

TRADE-OFFS BETWEEN FLOWER NUMBER AND INVESTMENT TO A FLOWER IN SELFING AND OUTCROSSING VARIETIES OF *IMPATIENS HYPOPHYLLA* (BALSAMINACEAE)¹

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Floral resource allocation was compared on a whole-plant basis between two varieties of *Impatiens hypophylla* that differ in flower size. There were significant negative correlations between flower number and investments to a flower at both the within-population and between-variety levels. In individual flowers, var. *hypophylla* with larger flowers invested significantly more resources to male and pollinator-attractive functions, whereas investments to female function did not differ between the varieties. In experimental populations placed in the field, pollinators preferred the larger flowers of var. *hypophylla* even within the same habitat of var. *microhypophylla*, which has smaller flowers. There was a significant lack of observed heterozygosity only in var. *microhypophylla*. Thus, the outcrossing variety had more attractive but fewer flowers, while the selfing variety had less attractive but more abundant flowers.

Key words: Balsaminaceae; flower number; *Impatiens hypophylla*; mating system; pollinator attraction; trade-off.

Through a series of theoretical studies, optimal resource allocation within a hermaphroditic flower has been shown to change with the mating systems (see Brunet, 1992, for review). All of these models assume a trade-off between allocation to male, female, and pollinator-attractive functions of a flower. However, some recent studies have shown that pollen production, ovule production, and petal size are not negatively but positively correlated (O'Neil and Schmit, 1993; Robertson, Diaz, and Macnair, 1994; Fenster and Carr, 1997). Therefore, the assumption of such a trade-off is not supported as far as the anthesis phase is considered. On the other hand, some of the studies in which seed production was measured as a part of female function have detected trade-offs between male and female functions (Silvertown, 1987; Stanton, Berezky, and Hasbrouck, 1987; Macnair and Cumbes, 1990; Rameau and Gouyon, 1991; Atlan et al., 1992; Garnier, Maurice, and Olivieri, 1993; but see Devlin, 1989; and Mossop, Macnair, and Robertson, 1994).

While most previous studies have concentrated on trade-offs within a flower, several authors considered the trade-off between an investment to a flower and the flower number (Morgan, 1993; Sakai, 1993; see also Lloyd, 1987a, and Fishbein and Venable, 1996, for relevant arguments). Among them, Sakai's model is unique in predicting that the evolutionarily stable allocation to attractive structures per flower is independent of the outcrossing rate. Whether there exists a trade-off between flower number and the reproductive investment to a flower remains, however, unanswered, since most of the studies to

date have measured reproductive resource allocation on a flower basis. The only empirical study of the trade-off between an investment to a flower and the flower number was made by Morgan (1998), using *Claytonia virginica*. Although he failed to detect any significant relationship between these two, the fact that *C. virginica* is a perennial may explain the absence of such trade-offs in his study. This is because plants with many large flowers may sacrifice further growth or survivorship. More rigorous testing of this kind of trade-off needs to be made using annual plants.

Impatiens hypophylla, chosen as our study material, is an annual species, which consists of two varieties. While the varieties are indistinguishable by vegetative traits, var. *hypophylla* has larger reddish-purple flowers, while var. *microhypophylla* has smaller white flowers. The objective of our study was to measure reproductive resource allocation on a whole-plant basis in the varieties of *I. hypophylla* and to examine whether there is a phenotypic trade-off between flower number and investment to a flower. The trade-off between flower number and investment to a flower can be documented, if one of the varieties has more flowers but a smaller investment to the flower than the other variety, and/or if there are negative correlations between flower numbers and investments to the flower among individuals within each variety. When such a trade-off was noted, we then also examined the prediction of Sakai (1993) that the evolutionarily stable allocation to pollinator attraction per flower is independent of the outcrossing rate.

The specific questions addressed in this paper were as follows. (1) Is there any phenotypic trade-off between flower number and the investment to a flower in the varieties of *I. hypophylla*? (2) Are larger flowers of var. *hypophylla* more attractive for pollinators? (3) Is the amount of pollinator attraction per flower independent of the outcrossing rate, as predicted by Sakai (1993)?

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MATERIALS AND METHODS

Biology of *Impatiens hypophylla*—*Impatiens hypophylla* Makino is a chasmogamous annual species, which occurs in forest understories and forest margins of eastern Japan (central Honshu, Shikoku, and central Kyushu). It is classified into two varieties, var. *hypophylla* and var. *microhypophylla* Nakai. Variety *microhypophylla* occurs around the range of var. *hypophylla*. From the late August to mid-October, they produce large, self-compatible flowers, which hang from several-flowered axillary racemes. Some of the flowers drop their androecium before abscission of the perianth, while others drop their androecium together with the perianth. Although vegetative organs of var. *hypophylla* and var. *microhypophylla* are indistinguishable, their flowers differ in size and the perianths differ in color, being slightly larger and pale reddish-purple in the former variety, but smaller and white in the latter (Akiyama, 1998). In this paper, dry masses were used to measure resource allocation. Dry mass of a flower and its energy give nearly equivalent allocation ratios in two species of *Impatiens* (Schemske, 1978).

