4. POLLINATION

4.1 GENERAL INTRODUCTION

In many plant-pollinator systems, flowers are visited by a variety of pollen vectors. As discussed earlier, not all visitors will be pollinators. Those that are will have different levels of effectiveness (Stebbins 1970, Herrera 1987, 1989). The successful transfer of pollen, in most cases, relies on contact between the reproductive organs of a flower and its visitor in such a way that pollen is picked up on the visitor’s body and deposited on the stigmatic surface of the host plant (Faegri and van der Pijl 1979). For obligate outcrossing species, pollinator movement between plants is also required. As a consequence the morphology of the flower and the behaviour of the visitor together determine the success of this process (Muchhala 2003).

Numerous approaches to identification of pollinators and assessment of their efficiency have been employed – none can be said to be the perfect test, and most add to the mounting evidence collected through observations of flower morphology and phenology as well as identifying candidate visitors. Observation of visitor behaviour and abundance has been the traditional approach, and remains the most frequently used technique to identify a plant’s pollinators (Hopper 1980, Sazima et al. 1996, Sakai et al. 1999, Yumoto 2000). In addition the relationship between floral structure, floral rewards and typical faunal associations (i.e. pollination syndromes) has been used to predict pollinators (Hansman 2001, Highan and McQuillan 2000, Hingston and McQuillan 2000); to discount some visitors as effective pollinators (Muchhala 2003); or even to replace field observations (Ibarra-Manriquez and Oyama 1992). Understanding flower structure, identifying the visitor array and observing visitor behaviour are all essential precursors to making testable predictions and designing useful pollination experiments. The difficulty with the approaches listed above is the failure to demonstrate actual pollen flow (Muchhala 2003).

Alternative techniques seek to track the movement of pollen as a measure of pollination success. Examples of these include the capture of visitors and quantifying of con-specific pollen loads (House 1989, Kato et al. 1995, Williams and Adam 1995, Bernal and Ervik 1996, Nagamitsu and Inoue 1997, Birkinshaw and Colquhoun 1998, Sakai and Inoue 1999); the use of fluorescent powders as a proxy for pollen and hence pollen movement (Kearns and Inouye 1993, Kwak and Vervoot 2000); and the deposition of pollen on pollen traps within a flower (Muchhala 2003). In this way, positive identification of pollen vectors and quantification of pollen moved can be made. Again, successful pollination is not confirmed. These techniques are also highly labour intensive, requiring extreme field and laboratory hygiene protocols to avoid pollen contamination (House 1989) and dependent on identification of donor plant species using pollen morphologies. For con-specific species, the latter can be very difficult (Pike 1956).

Manipulative exclusion experiments are designed to identify pollinators by partitioning visitor groups (e.g. day versus night visitors) and use signs of fertilisation (e.g. seed set) to identify and quantify successful pollination (Crome and Irvine 1986, Bernal and Ervik 1996). The design and execution of these experiments is determined by the question asked, and relies on some understanding of visitors and flower morphology. These techniques rely on pollinator arrays that can be partitioned in a testable way. The vagaries of post-fertilisation failure can reduce the success of these types of experiments (Crome and Irvine 1986).

The capacity of individual visitors to transfer viable pollen can also be assessed by examining pollen tube growth within a visited style (Nagamitsu and Inoue 1997). By opening bagged flowers to a single visitor only, examination of the style using fluorescence microscopy can demonstrate the success of that individual visitor (Wilfred Morawetz pers.
While this technique demonstrates successful pollen transfer and germination, it should not be mistaken for reproductive success, as post-fertilisation reproductive isolating mechanisms (Kenta et al. 2002) and predation are not accounted for.

4.2 POLLEN ON VISITORS

We know that visitors must move pollen between the male and female reproductive organs of a flower or flowers in order to be a pollinator. A visitor can be examined to see if indeed it is carrying pollen and is therefore a likely pollinator. This does require being able to identify the pollen of the species of interest. If a library of pollen exists, it may be possible to identify the array of plants visited by an animal. Pollen libraries can be established using the technique for collecting pollen described in Section 2.5.

