

DYNAMIC NECTAR REPLENISHMENT IN FLOWERS OF *PENSTEMON* (SCROPHULARIACEAE)¹

MARIA CLARA CASTELLANOS,^{2,4,5} PAUL WILSON,³ AND
JAMES D. THOMSON^{2,4}

²Department of Ecology and Evolution, State University of New York, Stony Brook, New York 11794-5245 USA; and ³Department of Biology, California State University, Northridge, California 91330-8303 USA

Plants that experience variation in pollinator visitation rates or fluctuations in weather conditions may be expected to have evolved homeostatic mechanisms that regulate their nectar offerings, thereby providing a more constant reward to the pollinators. A limited degree of such nectar homeostasis is reported here for *Penstemon*. First, nectar removal stimulates replenishment: when nectar was removed hourly for 6 h from *P. speciosus*, twice as much nectar was secreted cumulatively as when nectar was removed only at the beginning and end of the same 6-h period. Second, replacing artificial nectar in the nectaries of *P. speciosus* prevents replenishment. Third, the hummingbird-adapted *P. barbatus* made more nectar before leveling off than the bee-adapted *P. strictus*. Our work and previous studies with other plants imply mechanisms for dynamic regulation of nectar offerings, at least within broad limits. We speculate about the proximate physiology underlying this behavior and its evolutionary significance.

Key words: homeostasis; nectar replenishment; nectar resecretion; nectary; *Penstemon*; pollination; Scrophulariaceae.

The chemical composition of floral nectar, and the dynamic control of its secretion, should affect the reproductive success of plants visited by nectar-seeking pollinators. Its availability and quality can affect a pollinator's decision to visit or not to visit a flower and its behavior while visiting (Gass and Sutherland, 1985; Pyke, Day, and Wale, 1988; Thomson, 1988; Real and Rathcke, 1991; Hodges, 1995). This, in turn, may affect pollen removal from anthers or pollen deposition on stigmas (Mitchell, 1993; cf. Cresswell, 1999). Moreover, producing nectar involves at least some cost to the plant in terms of photosynthates and water that could otherwise be allocated to making more or healthier anthers or seeds (Pyke, 1991). These benefits and costs, we imagine, have been balanced by natural selection in varying ways for different species of plants (Zimmerman, 1988). For example, flowers differing in their pollination syndromes also often differ in the amount, concentration, and composition of the nectar that they produce. In particular, species adapted for hummingbird pollination produce more nectar that is more dilute and with a higher sucrose:hexose ratio than do congeners adapted for hymenopteran pollination (Baker and Baker, 1983). Based on studies of other species of plants, floral nectar is usually more concentrated than phloem sap and contains much more hexose, further indicating that the nectaries are not merely passive secretory organs (Baker, Hall, and Thorpe, 1978; Durkee, 1983). By extension, we expect that the different secretion profiles of related species arise from regulatory processes that constitute adaptations to the different suites of pollinators with which they covary.

Furthermore, ideal nectaries should be able (at least in a

crude way) to homeostatically regulate their nectar offerings by refilling nectar reservoirs after nectar has been removed or by readjusting the concentration of sugar as water evaporates. Fluctuations in pollinator abundances and weather conditions would make an inflexible nectar secretion schedule less apt than a plastic schedule could be. Without regulation, unvisited flowers would eventually fill to overflowing. Indeed, when flowers are caged, nectar can accumulate noticeably in some species (e.g., in *Impatiens capensis*; P. Wilson, personal observation), but such accumulation might be less than one would expect from continuous unregulated secretion. Here we demonstrate dynamic regulation of nectar replenishment in *Penstemon* flowers that have been drained. In addition, we show that *Penstemon* species vary in the volume at which nectar replenishment ceases and in the time it takes for nectar volume to level off.

Penstemon flowers are particularly appropriate for this type of study. Many species can experience impressively high levels of pollinator traffic. For example, *Penstemon strictus* flowers can routinely receive >100 bumble bee visits per day (Williams and Thomson, 1998). The pollination system is geared to such high visitation rates because pollen is released from anthers very gradually, and a large number of visits is beneficial to successfully moving pollen grains from anthers to stigmas (Thomson et al., 2000). Another feature of the genus is that some species are adapted for pollination by bees, whereas other species are adapted for pollination by hummingbirds (Straw, 1956; Clinebell and Bernhardt, 1998; Lange, Scobell, and Scott, 2000). Because nectar secretion profiles covary with pollination syndrome (Baker and Baker, 1983), we can study the adaptive divergence of the nectar regulatory mechanism. Finally, most *Penstemon* inflorescences have a convenient architecture for experimentation. At each flowering node, two matched cymes are produced opposite one another, so it is easy to find flowers that are closely paired. One flower can then be subjected to one treatment and the other can serve as a control. Although adjacent flowers do not necessarily share the same vascular supply, they do experience the same microclimate, and they are formed in the same position in the inflorescence.

The nectaries of *Penstemon* are on the outer bases of the

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⁴ Current address: Department of Zoology, 25 Harbord St., University of Toronto, Toronto, Ontario, Canada, M5S 3G5.

