Appendix A from M. C. Castellanos et al., "Anther Evolution: Pollen Presentation Strategies When Pollinators Differ"

(Am. Nat., vol. 167, no. 2, p. 000)

Measurement of Detailed Pollen Dispensing Schedules from Single Anthers

To study pollen presentation by anthers in the most realistic way, it would be ideal to have actual pollinators visit the flowers, but it is not feasible to have them visit naturally at regular intervals and to later count the pollen grains on their bodies that came from a particular visit. Earlier trials with dead honeybee thoraxes provided a relatively "natural" removal of pollen, but it was extremely difficult to count grains distributed throughout the deep pubescence of those complex three-dimensional structures. The velvet method used here might not remove pollen grains in a way identical to that of a bee's body or a bird's head, but it provides a standardized way of measuring pollen presentation in all types of flowers.

The velvet method uses acetate velvet fabric cut into 4 mm × 4 mm squares (or smaller squares for flowers with narrow corollas). The velvet material consists of a woven backing with soft acetate fibers protruding at right angles to form the velvet's pile. The fibers are soluble in acetone, but the backing is not. We use fine forceps to insert the velvet pieces into the flower, brushing the anther once going in and once again pulling out. We attempt to always use the same amount of pressure, touching the anthers just firmly enough with the velvet that they move slightly but perceptibly upward. The velvet square with pollen on it is immediately put on a microscope slide; two drops of a solution of basic fuchsin in acetone are added to stain the pollen and to dissolve the velvet nap. After the acetone evaporates, a drop of melted glycerin jelly is added, and a glass cover slip is pushed down on it until the jelly cools (Kearns and Inouye 1993). Our jelly is also stained with fuchsin and contains phenol as a preservative. We later count all the grains on the slides at 100 × with a compound microscope equipped with a computer-controlled motorized stage.

For the samples reported here, a different piece of velvet was brushed against the anther every half hour from the time the anther started dehiscing in the morning until 2200 hours. Examples of the cumulative release of pollen are shown in figure A1. At the end of the sampling period, the anther was picked with forceps and preserved in ethanol. The remaining grains were counted later using an Elzone particle counter (Micromeritics, Norcross, GA).

We studied pollen presentation in potted *Penstemon* plants grown from field-collected seed and kept in a growth chamber under controlled temperature and humidity. Each plant studied had been in the chamber for a few days before the day of sampling. Unable to obtain flowering *Penstemon barbatus* plants in the greenhouse, we sampled from this species under less controlled conditions in the field. Four plants were kept indoors during sampling, in Irwin, Colorado, where the temperature and humidity were lower and more variable than in the environmental chambers used in the greenhouse.

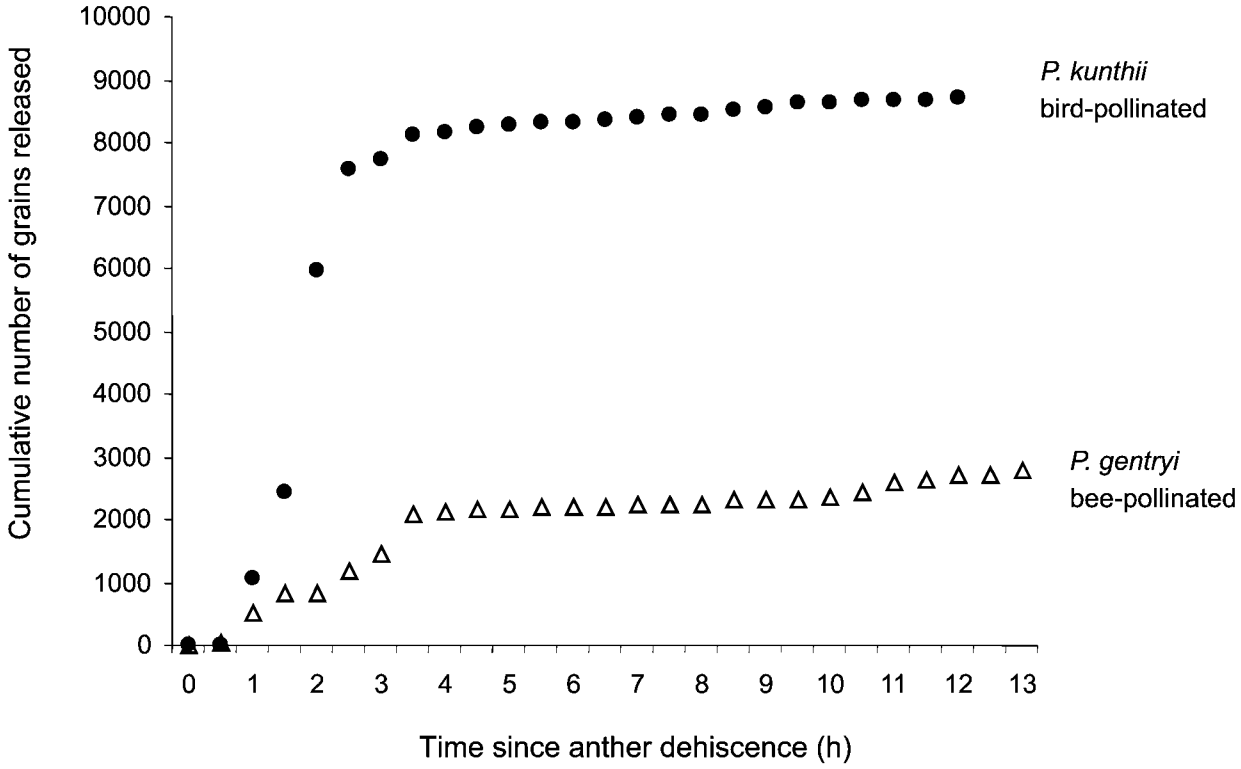

Figure A1: Examples of curves of cumulative numbers of pollen grains released during the sequence of velvet sampling, for one flower of *Penstemon kunthii* and one flower of *Penstemon gentryi*, close relatives. By the first 4.5 h of sampling, 87% of all grains in the anther of the bird-pollinated *P. kunthii* had been released, compared to 21% of those in the bee-pollinated *P. gentryi*.