

MORPH DIFFERENCES AND HONEYBEE MORPH PREFERENCE IN THE DISTYLOUS SPECIES *FAGOPYRUM ESCULENTUM* MOENCH

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The relatively low reproductive success of buckwheat (*Fagopyrum esculentum* Moench, Polygonaceae) is poorly understood. The question arises as to whether this distylous species is pollen or resource limited. We investigated the reproductive biology of buckwheat under controlled conditions in growth rooms and in the field in central Belgium in order to determine whether floral morph and pollination events may affect its reproductive success. In controlled conditions, flowering phenology and flower morphology of the two floral morphs did not differ. However, thrum flowers produced larger and fewer pollen grains and secreted more nectar, with a higher proportion of sucrose, than pin flowers. In the field, thrum flowers were preferentially visited by honeybees, but fewer pollen grains were deposited on their stigmas. However, numbers of pollen tubes growing in styles, seed set, and seed weight did not differ between morphs. Seed set was low under field conditions and did not increase after hand cross-pollinations, suggesting that there was no pollen limitation. These results indicate that factors other than floral morph or pollination events were governing female fertility in buckwheat.

Keywords: buckwheat, distyly, honeybees, morph differences, nectar, pollination, seed set.

Introduction

Buckwheat (*Fagopyrum esculentum* Moench) is a pseudocereal of the Polygonaceae family that has been cultivated for centuries in Asia, North America, and Europe for human and cattle consumption. Low seed set constitutes a major constraint to buckwheat culture in the main growing countries. The causes of this reduced fertility remain essentially unknown, although environmental stresses (high temperatures, water stress), resource limitations, inadequate pollination, and fertilization problems (defective reproductive organs, failure of fertilization due to self-incompatibility or seed abortion) have been investigated (Marshall and Pomeranz 1982; Björkman 1995b; Goodman et al. 2001; Taylor and Obendorf 2001; Halbrechq et al. 2005).

Fagopyrum esculentum is a sporophytic, self-incompatible, distylous annual crop (Nagamoto and Adachi 1985) pollinated by a diverse insect assemblage (Björkman 1995b; Goodman et al. 2001; A.-L. Jacquemart, C. Gillet, and V. Cawoy, unpublished manuscript). The nectariferous flowers are open for less than 1 d and have only one ovule. Buckwheat flowers are organized in racemes of cymes, and the flowering period at plant level is long lasting, depending on

the duration of activity of the reproductive meristems, which produce inflorescences, cymes, and flowers (Quinet et al. 2004). Pin flowers develop long styles that project 2–3 mm above the anthers. Thrum flowers present short styles reaching about the level of the middle of the filaments of the anthers. Fewer pollen grains were produced in thrum than in pin flowers (Ganders 1979; Namai and Fujita 1995), and pollen grains of the thrum flowers are larger than those of the pin flowers (Marshall and Pomeranz 1982; Samborska-Ciania et al. 1989; Namai and Fujita 1995).

As in many other distylous species, thrum and pin plants are found in equal frequencies in buckwheat populations (Quinet et al. 2004). This balanced polymorphism could favor reciprocal pollen transfer (i.e., cross-pollination between anthers and stigmas of equivalent height in the floral morphs; Barrett 2002). However, the efficiency of such a mechanism often depends on pollinator effectiveness (Cesaro and Thompson 2004; Ornelas et al. 2004). Since cross-pollination is necessary for reproductive success in both morphs, they should not differ in attributes that contribute to attracting or rewarding floral visitors, leading to equal reproductive success (Leege and Wolfe 2002). In a minority of heterostylous plants, however, additional differences were recorded in stalk length, flower number, corolla shape and size, floral-tube pubescence, stamen or stigma orientation, or color of pollen grains (Boyd et al. 1990; Lloyd and Webb 1992; Massinga et al. 2005). Several of these flower traits, including flower number, corolla size, and floral rewards, can influence

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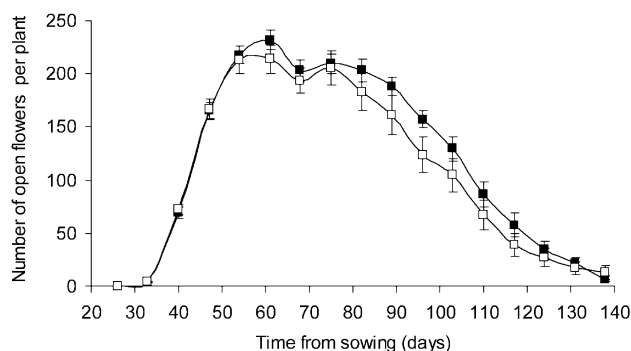


Fig. 1 Number of flowers reaching anthesis weekly on thrum (open squares) and pin (closed squares) plants of buckwheat cultivated in growth chambers. Means $\pm$ SE; $n = 15$ plants per morph.

pollinator attraction and thus pollination success (Zimmerman 1988; Goulson 1999; Pacini et al. 2003). Such differences have not been reported in *Fagopyrum* species.

Honeybees (*Apis mellifera*, Hymenoptera) are considered the main visitors on buckwheat in many countries. However, their abundance could greatly fluctuate according to the region or the season, and their effectiveness is poorly evaluated (Björkman 1995b; Goodman et al. 2001; A.-L. Jacquemart, C. Gillet, and V. Cawoy, unpublished manuscript).

A number of possible causes, not necessarily exclusive, can thus account for the limited reproductive success of buckwheat. In this work, our aim was to further investigate morph traits in buckwheat and to determine whether morph differences may affect honeybee behavior and reproductive success and whether low seed set in this species could be attributed to inadequate pollination (illegitimate or limited pollen deposits). Our objectives were thus (1) to examine in controlled conditions the variation between morphs in reproductive structures and rewards that operate to attract polli-

nators, (2) to observe honeybee behavior on both morphs in field trials, and (3) to evaluate the degree of pollen limitation and seed production for both morphs in field conditions.

Material and Methods

Plants

Buckwheat (*Fagopyrum esculentum* Moench) seeds of the cultivar La Harpe were obtained from Agri-Obtentions (Guyancourt, France). This cultivar is a facultative, short-day (Quinet et al. 2004), diploid plant developed by the Institut National de la Recherche Agronomique, Paris.