⁵ Author for reprint requests (e-mail: mcastel@zoo.utoronto.ca).

two lateral filaments. This position appears to be an innovation in the lineage leading up to the genus: plants in most of the related genera, such as *Keckiella*, have their nectaries at the base of the ovary (Straw, 1966). The positioning of the nectaries, one to either side of the staminode, is mechanically important: it makes a pollinator probe to one side of the staminode and then retract its body before probing into the other side (Walker-Larsen and Harder, 2001). In effect, the nectar is in two narrow tubes, functionally like nectar spurs, that channel the pollinator's movement. Such recessed nectaries have been claimed to promote floral diversification in other groups (Hodges, 1997). The secretory cells of *Penstemon* nectaries consist of densely packed, short trichomes (Straw, 1966), reminiscent of glandular hairs found on the inflorescences. In some species of *Penstemon*, the base of the ovary is slightly differentiated as a nonfunctioning remnant of the ancestral nectary (Straw, 1966). Thus, it seems the lineage leading up to *Penstemon* evolved a new kind of nectary and lost what was the ancestral nectary.

Nectar replenishment has been studied to a limited extent in *Penstemon* and many other plants. Cruden, Hermann, and Peterson (1983) worked on several Mexican species of *Penstemon*. They reported that, in the bee-adapted *P. gentianoides*, the amount of sugar contained in floral nectar decreased during the night and increased during the day in bagged flowers, implying nocturnal reabsorption of unconsumed nectar. They also suggested that removal of nectar stimulates replenishment in *Penstemon*, and they showed that substantial nectar is replenished in *P. kunthii* within 1 h after the nectaries have been emptied. Such a phenomenon has been found, to a limited extent, for many other plants. In *Heliconia imbricata*, removing nectar four times over 6 h increased secretion by 244%, compared to removing it once at the end of the same time period (Gill, 1988). In *Blandfordia nobilis*, removing nectar from flowers on four consecutive days produced 265% as much sugar as was produced by flowers bagged until the fourth day (Pyke, 1991). By removing nectar on four consecutive days, Navarro (1999) increased secretion in *Macleania bullata* flowers to 147% of the amount obtained by collecting once at the end of 4 d. Other studies showing that nectar replenishment can be stimulated by removal include those by Raw (1953); Feinsinger (1978); Galetto, Bernardello, and Juliani (1994); and Torres and Galetto (1998).

Our studies have improved on most past studies because of innovations in the way we handled nectaries. Most studies of nectar replenishment have used glass microcapillary tubes to remove nectar a few times, often as infrequently as once per day. With glass there is always a danger of damaging the nectary (Willmer, 1980). We therefore used paper wicks (McKenna and Thomson, 1988; Thomson, McKenna, and Cruzan, 1989) that allowed us to remove nectar more frequently, mimicking high pollinator visitation rates.

We have established that the amount of nectar removed by a wick can be determined by measuring the linear distance that the wick is moistened. The amount of sugar in the wick is determined with the anthrone reaction and spectrophotometry. Another improvement in technique, we believe, is to use mechanical micropipettes with minute plastic tips to add artificial nectar to nectaries. The plastic seems less likely to harm the nectary than the traditional steel-needled syringe.

In this paper, we explore the possibility that *Penstemon* flowers dynamically replenish nectar. We start with some descriptive floral biology of a focal species, *Penstemon specio-*

sus. Then we show that hourly removal of nectar stimulates replenishment of both water and sugar. Next we show that this replenishment can be inhibited by manually refilling the flowers with artificial nectar. Finally, we compare replenishment in the hummingbird-adapted *Penstemon barbatus* and the bee-adapted *Penstemon strictus*.

MATERIALS AND METHODS

Floral scheduling in *Penstemon speciosus*—Most of our data were gathered on *Penstemon speciosus* Lindley (Scrophulariaceae) in the southern Sierra Nevada of California at ~2000 m above sea level. This species has large vestibular flowers, with a mean length of 3.5 cm, that are mostly purple, with a white throat and a vestibule floor that is white with purple veins. The flowers are visited by several species of Hymenoptera (e.g., *Osmia*, *Pseudomasaris*) that visit right side up for a combination of nectar and pollen. Most *Bombus* individuals visit upside down, buzzing pollen out of the anthers. At some sites, hummingbirds also visit the flowers, although they do not remove much pollen, so we think they are relatively unimportant as pollinators. The concentration of nectar in *P. speciosus* is ~20–25% as measured by a refractometer from flowers that opened in bags. This is unusually low for a mostly hymenopteran-pollinated *Penstemon* (Thomson et al., 2000).

Like other species of *Penstemon*, *P. speciosus* has protandrous flowers. We established the scheduling of floral events in case there proved to be differences in nectar secretion between male and female phases. Between 27 June and 2 July 1999, when there was no rain and had not been for many days, we followed 52 flowers from bud to corolla detachment. Eight sequential stages were recognized: (1) corolla closed, (2) corolla opening, (3) forward anthers dehiscing, (4) rear anthers dehiscing, (5) the tip of the style bent noticeably but <45°, (6) style bent >45°, (7) style bent >90°, and (8) corolla abscised. In *Penstemon*, style bending coincides with stigmas becoming receptive (Chari, 2000). We wished to determine how long flowers stay on a plant and how long they take to reach each stage. All flowers were scored at 0700, 1100, 1500, and 1900 each day until stage 8 was reached. To keep this paper brief, we will present results as number of intercensus intervals, with the understanding that development can be faster during some of the intervals than others.

Baseline nectar production survey—Because variation in flower developmental stage could potentially cause variation in nectar secretion patterns, we wished to relate developmental stage to nectar accumulation in unvisited flowers. We were also interested in the amount of variation between pairs of flowers at a node relative to variation among plants. We selected 40 plants over 4 d (23–26 July 1999) under dry weather conditions. Pairs of flowers on each plant were tagged with paper clips, and the inflorescence was covered with a bridal veil bag. On 27 July, we unbagged one plant at a time and measured the volume of nectar that had accumulated using 5- μ L microcapillary tubes and measured the concentration of that nectar using a refractometer calibrated for small quantities. Developmental stage was also scored from stages 1 through 6.