4.2.1 Techniques for Examining Pollen Loads

See Appendix 1 for a list of equipment required.

Often, insects visiting flowers can have pollen on their body. To determine which species the pollen is from, and the quantity of pollen, pollen needs to be removed from the body and examined under a microscope. For insect visitors to Normanbya normanbyi, we used basic fuchsin jelly (see page 29 for recipe). Small lumps of the jelly can be speared onto a pin or other fine pointy instrument and dabbed over the insect's body. Transfer the jelly to a microscope slide. Place the slide on a slide warmer until the jelly is melted and cover with a cover slip. Leave to cool and then examine under a microscope, using the pollen library to help identify the pollen source. Refrigerate slides if they are to be kept for more than a few months.

4.3 EXCLUSION STUDIES

Exclusion studies offer a quantifiable method of assessing the success of a class or classes of visitors in pollinating a particular plant species (e.g. Crome and Irving 1986, Boulter et al. 2005). These techniques usually rely on being able to partition visitors into different classes. For example, we used exclusion studies to test the differing effectiveness of day and night visitors as well as visitors of different size classes.
Exclusion Trap Design

Our exclusion cages consisted of a collar constructed from a semicircle (radius 150 mm) of 0.2 mm thick plastic acetate. Two holes of diameter 70 mm were cut into the semicircle and circle mesh fabric was glued over these windows. Traps were assembled in-situ. The plastic cage was wrapped around the stem beneath each inflorescence to form a cone with the seam secured firstly with waterproof craft glue and then stapled. The bottom was sealed around the stem using waterproof tape. A cotton “make-up” pad was wrapped around the stem at the point of contact with the cage before placement to avoid damaging the stem. A cylinder of mesh fabric was attached to the top of the trap by gluing the long edge of a rectangle of fabric around the circumference of the trap and gluing and stapling the two shorter edges together. The bag was sealed by tying closed the cylinder with a length of string. The same type of cage was used for both Syzygium gustavioioides and S. sayeri. In the latter case, an additional curved wire stay was attached across the top of the trap to prevent the fabric touching the larger inflorescences. For Normanbya normanbyi, a cylindrical trap was constructed using acetate and plastic lids.

Figure 23: (a) Design of “bagging” cages, constructed from plastic acetate sheets with fine mesh windows; (b) assembled cages seen here on Syzygium sayeri during the breeding system experiment; and (c) the modified design used on inflorescences of (d) Normanbya normanbyi.
4.3.1 Size Class Exclusion Experiment

See Appendix 1 for a list of equipment required.

We have used exclusion cages with different levels of access to determine the capacity of different sized visitor groups to successfully pollinate a species. In this study, we designed cages that allow visual and physical access by visitors. Details of construction design are outlined on page 45 (Exclusion Trap Design). We used four treatments (i.e. four different mesh sizes) and two controls for our experiments. What you choose to use will depend on what animals you wish to differentiate between and the availability of suitable mesh fabric. For example, testing for the impact of a large introduced flower visitor (e.g. bumblebee) might be done with one mesh size that excludes only larger visitors including the bumblebee. For each treatment, the exclusion cage fabric mesh windows and the mesh at the top or ends of the cone / cylinder were replaced with one of four different sized mesh fabrics. Mesh hole diameters were 0.5 mm, 1.5-2.5 mm, 4.5 mm and 8-10 mm. A plastic cage with no mesh acted as the control for cage effect, and a non-manipulated inflorescence provided the control for natural out-crossing rates.