As a measure for investments to pollinator attraction, we used perianth biomass, because there are general observations that larger flowers are visited by pollinators more frequently than smaller flowers (pollinator-attraction hypothesis; Bell, 1985; Stanton and Preston, 1988). However, in addition to pollinator attraction, perianth size may also be determined by the mechanical fit around a pollinator body, which maximizes pollen receipt and deposition during the visit (pollinator-matching hypothesis; Stebbins, 1971; Galen and Newport, 1987; Nilsson, 1988; Galen and Stanton, 1989; Campbell et al., 1991). If this selection pressure is responsible for the differentiation in perianth size between the varieties of *I. hypophylla*, it would not be appropriate to regard perianth biomass simply as an investment to the pollinator attraction of a flower.

Preparation of potted plants—All observations were gathered from potted plants placed in the field in 1996 and 1997. In early June of each year, we collected seedlings of two varieties, numbering ~120 each, from natural populations in Oike (var. *hypophylla*) and Mt. Hane (var. *microhypophylla*), Kuju Highlands, Oita Prefecture, Japan. The Oike and Mt. Hane populations are ~25 km apart. To equalize the environmental conditions during their growth, we planted each seedling in an 18-cm pot and raised them to maturity in forest understories in the vicinity of the Kuju joint-training-center for national universities within the Kyushu area, at a point ~10 km from the natural population of var. *hypophylla* and ~15 km from that of var. *microhypophylla*.

Biomass estimation of potted plants—We estimated the biomass of potted plants for 1997 with multiple regression using their vegetative measurements as independent variables. For each potted plant, we measured height, stem diameter at the bottom, the number of leaves that were more than 1 cm in width, and the number of main branches. These measurements were performed on 21 August 1997, just prior to the reproductive phase of the plants. After the measurement, we harvested 22 plants of var. *hypophylla* and 24 of var. *microhypophylla* selected so as to cover the full range of plant sizes. These were then dried in a 60°C convection oven for at least 7 d, before being weighed to the nearest 10 mg.

Separate regressions were performed for each variety, employing the four measurements of vegetative organs as independent variable, and their dry masses as dependent variables. We employed an exhaustive search to find the “best” subset of variables for prediction, calculating the Akaike Information Criterion (AIC) of all possible combinations of the variables (Akaike, 1973). By “best” we mean some combination of parsimony and adequacy of prediction. This exhaustive search is favored for the stepwise procedure, which does not guarantee convergence upon “best” combination of predictor variables (Miller, 1990). Using AIC, only a predictor variable, i.e., “the number of leaves” was selected for the regression model that estimates the biomass of potted

plants of var. *hypophylla*. The value of AIC and coefficient of determination (r^2) of the model were 352 and 0.903, respectively. The resultant regression equation is: $y = 41.9x_1 - 77.3$, where y is the plant biomass, and x_1 is the number of leaves. In the case of var. *microhypophylla*, two predictor variables, “the number of leaves” and “plant height,” were selected for the model, and its value of AIC and coefficient of determination (r^2) were 366 and 0.949, respectively. The resultant regression equation is $y = 25.8x_1 + 55.6x_2 - 1156.7$, where y , x_1 and x_2 are plant biomass, number of leaves, and plant height, respectively. We applied resultant models to estimate the plant biomass of potted plants for 1997.

Measurement of reproductive resource allocation 1 (nondestructive measurement)—Thirty plants of each variety were arrayed in rectangular formation with 30-cm intervals. To minimize opportunities of hybridization that might produce artificial effects on their resource allocation patterns, such as a decrease in seed set by selective abortion of hybrid zygotes, the arrays of var. *hypophylla* and var. *microhypophylla* were separated by a distance of ~15 m, while the remaining plants were placed at a point ~300 m from there. The arrays were placed on the forest understory where the potted plants had been grown. Within a wide area (>10 km) around that point, no natural population of *I. hypophylla* has previously been found. Therefore, the experimental population did not have any “native benefits” such as pollen pools of a specific variety on pollinator bodies and/or a search image of pollinators (Heinrich, 1979) for flowers of their native variety, which would perhaps provide a reproductive advantage to one of the varieties. In order to equalize the environmental effects on the potted plants, we exchanged the positions both of the arrays and of the randomized positions of individual pots within each array after each of the daily observations.

We observed every individual within the experimental population every day until all of them were dead (from 22 August to 18 October). During each daily observation, all the flowers that were newly opened on that day were marked with combinations of paint dots on their peduncles for identification. For each flower, we recorded the dates of anthesis, androecium abscission, and perianth abscission. When a fruit was formed, we harvested it and counted the mature seeds within it. Each fruit was harvested ~2 or 3 d before it has completely matured, because upon maturing it spontaneously bursts, dispersing its seeds. The harvested seeds and pericarps were pooled by individuals, dried in a 60°C convection oven for at least 24 h, and weighed to the nearest 0.1 mg. The average dry mass of a seed and of a pericarp were calculated for each individual. During the observation period, one potted plant of var. *hypophylla* and seven of var. *microhypophylla* died. These plants wilted with mosaically withered leaves, probably the result of some unknown fungal infection. They were excluded from our analyses.