Evaporation effect—The volume and concentration of nectar present in a flower can be affected by evaporation (Corbet and Delfosse, 1984). To judge the magnitude of this effect independent of the regulating activity of the nectaries, we carefully placed a 5- μ L drop of a 22% sucrose solution between the base of a ventral filament and the corolla in much the same position as the nectar drops occupy relative to the lateral filaments. We did this to 31 flowers, making sure that the added nectar was not in contact with the nectaries. After 6 h, the artificial nectar was recovered with microcapillary tubes and the concentration was determined with a refractometer.

Nectar removal experiment, single vs. multiple collections—To investigate the effect of repeated emptying of nectaries on nectar secretion, we removed nectar hourly from one flower at a node and compared the amount of nectar we extracted over 6 h to the amount of nectar found in a paired flower at the same node that was allowed to accumulate nectar undisturbed over the same

6-h period. The experiment was replicated for flowers that were 1 d old ($N = 21$), 2 d old ($N = 17$), and 3 d old ($N = 20$). The work was done from 27 June to 3 July 1999, when there had not been any precipitation for many days. To prepare the flowers, we tagged pairs of unopened buds with paper clips, marked the inflorescence with label tape, and covered it with bridal veil bags to prevent visits by nectarivores. After 1, 2, or 3 d, all nectar in both flowers of a pair was removed using paper wicks (Whatman electrophoresis wick material) at ~ 1000 . We used a razor blade guided by a steel jig to cut strips 1.5 mm ($SD = 0.14$) wide and >30 mm long. Wicks were inserted into the nectaries to either side of the staminode and left there for 3 min. Pilot studies had shown that a wick placed in the correct position soaks up all the nectar in a flower such that dissection of the flower reveals only traces of moisture so small as to be unmeasurable by wicks or microcapillary tubes. Most nectar was usually absorbed by the first wicks. Nevertheless, a second set of wicks was inserted to be sure that all the nectar was removed. The length that each wick was moistened was measured to the nearest 0.5 mm with a ruler. We converted these measurements to microliters using a calibration curve for a 25% sucrose solution.

After all the nectar was removed from both flowers, one flower of each pair was randomly designated the "experimental" flower, and all the nectar was again removed from it hourly during the following 6 h, with rebagging between nectar extractions. The cumulative amount of nectar removed (not counting the initial emptying) was used in many analyses. The second flower was designated the "control," and it was not emptied again until the sixth hour. At that time, we recorded the developmental stage of the flowers and dissected both flowers to assure that all nectar had been absorbed by the final wicks. We used insect pins to mount all wicks for air drying. Later in the laboratory, the sugars in each wick were dissolved in 1 mL of boiling water and 2.0 mL of anthrone reagent, and then absorbance was measured at 540 nm with a spectrophotometer (McKenna and Thomson, 1988). Absorbances were then converted to moles of hexoses using a calibration curve constructed with a series of standards of known sugar concentration.

We will refer to amounts of nectar and sugar that are "replenished." By this term we mean the net production of nectar, or what Búrquez and Corbet (1991) called the "apparent secretion rate." At a cellular level, molecules might be moving in both directions, from inside to outside of plant cells and vice versa, and reabsorption might be taking place at a higher rate in control flowers (Corbet and Willmer, 1981). Here, we are only concerned with measuring the net production that is presented to pollinators.

Nectar addition experiment—Pairs of *Penstemon speciosus* flowers buds were marked and bagged, and nectar was removed using paper wicks not later than 24 h after the flowers opened. To the "experimental" flower of each pair, we added 5 μL of a solution of 22% glucose (w/w). We selected this concentration because it was the median concentration found in nectar of a sample of 1-d-old flowers. The "control" flower was left empty. Both flowers were immediately bagged, and 3 h were allowed for nectar replenishment. After that, nectar was recovered using microcapillary tubes, and the concentration was estimated with a refractometer. The experiment was done between 10 and 14 July 1999. Unfortunately, there was rain on 10, 11, and briefly around noon on 12 July. Many of the flowers were in wet bags before the experiment, and some were rained on during the experiment. This undoubtedly added to the results variance that would not have existed under the more usual dry conditions of California summers. It may also have caused the nectar to be less concentrated than normal.

Comparative refilling of *P. barbatus* and *P. strictus*—*Penstemon barbatus* (Cav.) Roth and *P. strictus* Benth. are two closely related species with morphologically different flowers. *Penstemon barbatus* produces a 1-m flowering stalk. The red flowers have a long narrow corolla tube ~ 30 mm long and 6 mm in diameter. The flowers are inclined downward in the inflorescence, and the anthers are exerted out of the corolla tube. *Penstemon barbatus* flowers are visited by hummingbirds. The purple-blue flowers of *P. strictus* have a broader corolla tube of 8–10 mm at their widest diameter. They are more or less horizontal in orientation, and they are visited by bees at very high rates (up to 100 visits \cdot flower $^{-1}$ \cdot d $^{-1}$) (Williams and Thomson, 1998). Nectar