Experiments Performed in Growth Chambers

Culture conditions. In July 2004, reproductive structures and nectar production were investigated using plants cultivated in growth chambers of the Department of Biology, Université Catholique de Louvain, Louvain-la-Neuve, Belgium. Seeds were sown in peat compost in seed trays. Germination occurred within 3–4 d and, 8 d after sowing, the more vigorous plants were single-planted into plastic pots (0.7 L) filled with the same substrate. Light was supplied by Philips HPIT 400-W lamps (Philips Lighting, Brussels). The day/night cycle was 16L/8D, and the light irradiance at the top of the canopy was $120 \pm 20 \mu\text{mol m}^{-2} \text{s}^{-1}$. Temperature was kept at $20^{\circ}\text{--}21^{\circ}\text{C}/16^{\circ}\text{--}18^{\circ}\text{C}$ (day/night) and relative humidity at $78\% \pm 5\%$. Axillary shoots were removed 28 d after sowing to facilitate plant accessibility and homogenize plant architecture.

Phenology and reproductive structure morphology. Flowering time was assessed by the number of days from sowing to first anthesis. From first anthesis to plant senescence, all flowers that had reached anthesis on all plant inflorescences were counted weekly (15 plants per morph), and when the experiment was discontinued after 5 mo of cultivation, the

Table 1
Flowering Time, Number of Reproductive Structures, and Flower Morphology in the Two Floral Morphs of Buckwheat Cultivated in Growth Chambers

	<i>n</i>	Thrum	Pin	<i>t</i>	<i>P</i>
Flowering time:					
Age at first anthesis (d)	25	35.3 $\pm$ 0.4	34.6 $\pm$ 0.5	1.08	0.286
Number of reproductive structures per plant:					
Inflorescences	25	12.2 $\pm$ 0.3	12.1 $\pm$ 0.3	0.52	0.609
Cymes	25	151.4 $\pm$ 6.1	161.5 $\pm$ 6.3	1.19	0.242
Flowers	15	1805 $\pm$ 122	1986 $\pm$ 80	1.24	0.224
Flower morphology:					
Perianth diameter (mm)	25	6.99 $\pm$ 0.09	7.01 $\pm$ 0.09	0.51	0.613
Nectar zone area (mm ²)	25	1.54 $\pm$ 0.05	1.47 $\pm$ 0.05	0.80	0.427
Stamen from the inner whorl:					
Length (mm)	25	2.51 $\pm$ 0.03	1.73 $\pm$ 0.02	22.00	<0.0001*
Pollen grain number	25	149.3 $\pm$ 2.9	207.7 $\pm$ 5.3	9.58	<0.0001*
Pollen viability (%)	25	97.1 $\pm$ 0.4	97.2 $\pm$ 0.8	0.90	0.371
Pollen grain size (μm)	25	50.18 $\pm$ 0.87	34.24 $\pm$ 0.22	18.32	<0.0001*
Gynoecium length (mm)	25	1.41 $\pm$ 0.02	2.58 $\pm$ 0.03	32.04	<0.0001*
Seed weight (mg)	25	26.43 $\pm$ 0.82	25.95 $\pm$ 0.65	0.45	0.656

Note. Morph data presented as means $\pm$ SE; n = number of plants per morph.

* $P < 0.05$ (*t*-test).

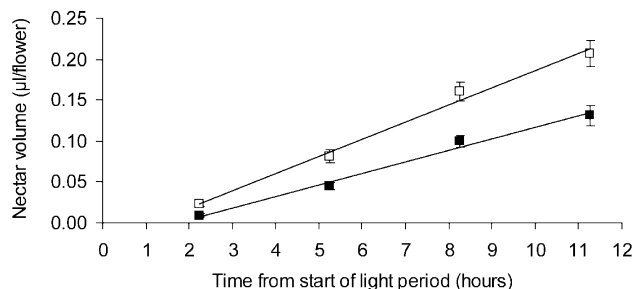


Fig. 2 Nectar production by thrum (open squares) and pin (closed squares) flowers of buckwheat as a function of time after the start of the light period in growth chambers. Nectar was collected from flowers of the fourth inflorescence on the fiftieth day after sowing. Means $\pm$ SE; $n = 12$ plants per morph. Thrum flowers: $y = 0.0211x - 0.0244$, $r^2 = 0.992$, $F = 236.65$, $P = 0.004$. Pin flowers: $y = 0.014x - 0.0237$, $r^2 = 0.990$, $F = 198.07$, $P = 0.005$.

total numbers of inflorescences and cymes per plant were recorded (25 plants per morph).

Forty-eight days after sowing, one open flower and one flower bud just before anthesis were collected on the fourth inflorescence (corresponding to tenth node counted from the cotyledonary node) of 25 plants per morph and fixed in FAA (70% ethanol–acetic acid–formaldehyde, 18 : 1 : 1). The open flowers were observed under a binocular light microscope, and perianth diameter, nectar zone area, length of stamens from the inner whorl, and gynoecium length (ovary base to the stigmas) were measured using a calibrated ocular micrometer. Pollen grains from one stamen of the inner whorl of flower buds collected before anthesis were dispersed on a glass slide in a drop of Alexander's solution (Alexander 1969) and observed under a light microscope. Viable pollen grains had a circular shape with red protoplasm, whereas aborted grains were oval and empty and exhibited green walls. After pollen viability percentage was estimated, the diameters of three viable pollen grains per flower were measured, still using a calibrated ocular micrometer.

Fifty-four and 56 d after sowing, two flowers from the fourth inflorescence were hand cross-pollinated by rubbing the anthers of one flower onto the stigmas of another compatible flower (25 plants per morph). Ripe seeds were harvested 6 wk later and oven-dried for 5 d at 60°C before dry weight measurement.

Nectar production and composition. Nectar was extracted using 1- μ L glass micropipettes (Hirschmann Laborgeräte, Eberstadt, Germany). The volume of the nectar collected was estimated by measuring the length of the nectar column in the capillary tube.

