Sexual Dimorphism in *Catasetum* Orchids: Forcible Pollen Emplacement and Male Flower Competition

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Orchids of the Neotropical genus *Catasetum* have sexually dimorphic flowers; that is, male and female flowers are distinctly different in shape and color. Male flowers forcibly attach a large pollinarium onto euglossine bees. *Euglossa* bees leave the male flower in response to pollinarium emplacement by *Catasetum ochraceum* and subsequently avoid male but not female flowers. This evidence suggests that sexual dimorphism promotes pollination. The aversion of the bee to pollinarium attachment and its avoidance of male flowers thereafter apparently reflect competition among male flowers that probably evolved concurrently with sexual dimorphism. Among *Catasetum* species, the extent of dimorphism is correlated with the degree of apparent aversion caused by the male flower (weight of the pollinarium). Thus, aversive pollinarium emplacement can account for widespread sexual dimorphism in *Catasetum* and for interspecific variations in its expression.

FLOWERS OF THE NEOTROPICAL ORCHID genus *Catasetum* are sexually dimorphic (Fig. 1). Plants are typically unisexual (*1*), and the sexes are so different that they once were assigned to different genera (*2*). Female flowers in closely related species are morphologically similar, whereas male flowers usually are quite different (*3*). *Catasetum* flowers produce no nectar and are pollinated exclusively by male euglossine bees, especially *Euglossa* and *Eulaema*, that collect aromatic hydrocarbons and volatile terpenes (such as cineole) (*4*). The strong sexual dimorphism of these orchids and the greater interspecific differences among males are apparently related to their interactions with euglossine bees and to competition among male flowers.

