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FLOWERING PHENOLOGY AND GENDER VARIATION IN *PENNISETUM TYPHOIDES*

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Because of the modular structure of pearl millet (an annual grass crop, Poaceae), different tillers of a plant share the same genotype but are subjected to different environmental conditions during their maturation. This allows investigation of the effects of tiller flowering phenology on allocation to resource-producing photosynthetic biomass, sexual functions, and thus tiller gender. All tillers of plants of two families collected from individual maternal plants (represented by 33 and 31 plants each) were analyzed. In both families, allocation to aboveground vegetative biomass decreased as flowering was delayed. On average, late-flowering tillers were 65% smaller than the first ones to flower. The proportion of biomass allocated to reproduction significantly increased with the flowering rank of the tillers, suggesting that translocations of assimilates occurred between early- and late-flowering tillers. In both families, late-flowering tillers produced significantly fewer pollen grains per stamen than early-flowering ones, and female reproductive allocation (expressed as seed mass per tiller) was also affected by flowering phenology. Tillers became increasingly female as flowering phenology progressed. This gender variation is possibly adaptive because pollination efficiency is maximized by plant height. Natural selection may favor a shift toward femaleness to maximize reproductive fitness in small, late-developing tillers.

Keywords: allocation to sexual functions, gender, modular structure, pearl millet, Poaceae, pollination regime, ecology, reproductive biology.

Introduction

Variability in allocation to sexual functions is a widespread phenomenon in higher plants. Among species, differences in life cycles are associated with variable allocation to reproduction and survival (Stearns 1992). Within a species, resource allocation to reproduction may vary with plant size. For example, larger plants allocate more biomass to reproduction (Samson and Werk 1986). Also, availability of water (Bawa and Webb 1983; Delph 1986), nutrients (Solomon 1985; Winn 1991; Havens et al. 1995), or light (Devlin 1988) and predation level (Marshall et al. 1985) can result in differences in allocation to reproduction. Seasonal variation (i.e., flowering phenology) may also play a role in variation in sex allocation, and sex allocation often decreases with plant age (Thomson and Barrett 1981; Cavers and Steel 1984; Handel and Mishkin 1984; Emms 1993; Klinkhammer et al. 1994).

If resources allocated to reproduction are limited, competition for these resources should occur between male and female functions in hermaphroditic angiosperms, resulting in a trade-off (Charnov 1982). Furthermore, reproductive phenology may strengthen the trade-off between sexual functions because allocation to male function (pollen production) most often occurs early in the reproductive season and allocation to female function (fruit and seed maturation) occurs later (Stanton and Galloway 1990). Therefore, it is important to distinguish between allocation to gametes (ovules vs. pollen) and allocation to mature seeds. A theoretical model (Burd and Head 1992) shows that investment in male function early in the reproductive season may later affect the amount of resources available to female function, resulting in a distribution of resources between the two sexual functions that significantly differs from the theoretically optimal 50 : 50 allocation. In this case, variation in sex-allocation patterns, and therefore variation in gender (i.e., relative contributions to reproductive fitness through male and female investments [Lloyd 1979]), may occur over the duration of a flowering season. Thus, if seed production is resource constrained, it may be adaptive for a plant to increase its gametic allocation toward male function.

Higher plants are modular organisms, with repeated units that do not mature and reproduce at the same time (Stanton and Galloway 1990). This modular structure is especially obvious in grasses (Poaceae), where resource translocation occurs between different tillers of the same genotype (Lupton and Pinthus 1969; Clifford et al. 1973; Lauer and Simmons 1988). If translocation is large enough, there may be no significant change of sex allocation over the duration of flowering phenology. If however, resources are constrained and translocation is limited, then tillers may change their gender over time. Also, gender changes may occur during the flowering period because of selective processes. Theory predicts that the fitness gain curve of male and female functions with plant size will differ (Bickel and Freeman 1993; de Jong and Klinkhamer 1994; Pannell 1997) depending on the pollination mode. In wind-

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pollinated species, natural selection should favor an increase in maleness with plant height since the male fitness gain should be proportional to plant height (pollen grains are better dispersed when released from a higher point). Therefore, we may expect that the female and male gain curves will not vary identically with changes in plant size, mediated by flowering phenology.