Traps are generally constructed around the inflorescence. Prior to trap construction, the number of buds on each inflorescence must be counted and recorded. Mark each trap with brightly coloured flagging tape on which the tree number and treatment type is recorded. Be sure to record the location of each tree. The cages are then left in place until all flowers on the inflorescence have opened and senesced. Cages can be removed at this stage. Retie the flagging tape to the stem of the inflorescence so it can be rechecked for successful pollination. In order to determine the success of pollinators reaching the flowers, signs of fertilisation need to be assessed. We chose the presence of a swollen receptacle in individual buds for *Syzygium gustavioides*, which had very large but low numbers of mature what, to avoid the effects of predation. In the case of other species, the development of fruit was rapid, and immature fruits could be used. We were able to return to manipulated inflorescences approximately eight to nine weeks after flowering to score for successful fertilisation (although regular observations should be made). The timing of scoring fertilisation should be based on the life history of the target plant.

In total, we used three to six sets of the six treatments on each of five to six trees for three of our target species. The resulting successful pollinations (i.e. swollen buds or fruits) were taken as a proportion of the original number of buds. Because the results of the experiment are a proportion, they must be arcsin transformed (arcsin √ proportion) for statistical analysis. We compared the various treatments and controls using ANOVA based on the transformed proportions (Sokal and Rohlf 1995).

4.3.2 Day / Night Exclusion

See Appendix 1 for a list of equipment required.

The exclusion experiment idea can also be used to test the different (if any) success rate of day versus night visitors. For this experiment, we use four treatments consisting of two controls – one with no cage (Control 1); the second with a cage that remained on for the entire experiment (Control 2) – and two treatments – first, diurnal pollination, for which the cage was removed at 06:00 hrs and reconstructed at 18:00 hrs, and second, nocturnal pollination, for which the cage was removed at 18:00 hrs and reconstructed at 06:00 hrs. For this experiment we simply used the “balloon cages” (described in Section 2.4.2), as they were very easy to remove and reapply.
Setting up the Experiment

For each set of treatments, select four similar sized inflorescences and count the starting buds. Choose buds that are close to opening. For Control 1, simply mark the inflorescence with brightly coloured flagging tape with the tree number and treatment number written on it in permanent marker pen. For Control 2, construct a trap around the inflorescence. This will stay on until the end of the experiment. Build traps on the inflorescences of the other two treatments. Label the three treatments with the tree number and the treatment (Control 2, day-pollinated or night-pollinated), again by writing these codes on brightly coloured flagging tape and tying around the stem of the inflorescence. This initial setting up can be done at any time of the day. Return the following day at dawn and remove cages from day-pollinated treatments. To remove the trap, undo the strings at top and bottom, remove the fabric sock, and remove the staples from the plastic cage. Leave only the flagging tape to denote the inflorescence and treatment. Return the same day at dusk, reconstruct the cages on the day-pollinated inflorescences and remove the cages from the night-pollinated treatments. Continue returning to the trees at dawn and dusk every day until most flowers have opened (this may take up to fourteen days). Do not miss any visitation or the experiment will be invalidated. At the end of the experimental period, remove any unopened buds and deduct from the original bud count, and reconstruct all cages to prevent any further pollinator access. The cages can be removed once all flowers have senesced. Leave the flagging tape on the inflorescence.
The size exclusion experiment on *S. sayeri* demonstrated that large visitors have an important role in its pollination. The results clearly show that the largest mesh size did not allow access to all visitors that contribute to the successful pollination of this species. When smaller insect visitors alone have exclusive access to these flowers, pollination still occurs, but at a level lower than naturally found among these trees. The largest mesh size used would be expected to completely exclude bats from feeding on flowers, although some honeyeaters were observed to feed on nectar by poking their beaks through the mesh (K. Goodall *pers comm.* July 2002). The results of the day and night exclusion experiment in this case offer no statistically significant results, however the general trend does suggest that both night and day time visitors contribute to the pollination success of *S. sayeri*, with night time possibly slightly greater. Interestingly, the number of birds visiting flowers per observation period was greater than recorded for any night visitor (Boulter et al. 2005).
4.4 POLLEN TUBE STUDIES

Obtaining definitive evidence that a visitor has pollinated a flower during its visit is extremely difficult, and often observations of behaviour and quantification of the success of a group of visitors is circumstantial at best. One fairly labour-intensive technique that provides greater evidence of pollination success looks at the growth of pollen tubes in the stigma following the visit of a single individual. The basic idea is to exclude all but one visitor to a flower and then test this flower for the successful transfer and germination of pollen in that flower. Once compatible pollen is deposited on the stigmatic surface, it can germinate, sending a tube down into the stigma to the ovaries. The tube can be examined by using one of a number of alternative staining techniques and microscopy.