Measurement of reproductive resource allocation 2 (destructive measurement)—The remainder of the plants from the experimental population were moved to forest understories, to a point ~300 m from the place where they had been grown, and used for destructive measurements, which might otherwise have produced artificial effects on the reproductive characters to be measured in the experimental population. We first examined biomass allocation patterns within a single flower. From each potted plant, we collected three floral buds that were just going to open. These buds were divided into three parts, perianth, stamen, and pistil, and these were then dried in a 60°C convection oven for at least 24 h, before being weighed to the nearest 0.1 mg.

Next, we measured the secretion rate and the concentration of floral nectar. After a worker of *Bombus diversus* visited a fully open flower, we immediately bagged the flower with an envelope of mesh to exclude visits by any more pollinators. One to two hours after the bagging, we took off the flower, recorded the bagging time, and immediately made a tiny hole at the end of its spur with a needle. Through this hole, the nectar in the spur was pushed out by blowing air through a 3 mm diameter silicon tube inserted into the entrance of the spur. The nectar

was extracted with 2- μ L microcapillary tubes from the side of the spur. We measured its volume with a digital caliper and its sugar concentration with a light refractometer BS-R70 (Bellingham & Stanley Ltd., Kent, UK). We examined the nectar from three flowers for each individual. These tasks were performed on sunny days in mid-September 1997, from 1300 to 1600 each day.

A nectar secretion rate was calculated by dividing the nectar volume by the bagging time. In this calculation, we assumed that *B. diversus* depleted all the nectar in the flowers. To confirm this assumption, we made another set of experiments with the potted plants. When a fully open flower was visited by *B. diversus*, it was immediately harvested and the nectar volume was measured by the above method. We examined ten flowers of each variety and found that the spurs of all of them were empty. This strongly supports the above assumption. For analyzing the energy cost of nectar secretion, the total mass of sugar in the nectar per flower and per day was determined from our measurements. Since the refractometer that we used reads as mass per total mass, its values were converted to mass per volume using the specific gravity of sucrose as listed in the CRC Handbook of Chemistry and Physics (1978–1979). Multiplying the sugar mass per volume by a nectar secretion rate, we obtained the total mass of sugars in the nectar per flower and per day. For the analysis presented below, we used individuals that had complete sets of data both regarding biomass allocation and nectar from three flowers.

Statistical analysis of resource allocation pattern—The differences in each reproductive character between the varieties was tested separately. Here, we were interested in genetic differentiations in the resource allocation pattern between the varieties. However, measurements of reproductive characters such as flower numbers can be expected to covary with the plant biomass, and so to identify which characters covary with the plant biomass, we calculated common regression coefficients with estimated plant biomasses as independent variables and individual averages of each measurement as dependent variables (Sokal and Rohlf, 1995, p. 493). In the model we used here, the two varieties shared a common regression coefficient while having distinct intercepts. Before calculating common regression coefficients, measurements of fruit set and concentration of nectar were angularly transformed to meet the assumption of normal distribution in regression. The significance of the common regression coefficients was examined by the *F* test. For reproductive characters where the common regression coefficients were significantly different from zero, differences between the varieties were tested by analysis of covariance (ANCOVA). ANCOVA enables us to exclude the effects of plant biomass from the results of our tests. In addition, assumptions of parallelism of the regression coefficients between the varieties were not rejected for any of the characters that were applied to ANCOVA in the present study. For reproductive characters where the common regression coefficients were not significantly different from zero, we tested their differences between the varieties with the Mann-Whitney *U* test. Throughout the study, we used two-sided levels of significance.

For each potted plant that was assigned to “nondestructive measurement,” we estimated per-flower averages of resource investments to males, females, and pollinator attraction by substituting variety averages of sugar secretion rates and dry masses of each floral part, which were measured as part of “destructive measurement.” Then, summing up the per-flower investments, total investment per flower was estimated for each potted plant. In the calculation, a male investment per flower equals the dry mass of a stamen; female investment per flower is $C (D \times E + F)$, where *C* is the fruit set, *D* is the seed set, *E* is the dry mass of a seed, and *F* is the dry mass of a pericarp; pollinator-attraction investment per flower is $L + I \times B$, where *L* is the dry mass of a perianth, *I* is the flowering days, and *B* is the sugar secretion rate of a flower per day. The resultant values were also applied to the above analysis to examine differentiation between the varieties.

We also calculated Pearson's correlation coefficients between the in-

dividual averages of reproductive characters within each variety. Reproductive characters that were measured as part of “nondestructive measurement” with the experimental population and those that were measured as part of “destructive measurement” with the other potted plants were analyzed separately. In addition, correlation coefficients between the flower number and estimated total investments per flower were calculated. For the characters of which common regression coefficient with plant biomass estimates as covariate was significant ($P < 0.05$), we used their residuals for our correlation analysis.

