2. **Protocols**

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Making casts of flower parts (Fig. 1, modified from Alison Reid's protocol)

Goal: prepare artificial experimental flowers for bee preference test.

Materials:

Elite HD+ light body dental wax
http://en.zhermack.com/Clinical/Clinical/Addition_Silicones/Elite_HD/C203030.klT
2-ton epoxy resin (2 hr. set) http://www.hobbystores.co.uk/default.asp?itemid=S-DV033
Petri dishes, wooden toothpicks, small plastic weigh boats, Humbrol paints (Hull, UK)

1. Mix the two wax cartridges (white and blue/a base and a catalyst) in a 1:1 ratio on a petri dish using a toothpick. Mix thoroughly and quickly to avoid setting.
2. When the mixture is even in color, press the flower surface (e.g. sepal) into the wax firmly. Do not to keep touching, just push it in making sure it's in contact with the wax.
3. Let the wax set for 10-15 min.

This wax is very resilient once set but can be cut with a blade if needed.

4. Once cast, make epoxy-resin replicas. The two solutions (resin and hardener) are mixed in a 1:1 ratio in a disposable dish (small plastic weigh boats) with a toothpick.

The resins need to be mixed thoroughly otherwise the casts won't set properly, and quickly so it doesn't set before pouring. Care should be taken not to introduce bubbles to the resin while mixing.

5. Once poured into the wax mold, leave the resin overnight at room temperature, or until set (touching before set will ruin the cast).
6. Remove casts of sepals, polish edges to remove any excess material, and arrange and stick around a center to make a flower prototype. Make the center separately, it should have a depression for "nectar".
7. Use the flower prototype to make 5 flowers of each type (flat and real).
8. Paint each flower type in a different color to allow the bumblebees to distinguish visually between different surface textures.

I used Humbrol paints No. 65 (green), 68 (purple) and 200 (pink) in a 1:3:7 ratio for flat and 3:1:7 for papillate flowers.

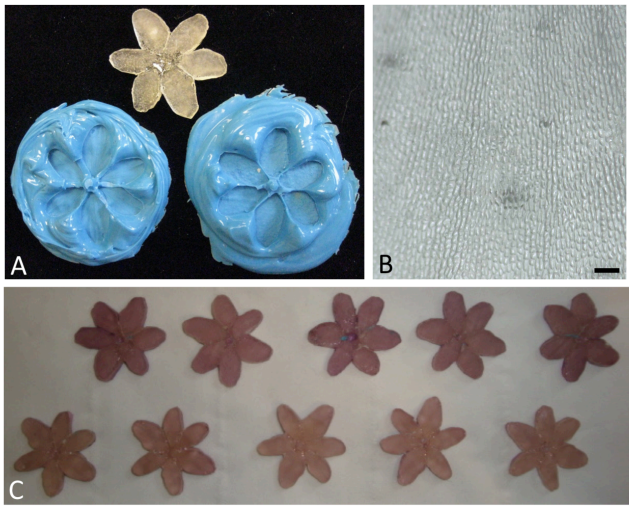

Fig. 1: A: resin replica (top) and wax molds of *T. thalictroides* "real" (papillate) and "flat" experimental flowers; B: papillate cell detail of replica (scale=100uM); C: painted experimental flowers: flat (purple, top) and papillate (pink, bottom).

Preference Test: (Fig. 2-3, Movie 1) (based on (Alcorn et al., 2012)

Materials:
Resin flower casts of 2 flower types, painted in slightly different colors and stuck to platform with double sided tape
Flower platforms made from 2 stacked 15ml tube caps joined together with parafilm
Platform shaker
Dressing paper material in bee-neutral color (to cover shaker)
Bumblebee colony attached to arena by tubing with gates to regulate bee flow (Fig. 2)
Bee-safe enamel bottles in various colors to mark bee foragers
30% sucrose solution
20 ul Pipettor and tips
Ethanol spray bottle
Tissue (for wiping with ethanol)
Lab notebook or spreadsheet

The goal of this particular preference test was to assess whether bumblebees prefer the papillate surface of *Thalictrum* sepals to a flat-cell surface. I placed the experimental flowers on a moving surface because prior work from the Glover lab had shown that papillate-conical cells have a grip function for pollinators that is especially evident in moving surfaces (Alcorn et al., 2012). In addition, a moving experimental flower is closer to the real-life situation of *Thalictrum* flowers, which are on delicate stems that move easily. The shaker had to be dressed with green paper. I took a spectrometer measurement of the paper material to check that the color was neutral to the bees (Ocean Optics with deuterium/halogen lamp; Spectrasuite software, Fig. 3), the position of the "green background" data point near 0 means the color is neutral to the bee. I also measure the white *T. thalictroides* sepals on both sides, the position of this data points in the top right quadrant indicates that the bees perceive it as blue.

Fig. 2: Experimental set-up for preference test.