Kearns and Inouye (1993) provide a number of alternative staining techniques useful for examining pollen tubes. The most commonly used of these methods is epifluorescence, but requires access to a fluorescence microscope. Alternative staining methods are available for use with a stereomicroscope, although can be more difficult to detect pollen tube growth. We used two methods and these are described here. The non-fluorescence staining technique proved to be sufficient under field conditions (e.g. during the pilot study), but where samples could be returned to the laboratory for examination, the latter method was preferred.

4.4.1 Pilot Study: How long does it take for pollen tubes to grow?

The rate of pollen tube growth varies among species. Some will take a matter of a few hours, others over 24 hours. Before commencing the pollen tube experiment, the length of time a pollen tube will take to grow in the species of interest needs to be established. This is done by examining artificially cross-pollinated flowers at different times following pollination to determine pollen tube growth. The following protocol can be used:

1. Bag an inflorescence of unopened buds (bagging prevents flower visitation by potential pollinators in order to avoid pre-existing tubes giving a false positive).
2. Visit flowers regularly and, when several are open, cross-pollinate using pollen from another tree (do this early in the morning to allow for sufficient return visits).
3. Re-bag the inflorescence.
4. Collect a pollinated flower every two hours for the rest of the day. Collect a final flower 24 hours after the initial pollination.
5. Place flowers immediately into a fixative to prevent further pollen tube growth.
6. Stain the stigmas (see the two alternative methods described in Section 4.4.2, Techniques for Viewing Pollen Tube Growth).
7. Examine the stigmas for pollen tube growth.

Using this protocol, you will be able to establish the time that elapses between the deposit of pollen and pollen tube growth.
4.4.2 Techniques for Viewing Pollen Tube Growth

See Appendix 1 for a list of equipment required.

**Method 1: Aniline blue epifluorescence**

This is a four-step process of fixing, softening, staining and viewing the styles to see pollen tube growth.

**Fixing:** Place flowers in a solution of 1:3 acetate:ethanol for 24 hours. Once fixed, the flowers can be transferred to 70% ethanol for storage until ready to process.

**Softening and clearing:** Remove the style from the flower. Softening can be done with sodium hydroxide (NaOH). The time required to soften will depend on the species. For *S. gustavioides*, we placed the styles in 8N NaOH for 24 hours, as these were fairly thick styles. We suggest monitoring the styles for signs of deterioration, particularly if they are fine.

**Staining:** Decolorise aniline blue by dissolving 0.1% mass / volume aniline blue in 0.1mol/L K$_2$HPO$_4$. Rinse the styles in tap water to remove the sodium hydroxide. Stain the styles in decolorised aniline blue for 24 hours. Mount in a drop of glycerol and cover with a cover slip, squashing gently. Store in a lightproof box until ready for examination.

**Viewing:** Finally, styles were viewed with a fluorescence microscope and UV filter (excitation wave length: 450-490 nm). Pollen-tube walls and callose plugs fluoresce a bright yellow-green (Figure 26).

Modified from Martin 1958; Kearns and Inouye 1993

**Method 2: Basic fuchsin / fast green**

This method does not require epifluorescence, and tubes can be viewed under white light.

**Fixing:** Place flowers in a solution of 1:3 acetate:ethanol for 24 hours. Once fixed, the flowers can be transferred to 70% ethanol for storage until ready to process.

