FINAL REPORT ON

THE AUSTRALIAN FLORA FOUNDATION PROJECT

Do introduced honey bees *Apis mellifera* disrupt breeding systems of bird-adapted Australian plants? A comparison in *Grevillea*

Insect adapted flowers of *Grevillea sphacelata*

Bird adapted flowers of *Grevillea acanthifolia*

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17 January 2006

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Abstract

Worldwide, the effects of honeybees *Apis mellifera* as exotic pollinators are contentious, and in Australia, the wide range of plant species potentially impacted calls for a strategic approach to conservation risk assessment. We conducted a comparative, experimental test of the hypothesis that introduced honey bees reduced effective pollination in bird-adapted (but not insect-adapted) plant species. Our results did not support this hypothesis.

Honey bees visited *Grevillea sphacelata* flowers 63 times more frequently than the combined visits of six native insects. Even though honey bees appeared to always contact pollen and stigmas (n=880 honeybee visits), less than 1% of visits (5) resulted in visible deposition of pollen on honey bees. The plant population studied was pollinator-limited in fruit production, produced 4–13 times more fruit following cross pollination than open-pollination (Mann-Whitney $P<0.001$).

Honey bees were the only potential pollinators we observed visiting *G. acanthifolia* inflorescences from which birds were excluded, a treatment which resulted in equivalent fruit set to open pollination in five of six experiments. However, in the remaining experiment, reproductive success (1.30±0.26 fruits per inflorescence) was significantly greater for open pollinated inflorescences (0.80±0.17, Wilcoxon Signed Rank $P<0.033$). Fruits were highly outcrossed regardless of whether birds were excluded (t_m= 0.92±0.08 and 0.86±0.09 SD for open pollination; and t_m=0.93±0.08 and 0.78±0.14 for bird-exclusion).

Keywords: Bird pollination; Exotic species; Alien species, Geitonogamy; Outcrossing, *Grevillea acanthifolia, Grevillea sphacelata,* Grey Spider Flower

1. Introduction

The Australian flora has a great diversity of pollinating organisms including mammals, such as gliding marsupials (Carthew and Goldingay 1997), birds (Ford and Paton 1986; Paton 1986) and thousands of species of solitary flies, bees and other insects (Michener 1965; Armstrong 1979). Around 100 Australian bird species forage for nectar (Ford et al. 1979) and it is estimated that about 1,000 Australian plant species possess adaptations (Gould and Vrba 1982, hereafter "adaptations") which promote pollination by birds (Stebbins 1974). Importantly, this diversity of Australian pollination systems is believed to have evolved for the past 40 million years in the absence of social pollinating insects (Michener 1965).

Since their introduction to Australia in the 1820s, honey bees have populated much of the temperate and subtropical zones in both domestic and feral hives. Honey bees collect pollen and/or nectar from more than 1,000 plant species across at least 200 plant genera (Paton 1996), and can remove in excess of 80% of the nectar or pollen produced by some species of plants (Paton 1990; Paton 1993). In many species adapted to bird or mammal pollination, the nectary, pollen and stigmas are too widely separated for honey bees to make concurrent contact while foraging for nectar (e.g. Ford and Paton 1986; Taylor and Whelan 1988; Paton 1993).

Because honey bees now interact with so many native Australian plant species, and so many Australian native plants are already threatened (Briggs and Leigh 1988), there is an urgent need to identify those types of plant species that are likely to face a significant threat from honey bees. The situation is further complicated because the honey industry based on commercial hives is very profitable (Gibbs and Muirhead 1998), feral honey bee control is prohibitively costly (Oldroyd 1998), honey bees may be supplementing pollination for some plant populations where native pollinator populations are reduced (Paton 2000), and honey bees may increase fruit set – which may increase populations of some plant species to the detriment of others (Paton 1997). It is clear that trying to study the effects of honey bees one plant species at a time is not strategic, and scientists should aim to provide policy makers and managers with tested principles which would enable them to balance the various risks and benefits of honey bees.

Many ecologists consider that honey bees will disrupt the pollination ecology of vertebrate-adapted plant species generally (e.g. Taylor and Whelan 1988; Vaughton 1992; Paton 1996; Pyke 1999). Selective exposure experiments have shown that honey bees can pollinate vertebrate-pollinated plants in several genera (such as *Banksia, Grevillea, Callistemon, Correa, Cyanthodes* and *Brachyloma*) but the quantity of fruit and/or seed produced was generally lower than when vertebrates also had access to flowers (e.g. Taylor and Whelan 1988; Paton 1996; Vaughton 1996; Faulks 1999; Higham and McQuillan 2000; Celebrezze and Paton 2004). Honey bees may also alter seed quality; England et al. (2001) detected a slight but significant increase in honey bee-mediated inbreeding for a self-compatible bird-adapted plant species. This may be because honey bees moved shorter distances while foraging for nectar than birds did (Ayre et al. 1994; Richardson et al. 2000), although we cannot rule out a simple increase in autogamy.

Despite the variety of studies illustrating that honey bees alter pollination systems in Australia, none has tested the hypothesis that vertebrate-adapted plants are more susceptible to negative impacts than insect-adapted plants through comparative experimental study of species with contrasting pollination adaptations within a plant family. The plant family Proteaceae is ideal for such a comparison. The 46 Australian genera encompass 1,100 species (CSIRO 1995), most with secondary pollen presentation (Yeo 1992). In species with this characteristic, pollen is deposited near the stigmatic surface prior to the bud opening to expose the pistil (Collins and Rebelo 1987; Ayre and Whelan 1989). The length and shape of the pollen presenter and the arrangement of up to 1,000 flowers in inflorescences, have evolved into configurations suited to mammal, bird and insect pollination (Collins and Rebelo 1987; Taylor and Whelan 1988). Several studies on the Proteaceae have linked pollinator behaviour and breeding systems with reproductive and genetic outcomes (Ayre et al. 1994; Richardson et al. 2000; England et al. 2001; Llorens 2004).