Nectar secretion as a function of the time during the day of flower anthesis was investigated on the fiftieth day after sowing. Nectar was collected 2, 5, 8, and 11 h after the light was switched on from flowers that were opening on 12 plants of both morphs.

The effect of plant age on nectar production was studied by collecting weekly the nectar of successive flowers that reached anthesis on the fourth inflorescence during the protracted flowering period of the plant. Nectar from 15 plants

of both morphs was harvested between 6.5 and 8.5 h after the light was switched on.

To investigate the influence of fertilization on nectar production, flowers from the fourth inflorescence were hand cross-pollinated 52 d after sowing (30 plants per morph). Seven hours after pollination, pollinated and unpollinated flowers (one flower per treatment and plant) were collected. Nectar was harvested, and flowers were fixed in FAA to check fertilization. After a water rinse, the flower styles were excised at their bases, treated with 1.0 M NaOH for 1 h, rinsed in distilled water, and crushed in 0.1% aniline blue solution (in KH_2PO_4 1.0 M, pH 9.0). Pollen grains and pollen tubes were examined under a fluorescent microscope. The callosic walls and plugs of the pollen tubes fluoresce heavily under UV excitation ($\lambda = 365$ nm).

Sugar composition and concentration of nectar were analyzed by HPLC. Samples were collected on 25 plants per morph 8.5–11.5 h after the light was switched on for five consecutive days from the fifty-fourth day after sowing. The nectar was harvested in all open flowers of a plant until a final volume of 1.5–2.5 μ L was obtained. The nectar samples were immediately frozen in liquid nitrogen and stored at -80°C until analysis. Before HPLC analysis, the samples were diluted in 200 μ L of ultrapure water and filtrated through a 0.2- μ m acrodisc (hydrophilic) polyvinylidene difluoride membrane. Aliquots were injected into a Bio-Rad (Hercules, CA) HPLC system (model 2700 Solvent Delivery System, with a Model 7010 sample injector equipped with a 20- μ L sample loop). The isocratic system was operated at 0.6 mL min^{-1} using degassed ultrapure water as the mobile phase. Carbohydrate separation was achieved using a Bio-Rad Aminex HPX-87C column (300 mm $\times$ 7.8 mm i.d.) maintained at 80°C. A Carbo-C guard column (30 mm $\times$ 4 mm i.d., Bio-Rad) was introduced between the injector and the analytical column. Detection was achieved via a Bio-Rad Refractive Index Monitor (model 1755). Retention time determination and quantification based on peak area were performed by Value Chrom software (Bio-Rad) integrating data from standard curves obtained using commercially

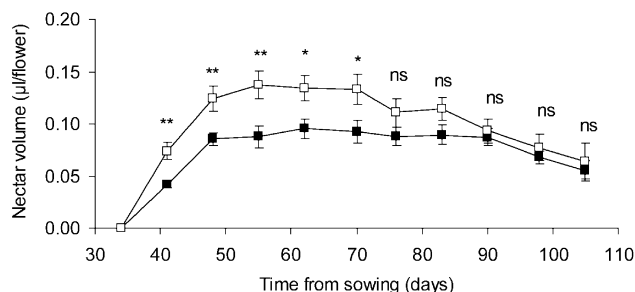


Fig. 3 Nectar production by thrum (open squares) and pin (closed squares) flowers of buckwheat as a function of plant age. Nectar was collected weekly, during a 2-h period extending from 6.5 to 8.5 h after the start of the daily light period, from flowers that reached anthesis on the fourth inflorescence of plants cultivated in growth chambers. Means $\pm$ SE; $n = 15$ plants per morph; t -test, $P < 0.05$. Statistical significance: ns = not significant; single asterisk indicates $0.05 < P \leq 0.01$; double asterisk indicates $0.01 < P \leq 0.001$.

Table 2
Sugar Composition of Nectar Produced by Thrum and Pin Floral Morphs in Buckwheat Cultivated in Growth Chambers

	<i>n</i>	Thrum	Pin	<i>t</i>	<i>P</i>
Sucrose (%)	20	16.81 ± 1.64	12.93 ± 0.81	2.12	0.041*
Glucose (%)	20	45.08 ± 0.96	47.45 ± 0.54	2.16	0.037*
Fructose (%)	20	38.10 ± 0.74	39.62 ± 0.33	1.86	0.070
Sucrose/hexose ratio	20	0.21 ± 0.02	0.15 ± 0.01	2.20	0.034*
Equivalent sucrose (mol L ⁻¹)	20	1.68 ± 0.09	1.45 ± 0.10	1.79	0.082
Sugar concentration (% w/v)	20	59.94 ± 3.28	51.62 ± 3.45	1.79	0.089

Note. Morph data presented as means ± SE; *n* = number of plants per morph.

* *P* < 0.05 (*t*-test).

available reference sugars (Sigma Chemical, St. Louis, MO). Because sucrose/hexose ratio is considered a good estimator of insect attractiveness, this ratio was calculated by dividing the sucrose percentage by the sum of the fructose and glucose percentages.

Experiments Performed in Fields or in a Garden Parcel

Growing conditions. Experiments concerning pollinator behavior, pollen transfer, and plant yields were performed during three growing seasons in fields located in Brabant Wallon, Belgium (Centre A de Marbaix, Chaumont-Gistoux, 50°40'20"N, 4°38'00"E). Sowings (June 6, 2001; May 28, 2003; and June 28, 2004) were made in 1100-m² plots at a density of 40 kg ha⁻¹. In 2004, seed set was additionally investigated using plants sown on June 2 in a garden parcel located in Biesme-sous-Thuin, Hainaut, Belgium (50°19'00"N, 4°17'60"E).

Honeybee behavior. In July 2001, honeybee behavior was observed in the course of 10-min observation censuses during four days on 1-m² quadrats located in the field trial. A total of 214 censuses were performed during sunny days (temperature >20°C). Times spent per flower, per inflorescence, per plant, and per quadrat, as well as the number of pin and thrum flowers visited per quadrat, were recorded.

In July 2003, 44 different honeybee trips were surveyed during four mornings on sunny days. The choice of a morph (pin or thrum) and the time spent on each morph during sequential visits were noted.