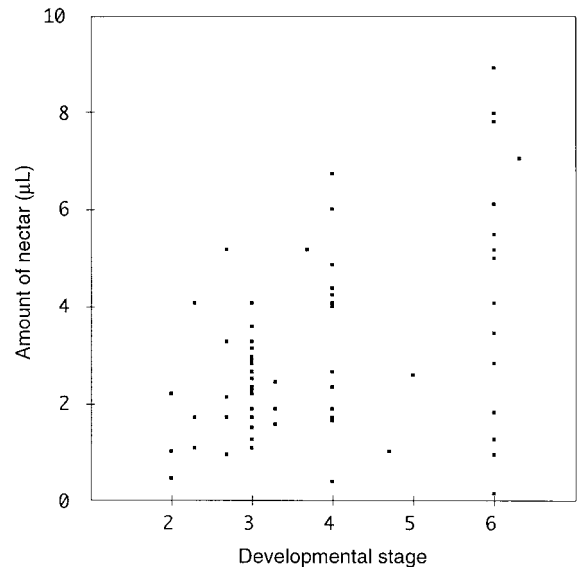


Fig. 1. The relationship between developmental stage and nectar volume for 80 flowers, two from each of 40 plants of *Penstemon speciosus*.

refilling was studied in 1998 for *P. strictus* and in 1999 for *P. barbatus*, in potted plants held indoors at the Rocky Mountain Biological Laboratory, Gothic, Colorado, at 2990 m asl. At various times, flowers were emptied of nectar with paper wicks. We resampled 44 *P. strictus* flowers and 43 *P. barbatus* flowers at intervals ranging from 12 to 500 min after the initial draining.

RESULTS

Floral scheduling in *Penstemon speciosus*—On average, it took 16.6 intercensus intervals (roughly 4.2 d) for flowers to go from just opening to falling off ($SE = 0.32$, $N = 52$). The percentage of a flower's life that was spent going from just opening to the forward anthers dehiscing was 5%, from just opening to rear anthers also dehiscing was 9%, from just opening to the style tip bent $<45^\circ$ was 30%, from just opening to style bent $>45^\circ$ was 38%, and from just opening to style bent 90° was 45% (for all values $SE \approx 1\%$). The last 55% of the life of a flower showed no outward signs of development, although with high visitation rates almost all of the pollen would have been removed from anthers by stage 6, and upon becoming receptive, stigmas also would have quickly become fully loaded.

Nectar production census—We studied variance in nectar production among 40 pairs of bagged *P. speciosus* flowers that varied in their developmental stage. An ANOVA showed that 42% of the variance was among plants; therefore, 58% was between flowers at a node on a plant. For 111 flowers, nectar volume and concentration were uncorrelated (Pearson's $r = -0.191$, $P = 0.09$). Furthermore, there was no significant correlation between concentration and developmental stage ($r = 0.061$, $P = 0.59$). There was, however, a significant relationship between the amount of nectar accumulated in a flower and the developmental stage ($r = 0.491$, $P < 0.001$). The scatterplot showed a triangular bivariate distribution (Fig. 1): early stages had small amounts of nectar, while older flowers ranged from having small amounts to having much larger quantities.

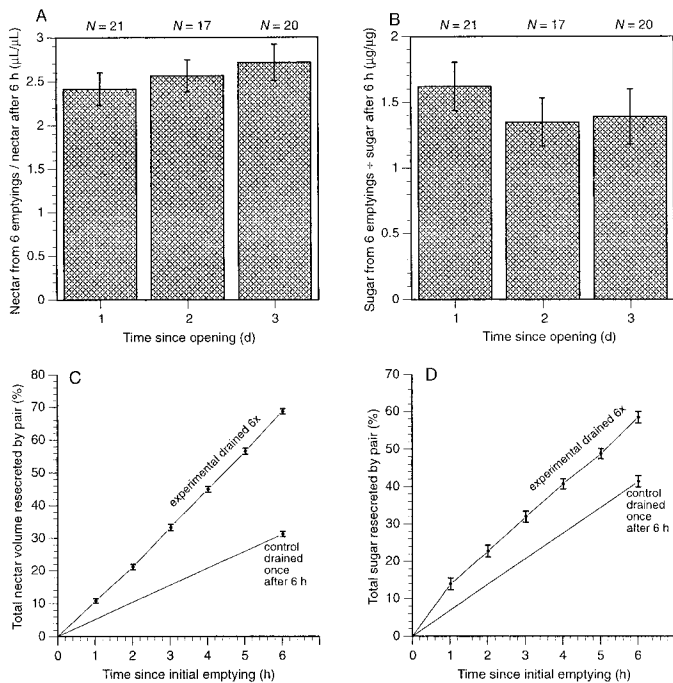


Fig. 2. The volume of nectar (A and C) and amount of sugar (B and D) replenished in flowers with six hourly removals compared to 6 h of accumulation in control flowers. Neither volume nor sugar significantly depended on how old the flowers were, as shown in (A) and (B). Therefore, in (C) and (D) the different age classes of flowers are pooled, with each datum representing the cumulative amount of nectar removed from a flower divided by the total amount of nectar replenished by both flowers collectively at the end of 6 h. The height of each bar thus represents the amount of additional secretion achieved by draining the flower repeatedly instead of only once. Notice that after ~ 3 h the flowers with hourly removal produced as much nectar as was found in the control flowers after 6 h and after 4 h had produced as much sugar as secreted by the controls. Values are means ± 1 SE.

Evaporation effect—Artificial nectar turned to syrup in 6 h. For most of the flowers we studied, the added nectar had evaporated to the point where it would not be easily drawn into a microcapillary tube and was more concentrated than the refractometer could measure, i.e., $>50\%$ sugar. Assuming that real nectar experiences similar rates of evaporation, nectaries must be continually adding water to keep the concentration in the range that we observed.