In this study, we tested the hypothesis that honey bees are detrimental to pollination systems of bird-adapted but not insect-adapted species. We compared the effects of honey bees on the reproductive success of two species of Proteaceae in the genus *Grevillea* with insect-adapted flowers (*G. sphacelata*) and bird-adapted flowers (*G. acanthifolia*) (Collins and Rebelo 1987). For each species, we determined the breeding systems using

experimental self- and cross-pollination and observed the behaviour of floral visitors. We then used experimental pollinator exposures to examine the effect of excluding birds (and other large pollinators) compared to open pollination. For the bird-adapted species, we also compared the genetic quality of seeds produced by exposure to all pollinators with those from which birds were excluded.

We predicted that:

(i) for the insect-adapted *Grevillea sphacelata*, pollination by honey bees would be as successful as experimental cross-pollination;

(ii) for the bird-adapted *G. acanthifolia*, exclusion of birds but not honeybees would result in significantly less successful pollination than open-pollination; and

(iii) *G. acanthifolia* seeds produced by exposure to honey bees alone would be significantly less outcrossed and display more biparental inbreeding than those produced through open-pollination.

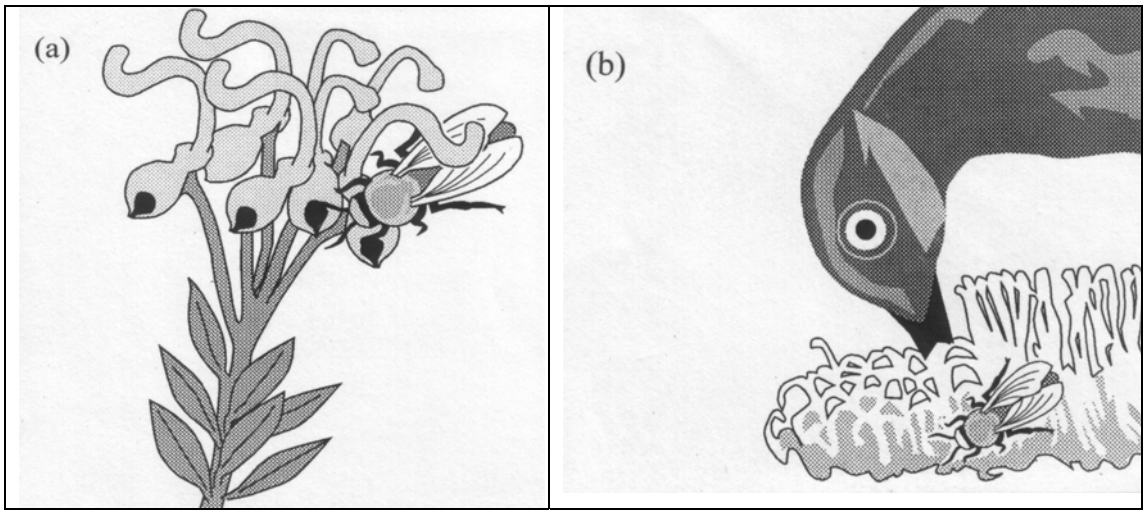
We did not assess the effect of honey bees on genetic seed quality in *G. sphacelata* because Richardson et al. (2000) showed that this species was self-incompatible and all seeds produced were the result of biparental inbreeding.

2. Methods

2.1 Study species and sites

Both study species are woody perennial shrubs. Each inflorescence of *G. sphacelata* is 1-3 cm in diameter and produces 10–20 flowers radiating from a central pedicel, hence the common name "spider flower". A gap of approximately 0.5 cm separates the nectary and the pollen presenter so insects longer than 1 cm from tip of head to end of thorax–including honey bees–are likely to brush the pollen presenter while collecting nectar (Fig. 1(a)). The red flowers of *G. acanthifolia* (Fig. 1(b)) are arranged in racemose inflorescences ("toothbrush") that are visited by nectar-feeding birds (Celebrezze, pers. obs.).

Fig. 1. The foraging behaviour of (a) honey bees at flowers of *Grevillea sphacelata* and (b) honey bees and birds at *Grevillea acanthifolia*. (a) Honey bees, as well as several species of native insects such as flies, collect nectar from the flowers of *G. sphacelata*, generally contacting the pollen presenter in the process. (b) Birds usually contact presenters as they probe *G. acathifolia* inflorescences for nectar. Honey bees sometimes contact pollen presenters while landing on or alighting from *G. acanthifolia* inflorescences, but not while crawling among flowers gathering nectar. Illustration by Robert Parkinson.



We observed pollinators and conducted experiments in two populations for each species. The *G. sphacelata* sites were located in the Royal National Park (population 1, 320 596.91 E, 6 224 460.80 N; population 2, 315 583.67 E, 6 220 589.2 N). The *G. acanthifolia* sites were in the Blue Mountains National Park (population 1, 251 089.55 E, 6 273 606.65 N; population 2, 255 410.68 E, 6 265 109.49 N).

2.2 Potential pollinators and their behaviour

To determine the range of native diurnal insect species that were potential pollinators, and their abundance relative to that of honey bees, we tagged 15–30 plants. We visited each plant at 4–6 one- or two-hour intervals between 07:00 to 19:00 by walking past the plants in the same sequence at these intervals. At each interval we recorded the number of honey bees and native insects visiting flowers on each plant. We recorded the family and morphospecies of native insects and, when possible, we measured the length (tip of head to

end of thorax) by holding a ruler near the insect. For *G. sphacelata*, we censused on three days in population 1 in 1997 (September 3, September 9 and October 3, *n*=25 plants), and in 1999 (August 29, September 14 and September 26, *n*=30 plants) and on one day in population 2 in 1999 (November 5, *n*=18 plants). For *G. acanthifolia*, we censused on three days in population 1 in 1998 (January 15 *n*=9, January 22 *n*=13 and February 13 *n*=25 plants) and six days in 2000 (January 24, 25, 26, 31 and February 1, 2, *n* = 15). We could not safely obtain pollinator observations from *G. acanthifolia* in population 2 because of rugged terrain. We summarized these data as the average percentage of plants being visited each day during each flowering season.