Observation of pollinator behavior—In both 1996 and 1997, we made two observation arrays within the vicinity of the Oike and Mt. Hane populations from which the seedlings of var. *hypophylla* and var. *microhypophylla* had been taken, respectively. Each of the observation arrays contained 32 potted plants in 1996 and 24 potted plants in 1997. Half the pots contained var. *hypophylla*, while the others contained var. *microhypophylla*. We arranged the plants of each variety into rectangular formation with 30-cm intervals and placed them side by side. Each year, we simultaneously observed pollinator visitation to all individuals in the two observation arrays for three successive days in the middle of their flowering period (19, 20, and 21 September 1996 and 1997). Observations were performed from 0700 to 1500, except for when it rained. Before each daily observation, potted plants were trimmed so that each individual plant had only three open flowers. We recorded the number of pollinators that entered the observation arrays and visited at least one flower, and identified their species. We also recorded the numbers of visits to each plotted plant, and calculated the visitation frequency for each individual plant on each of the days.

We calculated the visitation frequency for each day and for each individual, and applied nested-ANOVA with a mixed model (Sokal and Rohlf, 1995, p. 272) to them. The main effects in this model were the fixed effects of the varieties and its nested-term days. In addition, each day includes random nested-term individuals (error). The effect of variety was tested over days, and the effect of days was tested over individuals. For this analysis, visits by *Bombus diversus* and *Apis mellifera* were combined. Other observed insects such as members of the Sphingidae were omitted, because they were rarely observed and barely touched either the stigmas or the pollen.

Mating system estimation—To examine the mating systems of the varieties, we conducted electrophoretic analysis of allozyme for three natural populations of each variety. In late July 1996, we collected leaves from ~50 individuals of each population. The leaves were transported to our laboratory within 24 h and refrigerated for 3–5 d until electrophoresis was carried out. The eight enzyme systems resolved were as follows: aconitase (ACN) and malic enzyme (ME) using the number 2 buffer system of Soltis et al. (1983), and aspartate aminotransferase (AAT), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), phosphoglucomutase (PGM), phosphoglucose isomerase (PGI), and triosephosphate isomerase (TPI) using the number 8 buffer system. We followed the staining protocols of Soltis et al. (1983). Genotype frequencies of polymorphic loci were tabulated, and Wright's fixation index (denoted *F*) was calculated using the procedures of Wright (1977). Statistical significance of Wright's *F* were examined by chi-square test. We could not estimate the selfing rate of potted plants within the experimental population, because the polymorphic enzyme could not be stained with their seeds.

RESULTS

Pattern of reproductive resource allocation—Table 1A summarizes averages and standard deviations of “nondestructive measurement” for the experimental population and also shows common regression coefficients with the biomass estimates as covariate. The common regression coefficient of flower number was significantly

TABLE 1. Averages and standard deviations of measurements of the reproductive characters for the two varieties of *Impatiens hypophylla*. Common regression coefficients with the biomass estimates as covariate were also tabulated, and their deviation from 0 was tested. For those characters where the common regression coefficient was significantly deviated from 0, differences in the residual between the varieties are shown and these were tested by ANCOVA. For the other characters, differences in the averages between the varieties are shown and these were tested by Mann-Whitney *U* test.

A)		Biomass estimates (mg)		A		B		C		D		E		F	
Taxa and values	N			Flower number	Flower duration (d)	Fruit set ^a (fruit/flower)	Seed set (seed/fruit)	Dry mass of a seed (mg)	Dry mass of a pericarp (mg)						
<i>Var. hypophylla</i>	29	2458 ± 688		54.0 ± 21.4	2.6 ± 0.3	0.728 ± 0.167	1.61 ± 0.47	4.68 ± 1.74	6.11 ± 1.43						
<i>Var. microhypophylla</i>	23	2319 ± 721		65.5 ± 19.4	2.1 ± 0.3	0.733 ± 0.129	2.04 ± 0.46	3.94 ± 0.94	4.75 ± 0.93						
Common regression coefficients with biomass estimates as covariate				0.0182***	0.0000 ^{ns}	-0.0026 ^{ns}	-0.0002* ^{ns}	-0.0002 ^{ns}	-0.0005 ^{ns}						
Differences in residual or average between the varieties (<i>var. hypophylla</i> - <i>var. microhypophylla</i>)				-14.01**	0.52***	-0.005 ^{ns}	-0.40**	0.85***	1.36***						
B)		Biomass estimates (mg)		G		H		I		J		K		L	
Taxa and values	N			Dry mass of a stamen (mg)	Dry mass of a pistil (mg)	Dry mass of a perianth (mg)	Nectar secretion rate (μL/h)	Concentration of nectar (W/V) ^a	Sugar secretion rate (mg/d) [= J × K × 24]						
<i>Var. hypophylla</i>	19	3524 ± 1069		3.09 ± 0.48	0.28 ± 0.05	7.83 ± 1.18	0.33 ± 0.12	0.62 ± 0.3	4.83 ± 1.67						
<i>Var. microhypophylla</i>	19	3100 ± 939		1.99 ± 0.45	0.33 ± 0.06	4.49 ± 0.63	0.17 ± 0.07	0.63 ± 0.2	2.61 ± 1.04						
Common regression coefficients with biomass estimates as covariate				0.0001 ^{ns}	0.0000 ^{ns}	0.0000 ^{ns}	0.0000 ^{ns}	0.0000 ^{ns}	-0.0004 ^{ns}						
Differences in residual or average between the varieties (<i>var. hypophylla</i> - <i>var. microhypophylla</i>)				1.10***	-0.06*	3.35***	0.15***	-0.01 ^{ns}	2.23***						
C)		Biomass estimates (mg)		M		N		O		Total investment (mg/flower) [= M + N + O]					
Taxa and values	N			Investment to male (mg/flower) [= G]	Investment to female (mg/flower) [= CD × E + F]	Investment to attraction (mg/flower) [= I + B × L]									
<i>Var. hypophylla</i>	29	2458 ± 688		3.09 ± 0.48	9.12 ± 3.34	20.32 ± 1.51	32.49 ± 3.58								
<i>Var. microhypophylla</i>	23	2319 ± 721		1.99 ± 0.45	8.09 ± 2.55	9.89 ± 0.76	19.97 ± 2.82								
Common regression coefficients with biomass estimates as covariate				0.0001 ^{ns}	-0.0014*	0.0001 ^{ns}	-0.0008 ^{ns}								
Differences in residual or average between the varieties (<i>var. hypophylla</i> - <i>var. microhypophylla</i>)				1.10***	1.23 ^{ns}	10.44***	12.52***								