In cultivated pearl millet (*Pennisetum typhoides*, Poaceae), an annual hermaphroditic species, it is very easy to separate the different tillers within a genet and to distinguish between components of vegetative allocation (resource-producing, photosynthetic biomass) and reproductive allocation in a given tiller. There are large differences among tillers in the timing of reproduction. The aim of this research is to answer the following questions, using pearl millet as our study organism: Does the amount of resource-producing vegetative biomass per tiller change over the flowering season of the plant? Does plant gender vary with tiller flowering phenology? If so, does this variation occur because of limited resource availability that is associated with plant size? In this case, smaller tillers should shift toward increased male allocation because of resource constraints on seed production (the standard sex allocation prediction). Alternatively, does this variation occur because of fitness gains associated with plant height? If such is the case, smaller tillers should shift toward female allocation because gains though male function are reduced in short individuals of wind-pollinated species (Charnov 1982; Burd and Allen 1988).

Material and Methods

Biological Material

We studied the offspring of two naturally selfing mother plants (called 138 and 139) that had been randomly sampled in a population of cultivated pearl millet originating from North Africa. This geographical origin was chosen because these plants are not sensitive to the photoperiod variation that occurs in France (plants originating from tropical cultivars are, in general, short day plants). Plants had been grown for three consecutive generations in France to acclimate them to temperate environmental conditions and photoperiod.

Each family originally contained 50 offspring; some plants died before flowering, and we discarded those that had fewer than two tillers. Thirty-three plants from family 138 and 31 from family 139 were used. The total number of tillers analyzed was 163 in family 138 and 161 in family 139. Mean tiller number produced per plant was 5.33 in family 138 and 5.42 in family 139. A few tillers were damaged or lost before they matured; they were not included in the analysis.

The Experimental Plot

The 50 plants in each family were cultivated in a greenhouse at Université Paris-Sud, Orsay. Plants from each family were randomly chosen and distributed into two blocks. Seeds were sown on March 7, 1994, and seedlings were planted in soil on March 24. Plants were separated by 0.5 m from one another in all directions. Plants were not fertilized during the experiment. Plants were watered twice a week during the whole experiment. The male phase of flowering (which on average starts about 3 d after the female phase) started on May 4 and ended on June 21. Seed maturity occurred 5 wk after male flowering. From each tiller, seeds were harvested and biomass allocation was measured 5 wk after the end of the male phase of flowering.

On each tiller of all plants, the following measurements were carried out: seed mass (g) per tiller, which estimates female reproductive effort; seed : ovule ratio per spike (percentage of ovules that produced a viable seed, recorded on 50 randomly chosen hermaphroditic flowers); and dry mass (g) of the spike at seed maturity (including floral organs, seeds, stamens, and the stem to which they were attached), which measures total reproductive effort.

We also measured pollen production. The inflorescence of pearl millet is composed of spikelets; each spikelet includes a hermaphroditic flower (one ovule and three stamens) and a male flower (three stamens). On each tiller of all plants, a few days before male flowering, two hermaphrodite and two male flowers were randomly sampled at three different levels: 1 cm from the top of the spike, 1 cm from the base of the spike, and in the middle. The 12 stamens were collected and pooled for the four flowers from each level and stored in Eppendorf tubes. Pollen grains were extracted from these stamens by putting 120 μL pure sulfuric acid in each tube. Tubes were stored at 37°C for 1 wk and vortexed daily. Then 1.2 mL of 2% Nonidet P40 (detergent) water was added in each tube, and the mixture was centrifuged for 2 min at 1200 *g*. The pellet was suspended in 1 mL of 2% Nonidet P40 water and centrifuged for 2 min at 1200 *g*; this procedure was repeated twice. Finally, the pellet was suspended in 400 μL of 2% Nonidet P40 water (Dajoz and Sandmeier 1997).

For each level on the spike, pollen counts were carried out on two aliquots of 1 μL with a Malassez cell. We found no significant effect of level on mean number of pollen grains per anther (ANOVA, level effect: $F_{2,1952} = 2.28$, $P = 0.103$). From these data, we calculated the mean number of pollen grains per stamen estimated from the mean number of pollen grains per level.