To estimate the frequency with which honey bees contact *G. sphacelata* pollen presenters, we observed 18 individual honey bees during a total of 880 flower visits on September 26 and November 5, 1999. We noted the sequence of these visits and whether they resulted in the bright orange pollen adhering to the dorsal thorax of the honey bee.

To determine the mechanism by which honey bees were apparently pollinating *G. acanthifolia*, we observed the behaviour of 17 individual honey bees visiting a total of 38 inflorescences on January 15 1998 in population 2, and October 11, January 25 and February 1 2000 in population 1.

To determine whether birds were likely to pollinate *G. acanthifolia*, which species visited, and how frequently they visited, we observed bird behaviour over three days in 1999 in population 1 (January 15, January 22 and February 13) and six days in 2000 (January 24, 25, 26, 31 and February 1, 2). We observed groups of *G. acanthifolia* plants in populations 1 (25 plants in 1999, 86 plants in 2000) for 30-minute sessions to determine what proportion of plants were receiving visits. We divided our observations into four time periods (during which we made our 30 minute sessions) to analyse how bird behaviour varied through the day between morning (06:00–10:00), midday (10:00–14:00), afternoon (14:00–16:00) and early evening (16:00–dusk). We compared the average proportions of plants visited per 30 minute session among time periods using Kruskal Wallis test.

2.3 Breeding system

We assessed the breeding systems of both species by comparing fruit set of plants randomly assigned to self- and cross-pollination treatments.

To prevent pollination by insects or birds, we bagged 3–10 budding *G. sphacelata* inflorescences per plant in population 1 (n=12 plants) and population 2 (n=10 plants), and one inflorescence per *G. acanthifolia* plant in population 1 (n=20 plants in 1998 and n=13 plants in 2000) using nylon bags. Three to 11 days later we removed all visible pollen with a cotton tip and brushed either self- or cross-pollen over all stigmatic surfaces. We collected cross pollen from haphazardly selected plants five to 20 m from the treated plant. Neither species produced fruit in the first trial of this treatment so to increase the chance that the period of maximum stigma receptivity was intercepted (Goldingay and Carthew 1998) we repeatedly deposited pollen on stigmas during the flowering period in a later trial. For *G. sphacelata*, we treated inflorescences 2–7 times over six weeks in spring 1999 (in both populations) and for *G. acanthifolia* we treated flowers six times over two weeks in 2000 (population 1).

As is typical of the Proteaceae (Ayre and Whelan 1989), inflorescences of both species often fail to produce fruit, resulting in very low average fruit set with high standard error. Therefore, we compared the proportion of inflorescences that produced fruit ("fertile inflorescences"), the number of fruit per fertile inflorescence, and the average fruit set per inflorescence using Kruskal Wallis Test for both the breeding system tests and the selective pollinator exposure experiments.

We tested our assumption that animal pollinators were important to fruit production in both species by placing nylon bags around budding inflorescences and examining these for fruit produced by autogamy approximately 8 weeks later. The non-nil results were an order of magnitude lower than comparable open-pollination results in the same populations and years (Table 1). The proportion of inflorescences that produced fruit in the autogamy treatment for *G. sphacelata* was 0.02±0.2 in both population 1 in 1997 (from a total of 63 inflorescences on 5 plants) and population 2 in 1999 (6 inflorescences on 2 plants) and nil in spring 1999 in population 1 (46 inflorescences on 10 plants). In *G. acanthifolia*, the proportion of inflorescences that produced fruit in population 1 was nil in 1999 (15 inflorescences on 15 plants) and 0.21 in 2000 (n=5 inflorescences) and in population 2 was

0.2 in 1999 and (n=5) and 0.26 in 2000 (n=23 inflorescences). All results other that for 1999 in population 2 were significantly lower than open-pollination results (χ^2 test α=0.05).

For all breeding system experiments the results have been averaged by plant so as to prevent pseudoreplication of inflorescence data from the same plant.

2.5 Selective pollinator exposure experiments

We compared the effectiveness of all pollinators versus only insects by comparing the reproductive success of inflorescences exposed to all potential pollinators ("open pollination") with that of inflorescences from which large pollinators were excluded ("bird exclusion") by plastic mesh (15 mm^2 hole size). We measured the success of pollination as the fertility (proportion of inflorescences that produced fruit), as well as fruit set (the number of fruit per inflorescence).

For *G. sphacelata* in the first year, we used chicken-wire frames (20 cm diameter, 30-40 cm length) to hold the plastic mesh away from inflorescences. However, only a few inflorescences fitted into each of these frames. In the second year, we framed whole plants with polyvinyl chloride piping covered in plastic mesh and compared the reproductive success of these with whole open-pollinated plants. The experimental unit in both years was individual plants.

For *G. acanthifolia* in the first two years, we found no significant differences in fruit production between open and vertebrate-exclusion treatments. Whole-plant cages were not feasible because plants in these populations were large and spreading, with branches of neighbouring plants interspersed. Each inflorescence was caged separately. To increase experimental power, we paired these treatments on individual plants using all available inflorescences on each plant. We analysed these data (averaged by plant to prevent pseudoreplication) using Wilcoxon Signed Rank Test.

2.6 Genetic assessment

We used two microsatellite primers developed for other *Grevillea* species (Gm^{25} (England et al. 1999) and Gi^{9} (Hoebee 2002) to determine if *G. acanthifolia* seeds produced by exposure of flowers to insects only were less outcrossed than seed from open pollination. Screening of six other microsatellites developed for other *Grevillea* species

found little or no variation. We followed the DNA extraction and amplification procedures of England et al. (1999) and England et al. (2001).