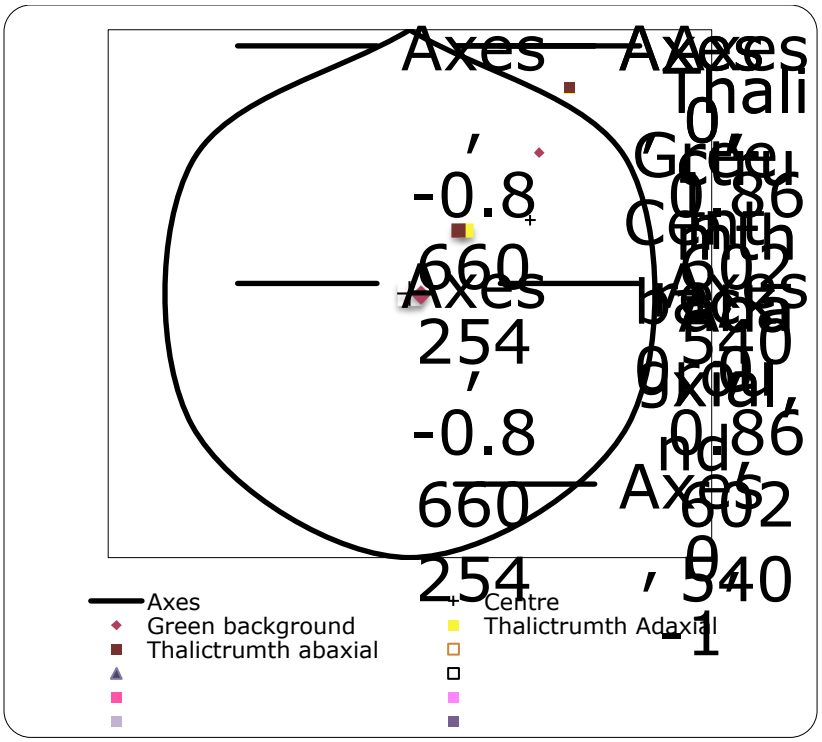

Fig. 3: Spectrometry measurements of flower surfaces and dressing material.

Procedure:

1. A few days before the start of the experiment: Feed the bees with a full 96 well plate of 30 % sucrose solution. Observe the colony to find and mark the good foragers: mark bees that go back and forth between the colony and the arena with 1-2 dots of special paint so that you can keep track of them.
2. The day before the experiment: reduce feeding.
3. On the day of the experiment: Let bees return to the colony and block their way out, until the arena is empty. Some bees will not go back, they will need to be removed from the arena.
4. Set up the dressed shaker, completely covered in bee-neutral material, and the cast flowers in a random array of the two types being tested (in this case: “real” and “flat”).
5. Fill flower centers with ~ 20 ul of sugar solution.
6. Allow one of the previously marked forager bees into the arena, record mark.
7. Observe and note: Type of flower where it lands (f=flat, r=real), and decision (a=abort, d=drink).
8. Every time the bee leaves a flower, refill the “nectar” (if it drank).
9. Once the bee returns towards colony, open gate to let it back in and proceed to clean the arena and flowers with ethanol to remove scent marks. Wipe arena surface, remove flowers and dab surfaces in ethanol, refill all flowers and re-arrange in a different way.
10. Once the arena is ready, allow a second bee in, or the same one. You will need 100 choices from each bee. Once your each 100 with a given bee, that bee is done, and won’t be allowed back into the arena.

11. Repeat for 10 bees, 100 choices each. This will typically take several days, depending on the level of activity of the colony.

Data analysis:

1. Input data into a spreadsheet.
2. Calculate averages for each bee total, for each bee per ten choices, and an overall average.
3. Perform a t-test to compare if the average of the first ten choices across all bees is statistically different from the average of the last ten choices across all bees.
4. Make a graph of all ten-choice-interval averages to see if there is any evidence that the bees have changed their preference between the start and end of their foraging, indicating whether there has been any learning.

References:

Alcorn, K., Whitney, H., and Glover, B. (2012). Flower movement increases pollinator preference for flowers with better grip. Funct. Ecol. *26*, 941–947.

Whitney, H.M., Chittka, L., Bruce, T.J.A., and Glover, B.J. (2009). Conical Epidermal Cells Allow Bees to Grip Flowers and Increase Foraging Efficiency RID B-8523-2009. Curr. Biol. *19*, 948–953.

Movies:
(you can view these movies by clicking the links below, or by going to http://edenrcn.com/protocols/)

Movie 1: Experimental set-up for preference test with movement.
http://edenrcn.com/protocols/Individual%20Protocols/DiStilio_Protocol_Movie1.MPG

Movie 2: bumblebee behavior with *T. thalictroides* plant. Flowers are naturally nectarless, the bumblebee is collecting pollen.
http://edenrcn.com/protocols/Individual%20Protocols/DiStilio_Protocol_Movie2.MPG