Pollen limitation. To estimate open pollination, 66 flowers were collected in the field at different times (three periods: before 9:00 a.m., 9:00 a.m.–1:00 p.m., and after 1:00 p.m.) during three sunny days in July 2004. These flowers, conserved in FAA, were used to record pollen grains on stigmas and pollen tube growth in styles (as described above). The compatibility percentage was estimated by counting flowers that presented compatible pollen tubes at the base of their styles.

To test for pollen transfer limitation, supplementary hand cross-pollinations were performed in the field. Ten individual inflorescences of both morphs were selected and tagged (ninth node), and freshly open flowers were pollinated daily during 12 successive days in July 2003 and six days in July 2004, using a thin paintbrush. Cross-pollination refers to flowers that were pollinated with a mixture of pollen from three to six compatible donor plants, i.e., of the opposite morph. To compare open- and hand-pollination effects during the same period, unpollinated open flowers were removed from the inflorescences before and at the end of the pollination experiment. Ten other inflorescences from both morphs were open pollinated (left unmanipulated). All inflorescences were collected 1 mo later to count developing fruit under a dissecting microscope.

Seed set. Under field conditions, mean yield per whole plant was estimated in October 2003 (total numbers of brown and green seeds per plant) on 16 plants per morph. For the experiment performed in the garden parcel in 2004, the number of successful pollinations resulting in seed set was estimated in October on 20 plants per morph. Plants were oven-dried for 7 d at 60°C before reproductive structures were counted and seed dry weight was measured.

Statistical Analyses

All statistical analyses were performed with the SAS statistical package (SAS Institute 1993). Analyses of variance in normally distributed data were performed with the general linear method procedures in SAS, version 9.1. Honeybee

Table 3
Honeybee Foraging Activity in a Buckwheat Field as a Function of Time of Day, July 2001

Time of day	<i>n</i>	Time (s) per				Number of visited flowers per quadrat
		Flower	Inflorescence	Plant	Quadrat	
Before 9:00 a.m.	36	3.2 ± 0.3	3.9 ± 0.4	6.7 ± 1.0	24.3 ± 4.5	7.5 ± 1.1 ^A
10:00 a.m.	77	2.7 ± 0.2	4.5 ± 0.5	9.0 ± 1.2	25.4 ± 4.6	8.0 ± 0.8 ^A
11:00 a.m.	62	2.9 ± 0.2	4.5 ± 0.4	8.7 ± 0.8	21.9 ± 2.9	7.8 ± 0.9 ^A
Noon	28	3.2 ± 0.3	4.6 ± 1.1	7.4 ± 1.8	17.9 ± 5.3	4.6 ± 1.0 ^{AB}
After 1:00 p.m.	11	2.8 ± 0.4	6.3 ± 1.2	8.6 ± 1.7	9.7 ± 2.0	3.5 ± 0.5 ^B
<i>F</i>		1.07	1.20	0.59	1.18	3.27
<i>P</i>		0.3718	0.3098	0.6086	0.3219	0.0125*

Note. Data presented as means ± SE; *n* = number of 10-min censuses. Letters refer to significant differences.

* *P* < 0.05 (one-way ANOVA).

Table 4
Honeybee Preference among Pin and Thrum Flowers of Buckwheat, before and after 9:00 a.m., during Four Consecutive Days in July 2003

Period of the day	<i>n</i>	Total foraging time on flowers (s)		Visit time on thrum flowers (%)	Number of flowers visited per inflorescence	
		Pin	Thrum		Pin	Thrum
Before 9:00 a.m.	24	98.3 ± 19.5	163.3 ± 25.2	64.7 ± 2.6	1.14 ± 0.03	1.30 ± 0.04
After 9:00 a.m.	20	86.3 ± 12.4	107.3 ± 16.0	55.2 ± 3.6	1.33 ± 0.06	1.39 ± 0.05
Between periods of the day:						
<i>t</i>		0.25	3.19	4.69	7.42	1.73
<i>P</i>		0.621	0.081	0.036*	0.009*	0.196
Between morphs:						
<i>t</i>			2.82	4.72		4.14
<i>P</i>			0.007*	<0.0001*		0.0002*

Note. Data presented as means ± SE; *n* = number of 10-min observations.

* *P* < 0.05 (*t*-test).

behavior (time spent and number of visited flowers) on thrum and pin flowers was analyzed using paired-sample *t*-tests. Correlation and regression analyses were performed to compare flower production and floral nectar secretion on both morphs and to examine the relationship between flowering phenology and nectar production. Means are given with their standard errors.

Results

Flowering Phenology, Floral Morphology, and Seed Weight

In both morphs, flowering phenology was similar (fig. 1; $r^2 = 0.981$, $F = 732.74$, $P < 0.0001$). The first flowers opened ca. 1 mo after sowing, and the number of flowers reaching anthesis on a plant increased regularly during a further month before decreasing progressively until the end of the flowering period, 5 mo after sowing. No differences between morphs were detected in the number of inflorescences, cymes, and flowers (table 1). Thrum flowers had taller stamens and produced larger but fewer pollen grains than pin flowers (table 1). Pollen viability was high and similar for the two morphs (97%). No significant differences were observed in perianth diameter or nectary zone area between morphs. Seed weight was also similar.

Nectar Secretion and Composition

In flowers of both morphs, nectar flow increased constantly during the light period, until flower wilting (fig. 2), making possible comparisons of the two morphs by collecting nectar during the same limited period of time during the day. Consequently, in subsequent experiments, nectar harvest was always performed during a 2-h period starting 6.5 h after the light was switched on.

Nectar secretion by successive flowers reaching anthesis on the fourth inflorescence increased from the start of the flowering period of the plant, 1 mo after sowing, and culminated at flowering peak (fig. 3). It was positively correlated with number of flowers per plant reaching anthesis weekly (pin flowers: $y = 0.0098 + 0.0033x$, $r^2 = 0.916$, $F = 98.84$, $P < 0.0001$; thrum flowers: $y = 0.0097 + 0.005x$, $r^2 = 0.935$,

$F = 130.61$, $P < 0.0001$) and evolved similarly in both morphs ($r^2 = 0.719$, $F = 20.51$, $P = 0.002$).