We pooled seed from all years of the experiment to estimate outcrossing rates. For population 1, we determined the genotypes of 24 progeny arrays resulting from open pollination (n=98 seed) and 15 progeny arrays resulting from the bird exclusion treatment (n=58 seed). To improve estimates of pollen allele frequencies for the population, we genotyped an additional 30 neighbouring adults. For population 2, the sample sizes were 24 open pollinated progeny arrays (n=61 seed), 13 bird exclusion progeny arrays (n=25) and 25 additional adults.

We used Ritland's revised multilocus estimation program (Ritland 2002) to estimate outcrossing rates. This approach compared the genotypes of seed with expectations (assuming random mating) given the genotypes of the maternal parents and the estimate of allele frequencies within the pollen pool. Values of t should normally range from 0 to 1, where zero is the expectation for complete self-fertilization and one is the expectation for random mating. Values of greater than one may reflect the level of accuracy of estimation but may also result from negative assortative matings where outcrosses with more distantly related individuals are favoured. We also obtained an estimate of "detectable outcrossing" by comparing genotypes of progeny arrays with their maternal parents. We also report the value t_m-t_s calculated by MLTR; a large and positive value would suggest that there is a high level of biparental inbreeding. We used the linkage disequilibrium function in GENEPOP (V 1.2) (Raymond and Rousset 1995) to test the assumption that genotypes were assorting independently from genotypes at the other locus. We used GENEPOP to indirectly estimate the breeding system that had generated the adult population by calculating the indirect fixation index (F_{IS}), the difference between the heterozygosity observed and that expected under random mating. F_{IS} ranges from –1 to +1; where 0 is the expectation for random mating, positive values imply positive assortative mating or inbreeding, and negative values imply negative assortative mating.

Multilocus outcrossing estimates rely upon several assumptions including the expectations that (i) each adult contributes equally to the pollen pool (and hence pollen allele frequencies should equal adult allele frequencies) and, (ii) maternal adults are a random sample of the population. To test the first assumption, we used χ^2-tests to compare

allele frequencies of the adult plants with pollen allele frequency estimates produced by MLTR. We then tested whether the maternal adult population was a random sample of the larger population by comparing allele frequencies of the maternal adults with the allele frequencies of all of the adults sampled using χ^2-tests.

3. Results

3.1 Potential G. sphacelata pollinators and their behaviour

Honey bees visited 6.3% of the *G. sphacelata* plants censused throughout each day, significantly more than native insects (0.1% of plants observed, Mann-Whitney Z=3.772, P<0.000). From 10:00 to around 16:00, honey bees visited an average of 2–16% of censused plants per hour (Fig. 2). Six native insect species (four hymenopterans, one dipteran and an hemipteran) were likely pollinators, but collectively they were infrequent visitors (2–4% of plants visited per hour).

Honey bees collected nectar from both study species but we never observed them actively collecting pollen. Honey bees always contacted pollen presenters while foraging (100% of 880 visits observed) and typically crawled among all open flowers on each inflorescence before taking flight. Only five of 880 visits to flowers (0.6%) resulted in visible pollen deposition on honey bees. On average, this pollen was no longer visible after the honey bee visited 26±13 additional flowers (at 9.2±3.4 inflorescences), generally on one or two individual plants (1.7±0.2 plants visited). One in five flights (21%) were between plants.

3.2 Potential G. acanthifolia pollinators and their behaviour

When foraging on *G. acanthifolia* inflorescences, honey bees visited an average of 9.1% of plants each hour (averaged hourly percentage) (Fig. 3).

Fig. 2. Average proportion of *Grevillea sphacelata* plants observed being visited by honey bees (black) and native insects (grey) in population 1 in 1997 and 1999 and population 2 in spring 1999 (± standard error). The number of days of observations and total number of plants censused, respectively, are shown along the x-axis in parentheses.