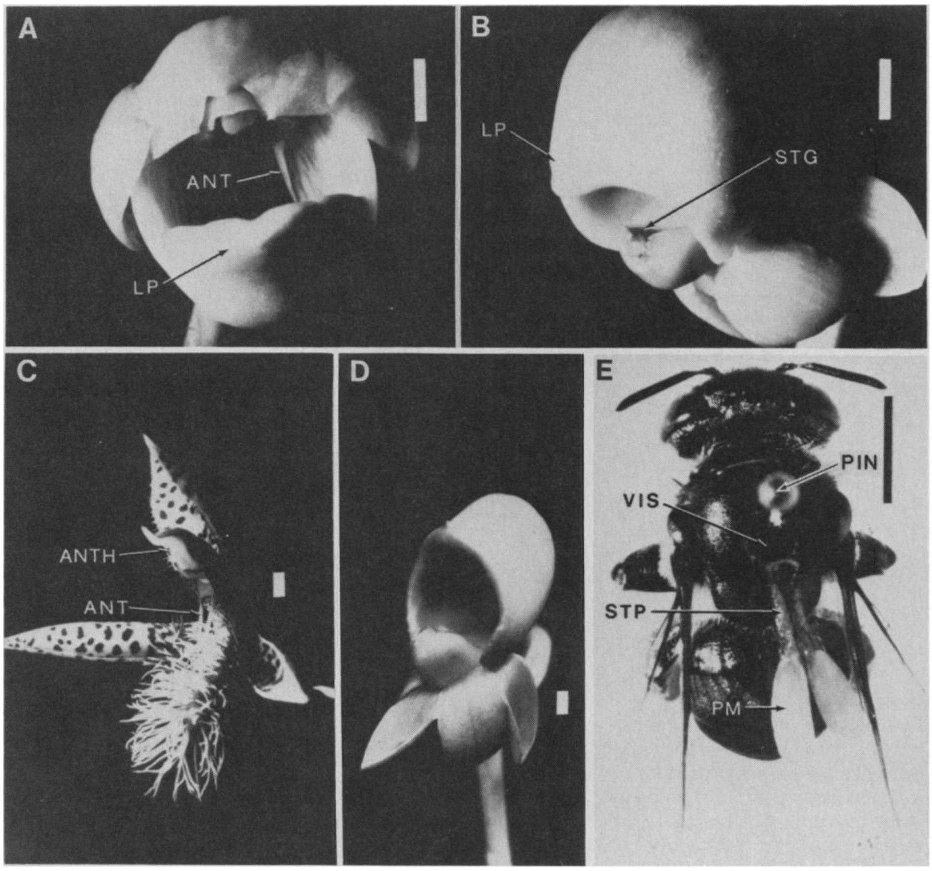

Fig. 1. (A to D) Sexual dimorphism in *Catasetum* from Puerto Ayacucho, Venezuela: (A) *C. ochraceum* male (a "hooded" male flower), (B) *C. ochraceum* female, (C) *C. barbatum* male (an "open" male flower), (D) *C. barbatum* female, and (E) bee with pollinarium. The single pollinarium of each male flower includes two pollen masses, an adhesive viscidium, and an elastic stipe that is stretched taut during the flower's development (*1*). The entire pollinarium and the anther are expelled when the tension on the stipe is released (*1, 4*). Abbreviations: ANT, antenna; ANTH, anther covering the pollen masses; LP, lip; PIN, head of a pin; PM, pollen mass; STG, stigmatic cleft; VIS, viscidium; and STP, stipe. Scale bar, 5 mm.

Euglossa gaianii and *E. modestior* were observed foraging at terrestrial *Catasetum ochraceum* plants near Puerto Ayacucho, Venezuela (*5*). A bee typically hovered at a male flower (Fig. 1A), landed right side up on the flower's hooded lip, collected chemical attractants from inside the lip with the front legs, and then hovered while transferring the chemicals to the hollow hind legs. After several such sequences, the bee fully entered the lip and pushed the flower's antenna (Fig. 1A), triggering the rapid ejection [at up to 323 cm/sec in *C. fimbriatum* (*6*)] of the pollinarium and anther cap (Fig. 1). These struck the bee's dorsum where the pollinarium adhered by the viscidium (Fig. 1E). The anther cap later dried and dropped. Discharged male flowers wilted within a day. In their approach, bees behaved similarly at female flowers. However, as female flowers are inverted relative to male flowers (Fig. 1B), the bees foraged upside down inside the females' lips. Pollination could occur when a pollinarium-bearing bee (loaded bee) exited the female upside down. In this position the pollinarium would pivot down and a pollen mass could lodge in the stigmatic cleft (Fig. 1B) (*1, 5*).

Bees responded negatively to pollinarium attachment. We observed 13 *C. ochraceum* pollinarium emplacements. The bees left immediately in seven cases, left after unsuccessfully trying to remove the pollinarium in five, and died after wing-fouling in one (*5*). In contrast, no bees ceased foraging at male flowers before pollinarium emplacement (except after aggressive interactions among bees). Thus, emplacement was aversive. The aversive stimulus was the forceful emplacement of the pollinarium rather than another change in the male flower, as naïve bees (bearing no pollinaria) sometimes foraged at recently discharged male flowers.

The aversion of loaded bees to male flowers affected subsequent foraging. Loaded bees rarely landed on male *C. ochraceum* flowers but foraged as freely at females as at cineole baits (Table 1). Further, although naïve bees usually triggered male flowers, none of the 86 loaded bees had fully entered a second male flower as none bore two pollinaria (*7*). In contrast, the bees had had many chances to visit male flowers and inflorescences: the mean daily floral sex ratio was 1.6 males per female (*8*), and the sea-

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sonal inflorescence ratio was 4.5 males per female (*9*). If loaded bees had visited flowers randomly, many would have visited a second male flower and would have borne two pollinaria. Hence, avoidance of male flowers by loaded bees must have been pronounced.

Since loaded bees avoided male but not female flowers, sexual dimorphism could have promoted pollination. Conspecific male and female *Catasetum* flowers produce the same chemical attractants (*4*), and, although loaded bees seldom landed on male *C. ochraceum* flowers, they approached flowers of both sexes at similar rates (*10*). Consequently, the discrimination against male *C. ochraceum* flowers by loaded bees was based on short-range, nonchemical cues, probably on visually perceived differences in shape and color. Moreover, bees apparently learned from a single emplacement to recognize and avoid male flowers.

