as biomass of stolons produced by female and male plants in the second growing season did not significantly differ between the sexes. Furthermore, observations of marked plants in the garden experiment indicated that sex expression is stable in the course of time. This is in accordance with observations from earlier experiments, in which female and male plants did not change their sexual state after treatments such as removal of leaves or crown pruning, cloning, and hormone application (Glawe and de Jong 2005).

Because *U. dioica* female and male plants show little sexual dimorphism for the life history traits measured in this study, these characters are unlikely to have a major effect on the sex ratio. Additionally, natural enemies such as parasitic plants appear to have very little effect on the sex ratio of natural *U. dioica* populations (Koskela 2002). Also, there was no difference of simulated herbivory on above and below ground (rhizomes and roots) plant biomass between the sexes (Mutikainen et al. 1994). If sexual dimorphism in life history traits and/or spatial segregation of the sexes play a major role in this plant species, we would expect the degree of sex ratio bias in natural populations to vary with ecological conditions, i.e. with habitats (e.g. dry sand dunes and moist meadows). At least, no indications have been found in the populations studied here. However, it

should be noted that our experimental research concentrated on the investigation of vegetative growth and mortality of young mature individuals only (up to the third growing season). Due to the fact that *U. dioica* is a long-lived perennial we cannot exclude the possibility that the time frame during which the bias develops stretches beyond the three years of our study.

On the other hand, previous studies on primary sex ratio in *U. dioica* have revealed that the sex ratio already can be biased in the seeds (de Jong et al. 2005, Glawe and de Jong 2005). Therefore, it should be not surprising if natural *Urtica* populations exhibit biased sex ratios among flowering individuals. Interestingly, both the frequency distribution of the seed sex ratio and the frequency distribution of the flowering shoot sex ratio are very similar (see de Jong et al. 2005 for comparison).

There are indications from some dioecious plant species where biases found in natural populations may be a consequence of the primary sex ratio (Taylor 1999, de Jong and van der Meijden 2004, Hettwer and Gerowitt 2004, Stehlik and Barrett 2005). In *Cirsium arvense*, female-biased sex ratios are frequently reported for natural populations (e.g., Oesau 1998, Hettwer and Gerowitt 2004). Hettwer and Gerowitt (2004) estimated the sex ratio from seeds collected from individual maternal plants in the field and, indeed, found female-biased sex ratios in all of the progenies. While other studies have shown that the average sex ratio in the seeds generally corresponded to the sex ratio of flowering conspecifics in the field, Taylor (1999) is the only one who compared individual population sex ratios with primary sex ratios produced by local female and male individuals. He found that most of the wide variation in sex ratio observed between the populations could be explained by the primary sex ratio yielded by local plants. Given the heritable component in seed sex ratio in U. dioica (de Jong et al. 2005), it thus may be not surprising to find such a pattern as well. De Jong et al. (2005), however, found no correlation between the progeny sex ratio produced by local *U. dioica* plants and the sex ratio in the field. They sampled large individuals from a population that established more than 10 years ago and in which the plants grew 2 m to 100 m apart, with the intermingling of other species. They suggested that the genetic factors responsible for biased progeny sex ratios must be well mixed in the population. In contrast, the present study examined *U. dioica* in vegetations, in which the

plants were relatively young and grew in compact communities without the intermingling of other species. For that reason, de Jong et al. (2005) may have missed small scale variation in flowering sex ratio.

The cause of the enormous variation in the seed sex ratio of *U. dioica* remains unclear. Sex allocation theory predicts that, if the population sex ratio is not equal, selection will favour individuals that produce a greater number of offspring of the sex that is in a minority. Once the population equilibrium is reached, selection ceases to act. Such a population may consist of plants that all produce equal numbers of female and male offspring, or it may be composed of equal numbers of individuals that produce female- and male-biased seed sex ratios. According to Bulmer and Taylor (1980) it is basically possible to obtain biased sex ratios in both directions, if we look at the relative dispersal distance of seed and pollen from different dioecious species. A comparative study, however, grouping dioecious species with known seed sex ratios according to seed and pollen dispersal, showed that the data available did not support the prediction (de Jong and Klinkhamer 2005).

In conclusion, while in some dioecious plant species the observed bias may be a consequence of differential response of males and females to selective forces acting on certain life history traits, in other species the sex ratio bias is more likely to be a result of selection acting directly on the seed sex ratio.

ACKNOWLEDGEMENTS

We are grateful for the assistance in the garden by Henk Nell. Nicole van Dam gave valuable comments on an earlier version of the manuscript.