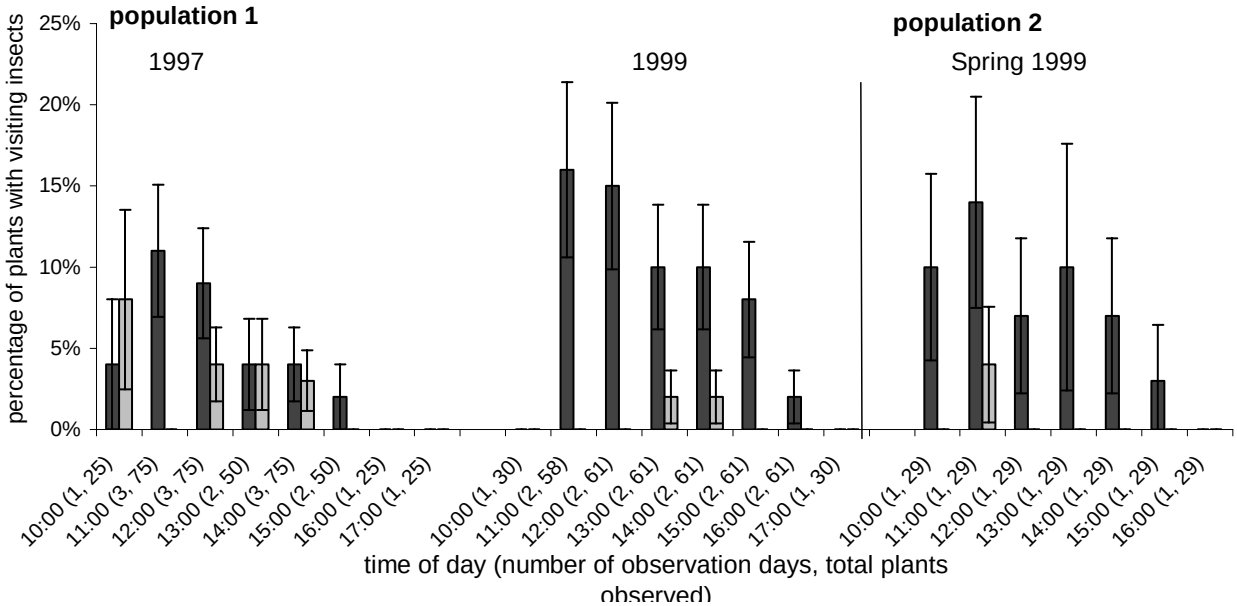
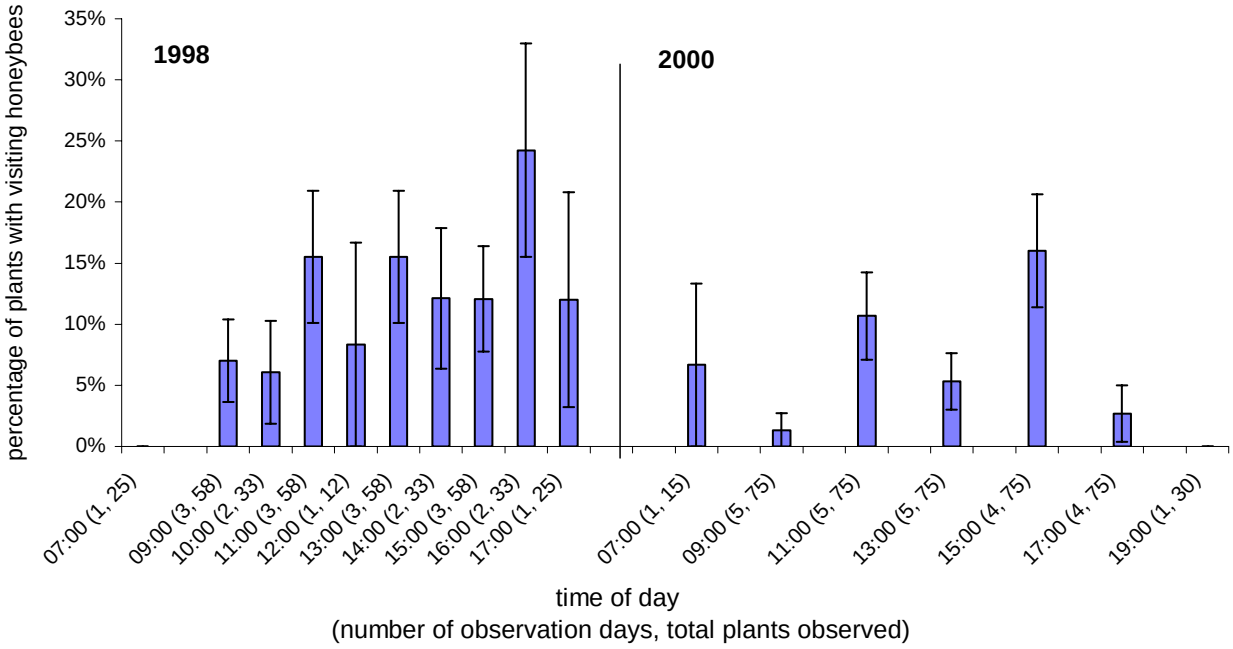


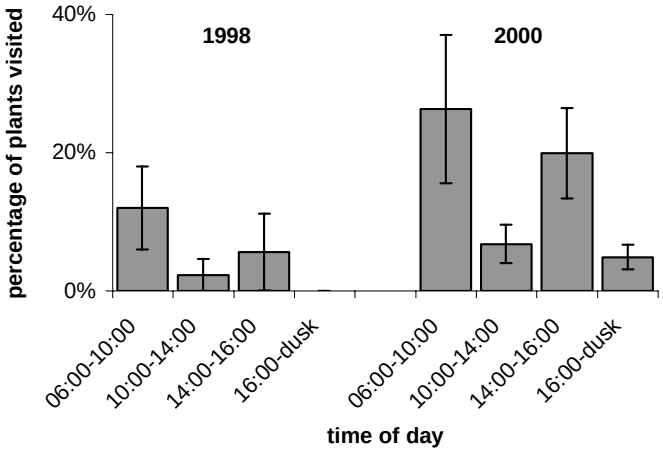
Fig. 3. Average proportion of *Grevillea acanthifolia* plants observed being visited by honey bees in population 1 in 1998 (left) and 2000 (right) (± standard error). The number of days of observation and total number of plants censused, respectively, are shown along the x-axis in parentheses.



Honey bees typically landed on *G. acanthifolia* inflorescences from above, collected nectar from flowers or buds on the same inflorescence, and then flew to another inflorescence, generally on another plant (70%). When arriving at or departing from inflorescences, 6 of 17 honey bees observed (42%) touched at least one pollen presenter, and three of these subsequently touched a pollen presenter on another plant (although visible amounts of pollen were not brushed onto these individuals). Honey bees collected nectar but we never observed them actively collecting pollen. On one occasion, a flying native insect (a butterfly) was observed at *G. acanthifolia* flowers, and brushed pollen presenters during its visit. However, this insect was too large to cross the vertebrate exclusion treatment in our selective pollinator exposure experiments, so it does not affect our assumption that this treatment measures pollination caused by honey bees only.

New Holland honeyeaters *Phylidonyris novaehollandiae* Meliphagidea, foraged for nectar at *G. acanthifolia* inflorescences throughout the day (Fig. 4), with significantly greater frequency in the early morning (06:00–10:00) than at any other time (Kruskal-Wallis test χ^2=12.77, P=0.005; Tukey HSD P<0.05).

Fig. 4. Proportion of *Grevillea acanthifolia* plants visited by New Holland honeyeaters during half-hour observations in population 1 over three days in 1998 and six days in 2000 (± standard error).



Honeyeaters typically brushed against whole rows of open flowers while probing red flower buds (which held 83% of the nectar available (data not presented)), so that pollen

presenters were brushed on the chin, head or cheeks. Honeyeaters nearly always moved between plants (89% of movements) when moving between inflorescences.