Thrum flowers produced more nectar than pin flowers (figs. 2, 3). This higher production was recorded only during the first 5 wk after plants started to flower (fig. 3). Thereafter, nectar production decreased, and differences between the two morphs were no longer statistically significant.

Nectar production did not significantly differ between pollinated and unpollinated flowers (pin flowers: 0.089 ± 0.008 and 0.089 ± 0.010 μL , respectively, $t = 0.03$, $P = 0.975$; thrum flowers: 0.110 ± 0.012 and 0.124 ± 0.011 μL , respectively, $t = 0.85$, $P = 0.398$).

Nectar contained sucrose, glucose, and fructose and was hexose dominant (table 2). Total sugar concentration was

Table 5
Pollen Deposition and Pollen Tube Growth in Thrum and Pin Flowers of Buckwheat Cultivated in the Field at Different Times during the Day, July 2004

Floral morph, period	<i>n</i>	Pollen grain number	Pollen tube number	Compatibility (%)
Thrum:				
Before 9:00 a.m.	9	1.9 ± 1.4	1.5 ± 0.5	50.0 ± 5.0
9:00 a.m.–1:00 p.m.	7	9.3 ± 5.1	3.3 ± 3.3	100 ± 0
After 1:00 p.m.	14	18.6 ± 3.8	3.7 ± 0.4	100 ± 0
Pin:				
Before 9:00 a.m.	14	10.7 ± 2.7	2.7 ± 0.3	63.6 ± 15.2
9:00 a.m.–1:00 p.m.	6	16.5 ± 7.3	2.7 ± 0.5	100 ± 0
After 1:00 p.m.	13	43.1 ± 6.2	4.2 ± 0.3	100 ± 0
Among periods:				
<i>F</i>		15.26	12.95	7.91
<i>P</i>		<0.0001*	<0.0001*	0.001*
Between morphs:				
<i>F</i>		15.36	0.95	0.08
<i>P</i>		0.0002*	0.335	0.786

Note. The compatibility percentages were estimated by recording the proportion of flowers that presented compatible pollen tubes at the bases of their styles. Data presented as means ± SE; *n* = number of flowers.

* *P* < 0.05 (two-way ANOVA, linear model).

Table 6
Seed Set after Open Pollinations or Hand
Cross-Pollinations in Buckwheat Plants
Cultivated in the Field

Year, pollination treatment, morph	<i>n</i>	Seed set (% of open flowers)
2003, open:		
Thrum	10	15.1 ± 1.8
Pin	10	14.6 ± 3.3
2003, hand:		
Thrum	8	15.8 ± 3.0
Pin	9	17.4 ± 5.3
2004, open:		
Pin	8	15.1 ± 3.6
2004, hand:		
Pin	8	13.9 ± 2.4
Between years:		
<i>F</i>		0.19
<i>P</i>		0.668
Between treatments:		
<i>F</i>		0.37
<i>P</i>		0.549
Between morphs:		
<i>F</i>		0.02
<i>P</i>		0.877

Note. Data were collected in two consecutive years and are presented as means ± SE; *n* = number of inflorescences (three-way ANOVA, linear model, significance at *P* < 0.05).

similar in the two morphs, but sucrose concentration was significantly higher in nectar of thrum flowers. The sucrose/hexose ratio was therefore higher for thrum plants (table 2).

Honeybee Behavior

Honeybee foraging activity (time spent per flower, inflorescence, plant, or quadrat) was not significantly affected by time of day, although fewer flowers (mainly, fewer pin flowers) were visited per trip after midday (table 3). Honeybees spent more time on thrum than on pin flowers (an average of 61.4% of total foraging time; paired *t*-test: *t* = 4.72, *P* < 0.0001), particularly in the morning, visiting more thrum inflorescences (*t* = 4.14, *P* = 0.0002) and more thrum flowers per inflorescence (*t* = 2.82, *P* = 0.0073) (table 4).

Pollen Deposition

Pollen deposition onto the stigmas and pollen tube growth greatly varied with time of day ($F_{3,59} = 14.23$, *P* < 0.0001), increasing during the day (table 5). Although fewer pollen grains were counted on stigmas of thrum flowers, similar numbers of pollen tubes were recorded in the two morphs.

Pollen Transfer Limitation

No significant differences ($F_{3,46} = 0.19$; *P* = 0.900) were observed in seed set between open-pollinated and hand cross-pollinated flowers (table 6). Seed set was not affected by year or morph. It was consistently low (ca. 15%).

Seed Production

Seed production was far less in the garden parcel than in the field because of poor soil conditions. Nevertheless, in

both locations, no significant differences were detected between morphs, even if pin plants produced more mature seeds than thrum plants (table 7).

Discussion

Floral Morph Differences

A number of differences between the two floral morphs of buckwheat have been quantified in this study (fig. 4). Thrum flowers have larger and fewer pollen grains than pin flowers, and our values were consistent with those reported in other studies on buckwheat or even on other distylous species (Ganders 1979; Marshall and Pomeranz 1982; Samborska-Ciania et al. 1989; Namai and Fujita 1995). Our results also indicated that thrum flowers produce more nectar than pin flowers. Sexual differences in nectar production have been found in several monoecious, dioecious, and protandrous species, but usually no difference in nectar production has been found between morphs in distylous species (Sobrevila et al. 1983; Pérez-Nasser et al. 1993; Passos and Sazima 1995; Ree 1997; Contreras and Ornelas 1999; Legee and Wolfe 2002; Lau and Bosque 2003). Only Ornelas et al. (2004) and Teixeira and Machado (2004) showed that pin flowers of *Palicourea padifolia* and *Psychotria barbiflora* secreted more nectar than thrum flowers.

In our growth chamber experiments, total sugar concentration of flower nectar was similar for the two morphs, averaging 55%, which is higher than reported for field-caged plants (Lee and Heimpel 2003). Sucrose, glucose, and fructose were detected by HPLC. The two hexoses were the major components in both morphs, and sucrose concentration was higher in thrum flowers than in pin flowers. Buckwheat nectar could thus be classified as a hexose-rich type according to Percival's classification (Percival 1961). *Muehlenbeckia complexa*, the only other Polygonaceae in which nectar composition has been analyzed, is also a hexose-rich type (Percival 1961).