^a Common regression coefficients were calculated and tested using angularly transformed data.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns: not significant.

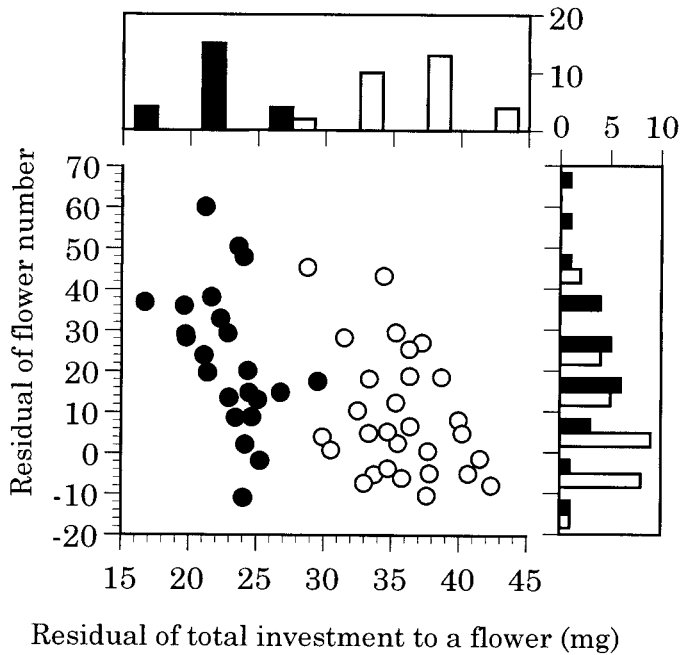


Fig. 1. Plot of the residuals of the regression of total investment to a flower on biomass estimates (x-axis) against the residuals of the regression of flower number on biomass estimates (y-axis), and histogram of each residual. Open circles and bars indicate var. *hypophylla*, solid circles and bars indicate var. *microhypophylla*. Variety *hypophylla* had significantly larger values in the residual of total investment to a flower ($P < 0.001$), but significantly lower values in the residual of flower number ($P < 0.01$) compared to var. *microhypophylla*. Pearson's correlation coefficients between the residuals were 0.36 ($P = 0.057$) in var. *hypophylla*, and 0.45 ($P < 0.05$) in var. *microhypophylla*.

larger than zero ($F_{1,49} = 30.8, P < 0.001$), while that of seed set was significantly smaller than zero ($F_{1,49} = 4.6, P < 0.05$). Taking the effect of biomass into consideration, var. *hypophylla* had significantly larger values for flower duration, dry mass of a seed, and dry mass of a pericarp than var. *microhypophylla*, while var. *hypophylla* had significantly smaller values for flower number and seed set. The distribution of flower number after removing the correlations with biomass estimates is shown in Fig. 1.

Table 1B shows results from the "destructive measurement." Since none of the common regression coefficients were significantly different from zero, all the characters were subject to the Mann-Whitney *U* test. Variety *hypophylla* had significantly larger values for dry mass of a stamen, dry mass of a perianth, nectar secretion rate, and sugar secretion rate, while having a significant smaller value for dry mass of a pistil than var. *microhypophylla*.

Table 1C shows the results of per-flower investments. Common regression coefficients were significantly smaller than zero in per-flower investment to females ($F_{1,49} = 5.8, P < 0.05$). Removing the effect of biomass by ANCOVA, var. *hypophylla* had significantly larger values in per-flower investments to males, pollinator attraction, and total investment to a flower. Distribution of the total investment to a flower after removing the correlations with biomass estimates is shown in Fig. 1.