3.3 Breeding systems

As predicted (Richardson et al. 2000), *Grevillea sphacelata* was effectively self-incompatible (Table 1). Self-pollination resulted in no fruit set, while 16 to 52% of cross-pollinated inflorescences produced at least one fruit.

Table 1. Breeding systems

The breeding systems of *Grevillea sphacelata* and *G. acanthifolia* from cross- and self-pollination experiments, showing the percentage of inflorescences treated which produced at least one fruit ("fertile inflorescences") and the number of fruits produced by fertile inflorescences. Cross pollen was obtained from haphazardly selected plants five to 20 m from the treated plants. Standard errors are shown for *G. sphacelata* because more than one inflorescence was sampled on each plant, whereas only one inflorescence was used in each *G. acanthifolia* plant.

		percentage fertile inflorescences				fruits per fertile inflorescence			
population		self	*n*	cross	*n*	self	*n*	cross	*n*
G. sphacelata									
1	Spring 1999	0	6	52 ± 8%	4	no data	0	1.6 ± 0.5	4
2	Spring 1999	0	5	16 ± 7%	5	no data	0	1	3
G. acanthifolia									
1	2000	28%	7	25%	6	1.5 ± 0.5	2	1.7 ± 0.3	3

The results of autogamy tests indicate that *Grevillea acanthifolia* was at least partly self-compatible in both populations. Although cross- and self-pollination of ten inflorescences resulted in no fruit set resulting from these experimental treatments in 1998, two of seven self-pollinated *G. acanthifolia* inflorescence produced fruit in population 1 in 2000 (Table 1).

3.4 Selective pollinator exposure experiments

As expected, we did not find a significant difference (α=0.05) in the percentage of *G. sphacelata* inflorescences that produced fruit between selective pollinator exposure treatments (Table 2); less than one in five open pollinated inflorescences produced fruit. Inflorescence fertility varied by an order of magnitude among seasons; in 1999 less than one in 20 inflorescences produced fruit. Fertile inflorescences generally produced only one fruit each.

For *G. acanthifolia*, when treatments were paired on plants in 2000 (Table 2), bird exclusion resulted in significantly fewer fruits per inflorescence in population 2 than open pollination (Wilcoxon Rank Sum Test Z=-2.14, P=0.03). The same comparison in population 1 in 2000 bordered on significance (Z=-2.93, P=0.054). This resulted from both a lower percentage of inflorescences producing fruit, and a lower number of fruit per fertile inflorescence (Table 2), although neither of these factors alone was significantly different between treatments. There were also no significant differences in fruits per inflorescence for *G. acanthifolia* between selective pollinator exposure experiments in 1998 and 1999.

Table 2. Selective pollinator exposure experiments

The effect of excluding large pollinators on reproductive success in *Grevillea sphacelata* and *G. acanthifolia* (*n*=plants treated). Large pollinators were excluded using 15 mm^2 plastic mesh. The average number of fruits per inflorescence, the percentage of inflorescences treated which produced at least one fruit ("fertile inflorescences") and the number of inflorescences produced by fertile inflorescences were compared using non-parametric ANOVA. There were no significant differences ($\alpha = 0.05$) except in *G. acanthifolia* in 2000, where treatments were paired on individual plants using all available inflorescences to increase power (fruits per inflorescence, population 1, Z=-2.93, P=0.054; population 2, Z=-2.14, P=0.03). Results of each treatment on each plant were averaged to avoid pseudoreplication and analysed using Wilcoxon Signed Rank Test. Figures are ± standard error.

	fruits per inflorescence				percentage of fertile inflorescences per plant				fruits per fertile inflorescence			
	open-pollinated	*n*	bird-excluded	*n*	open-pollinated	*n*	bird-excluded	*n*	open-pollinated	*n*	bird-excluded	*n*
G. sphacelata												
population 1												
1997					20 ± 10%	6	40 ± 10%	5	1	12	1	4
Spring 1999					4 ± 2%	9	1 ± 3%	10	1	3	1.5 ± 0.3	4
population 2												
Autumn 1999					20 ± 20%	2	0	2	1	1		0
Spring 1999					4 ± 2%	10	9 ± 3%	9	1	4	1	5
G. acanthifolia												
population 1												
1998					34%	38	36%	14	2 ± 0.3	13	2.6 ± 5	5
1999					55%	20	44%	15	1.9 ± 0.3	11	1.4 ± 0.2	7
2000					48%	27	32%	28	1.5 ± 0.3	13	1.3 ± 0.3	9
population 2												
1998					57%	7	83%	6	1.8 ± 0.8	4	1	5
1999					33%	15	62%	8	1.4 ± 0.6	5	2 ± 0.8	5
2000					76%	25	65%	23	1.9 ± 0.3	25	1.1 ± 0.2	15

3.5 Outcrossing rates in G. acanthifolia

Single and multi-locus estimates of *t* indicate that *G. acanthifolia* seeds were highly outcrossed, with multi-locus estimates of *t* approaching one for both populations regardless of treatment (Table 3). Seed produced by bird-exclusion in population 2 had the lowest multilocus outcrossing rates, but this was within two standard deviations of open pollination outcrossing rates. A simple estimate of levels of outcrossing based on the number of detectable outcrosses revealed no significant effect of treatment with 50% or more of seed displaying at least one non-maternal allele (Table 3; $P>0.20$ Mann-Whitney U-Test).

3.6 Genetic assumptions

The genetic data did not appear to depart from the assumptions of the multilocus outcrossing estimate procedure. Our results provide little evidence of biparental inbreeding in both bird excluded and open pollinated treatments for either populations (t_m-$t_s \leq 0.07$) (Table 3). The Gm^{25} and Gi^{9} loci did not exhibit significant linkage disequilibrium in either population 1 ($P=0.052$) or population 2 ($P=0.44$). The adult population genotypes and pollen pool estimates produced by MTLR did not differ significantly, consistent with the assumption that adults contributed equally to the pollen pool. Maternal allele frequencies did not differ significantly from the allele frequencies of the non-maternal adults genotyped, suggesting that the maternal plants were a random sample of the adult population.