We did not find any other significant differences between the two floral morphs of buckwheat, although Komenda et al. (1986) mentioned that the perianth size was generally

Table 7

Number and Weight of Seeds Produced by Thrum and Pin
Morphs of Buckwheat Plants Cultivated in the Field in
2003 and in a Garden Parcel in 2004

Year, parameter	<i>n</i>	Thrum	Pin	<i>t</i>	<i>P</i>
2003:					
Aborted seeds	16	115.2 ± 21.1	138.7 ± 19.1	0.83	0.414
Mature seeds	16	325.9 ± 58.2	368.8 ± 41.0	0.60	0.552
2004:					
Unfertilized flowers	20	348.2 ± 27.7	331.0 ± 29.7	0.42	0.674
Aborted seeds	20	47.3 ± 5.3	50.6 ± 5.3	0.43	0.668
Mature seeds	20	24.5 ± 3.1	35.8 ± 4.7	2.00	0.053
Total	20	420.1 ± 31.9	417.4 ± 32.5	0.06	0.952
Seed dry weight (mg)		23.6 ± 0.7	22.6 ± 0.6	1.01	0.318

Note. Data presented as means ± SE; *n* = number of plants per morph (*t*-test, significance at *P* < 0.05).

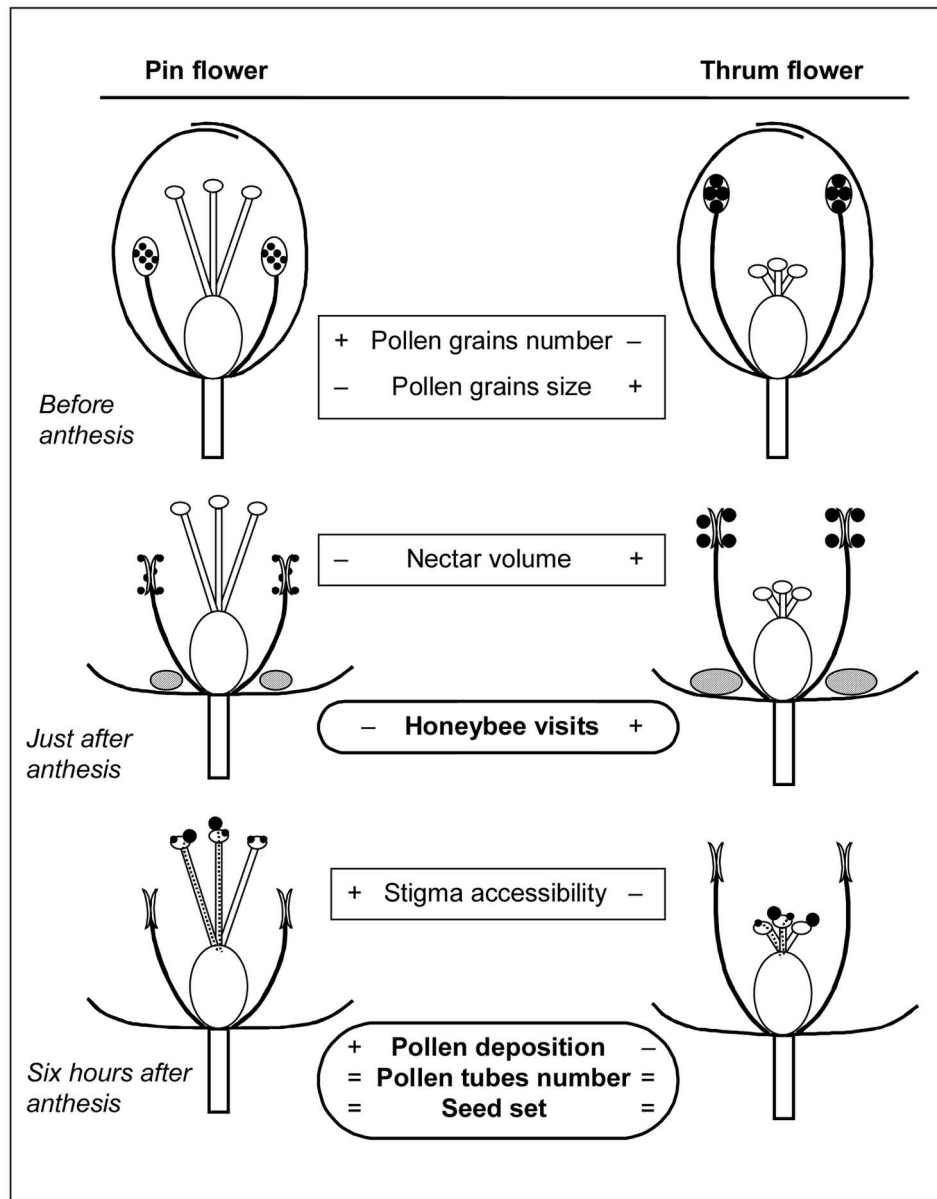


Fig. 4 Schematic representation of morph-related traits in buckwheat as quantified in this study.

larger in thrum flowers than in pin flowers. Some authors also reported that thrum plants produced more seeds or heavier seeds than pin plants (Komenda et al. 1986; Lee 1986; Namai 1990).

Morph Differences and Honeybee Behavior

Honeybees preferred thrum flowers: they spent more time per flower and visited more flowers per inflorescence on thrum than on pin plants. In this study, honeybee foraging activity was apparently correlated with nectar production, which was higher in thrum flowers and increased during the course of a plant's life, culminating at full blooming, when nectar secretion was found to be maximal in this study. Flowers producing

higher quantities of nectar are expected to attract higher numbers of visitors (Ornelas et al. 2004). Greater attractiveness of thrum flowers compared to pin flowers could also be related to their production of larger pollen grains with higher starch content (Tatebe 1954). No direct relationship has been established between particular sucrose/hexose ratios or total sugar concentration and honeybee attraction (Percival 1961; Dafni et al. 1988). According to Eickwort and Ginsberg (1980), however, Apidae seem to prefer 30%–50% sugar in nectar. However, these insects could also be attracted by more dilute nectars (Baker and Baker 1983). Moreover, Percival (1961) did not find honeybee preference among several sucrose-fructose-glucose combinations.