Within each variety, there were significant correlations between reproductive characters after removing the effects of plant biomass (Table 2). Flower numbers were negatively correlated with seed set and dry mass of a pericarp in var. *hypophylla* ($P < 0.01$) and also negatively correlated with dry mass of a seed and that of pericarp in var. *microhypophylla* ($P < 0.01$). Dry masses of a pericarp were positively correlated with fruit set and seed

TABLE 2. Matrices of Pearson's correlation coefficients among reproductive characters across all individuals within each variety. Reproductive characters that were measured with the experimental population and those measured with other potted plants were analyzed separately. They are shown in (A) and (B), respectively. In both cases, correlation coefficients of var. *hypophylla* are presented in the upper right section, while those of var. *microhypophylla* are presented in the lower left section. The sample numbers of matrix (A) are 29 for var. *hypophylla* and 23 for var. *microhypophylla*. In matrix (B), the sample number is 19 for both varieties.

	Flower number ^a	Flower duration	Fruit set ^b	Seed set ^a	Dry mass of a seed	Dry mass of a pericarp
A)						
Flower number ^a		0.01	-0.16	-0.48**	-0.02	-0.55**
Flower duration	-0.05		-0.23	0.18	0.06	-0.11
Fruit set ^b	-0.10	-0.08		0.21	0.21	0.41*
Seed set ^a	-0.15	0.23	-0.02		-0.15	0.51**
Dry mass of a seed	-0.54**	0.23	0.61**	-0.08		0.12
Dry mass of a pericarp	-0.56**	0.41	0.26	0.62**	0.50*	
	Stamen	Pistil	Perianth	Nectar secretion rate	Concentration of nectar [†]	
B)						
Stamen		0.49*	0.14	0.30	-0.13	
Pistil	0.29		0.63**	0.22	0.03	
Perianth	0.44	0.35		0.39	-0.11	
Nectar secretion rate	0.28	0.45	0.14		-0.41	
Concentration of nectar [†]	0.07	-0.07	-0.06	-0.06		

^a Since common regression coefficients with the estimated biomass as covariate were significant, correlation coefficients were calculated using its residual.

^b Correlation coefficients were calculated using angularly transformed data.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

TABLE 3. Number of pollinator arrivals observed at two observation arrays over 2 yr. Observations were performed by two observers simultaneously on 19, 20, and 21 September in both 1996 and 1997. "Oike" and "Mt. Hane" are located within the vicinity of a natural population of var. *hypophylla* and var. *microhypophylla*, respectively.

Observation site	Visitor species	Observation year	
		1996	1997
Oike	<i>Bombus diversus</i>	8	51
	<i>Apis mellifera</i>	276	1
	Other species	0	15
	Total observation time (min)	(1395)	(1401)
Mt. Hane	<i>Bombus diversus</i>	258	183
	<i>Apis mellifera</i>	0	0
	Other species	0	23
	Total observation time (min)	(1282)	(1440)

set in var. *hypophylla* ($P < 0.05$ and $P < 0.01$, respectively), and also positively correlated with seed set and dry mass of a seed in var. *microhypophylla* ($P < 0.01$ and $P < 0.05$, respectively). Fruit set and dry mass of a seed were positively correlated in var. *microhypophylla* ($P < 0.01$). Among the reproductive characters that were measured by "destructive measurement," significant positive correlations were found only in var. *hypophylla*, between the stamen and the pistil ($P < 0.05$) and between the pistil and the perianth ($P < 0.01$).

Flower number and total investment to a flower were negatively correlated within each variety, after removing the effect of the estimated biomass (Fig. 1). Pearson's correlation coefficient of var. *hypophylla* was 0.36 ($P = 0.057$), while that of var. *microhypophylla* was 0.45 ($P < 0.05$).

Observation of pollinator behavior—In the Oike array, which was located within the vicinity of a natural population of var. *hypophylla*, both pollinator compositions and pollinator availability varied considerably between 1996 and 1997 (Table 3): the flowers were exclusively visited by *Apis mellifera* in 1996 and by *Bombus diversus* in 1997, and the number of pollinator arrivals was more than 5 times greater in 1996 than in 1997. In the Mt. Hane array, which was located within the vicinity of a natural population of var. *microhypophylla*, pollinator compositions and pollinator availability were relatively more stable (Table 3): the flowers were mainly visited by *B. diversus* in both years, and the number of pollinator arrivals was ~1.4 times greater in 1996 than in 1997.

Visitation rates of pollinators were significantly higher in var. *hypophylla* than in var. *microhypophylla*, except for the Oike population in 1997 (Table 4). However, the degrees of preference varied considerably between the two years and between the two observation sites. Furthermore, in the Oike array in 1996 and 1997, visitation rates also varied significantly among the three days of observation (Table 4).

Mating system estimation—Of eight enzymes examined in three natural populations of each variety, polymorphic loci were found in all the populations of var. *hypophylla*, but in only one of the populations of var. *microhypophylla* (Table 5). Wright's F indices did not significantly deviate from zero in any of the polymorphic loci of var. *hypophylla*, but they were significantly greater than zero in the Mt. Hane population of var. *microhypophylla* (chi-square test, $P < 0.001$; Table 5).