Nectar removal experiment, single vs. multiple collections—The cumulative amount of nectar produced in the flowers emptied six times ($6\times$) exceeded that in “control” flowers after the same 6 h. For first-day, second-day, and third-day flowers, there was uniformly about twice as much nectar produced in the flowers with hourly removal (Fig. 2A). Paired t tests were highly significant for each flower age (all $P < 0.001$). A single-classification ANOVA on the ratio of cumulative nectar volume in $6\times$ -emptied flowers divided by nectar in once-emptied flowers revealed no effect of flower age ($F_{2,55} = 0.63$, $P = 0.54$). In the initial removal, the mean amount of nectar in the flowers was $11.00 \pm 0.783 \mu\text{L}$ (mean ± 1 SE; $N = 116$). The mean amount of nectar in the once-emptied flowers after 6 h was $9.91 \pm 0.391 \mu\text{L}$ ($N = 58$), while the mean cumulative amount of nectar in the $6\times$ -emptied flowers after the same period of time was $24.11 \pm 0.964 \mu\text{L}$ ($N = 58$). The same trend was significant for the total amount of sugar re-

plenished (Fig. 2B), although the gain was not quite as high as for volume. Sugar values in two of the 58 pairs were extreme outliers that probably reflected some problem in the anthrone assay. We removed these pairs from the statistical analyses. (This was conservative, in that these points would have exaggerated the treatment effects that we report here. These pairs were not removed from Fig. 2D; deleting them would change the figure imperceptibly.)

The mean (± 1 SE) amount of sugar in the nectar at the initial removal in all flowers was $2369.1 \pm 181.02 \mu\text{g}$ ($N = 112$). Six hours after that, the once-emptied flowers had produced on average $1163.0 \pm 96.16 \mu\text{g}$ ($N = 56$), while the flowers that had experienced hourly removal had secreted a cumulative mean of $1538.38 \pm 76.42 \mu\text{g}$ of sugar ($N = 56$). Again, an ANOVA showed no differences among flower ages ($F_{2,55} = 1.03$, $P = 0.36$), and a paired t test for all flowers confirmed that the cumulative amount of sugar in “visited” vs. control flowers was significantly higher ($t = 2.8$, $P = 0.006$). The mean ratio of cumulative sugar amount in nectar in experimental flowers vs. control flowers was 1.46 ± 0.6 . This mean excludes two flowers that were extreme outliers. If those two flowers are included, the mean becomes 2.36 ± 5.6 .

A good way to visualize the data is to plot curves of cumulative nectar removed expressed as a percentage of the total nectar replenished from both flowers taken as a pair (i.e., nectar removed after the initial removal). Figure 2C shows the graph for nectar volume and Fig. 2D for sugar. Notice that after ~ 3 h the experimental flowers had produced about as much nectar as was found in the once-emptied flowers at the end of the experiment, and after ~ 4 h they had secreted as much sugar. Also, on these graphs, there is no suggestion of any deceleration such as would be due to nectary exhaustion. Assuming nectaries experience similar rates of evaporation to the ones seen in our evaporation study, they must be continually adding water to keep the concentration within the narrow range that we observed.

Nectar addition experiment—In analyzing the data from the nectar addition experiment, our null hypothesis was that replenishment would be unaffected by the artificial nectar we had put in the flowers. For the amount of nectar, we subtracted the $5 \mu\text{L}$ that we had injected from what they contained after 3 h. To calculate a paired t test, we then took the difference between this value and the amount of nectar found in the paired control flower. On average, the experimental flowers had $0.88 \mu\text{L}$ less nectar than control flowers (SE = 0.267, paired t test $P < 0.022$, $df = 37$; Fig. 3A). We did similar calculations on the amount of sugar. First, we calculated the amount of sugar we found after 3 h in each flower. For this measurement, we used Table 88 in the *CRC Handbook of Chemistry and Physics* (CRC, 1978) to convert refractometer readings into sugar mass (see Bolten et al., 1979, for explanation). Next, we subtracted the amount we had added, $119.9 \mu\text{g}$ (the sugar present in $5 \mu\text{L}$ of a 22% w/w sucrose solution). Then, we calculated the difference between the “experimental” flowers and the “control” flowers in a pair. On average, the experimental flowers had secreted $43.68 \mu\text{g}$ less sugar than their paired controls (SE = 4.206, paired t test $P < 0.001$, $df = 37$; Fig. 3B). In fact, the experimental flowers had significantly less sugar in them than we had injected (one-sample t test $t = 4.04$, $df = 37$, $P < 0.001$; Fig. 3B), indicating that some of it must have been absorbed.

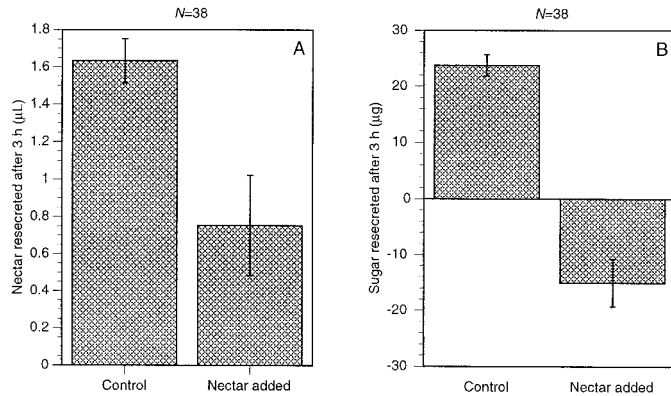


Fig. 3. Nectar secreted after addition of 5 μL of 22% sucrose artificial nectar to experimental flowers compared to that secreted by untouched paired control flowers. (A) Mean \pm 1 SE volume of nectar secreted after 3 h. The value for the experimental flower (right bar) was calculated by subtracting the 5 μL added. (B) Mean \pm 1 SE amount of sugar in nectar secreted. After 3 h, less sugar than that added was found in experimental flowers. $N = 38$.