Pollination and Seed Set

In our experiment, despite their better pollinator attraction, thrum flower stigmas captured fewer pollen grains than those of pin flowers (fig. 4). Such asymmetric pollen deposition is common in distylous species, with a majority of them showing greater compatible pollen deposition on pin stigmas (Ganders 1979; Dulberger 1992; Björkman 1995a). Pin stigmas are usually considered to be more accessible to pollinators (Ganders 1979; Dulberger 1992; Massinga et al. 2005).

In our experiments, hand pollinations did not increase seed set compared to open pollinations, suggesting that pollen transfer was not limited in buckwheat in the field. This result was unexpected because a majority of self-incompatible species are affected by pollen transfer limitation, showing an increase in seed/ovule ratios after hand cross-pollinations (Baker et al. 2000; Griffin and Barrett 2002; Ashman et al. 2004; Darrault and Schlindwein 2005). That seed set in the field remained poor (ca. 15%), even after hand cross-pollinations, was surprising. Factors other than the total amount of compatible pollen delivered to stigmas have yet to be considered to account for this reduced fertility. Prior pollination events by pollinators or prior self-pollination, which have been reported to prevent subsequent hand cross-pollination success in various species (Waser and Price 1991; Broyles and Wyatt 1993; Barrett 2002), could not apply in our study because cross-pollinations were performed early in the morning and increases in pollen deposition and pollen tube growth were still observed at the beginning of the afternoon. An alternative explanation for the low seed set could be linked to the status of the unfertilized flowers. Indeed, it could be that not all ovules produced were viable and therefore capable of being cross-fertilized. According to Taylor

and Obendorf (2001), ca. 20% of megagametophytes appeared to be defective in buckwheat at anthesis, and fertilization is a limiting process. Structural and functional assays of ovules and early embryo development are needed to determine whether such impediments to seed production similarly occur under our cultural conditions. Finally, resource limitation has been shown to contribute to reduced seed set in a number of outcrossing species (Campbell and Halama 1993; Morales and Galetto 2003) and could thus be implicated in the low seed production recorded in buckwheat.

In conclusion, this study established that, despite induced honeybee preference for thrum flowers, seed set in the two morphs of buckwheat was similar. Since seed set remained at a low level, although there was no pollen limitation, further studies on factors governing female fertility are required. These studies should include manipulations of resource levels and analyses of ovule viability or possible cryptic early abortion processes.

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Literature Cited

- Alexander MP 1969 Differential staining of aborted and nonaborted pollen. *Stain Technol* 44:117–122.
- Ashman T-L, TM Knight, JA Steets, P Amarasekare, M Burd, D Campbell, M Dudash, et al 2004 Pollen limitation of plant reproduction: ecological and evolutionary causes and consequences. *Ecology* 85:2408–2421.
- Baker AH, SCH Barrett, JD Thompson 2000 Variation in pollen limitation in the early flowering Mediterranean geophyte *Narcissus assoanus* (Amaryllidaceae). *Oecologia* 124:529–535.
- Baker H, GI Baker 1983 Floral nectar constituents in relation to pollinator type. Pages 117–141 in CE Jones, RJ Little, eds. *Handbook of experimental pollination biology*. Van Nostrand Reinhold, New York.
- Barrett SCH 2002 The evolution of plant sexual diversity. *Nat Rev Genet* 3:274–284.
- Björkman T 1995a The effectiveness of heterostyly in preventing illegitimate pollination in dish-shaped flowers. *Sex Plant Reprod* 8: 143–146.
- 1995b The role of honeybees (Hymenoptera: Apidae) in the pollination of buckwheat in eastern North America. *J Econ Entomol* 88:1739–1745.
- Boyd M, J Silvertown, C Tucker 1990 Population ecology of heterostyly and homostyle *Primula vulgaris*: growth, survival and reproduction in field populations. *J Ecol* 78:799–813.
- Broyles SB, R Wyatt 1993 The consequences of self-pollination in *Asclepias exaltata*, a self-incompatible milkweed. *Am J Bot* 80:41–44.
- Campbell DR, KJ Halama 1993 Resource and pollen limitations to lifetime seed production in a natural plant population. *Ecology* 74: 1043–1051.
- Cesaro AC, JD Thompson 2004 Darwin's cross-promotion hypothesis and the evolution of stylar polymorphism. *Ecol Lett* 7:1209–1215.
- Contreras PS, JF Ornelas 1999 Reproductive conflicts of *Palicourea padifolia* (Rubiaceae) a distylous shrub of a tropical cloud forest in Mexico. *Plant Syst Evol* 219:225–241.
- Dafni H, Y Lensky, A Fahn 1988 Flower and nectar characteristics of nine species of Labiateae and their influence on honeybee visits. *J Apic Res* 27:103–114.
- Darrault RO, C Schlindwein 2005 Limited fruit production in *Hancomia speciosa* (Apocynaceae) and pollination by nocturnal and diurnal insects. *Biotropica* 37:381–388.
- Dulberger R 1992 Floral polymorphisms and their functional significance in the heterostylous syndrome. Pages 40–84 in SCH Barrett, ed. *Evolution and function of heterostyly*. Springer, New York.
- Eickwort GC, HS Ginsberg 1980 Foraging and mating behaviour in Apidae. *Annu Rev Entomol* 25:421–446.
- Ganders FR 1979 The biology of heterostyly. *NZ J Bot* 17:607–635.
- Goodman R, G Hepworth, P Kaczynski, B McKee, S Clarke, C Bluett 2001 Honeybee pollination of buckwheat (*Fagopyrum esculentum* Moench) cv. Manor. *Aust J Exp Agric* 41:1217–1221.
- Goulson D 1999 Foraging strategies of insects for gathering nectar and pollen, and implications for plant ecology and evolution. *Perspect Plant Ecol Evol Syst* 2:185–209.