DISCUSSION

Trade-offs between flower number and the investment to a flower—Trade-offs between flower number and the investment to a flower in *Impatiens hypophylla* were detected by two different sources of observations. One source derived from a comparison of reproductive resource allocation between the varieties: var. *hypophylla* allocated greater amounts of biomass to a flower, yet it had fewer flowers than var. *microhypophylla*. Since the difference in flower size between the varieties is genetic, it is highly plausible that the size-number trade-off we found has a genetic basis. The other source of evidence was obtained from a comparison of reproductive resource allocation among individuals within each variety: there was a negative correlation between total investment to a flower and flower number within each variety. It remains uncertain whether or not this negative correlation also has a genetic basis.

Although further quantitative genetic studies are needed, the present study is the first to have detected trade-offs between flower number and investment to a flower. Thus, our finding clearly supported the theoretical framework of Sakai (1993), who stressed the importance of the size-number trade-off in the evolution of floral resource allocation.

Recently, it has become widely known that genetic correlations among pollen, ovule, and petal biomass are not negative but positive (see Fenster and Carr, 1997, for review), although such correlations could be expected un-

TABLE 4. Averages and standard deviations of the number of pollinator visitations to individual plants per hour, at two observation arrays over 2 yr. Their variance components were estimated and tested by nested ANOVA. Each individual plant had three open flowers. Observations were performed by two observers simultaneously on 19, 20, and 21 September in both 1996 and 1997. "Oike" and "Mt. Hane" are located within the vicinity of a natural population of var. *hypophylla* and var. *microhypophylla*, respectively.

Observation site	Observation year	Visitation rates (visits/hour)		Variance components of visitation rate (%)		
		Var. <i>hypophylla</i>	Var. <i>microhypophylla</i>	Among varieties	Among dates	Among individuals
Oike	1996	2.52 ± 0.47	0.12 ± 0.09	76.1**	3.2*	20.7
	1997	0.56 ± 0.22	0.71 ± 0.21	-36.3 ^{ns}	113.0***	23.3
Mt. Hane	1996	7.23 ± 1.71	4.65 ± 1.12	29.6*	3.9 ^{ns}	66.5
	1997	5.55 ± 0.70	1.98 ± 0.39	89.6***	-0.2 ^{ns}	10.6

* $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$; ns: not significant.

TABLE 5. Observed numbers of genotypes, their allele frequency, and Wright's *F* in natural populations of *Impatiens hypophylla*. Three natural populations of each variety were analyzed with eight enzyme systems, and only polymorphic loci were tabulated. Alleles "F" and "S" represent, respectively, relatively fast and slow anodally migrating alleles. Significance of Wright's *F* was determined by chi-square test.

Variety and population	Locus	Genotypes			Frequency of "F" allele	Wright's <i>F</i>
		FF	FS	SS		
Var. <i>hypophylla</i>						
Sindo (<i>N</i> = 50)	PGI	28	19	3	75%	-0.01 ^{ns}
Oike (<i>N</i> = 48)	PGI	38	10	0	90%	-0.12 ^{ns}
	MR	0	17	31	18%	-0.22 ^{ns}
Kamijo (<i>N</i> = 50)	PGM	30	18	2	78%	-0.05 ^{ns}
Var. <i>microhypophylla</i>						
Mt. Hane (<i>N</i> = 50)	ACO	35	9	6	79%	0.46**

** *P* < 0.01; ns: not significant.

less differences in total reproductive allocation are controlled. Based on these studies, Fenster and Carr (1997) suggested the need to examine the trade-off between pollen production and seed production. Actually, a few studies have shown a negative genetic correlation between pollen and seed productions of a flower (Rameau and Gouyon, 1991; Atlan et al., 1992). However, Devlin (1989) and Mossop, Macnair, and Robertson (1994) failed to find this, thus casting doubt on the generality of the trade-off between pollen and seed productions. In *I. hypophylla*, var. *hypophylla* had significantly heavier stamens, whereas investment to seed production per flower did not differ between the varieties (see Table 1C). Thus, there was no trade-off between pollen production and seed production of a flower. On the other hand, var. *hypophylla* produced significantly fewer flowers, indicating that there was a trade-off between flower number and pollen production of a flower. Within each variety, flower number was negatively correlated with seed and/or fruit mass. Based on these findings, we suggest that a trade-off between flower number and flower size is a more general picture of floral resource allocation than the trade-off between pollen and seed production of a flower, although observed trade-off between size and number does not negate the requirement for trade-off between genders (Houle, 1991). In future studies into floral resource allocation, the relationship between these two trade-offs will need to be more carefully examined.

Differentiation in investment to pollinator attraction per flower between the varieties—Compared to var. *microhypophylla*, var. *hypophylla* had larger perianths, larger floral duration, higher sugar secretion rate, and thus larger investment to pollinator attraction per flower. On the other hand, flowers of var. *hypophylla* were visited more frequently by effective pollinators than were those of var. *microhypophylla*. Since the pollinators preferred var. *hypophylla* even within the habitat of the var. *microhypophylla*, "pollinator's specialization" (Heinrich, 1979) in their native flowers does not explain the result. The pollinator-matching hypothesis predicts a positive correlation between perianth size and pollinator body size. However, var. *microhypophylla* with smaller flowers was exclusively visited by *Bombus diversus*, while var. *hypophylla* with larger flowers was visited by a smaller pollinator, *Apis mellifera*, in addition to *B. diversus*. Although more careful observations over several years are

desirable to determine pollinator species that the varieties depend on, a pollinator-matching hypothesis can hardly explain the observed association of var. *microhypophylla* and *B. diversus*. Thus, our observation supports only the pollinator-attraction hypothesis, which considers pollinator attraction to be the main selective force on the evolution of flower size.