Finally, to measure vegetative biomass, the stems and leaves of each tiller were collected at seed maturity, dried in an oven for 72 h at 80°C, and then weighed. From these data, the vegetative dry mass (g) of aboveground vegetative structures for each tiller was calculated. Since vegetative biomass and tiller height were strongly correlated ($r = 0.743$, $P = 0.0001$; $n = 326$), we consider vegetative biomass to be a good estimate of tiller height.

Also, before drying the leaves, we measured their surfaces with a planimeter. From this, we estimated leaf area per tiller (cm^2) for each tiller. However, since the values of vegetative biomass and leaf area were strongly correlated ($r = 0.875$, $P = 0.0001$, $n = 256$), we chose to use only vegetative biomass as our estimate of vegetative size in our analyses.

Statistical Analyses

Statistical analyses were performed using SAS (SAS Institute 1989). Some of the variables studied were not normally distributed and had to be transformed to achieve normality. Spike mass, seed mass per tiller, leaf area, and vegetative biomass

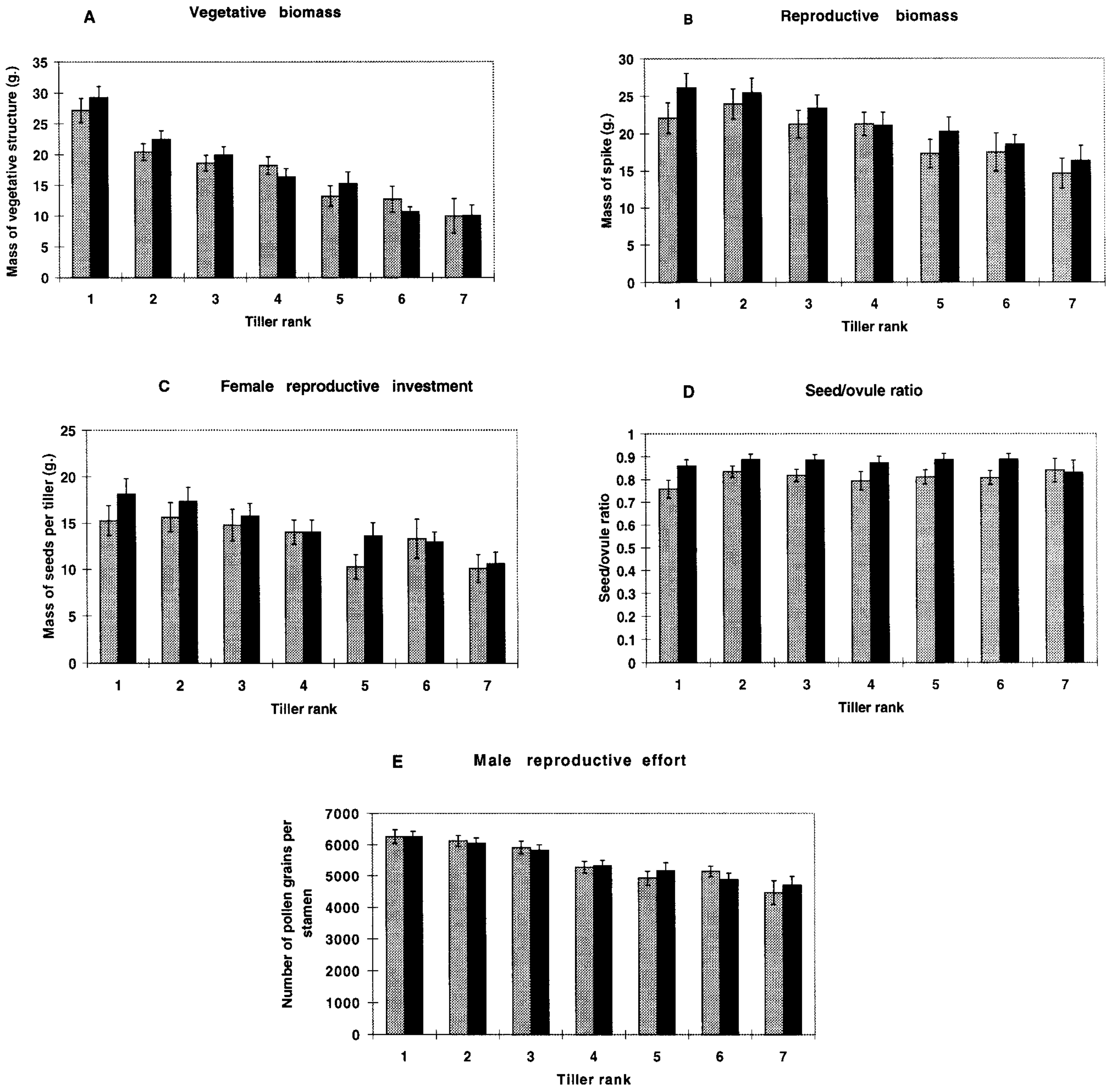

Fig. 1 Mean allocation to vegetative and reproductive traits for the different tiller ranks and for each family of *Pennisetum typhoides* (138 = gray bars, 139 = black bars). Error bars: ±1 standard error of the mean. *A*, Vegetative biomass: total dry mass (g) of aboveground vegetative structure. *B*, Reproductive biomass: dry mass (g) of spike. *C*, Female reproductive investment: mass of seeds (g) per tiller. *D*, Female fertility: seed/ovule ratio per tiller. *E*, Male reproductive investment: number of pollen grains per stamen.

were square-root transformed, and seed/ovule ratio per spike was arcsine square-root transformed.

A mixed hierarchical ANCOVA (with tiller rank as covariate) was carried out to test for the effects of family, tiller, plant (family), and the tiller × family interaction on the estimates of total reproductive effort, male and female reproductive effort, and vegetative biomass. Family and plant were treated as fixed and random variables, respectively. There was no significant block effect.