- Griffin SR, SCH Barrett 2002 Factors affecting low seed : ovule ratios in a spring woodland herb, *Trillium grandiflorum* (Melanthiaceae). *Int J Plant Sci* 163:581–590.
- Halbrech B, P Romedenne, JF Ledent 2005 Evolution of flowering, ripening and seed set in buckwheat (*Fagopyrum esculentum* Moench): quantitative analysis. *Eur J Agron* 23:209–224.
- Komenda K, J Komenda-Ronka, B Komenda 1986 Study of buckwheat varieties collection (*Fagopyrum* spp.). Pages 68–70 in Institute of Soil Science and Plant Cultivation, ed. Buckwheat research 1986: proceedings of the 3rd International Symposium on Buckwheat. Vol 2. Laboratory of Science, Pulawy, Poland.
- Lau P, C Bosque 2003 Pollen flow in the distylous *Palicourea fendleri* (Rubiaceae): an experimental test of the disassortative pollen flow hypothesis. *Oecologia* 135:593–600.
- Lee JC, GE Heimpel 2003 Nectar availability and parasitoid sugar feeding. Pages 220–225 in RG Van Driesche, ed. Proceedings of the 1st International Symposium on the Biological Control of Arthropods. USDA Forest Service, Morgantown, WV.
- Lee MS 1986 Studies on heterostylism, fertility, and embryological characteristics in buckwheat, *Fagopyrum esculentum*. *Korean J Crop Sci* 31:129–142. (In Korean.)
- Leege LM, LM Wolfe 2002 Do floral herbivores respond to variation in flower characteristics in *Gelsemium sempervirens* (Loganiaceae), a distylous vine? *Am J Bot* 89:1270–1274.
- Lloyd DG, CJ Webb 1992 The selection of heterostyly. Pages 179–205 in SCH Barrett, ed. Evolution and function of heterostyly. Springer, Berlin.
- Marshall HG, Y Pomeranz 1982 Buckwheat: description, breeding, production and utilization. Pages 157–210 in Y Pomeranz, ed. Advances in cereal science and technology. Vol 5. American Association of Cereal Chemists, St. Paul, MN.
- Massinga PH, SD Johnson, LD Harder 2005 Heteromorphic incompatibility and efficiency of pollination in two distylous *Pentstemon* species (Rubiaceae). *Ann Bot* 95:389–399.
- Morales CL, L Galetto 2003 Influence of compatibility system and life form on plant reproductive success. *Plant Biol* 5:567–573.
- Nagamoto T, T Adachi 1985 *Fagopyrum esculentum*. Pages 1–8 in AH Halevy, ed. Handbook of flowering. Vol 3. CRC, Boca Raton, FL.
- Namai H 1990 Pollination biology and reproductive ecology for improving genetics and breeding of common buckwheat, *Fagopyrum esculentum*. 1. *Fagopyrum* 10:23–46.
- Namai H, Y Fujita 1995 Floral characteristics associated with seed productivity in common buckwheat, *Fagopyrum esculentum* Moench. Pages 425–435 in T Matamo, A Ujihara, eds. Current advances in buckwheat research: proceedings of the 6th International Symposium on Buckwheat. Shinshu University Press, Shinshu, Japan.
- Ornelas JF, C Gonzáles, L Jiménez, C Lara, AJ Martinez 2004 Reproductive ecology of distylous *Palicourea padifolia* (Rubiaceae) in a tropical montane cloud forest. II. Attracting and rewarding mutualistic and antagonistic visitors. *Am J Bot* 91:1061–1069.
- Pacini E, M Nepi, JL Vesprini 2003 Nectar biodiversity: a short review. *Plant Syst Evol* 238:7–21.
- Passos L, M Sazima 1995 Reproductive biology of the distylous *Manettia luteo rubra* (Rubiaceae). *Bot Acta* 108:309–313.
- Percival MS 1961 Types of nectar in angiosperms. *New Phytol* 60: 235–281.
- Pérez-Nasser N, LE Eguiarte, D Piñero 1993 Mating system and genetic structure of the distylous tropical tree *Psychotria faxlucens* (Rubiaceae). *Am J Bot* 80:45–52.
- Quinet M, V Cawoy, I Lefèvre, F Van Miegruet, A-L Jacquemart, JM Kinet 2004 Inflorescence structure and control of flowering time and duration by light in buckwheat (*Fagopyrum esculentum* Moench). *J Exp Bot* 402:1509–1517.
- Ree RH 1997 Pollen flow, fecundity, and the adaptive significance of heterostyly in *Palicourea padifolia* (Rubiaceae). *Biotropica* 29:298–308.
- Samborska-Ciania A, E Januszewicz, T Ojczyk 1989 The morphology of buckwheat flowers depending on the course of plant flowering. *Fagopyrum* 9:23–26.
- SAS Institute 1993 SAS user's guide. SAS Institute, Cary, NC.
- Sobrevila C, N Ramírez, N Xena de Enrech 1983 Reproductive biology of *Palicourea fendleri* and *P. petiolaris* (Rubiaceae), heterostylous shrubs of a tropical cloud forest in Venezuela. *Biotropica* 15:161–169.
- Tatebe T 1954 Physiological studies on fertility in buckwheat. IV. On the mechanism of heterostylism in short-styled flowers. *Jpn J Breed* 4:127–131. (In Japanese.)
- Taylor DP, RL Obendorf 2001 Quantitative assessment of some factors limiting seed set in buckwheat. *Crop Sci* 41:1792–1799.
- Teixeira LAG, IC Machado 2004 Pollination biology and breeding system of *Psychotria barbiglora* DC. (Rubiaceae). *Acta Bot Bras* 18: 853–862. (In Portuguese.)
- Waser NM, MV Price 1991 Reproductive costs of self-pollination in *Ipomopsis aggregata*: are ovules usurped? *Am J Bot* 78:1036–1043.
- Zimmerman M 1988 Nectar production, flowering phenology, and strategies for pollination. Pages 157–158 in J Lovett Doust, L Lovett Doust, eds. Plant reproductive ecology: patterns and strategies. Oxford University Press, New York.