In population 2, the pollen pool appeared to be a random sample of available alleles because allele frequencies did not differ between adults and pollen (the latter estimated from open-pollinated seed). However, in population 1, allele frequencies of adults and open-pollinated seed for Gm^{25} differed significantly ($\chi^2=20.5$, df=8, $P<0.01$)**.** This result might have arisen from disproportionate contribution of Gm^{25} genotype by maternal plants, because the Gm^{25} genotypes of the two adults with the largest progeny arrays of open pollinated seed contained the same two alleles that differed the most in frequency between the adult and seed arrays (allele 238, 13% in adults, 23% in seed; allele 232, 24 versus 32%). In contrast, the genotypes of Gi^{9} for these two plants included the most common allele.

Table 1 Outcrossing rates

Proportion of *Grevillea acanthifolia* seed that was detectably outcrossed and outcrossing rate estimates (*t),* generated using MLTR (Ritland, 1981 104) for seed produced through open-pollination and bird-exclusion in two populations. All seed were used in estimates of detectable outcrossing, but a few seed could not be genotyped for one or the other allele, so single locus, t_s and multilocus outcrossing estimates, t_m differ slightly (sample sizes shown). Frequency of detectable outcrosses did not differ significantly between treatments in either population (Mann-Whitney test $P>0.20$). Arrays refer to the number of sets of maternal plants with seeds. Additional adults (36 in population 1 and 25 in population 2) were genotyped and included in MLTR analyses to provide a more accurate estimate of pollen allele frequencies for each locus.

	total sample (arrays or adults)	*n* (seeds)	proportion detectably outcrossed	t_m (± S.D.)		t_s (± S.D.)				t_m-t_s (± S.D.)
				Gi^{9} *and Gm*^{25}	*n*	*Gi*^{9}	*n*	*Gm*^{25}	*n*	
population 1										
open pollination	24	106	0.67	0.92 ± 0.08	98	1.31+ 0.31	99	0.77 + 0.08	105	0.012 + 0.031
bird exclusion	15	65	0.58	0.93 ± 0.08	58	0.93 + 0.23	61	0.78 + 0.10	62	-0.018 + 0.107
total maternal plants	36				25					
population 2										
open pollination	24	77	0.53	0.86 ± 0.09	61	0.87 + 0.13	68	0.64 + 0.15	70	0.069 + 0.029
bird exclusion	13	29	0.5	0.78 ± 0.14	25	0.53 + 0.22	27	0.95 + 0.29	27	0.037 + 0.068
total maternal plants	29				21					

3.7 Random mating and population structure

Our estimates of the indirect fixation index for adult plants in both populations were consistent with random mating (for Gm^{25} and Gi^{9} respectively, F_{IS}=-0.116 and 0.126 in population 1 and 0.105 and –0.114 for population 2).

The genetic structure of the adult populations were near what would be expected for Hardy-Weinberg equilibrium; F_{IS} was small and did not depart consistently from zero (Table 4). Tests based upon Markov chain resampling (using GENEPOP) also indicated that in all cases, adult genotype frequencies did not depart significantly from expectations for Hardy-Weinberg equilibrium (P>0.10). Although these tests are not direct measures of pollen movement, they indicate that inbreeding in previous generation(s) did not contribute significantly to the genetic population substructure of the current crop of adult plants.

Table 4. Observed heterozygosity (H_o) in adults for two *Grevillea acanthifolia* populations, expected values for a population at Hardy –Weinberg equilibrium (H_E), and the inbreeding coefficient ($F_{IS} = H_o - H_E/H_E$). *n* is the number of adults used in the estimates.

population 1		Gm^{25}	Gi^{9}
	H_o	0.719	0.804
	H_E	0.813	0.714
	F_{IS}	-0.116	0.126
	n	60	50
population 2			
	H_o	0.756	0.628
	H_E	0.684	0.709
	F_{IS}	0.105	-0.114
	n	44	40

4. Discussion

In our comparison of *Grevillea sphacelata* and *G. acanthifolia*, we found little evidence that bird-adapted pollination systems are disrupted by honey bees and insect-adapted systems are not. Instead, honey bees appeared to severely restrict fruit set in *G.*

sphacelata flowers, which are visited by variety of large native flies, bees and other insects, but had little detectable effect on reproductive success or inbreeding in the bird-adapted *G. acanthifolia.*

4.1 Grevillea sphacelata

Although honey bees were 63 times more abundant on flowers than were other potential pollinators, they did not provide full pollination services to *G. sphacelata*. This counterintuitive outcome may have resulted from the interaction of honey bee-mediated geitonogamy (de Jong et al. 1993) and a self-incompatible breeding system. Cross pollinated *G. sphacelata* flowers produced on average 4-13 times more fruit than open pollination, which suggests that fruit production was limited by effective animal pollination. Honey bees always contacted the pollen presenter of *G. sphacelata* while foraging for nectar. Nevertheless, individual honey bees visiting flowers rarely accumulated visible pollen (5 of 880 visits) and typically visited 14 flowers on the same plant before flying to another nearby plant. The likelihood of honey bees transferring pollen to receptive flowers was probably further decreased by periodic grooming (Celebrezze pers. obs.; Thomson 1986). We would expect that pollination by native insects in the absence of honey bees would result in greater fruit set, because native insects would be much more likely to transfer pollen among plants.

The methodical behaviour of honey bees has been implicated in fruit set limitation in several other Australian plant species (Gross and Mackay 1998; Celebrezze 2002; Faulks 1999). If native insects were erratic and less frequent floral visitors, they might be expected to be more effective pollinators of a self-incompatible plant with long-lived flowers, such as *G. sphacelata*. An alternative (but not exclusive) hypothesis is that native pollinators have suffered from competition with honey bees, and their depressed populations are no longer providing effective pollination service. Both this and our preferred hypothesis could be tested through the removal of honey bees from study populations.