**Staining:** Stain the styles in 1% basic fuschin:1 % fast green (4:1) for at least 24 hours.

**De-stain and soften:** De-stain and soften tissues in lactic acid for twelve hours. Mount on a microscope slide and squash under a cover slip. Pollen tubes will stain maroon against a white background under white light.

Source: Kearns and Inouye 1993.

**Setting up the Experiment**

Bag a number of inflorescences of unopened flowers using a “balloon cage” and fine mesh sock (see Section 2.4.2 for a description of these cages). Visit the inflorescences daily and search for open flowers. If open flowers are found, remove the bag and cage. Observe flowers until the first visitor arrives (we recommend using a data sheet for observations similar to that shown in Figure 20). Note which flower is visited and the behaviour of the visitor. Once the visitor has departed, tag the visited flower / flowers using numbered retail swing tags and record the flower number next to behaviour observations and the time of the visit. Re-bag the inflorescence to prevent any further visitation. Collect the flower at the allotted time required for the pollen tube to grow (established in the pilot study), and treat using the chosen method of staining (Method 1 or Method 2, above). Flowers can be immediately fixed in 1:3 acetate:ethanol for both methods, but be sure to transfer to 70 %
ethanol after 24 hours if not able to complete the staining protocol immediately. Control flowers should also be sampled. Two types of controls can be made. First, simply collect unvisited flowers, and second, artificially cross-pollinate flowers. In the former, no pollen tubes are expected to grow and the latter should show pollen tube growth.

**Examining Pollen Tubes**

Following staining of the styles, they should be mounted on microscope slides in preparation for examination. Label each slide using a small sticker, recording the visitor number, date, time of visit and visitor taxa. Note, slides for epifluorescence should be kept in a lightproof box until they are ready to be examined, as the fluorescence has a limited excitation time and will fade after a period of exposure to UV light. The visitor is scored as “successful” if pollen tubes can be seen growing down the style.
Results of Pollen Tube Study on *Syzygium gustavioides*

**Table 9:** Proportion of styles from *Syzygium gustavioides* flowers, demonstrating the presence of pollen tubes following a single insect visit.

<table>
<thead>
<tr>
<th>Visitor</th>
<th>Pollen Tubes Present (Percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artificial Cross</td>
<td>100 (n = 5)</td>
</tr>
<tr>
<td>No Visitors</td>
<td>0 (n = 5)</td>
</tr>
<tr>
<td><em>Apis mellifera</em> (Feral Honeybee)</td>
<td>90 (n = 20)</td>
</tr>
<tr>
<td>Small Hymenoptera</td>
<td>86.36 (n = 44)</td>
</tr>
<tr>
<td>Native Bees</td>
<td>100 (n = 8)</td>
</tr>
<tr>
<td>Nitidulidae sp.</td>
<td>100 (n = 3)</td>
</tr>
<tr>
<td>Thysanoptera</td>
<td>100 (n = 1)</td>
</tr>
<tr>
<td>Small Diptera</td>
<td>66.67 (n = 3)</td>
</tr>
<tr>
<td>Large Hymenoptera</td>
<td>100 (n = 2)</td>
</tr>
<tr>
<td>Curculionidae sp.</td>
<td>50 (n = 2)</td>
</tr>
</tbody>
</table>

**Figure 26:** (a) Pollen tube germination; and (b) multiple pollen tube growth in *Syzygium gustavioides*. Style examined using fluorescence microscopy (100x magnification).

We bagged over one hundred *S. gustavioides* inflorescences and observed visitors to individual flowers from within those cages over the period from November 2002 to February 2003. In addition, five flowers were collected and examined that had not been exposed to visitations, and a further five were artificially cross-pollinated and collected as above, for scoring of pollen tube growth. We see from the results that all visiting taxa were capable of pollinating this species. The controls (‘artificially cross-pollinated’ and ‘no visitors’) suggest that this technique is a good indication of the pollination success of the treatment.