Comparative refilling of *P. barbatus* and *P. strictus*—To estimate replenishment parameters, we fit hyperbolic tangent functions to refilling data for each species: $\text{NECTAR} = a \times \tanh[b \times \text{TIME}/a]$. This function provides a conveniently fit model for a saturating function where a is the asymptote up to which nectaries fill, and b is the initial slope of the line out of the origin. Therefore, we can obtain separate estimates and standard errors for the initial refilling rate and the “full” mark. NECTAR was in units of millimeters of wick moistened, and TIME in minutes (Fig. 4). The hummingbird-adapted species *P. barbatus* had an asymptote of $a = 15.98$ (SE = 1.127), whereas the bee-adapted *P. strictus* had an $a = 2.192$ (SE = 0.166). In other words, the bird flower refills its nectaries to a greater volume than the bee flower ($t \approx 12.1$, $df = 85$, $P < 0.05$). These species also seemed to differ in the rate of secretion just after emptying, with $b = 0.127$ (SE = 0.013) in *P. barbatus* and $b = 0.024$ (SE = 0.0057) in *P. strictus* ($t \approx 7.2$ with, $P < 0.05$). If we scale the initial refilling rate for each in terms of the asymptote (the “full load”) for that species (i.e., b/a), *P. strictus* actually begins filling more quickly, in the sense of approaching fullness 1.38 times faster. It is also of importance to the pollinators that it took 186 min for *P. barbatus* to become 90% full compared to 135 min for *P. strictus*.

DISCUSSION

We interpret nectary function in *Penstemon* as follows. Around the time the flower opens, nectaries begin secreting. If unvisited, they fill up in a few hours. Once full, secretion slows down to not much more than replacing evaporative loss, although this is done with imperfect fidelity. When nectar is removed, the nectaries are quickly stimulated to refill themselves. In *P. speciosus*, this takes ~ 3 –4 h. The ability to replenish nectar persists throughout the life of the flower, including male and female phases, until the corolla abscises. The various species of *Penstemon* have probably diverged adaptively to replenish at different rates and fill up to different levels. The differences between *P. barbatus* and *P. strictus* are consistent with the view that the regulatory machinery of nectaries has diverged adaptively; the quick but shallow replenishment of *P. strictus* is consonant with its high rate of visi-

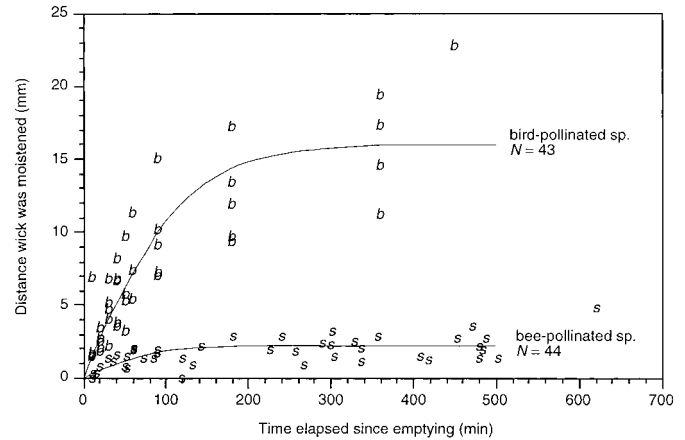


Fig. 4. Nectar refill curves for *Penstemon barbatus* (b) and *P. strictus* (s). Nectar was sampled from different flowers at different time intervals after an initial emptying. Curves fitted to points as hyperbolic tangents.

tion by bees, which in turn is necessary for the plant to reap the maximum possible fitness gains from its more gradual schedule of pollen presentation (Thomson et al., 2000). Of the other species studied to date, bee-adapted species quickly replenish a small amount of concentrated nectar, and hummingbird-adapted species refill their nectaries to a higher level with more dilute nectar (P. Wilson, unpublished data). There is some suggestion that hummingbird-adapted species may produce nectar during periods of the day just before birds are active (Cruden, 1972; Cruden, Hermann, and Peterson, 1983). It seems that *Penstemon* nectaries have ample ability to respond to the rate at which nectar is being consumed by nectarivores and to encourage pollinators to continue visiting many times over the course of a flower's life.

Our study and other studies of replenishment—This study offers some refinements that most previous studies lack. First, our experimental flowers were paired to controls, allowing us to account for flower-to-flower and plant-to-plant variation. Although we have chosen to emphasize that these flowers have “behavioral” mechanisms for regulating their nectar offerings, this emphasis should not obscure the great variation among flowers and among plants in a population (Hodges, 1993). Second, we sampled on a fine time scale that seems ecologically relevant for *Penstemon* flowers. Although hourly nectar removal does not approach the very high rates experienced by some *Penstemon* species in natural conditions, it comes closer than the schedules in many other studies. For example, Pyke's (1991) tripling of nectar production came from removing nectar only once per day. How much more nectar might have been produced if the flowers had been emptied at realistic rates? Similarly, Raw (1953) removed nectar in *Rubus idaeus* and *R. fruticosus* twice every 24 h, and Galetto and Bernardello (1992) removed nectar twice a day.