4.2 Grevillea acanthifolia

The exclusion of birds from *G. acanthifolia* flowers did not measurably affect the quantity or quality of seed produced relative to that produced on open-pollinated flowers visited by birds and honey bees. This contrasts with the results of comparable experiments with *G. macleayana*, a self-compatible species with inflorescences nearly identical in morphology to *G. acanthifolia*. In *G. macleayana*, honey bees collected pollen but produced half the fruit set obtained through exposure to all potential pollinators (Vaughton 1996) and so reduced outcrossing (England et al. 2001). Our results do not support the hypothesis that honey bees should restrict fruit set and outcrossing in bird-adapted plants, but there are at least two other ways in which honey bees may be altering *G. acanthifolia* pollination systems; through interference effects, and through differences in pollen quality.

4.3 Interference effects

Honey bees may remove large quantities of pollen and/or nectar, changing the frequency or effectiveness of bird visits. For example, Paton (1996) demonstrated that the sizes of bird territories increased with an increased abundance of honey bees; this kind of interference might decrease the frequency of bird visits to flowers, while increasing the movement distance among them. If such an effect were occurring in *G. acanthifolia*, we would have expected cross-pollination tests to result in greater fruit set than open pollination. Cross-pollination produced significantly lower fruit set than open pollination, which may have resulted from applying pollen to stigmas that were not receptive, or might indicate that resources available to the plant, rather than pollen, limited the fruit set of these inflorescences. Further cross-pollination tests could be performed by applying mixed pollen loads when stigmas are receptive.

4.4 Genetic assessment

We found no difference in estimated multilocus outcrossing rates among seeds produced by open pollination or exclusion of birds. Outcrossing rates were very high regardless of treatment, supporting the view that pollinators are moving pollen among plants and there is little or no biparental inbreeding. The multilocus outcrossing estimates

are based upon the assumption that the loci are independently assorting, which is supported by the lack of evidence of biparental inbreeding (Table 3).

Although we sampled only one section of each of the two populations our sample was sufficient to produce relatively tight estimates of outcrossing for those sets of plants. In addition to sampling sufficient parents and their seed the critical issue in estimating outcrossing rates is to have good estimates of allele frequencies in the surrounding potential pollen donors. Our results suggest that our sampling achieved this aim be we concede that outcrossing rates may vary within each population. This may be especially true for population 1 which we estimate consists of approximately 1000 flowering plants but should be less of an issue for population 2 with only 100 plants.

Another potentially confounding factor was that we pooled results across seasons for genetic analysis (in order to obtain a reasonable sample size). This may have obscured real differences in genetic outcomes among seasons, if, for example, honey bees have inbreeding effects under conditions which vary temporally. For example, nectar flows may be different in drought years than in wetter years, and honey bee behaviour is known to vary depending upon nectar quality and volume (Wells and Wells 1986). By pooling results across years, any such effects would be obscured by results of years when honey bee behaviour was different. Although no such difference is apparent in our study (Fig. 3), our samples sizes were small.

Although we believe that the assumptions of the multilocus estimation procedure have been satisfied, the differences in allele frequencies observed among adults and open-pollinated seed for Gm^{25} may have resulted from the adult allele frequencies being unrepresentative of the population as a whole. For this locus, the pollen pool included two alleles not detected in the adult population. This is not surprising, since the majority of adult plants were not genotyped. Future studies using more powerful genetic assessment, such as paternity analysis using more loci, could assess the movement of individual pollen grains to determine whether there are differences in the distance pollen is moved by different suites of pollinators.

4.5 Significance for plant conservation

This study demonstrates, through experimental comparison of two closely related plant species, that apparent floral adaptations alone do not determine the effects of honey bees on fruit set and seed quality in *Grevillea acanthifolia* and *G. sphacelata*. There is nevertheless still a need to identify general characteristics of Australian plants which might make them vulnerable to negative effects of honey bees. Such general characteristics would help identify specific management actions for plant species at risk from honey bees, without inappropriately limiting the economic benefits of domestic honey bees or going to great expense to remove feral honey bees unnecessarily. In our view, further study of potential honey bee effects should focus on the role of breeding systems to test the hypothesis that honey bees limit the fruit set of self-incompatible species but not species with mixed breeding systems. An ideal model for such a study would be a plant species which has both self-incompatible and self-compatible populations, and from which honey bees collect pollen. Comparing sites from which feral honey bees were removed with sites where honey bees are abundant would greatly strengthen such comparisons. We would expect other plant life history characteristics to interact negatively with honey bee effects, including an inability of adult plants to survive fires, short seed viability, and long juvenile period, thus having important ramifications for the conservation of Australian plant species. Precautionary options for managers and policy makers would be to control or eliminate feral honey bees and restrict domestic honey bees in some populations of all plant species visited by honey bees. For example, some plans of management for National Parks in New South Wales have identified feral honey bee control as a management priority. The results of this study demonstrate the importance of testing the assumptions of such management actions. Considering the expense of honey bee removal, implementation of this management priority where it has been identified should be coordinated with pre- and post-implementation ecological studies. In addition to comparative studies of plant species, insect population and behaviour studies might shed more light on the resilience native pollination systems following changes in honey bee populations.

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Acknowledgements

This research was supported by an Australian Postgraduate Research Scholarship, a University of Wollongong Postgraduate Award, with additional support from the Australian Research Council, the Australian Flora Foundation and the University of Wollongong's Institute for Conservation Biology. The New South Wales National Parks and Wildlife Service provided a permit for field studies. P. England and S. Hoebee developed the microsatellite markers used in this study. Laboratory work was assisted by D. Roberts and advice from M. Dowton. R. Parkinson donated the illustration and S. Lloyd and T. Llorens provided constructive advice on the draft.