A number of previous studies have shown that obligatory outcrossing species have a larger flower size than selfing or mixed mating species (Schoen, 1982; see Brunet, 1992, for reviews). Although these earlier studies never empirically measured the degree of pollinator attraction of the flowers (but see Inoue, Maki, and Masuda, 1995), the observed patterns have been interpreted as evidence of a positive correlation between the outstanding rate and the amount of investments to pollinator attraction per flower. However, most of these studies had several drawbacks. First, some studies compared several species that were not randomly sampled due to a phylogenetic relationship (Doust and Cavers, 1982; Cruden and Lyon, 1985). To test the significance of the correlations observed, we need to use recently developed methods of interspecific comparison in which phylogenetic relationships are taken into consideration (Harvey and Pagel, 1991). Secondly, some of the other studies compared species that were visited by different pollinator species (Spira, 1980; Inoue, Maki, and Masuda, 1995). In this case, the mating system would not be the only factor determining the evolutionary outcomes of investment to pollinator attraction, since different pollinator species are likely to exhibit different preferences for floral attractive structures (Stanton et al., 1991; Inoue, Maki, and Masuda, 1995; Conner and Rush, 1996). By comparing a pair of closely related taxa that were visited by common pollinator species, our study clearly showed the existence of a positive correlation between the mating system and investments to pollinator attraction per flower.

Evolutionary causes of floral differentiation between the varieties—In the present study, we showed that relative allocations to males and pollinator attractive functions per flower were greater for var. *hypophylla* than for var. *microhypophylla*. This difference between two varieties of *I. hypophylla* agreed with the general pattern of differentiation in reproductive resource allocation within a flower that is found among animal-pollinated hermaphrodites: predominantly outcrossing species have a greater

allocation to males and pollinator attractive functions per flower (see Brunet, 1992, for review), than selfing or mixed-mating species.

A number of theoretical applications have attempted to explain this general pattern of differentiation in floral resource allocation. Charnov (1982) employed local mate competition theory (LMC) to examine the evolutionarily stable allocation to male and female structures of hermaphroditic plants. He predicted that the evolutionary stable allocation ratio to male function increases with the outcrossing rate and claimed that the prediction from LMC is supported by empirical evidence. However, his model did not predict the evolutionarily stable resource allocation to attractive structures in the absence of the a priori rule of their gender. Lloyd (1987b) and Charlesworth and Charlesworth (1987) constructed an evolutionary stable resource allocation model that treats attractive structures separately from male and female functions. They predicted that an evolutionary stable allocation to males and attractive structures increases with the outcrossing rate, when probabilities of pollen being picked up and of ovules being fertilized increase with the resource amount allocated to attractive structures. These predictions correspond with the general patterns of reproductive resource allocation of hermaphroditic plants, including the varieties of *I. hypophylla*.

Sakai (1993) developed a model with some new assumptions, including a trade-off between flower number and reproductive allocation to a flower. His model predicted that evolutionarily stable allocation to male function increases with the outcrossing rate, while an evolutionarily stable investment to pollinator attraction per flower is independent of the outcrossing rate. Both generally observed patterns of resource allocation of hermaphroditic plants and our results agreed with his prediction regarding the allocation to male function, but did not agree with his prediction regarding the investment to pollinator attraction per flower. This finding indicates that the conditions assumed in Sakai's model are insufficient to explain the evolution of floral resource allocation.

Sakai (1993) suggested that evolutionarily stable allocation to pollinator attraction per flower could depend upon the outcrossing rate, if resource allocation per flower is constrained to some level, if male fertility depends on the product of male and attractive returns, or if pollen and ovule productions are nonlinear functions of male and female allocation. In addition to these possibilities, we suggest that geitonogamy could be another factor causing the observed pattern of resource allocation: when the model of Sakai (1993) is modified so that the number of ovules fertilized per visit and the quantity of pollen exported to other individuals during a pollinator visit become decreasing functions of the flower number of an individual plant, evolutionarily stable allocation to pollinator attraction per flower increases with the outcrossing rate. This hypothesis is supported by some recent observations regarding several plant-pollinator systems. It has been shown that an increase in the flower number of any individual gives rise to negative effects on the efficiencies of pollen export and pollen donation to other individuals, because geitonogamous pollination increases with the flower number (de Jong, Waser, and Klinkhamer, 1993; Harder and Barrett, 1995; Snow et al., 1995).

Of course, Sakai's suggestion and ours are not mutually exclusive. In future studies, the relative importance of these hypotheses will need to be evaluated by both theoretical and empirical approaches. Also, for precise examination of the model of Sakai (1993) in *I. hypophylla*, we would need to estimate the degree of inbreeding depression and the relationships between investment to pollinator attraction and the reproductive return from male and female functions of the varieties.

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