By removing nectar, we stimulated replenishment of sugar as well as the amount of fluid. This has not been the case in all other studies. Galetto, Bernardello, and Juliani (1994), after removing nectar two to three times over a 24-h period, observed that flowers produced a higher volume than controls, but the cumulative amount of sugar produced was not significantly higher in flowers with repeated removal. Likewise, Gautian, Navarro, and Gautian (1995) found that nectar re-

removal once a day increased the total volume secreted by a flower but not the amount of sugar, presumably because renewed nectar was more dilute. We found that sugar was replenished more slowly than water (compare Fig. 2C with Fig. 2D), but some sugar replenishment was stimulated by removal. Other studies, such as Navarro's (1999), have also increased secretion without changing concentration.

Nectar removal does not stimulate replenishment in all plants. Some studies have found no effect of removal on nectar production (Pleasants, 1983; Gryj, Martinez del Rio, and Baker, 1990; Galetto and Bernardello, 1993). Others have found that removal inhibits further secretion, as in *Nicotiana* (Galetto and Bernardello, 1993) and *Justicia* (Corbet and Willmer, 1981). In the protandrous *Ligaria cuneifolia*, nectar removal reduced the rate of nectar production in male-phase flowers but not in female-phase flowers (Rivera, Galetto, and Bernardello, 1996). One could argue that for some plants, once a flower has had its nectar removed by a pollinator, it has probably had its pollen removed. In such a case, halting further nectar production would save calories and water (Aizen and Basilio, 1998). This argument would seldom apply to *Penstemon*, because even hummingbird-pollinated flowers seem to have pollen removed over the course of many visits. It certainly would not apply to bee-adapted species like *P. strictus* and *P. speciosus* that have narrowly dehiscent anthers. At any rate, we can imagine situations in which nectar secretion might be aptly halted by nectar removal, pollen removal, or the germination of pollen grains on a flower's stigma.

Nectar production in many species often depends on developmental stage (Devlin and Stephenson, 1985; Klinkhamer and de Jong, 1990). In *Alstroemeria aurea*, Aizen and Basilio (1998) found that male-phase flowers secreted 3.1 times more sugar than female-phase flowers. These authors gave an adaptive explanation based on sex-phase differences in the need for pollination by bumble bees: 3.1 is close to 3.3, which is the number of pollinator visits it takes to saturate male function (12.0) divided by the number it takes to saturate female function (3.6). In *A. aurea*, protandry is synchronous, i.e., all the flowers on a ramet pass through male and female phase together. Presumably, they appeal to pollinators as a unit, so the attraction hypothesis is plausible. In contrast, Thomson, McKenna, and Cruzan (1989) found no consistent differences in sugar output between male- and female-phase flowers of *Aralia hispida*, another bumble-bee-pollinated plant that shows several waves of synchronous protandry. Thomson (1988) showed that bees develop persistent spatial memories for rewarding *A. hispida* plants, so that nectar secreted during one sexual phase also enhances visitation in the succeeding phase. The argument of Aizen and Basilio may be too simple if pollinator responses are integrated over more than one sexual phase. In *Penstemon*, male- and female-phase flowers are intermingled, so nectar in a female-phase flower may actually be serving male function in an adjacent flower even after its own stigma has been saturated. Therefore, there is little reason to expect an association between nectar production and sexual phase. Indeed, 1-, 2-, and 3-d-old flowers resulted in 6-h nectar replenishment curves that are remarkably invariant and linear (Fig. 2C–D).

Proximate mechanisms and evolutionary strategies for nectar replenishment—The field is open for future research on both the proximate physiological mechanisms that regulate nectar secretion and the ultimate evolutionary reasons why

plants present various patterns of secretion. Existing studies are tantalizing but too scattered and incomplete to draw firm conclusions. Still, they invite speculation. Most studies on nectar replenishment, ours included, do not go much further than showing whether or not replenishment is stimulated by nectar removal. The data also often suggest that there is some regulation of both the amount of nectar and the concentration. In some cases, very careful regulation has been demonstrated, for example in plants producing nectar exposed to very dry air (Nicolson, 1993). There are hints that sugar can be dynamically reabsorbed (Cruden, Hermann, and Peterson, 1983; Nicolson, 1995; Rivera, Galetto, and Bernardello, 1996; present study). It would be most interesting to do a series of nectar-addition experiments refilling nectaries with artificial nectar of various concentrations for various time intervals (as in Findlay, Reed, and Mercer, 1971, but with flowers still attached to plants). Although evaporation would present a complication, such experiments could help show how sugar is regulated independent of water. For instance, if a large amount of dilute artificial nectar stimulated nectaries to add sugar but not water while a small amount of concentrated artificial nectar stimulated nectaries to reabsorb sugar and add water, then one might conclude that the nectaries can separately regulate concentration and volume.