To test for the existence of changes in tiller gender with flowering phenology, we first carried out linear regressions between reproductive and vegetative biomass and, separately, between male and female reproductive effort and vegetative biomass. Residuals from these regressions give the reproductive effort or the male or female reproductive effort, after controlling for tiller size. We then regressed these three measures of reproductive effort against tiller rank. For male reproductive effort, because the ANCOVA revealed a significant

Table 1

Results of a Mixed Hierarchical ANCOVA (with Tiller Rank as Covariate) Model (Plant Effect Is Random and Family Effect Is Fixed) to Test for Effects of Family, Tiller, Plant (Family), and Tiller × Family Interaction on the Following Estimates of Male and Female Reproductive Effort

	Traits				
Source of variation	Vegetative biomass	Reproductive biomass	Mass of seeds per tiller	Seed/ovule ratio per spike	Number of pollen grains per stamen
Family:					
F values	$F_{1,94} = 1.57$	$F_{1,90} = 2.22$	$F_{1,98} = 1.67$	$F_{1,191} = 8.93$	$F_{1,141} = 0.94$
P	0.213	0.140	0.199	0.003	0.335
Plant (family):					
F	$F_{61,247} = 14.44$	$F_{62,255} = 17.29$	$F_{62,255} = 13.97$	$F_{62,256} = 4.01$	$F_{62,252} = 6.51$
P	0.0001	0.0001	0.0001	0.0001	0.0001
Tiller:					
F	$F_{1,247} = 490.79$	$F_{1,255} = 95.28$	$F_{1,255} = 62.29$	$F_{1,256} = 0.34$	$F_{1,252} = 199.51$
P	0.0001	0.0001	0.0001	0.558	0.0001
Tiller × family:					
F	$F_{1,247} = 2.10$	$F_{1,255} = 1.79$	$F_{1,255} = 0.95$	$F_{1,256} = 2.96$	$F_{1,252} = 5.36$
P	0.149	0.182	0.329	0.087	0.021

Note. Family differences were tested against a linear combination of plant (family) and error mean square.

tiller × family interaction (see "Results"), the two families were analyzed separately.

Results

Variation in Vegetative Biomass

We found significant variation among plants within families and a significant effect of tiller rank on vegetative biomass. Late-flowering tillers were smaller than early-flowering ones (fig. 1*A*). In contrast, families were not significantly different, nor did the effect of tiller rank differ between families (tiller × family interaction, n.s.) (table 1).

On average, in family 138, mean vegetative biomass for rank-7 tillers represented 36.8% of the biomass of rank-1 tillers. In family 139, mean vegetative biomass for rank-7 tillers represented 34.4% of the biomass of rank-1 tillers.