Forcible attachment (*11*) of a large pollinarium apparently led bees to avoid males of a second species of *Catasetum*: 21 *Eulaema cingulata* caught at chemical baits had visited at least one male *C. pileatum* flower (each bore one pollinarium), 8 of these had apparently visited a female flower (they bore a stipe with one or both pollen masses missing), but apparently none had visited a second male (none bore two pollinaria).

The extent of dimorphism in *Catasetum* species in southern Venezuela varied with visiting bee size and with the weight of the pollinarium load discharged onto the bees (Table 2). For *C. ochraceum*, the only moderately dimorphic *Euglossa*-pollinated species, the relative load weight was distinctly less than for the strongly dimorphic *Euglossa*-pollinated species (10% versus 17 to 23% of bee weight). Similarly, the least dimorphic (sexes alike in coloration) of the *Eulaema*-pollinated species had the smallest relative load (10% versus 18 to 24%). Finally, *C. pileatum*, the only strongly dimorphic *Eulaema*-pollinated species had the greatest load weight for any species, both absolutely (Table 2) and relatively (24% of bee weight) and was the only species in which the pollinarium struck the bee in flight (*5*). Thus, for orchids served by a given genus of bees, the extent of dimorphism increased with load size. However, for *Catasetum* species pollinated by the larger-bodied *Eulaema* relatively greater loads were associated with a given level of dimorphism than *for Catasetum* pollinated by the smaller *Euglossa*. The bees of different genera apparently reacted differently to loads of a given size. In fact, *Eulaema* sometimes continued foraging among the hooded males of *C. maculatum* after a pollinarium was attached and, at other times, bore three pollinaria from the hooded males of *C. macroglossum*, whereas *Euglossa* never continued foraging after pollinarium discharge by the hooded males of *C. ochraceum* or the open males of *C. barbatum* (*12, 13*). Thus, aversive emplacement can also account for interspecific variations in the intensity of sexual dimorphism in *Catasetum*.

Table 1. Percentage of bees bearing *C. ochraceum* pollinaria (= loaded) and total number of bees foraging at different sources. *Euglossa* collecting chemical attractants were counted with binoculars for 1 of every 15 minutes from 0800 to 1200 on 20 days at cineole-saturated cotton wicks and from 0900 to 1100 on 5 days for each of 20 male and 20 female inflorescences in July and August 1984. Only *C. ochraceum* was in flower. ($\chi^2 = 31.48$, $P < 0.005$ for male versus female.)

Source	Bees	
	% Loaded	Total no.
C. ochraceum inflorescences		
Male	1.3	389
Female	10.8	592
Cineole baits	8.2	147

Cycnoches, the only other orchid genus typically having unisexual flowers (*14*), is closely allied to *Catasetum* and also has large, somewhat forcibly emplaced pollinaria (*15*) and male flowers that are interspecifically more distinct than those of females. *Euglossa*-pollinated *Cycnoches* species generally are more sexually dimorphic than *Eulaema*-pollinated *Cycnoches* species (*4, 13*). The selective factors inducing sexual dimorphism in *C. ochraceum* apparently also operate in *Cycnoches*.

Competition among male flowers may explain the origin of this system. Both genera have unisexual flowers, environmentally determined sex, and, typically, strongly male-biased sex ratios (*1, 5*). Pollination in both depends on positioning a pollinarium so that a pollen mass lodges in the stigmatic cleft; the cleft swells shut within minutes after pollination (*1, 5*). Consequently, each female flower usually receives pollen from only one male flower. This and the male-biased sex ratio have led to strong competition among male flowers (*16, 17*) that is intensified by the likelihood of mechanical interference by more than one pollinarium on the same bee. For *C. ochraceum*, we estimate that the viscidium (Fig. 1E) of a second pollinarium would mechanically interfere with the first pollinarium one-third to two-thirds of the time (*18*). This interference contrasts with the previous reports of prepollination competition in which pollen donors compete by producing more pollen or advertising it better (*19*). Competition for the chance to pollinate female flowers that are fewer in number and that accept only one pollen mass and the potential for interference by an additional pollinarium both make it advantageous for a male flower to discourage visits to subsequent male flowers by aversive emplacement. However, aversion by loaded bees would also cause them to avoid female flowers unless appreciable sexual dimorphism exists or evolves concurrently. The correspondence between relative load and degree of dimorphism suggests concurrent evolution.