What might be the cellular basis for such homeostatic nectar secretion? Sugar secretion in nectaries probably occurs via direct membrane transport or secretion of endoplasmic reticulum or Golgi-derived vesicles from symplast to apoplast (Fahn, 2000). Removal of nectar in our experiments is analogous to sugar retrieval from the apoplast of sink tissues in plants. Removal of sugars in sink tissues increases the water potential of the sink apoplast and, therefore, the subsequent movement of water into it (Patrick, 1997). In addition, nectaries ought to have "sugar sensing" mechanisms for regulating the concentration of nectar. As in other plant tissues, sugar secretion could happen passively following the concentration gradient, while regulation of concentration in the apoplast could be achieved by sucrose hydrolysis (which would maintain the gradient and allow further secretion; Fahn, 2000). Active reabsorption of sugars may also occur (Bieleski and Redgwell, 1980). A mechanism for reabsorption has not been elucidated, but it could be a response to changes in cell turgor (Wyse, 1986), which in turn responds rapidly to changes in osmolality (Patrick, 1997). It is also possible, however, that the regulation happens before secretion, if sugar moves from symplast to apoplast by facilitated or active transport (Robards and Stark, 1988). To explain the response of flowers to nectar removal, as well as the differences between insect and hummingbird-adapted flowers, we also must postulate some mechanism for regulating the amount of nectar present in a flower. Receptors, "sugar sensing" or other, might be located at the appropriate place in the flower to stop nectar production in a manner analogous to a float valve.

Turning to evolutionary causes, we postulate that there has been ongoing adaptation in the regulatory mechanism. For *Penstemon centranthifolius*, hummingbirds have been shown to prefer individuals with more nectar, and at least under garden conditions there is significant heritability for nectar accumulation during the first day after the flower opens (estimated heritability $h^2 = 0.38$ for nectar volume and $h^2 = 0.37$ sugar: Mitchell and Shaw, 1993; Mitchell, Shaw, and Waser, 1998). Thus, there are reasons to think that nectar production rates can evolve under the action of natural selection. It seems

the same must be true of the “full mark” that nectaries fill up to and the speed with which they refill themselves after being emptied. Otherwise, we would not have found differences between closely related bird- and bee-syndrome flowers.

One adaptive reason for not secreting nectar on a fixed schedule is conservation of water and energy when pollinators are scarce. Our study shows that using only 24-h nectar accumulation would underestimate costs to the plant under some natural conditions. If one were interested in calculating the energy invested in nectar, which has been shown to be an important sink of photosynthates in some plants (Southwick, 1984), it would be important to take into consideration that visitation can determine the amount expended (Pyke, 1991). Additionally, even if replenishment in response to visitation is taken into account, measuring only volumes of nectar is not a good estimation of the sugar invested by the plant in nectar. In *Penstemon*, when visitors are abundant, nectar costs must be substantial, with scores of flowers each secreting several micrograms of sugar in tens of microliters of water.

The way in which selection acts on nectar secretion may greatly depend on the type of pollination system. Imagine a species in which anthers and stigmas mature rapidly and are usually pollinated during a short period of time, such as shortly after dawn. Such a plant would gain nothing from replenishing its nectar, since the stigmas of the day would already have been pollinated. Several factors could select plants away from this extreme. Replenishing at least the water in nectar could be favored when plants are subject to low pollinator visitation rates in environments where nectar tends to evaporate. Replenishment of sugar and water may be favored if male or female reproductive success increases with an extended number of pollinator visits or number of probes by the pollinator (Mitchell, 1993). Thus, species such as *Penstemon strictus* dose out pollen to visitors in small amounts (Thomson et al., 2000; M. C. Castellanos, unpublished data), and one would expect a matching schedule for nectar replenishment (Thomson, McKenna, and Cruzan, 1989). Finally, secreting small quantities of nectar with time between emptying and replenishment may encourage pollinators to revisit plants while keeping low the rate of geitonogamy (Harder and Barrett, 1996).

Conclusions—Our study and others show some degree of homeostasis in nectar production, and such homeostasis can plausibly be adaptive. However, this homeostasis is almost certainly imprecise. For several reasons, we doubt that natural selection acting through pollinator behavior and pollination success will be consistent enough to produce flowers that precisely maintain volumes and concentrations in standing crops of nectar. First, the physiology and anatomy of nectaries constrain the speed of nectar replenishment. Immediate refilling would seem to require some kind of storage bladder or vesicles that could squeeze out a new aliquot of prepared nectar after a visit, but this is not how nectaries work. Second, even if such complex mechanics were obtainable, it would not be advantageous for a flower to refill immediately, because then flower-feeders would learn to wait at the flower for more nectar. Some refractory period is necessary (Feinsinger, 1978). Third, a regulation system that precisely buffered nectar levels against not only abrupt removals by animals but also against temperature, evaporation, water stress, light stress, etc., would presumably be metabolically expensive. Fourth, the nectar level that is optimal with respect to influencing pollinator behav-

ior would depend on the number and the developmental stages of flowers on a whole plant or even the patch of plants, whereas nectar secretion dynamics would presumably be determined within each flower module. Fifth, pollinators' responses to a given nectar level surely vary with the species of animal, the satiety of the individual forager, and the availability of food in other plants. Sixth, pollinators' choices and movements depend not only on nectar but also on pollen, as well as environmental factors unrelated to flowers. Given all these considerations, it is not surprising that some flowers sometimes overflow with nectar, that others sometimes dry up, or that nectar is sometimes very scanty or dilute (Bertsch, 1983; Corbet and Delfosse, 1984; Boose, 1997). Indeed, it may be more surprising that nectar frequently is maintained more or less at a steady state of quantity and concentration (Zimmerman, 1988 for review; Corbet, Unwin, and Prs-Jones, 1979; Pleasants, 1983; Southwick and Southwick, 1983; Rathcke, 1992; Wyatt, Broyles, and Derda, 1992). Such regulation, even if sloppy, poses proximate and ultimate questions that we have only begun to examine.

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