Variation in Reproductive Biomass

Variation in reproductive biomass also showed a strong plant (within family) effect and a strong effect of tiller rank (table 1). Reproductive biomass also decreased with the increasing tiller rank (fig. 1*B*). Mean reproductive biomass of rank-1 tillers was 22.1 g in family 138 and 26.1 g in family 139. The reproductive biomass of rank-7 tillers was 14.6 g (66.2%) in family 138 and 16.3 g (62.5%) in family 139.

Variation in Allocation to Male and Female Function

In both families, there were significant differences among plants (within family) and tillers in allocations to male (pollen grain per tiller) and female (mass of seeds per tiller) functions. However, there were no significant differences between families (table 1). Late-flowering tillers produced fewer pollen grains per stamen (fig. 1*E*) and lower seed mass than early-flowering ones (fig. 1*C*). For example, in family 138, the mean values of pollen grains per stamen for rank-7 tillers represented 71.3% of pollen grains per stamen for rank-1 tillers. In family 139, mean values of pollen grains per stamen for rank-7 tillers represented 75.3% of pollen grain per stamen for rank-1 tillers. Late-flowering tillers produced less female reproductive effort (mass of seeds per spike) than early-flowering ones. Mean values of mass of seeds per spike for rank-7 tillers represented 66.1% (family 138) and 62.5% (family 139) of mass of seeds for rank-1 tillers. A significant interaction between family and tiller was found for the allocation to male function but not for the allocation to female function (table 1).

There were significant family and plant (within family) effects on seed : ovule ratio, but no significant tiller effect or interaction between family and tiller (table 1; fig. 1*D*). Therefore, differences observed between early- and late-flowering tillers in allocation to female function were not caused by a decrease in seed : ovule ratio.

Is the Relative Reproductive Effort Greater in Late-Flowering Spikes?

Reproductive and vegetative biomass both decreased in late-flowering spikes, with a greater decrease in vegetative biomass. Relative reproductive effort (calculated as the ratio reproductive [mass of spike] : vegetative biomass; Samson and Werk 1986) increased from 0.86 to 1.76 (138) and from 0.94 to 1.73 (139) from rank-1 tillers to rank-7 tillers. We found significant positive relationships between the residuals of the linear regression of reproductive biomass (mass of spike) on vegetative biomass and tiller rank (table 2). Relative reproductive effort was significantly more important in the late-flowering spikes than in early-flowering ones.

Does Tiller Gender Vary with Flowering Phenology?

In both families, there was a significant negative relationship between the residuals of the linear regression between number of pollen grains per stamen and vegetative biomass and tiller

Table 2

Linear Regression Carried Out between the Residuals of Linear Regressions (between Allocation to Female or Male Function and Total Aboveground Vegetative Dry Mass) and Tiller Rank

Traits used to obtain residuals	F	P	Slopes of linear regression ± SE
Reproductive biomass	$F_{1,\,310} = 9.73$	0.002	0.083 ± 0.026
Mass of seeds per tiller	$F_{1,\,309} = 5.92$	0.0155	0.070 ± 0.028
Number of pollen grains per stamen 138	$F_{1,\,149} = 13.89$	0.0003	−185.2 ± 49.7
Number of pollen grains per stamen 139	$F_{1,\,155} = 14.95$	0.0002	−166.3 ± 43.0

Note. The slopes are given for the linear regression between residuals and tiller rank.

rank (table 2). Late-maturing tillers invested significantly less in male function than early-maturing ones. However, residuals of variation in allocation to female function increased with flowering rank of tillers (table 2). Therefore, there was a change in tiller gender toward an increase in femaleness with flowering phenology.

Discussion

One of the consequences of the modular structure of grasses is that the different shoots of a plant share the same genotype but are subjected to different environmental conditions during their maturation. This has enabled us to show that there is a significant effect of tiller flowering phenology on the allocation to vegetative biomass and the allocation to sexual functions, and thus the functional gender of a tiller. There are two possible explanations for this finding. Gender variation may result from either physiological constraints or from selective processes associated with height. These results are discussed in more detail below.

Effects of Flowering Phenology on Vegetative Biomass

There was a significant decrease in vegetative biomass allocation with flowering phenology. The earliest-maturing tillers were the heaviest with the highest proportion of vegetative biomass in both families. This trend has also been recorded in other species of Poaceae, cultivated or not (Lauer and Simmons 1985; Rathcke and Lacey 1985; Chafai-Elalaoui and Simmons 1988), and is related to environmental changes such as nutrient availability and source-sink relationships among tillers. Early-maturing tillers with a high proportion of vegetative biomass often behave as a source of photosynthetic assimilates for late-flowering tillers that act as nutrient sinks.