The greater divergence of male flowers may also reflect interactions with pollinators. Euglossine bee life-spans (*17, 20*) exceed those of *Catasetum* inflorescences (*1, 5, 17*) and overlap the flowering seasons of more than one *Catasetum* species (*5*). Bees foraging on female flowers may learn to orient themselves upside down to collect fragrances more efficiently. Since an upside-down position is required for pollination, they would coincidentally become more effective pollinators. Increased effectiveness could select for nondivergence or convergence among female flowers of different species. However, loaded bees that avoid male flowers of one species may also avoid male flowers of similar species. This aversion would select for divergence from earlier blooming species visited by the same bee (*21*); consequently male flowers should diverge more than female flowers. Note that

Table 2. Pollinator size, extent of sexual dimorphism, and load* for *Catasetum* species from southern Venezuela. Values are means ± 95% confidence interval. One-tailed Fisher's exact test (*24*) ($P < 0.04$) was conducted on the number of species for level of dimorphism by pollinator taxon.

Pollinators		*Catasetum* dimorphism			
		Moderate dimorphism: hooded male flowers		Strong dimorphism: open male flowers	
Taxon	Live weight (mg)	No. of species	Load (mg)	No. of species	Load (mg)
Euglossa spp.	93.2 ± 3.4	1	9.6 ± 0.4	5	16.2 ± 0.9 to 21.7 ± 1.5
Eulaema cingulata†	615.1 ± 30.8	5	64.3 ± 1.6 to 129.7 ± 0.6	1	149.4 ± 2.1

*Mean wet weight of pollinarium and anther immediately after discharge. †The only frequent pollinator of these six species.

neither learning by bees of how to forage at female flowers nor male divergence in form and color will reduce natural hybridization (*22*). Reproductive isolation in these orchids apparently depends primarily on differences in fragrances (*23*) and flowering times (*5*) rather than on form.

Since it should be advantageous for male flowers to discourage subsequent visits to other males in any animal-pollinated species having unisexual flowers, similar evolutionary processes should occur in other plants.

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7. An obvious stipe persists after pollination.
8. July–August 1984 ($\chi^2 = 6.64$, $P < 0.01$); outlying plants not included were all males.
9. Some wild *C. ochraceum* plants changed sex (some plants with male flowers bore only female flowers the next year and vice versa) yielding a ratio of 30 male per 1 female inflorescence in 1985 (*5*).
10. *Euglossa* hovering within 10 cm of a flower were counted from 0900 to 1100 on 1 day for ten male and ten female inflorescences in July 1984. Four of 48 approaching males were loaded as were 2 of 34 approaching females ($\chi^2 = 0.176$, $P > 0.5$).
11. Forcible emplacement and attachment of pollinarium is characteristic of *Catasetum*, except for the *C. discolor* alliance (*1*).
12. D. H. Janzen, personal communication concerning *C. maculatum* in Costa Rica; Romero (*5*) for *C. ochraceum*; G. Carnevali, personal communication concerning *C. barbatum* in Venezuela.
13. C. H. Dodson and P. G. Frymire [*Mo. Bot. Gard. Bull.* **49**, 133 (1961)] for *C. macroglossum* in Ecuador.
14. Some *Mormodes* species have unisexual flowers (*1*).
15. The viscidium (Fig. 1E) is "violently" discharged onto the bee, thus attaching the pollinarium and anther cap (*4*).
16. The high pollen-donation success suggested for *Catasetum maculatum* in Costa Rica [D. H. Janzen, *Biotropica* (suppl.) **13**, 77 (1981)] is numerically impossible in populations with strongly male-biased sex ratios (*1, 5*).
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18. Calculated as the area within which a second viscidium would contact the first pollinarium divided by the area within which most pollinaria strike.
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21. Selection on simultaneously blooming males would be more complex (*5*) and might augment variability.
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Estrogen-Induced Factors of Breast Cancer Cells Partially Replace Estrogen to Promote Tumor Growth

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The hormone 17β-estradiol acts through its receptor system to induce MCF-7 human breast cancer cells to form tumors in athymic mice. In vitro studies have identified the production of estrogen-induced growth factors from MCF-7 cells that may have a role in growth control. These induced growth factors were sufficient to stimulate MCF-7 tumor growth in ovariectomized athymic mice, thus partially replacing estradiol. Growth factors may act as estrogen-induced "second messengers" in estrogen-responsive growth of human breast cancer.

BREAST CANCER, ONE OF THE MOST prevalent of all cancers, is characterized by marked hormonal control of its growth in many species. In women, a high proportion of primary breast cancers contain the estrogen receptor and require 17β-estradiol (E_2) or other estrogenic activities for tumor growth. Past and current therapies have been directed toward interruption of estrogen action by ovariectomy (or hypophysectomy) or the use of antiestrogens such as tamoxifen. However, antiestrogen resistance has limited these therapeutic approaches (*1*). Several estrogen-responsive cell lines have been isolated; MCF-7 and ZR-75-1 are among the most widely studied (*2*). In vitro, the growth of these cell lines and the release of several major proteins (including proteases such as plasminogen activator), are estrogen-stimulated (*3*). In one in vivo model system, the athymic (nude) mouse, formation of tumors by MCF-7 cells is completely dependent on estrogen stimulation (*4*). We and others have recently presented evidence that growth factor activities, some of which are estrogen-induced, are released by these cell lines into their serum-free conditioned medium (*5–7*). We have identified one of these, a 30-kilodalton transforming growth factor α (TGFα)–like activity that binds to the epidermal growth factor (EGF) receptor (*6*). Insulin-like growth factor I (IGF-I) is also released by MCF-7 cells, but its regulation by estrogen is much less marked (*6, 7*). The aim of this study was to determine if an estrogen-induced growth factor (or factors) is sufficient to replace estrogen itself in induction of tumor formation by MCF-7 cells in vivo in the nude mouse.

Initial experiments were carried out to compare growth-promoting activities of serum-free conditioned medium from MCF-7 cells (CM) with CM obtained from MCF-7 cells that had been treated with E_2 (CME_2). Acid dialysis of CME_2 samples, a typical extraction procedure in the initial isolation of many growth factor activities, allowed complete (>99.98%) removal of residual E_2. This was demonstrated by the addition of ^{125}I-labeled E_2 (10^7 dis/min; New England Nuclear) to three CME_2 samples. After concentration and acid extraction only 2×10^2 to 2×10^3 dis/min remained in the medium. Removal of E_2 was further verified by bioassay [acid-extracted CME_2 was infused into athymic nude mice as in Table 1; no stimulation of uterine wet weight was observed and E_2 was <25 pg/ml blood by radioimmunoassay (*8*)]. Acid-extracted CM preparations were used in all experiments of the current study, both in vitro and in vivo. CM from E_2-treated MCF-7 cells contained sufficient growth-promoting activity to reduce MCF-7 cell-doubling times in vitro from 40 to 21 hours (Table 1, A). In contrast, CM from untreated MCF-7 cells was only weakly growth-stimulating, reducing MCF-7 cell-doubling time to 33 hours.

We then tested the biological activity of CM and CME_2 samples for their ability to stimulate the development of MCF-7 cell implants into tumors grown in ovariectomized athymic mice. Highly concentrated extracts of CME_2 were nearly as active as E_2 itself over a 14-day treatment period in inducing tumor development (Table 1, A). The CME_2 sample induced an average of one tumor per animal (or 25% incidence, since four separate fat pad sites were implanted with MCF-7 cells). This was a significant incidence of tumor induction as compared to the CM group ($P < 0.05$ by χ^2 analyses). On histologic analysis of tumors

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