Effects of Flowering Phenology on Sex Allocation

Flowering phenology had different effects on allocation to male and female function. Male reproductive biomass and allocation to male function (controlled for tiller size) significantly decreased with a delay in flowering, in both families, whereas allocation to female function increased.

We also found a significant increase in reproductive effort after controlling for tiller size (using mass of spike, cf. table 2). These results agree with those already found in pearl millet (Dajoz and Sandmeier 1997), where relative reproductive effort was found to be greater in small tillers than in large ones. Also, the proportion of reproductive biomass decreased with increasing plant biomass. Similarly, in *Poa annua* (Wagner 1989), there is a significant positive correlation between plant size and sex allocation, but no measurements were carried out at the tiller level. In families other than the Poaceae, positive correlations between plant size and reproductive allocations are commonly found (Chaplin and Walker 1982; Dudash 1991; Klinkhamer et al. 1994).

Late-flowering tillers invested proportionally more in reproduction than early-flowering tillers. One possible explanation for this is that surplus resources from early tillers are translocated to late-maturing ones. Resource translocations between tillers have been recorded in other species of Poaceae. For example, in *Hordeum vulgare*, ^{14}C-labeled assimilates are translocated from the main shoot to the youngest ones (Lauer and Simmons 1985). In cultivated wheat, resource exchanges from the main shoot are most important at the beginning of the development of secondary shoots (Quinland and Sagar 1962; Lupton 1966). Thus, assimilate translocations between tillers, known in Poaceae, may explain our observed patterns of increasing relative reproductive allocation.

Functional Gender of a Tiller

The functional gender of tillers changed as flowering progressed. One possible explanation could be that this gender change has evolved in response to physiological constraints. Indeed, evidence for this hypothesis has been reported in several dicotyledonous insect-pollinated species, where estimates of allocation to female function such as ovule number, ovule diameter, seed number per flower, or seed set all decrease with flowering phenology (Devlin 1988; Emms 1993; Frazee and Marquis 1994). As Winn (1991, p. 843) points out, “seed parents are not capable of producing a uniform seed crop in the face of change in resource availability in the course of seasons.” Such an argument, however, does not explain our data. In our experiment, tillers became increasingly female. It is possible that translocation of assimilates occurs between early- and late-flowering tillers since late-flowering tillers invest proportionally more to reproduction than early-flowering tillers. This has been reported in several other Poaceae species.

An alternative explanation is that this gender change has evolved in response to the effect of height on pollen success. Theoretical predictions are available concerning the direction of gender change with plant size. In hermaphroditic plants, the fitness gain curve of male and female functions may not vary the same way with plant size (Bickel and Freeman 1993; de Jong and Klinkhamer 1994; Pannell 1997). In animal-pollinated species, the fitness gain through male function may saturate more quickly than in wind-pollinated species, because large plants attract more pollinators and may suffer from an increase in geitonogamy (Harder and Barrett 1995). Therefore, evolutionary theory predicts that natural selection should favor an increase in femaleness with size (measured as plant mass), which is indeed the case in several insect-pollinated species (Rademaker and de Jong 1998). However, in wind-pollinated species, the saturation of male fitness gain should be less important (Charnov 1982) and should be proportional to plant height (Burd and Allen 1988; de Jong and Klinkhamer 1994). In this case, theory predicts that natural selection should favor an increase in maleness with height.

Experimental evidence for this gender change with height has been found in grass species such as *Zea mays* (Burd and Allen 1988). In pearl millet, within a plant, tiller size significantly decreases with a delay in time. If pollination efficiency is maximized by plant height, natural selection may favor a shift toward femaleness to maximize reproductive fitness in the small, late developing tillers. On the contrary, large, early-flowering tillers may have the highest reproductive fitness when investing the most in male function. A similar trend has been recorded in *Zizania aquatica* (Poaceae), where floral sex ratio (number of male/number of female flowers) decreases with flowering phenology (Wilson and Ruppel 1984).

Overall, our results indicate that in pearl millet, the changes in reproductive allocation and gender observed within plants result from resource translocations among tillers and may be the result of natural selection.

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