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Do introduced honeybees affect seed set and seed quality in a plant adapted for bird pollination?

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Abstract

Aims: Worldwide, evidence suggests that exotic pollinators can disrupt plant mating patterns. However, few studies have determined if pollination by the honeybee Apis mellifera (the world's most widely introduced pollinator) reduces offspring quality when compared with pollination by native birds. The Australian Proteaceae provide an excellent opportunity to test the impact of honeybees in pollination systems that are adapted to birds and non-flying mammals.

Methods: We compared the frequency of flower visitation and foraging behaviour of birds and insects within seven populations of Banksia ericifolia. Banksia ericifolia is hermaphroditic and has large nectar-rich, orange inflorescences typical of bird and mammal pollinated species. For a subset of the study populations, we compared the quality of seed produced via an exclusion treatment (that only allowed invertebrates to access flowers) with an open-pollination treatment (potentially visited by mammals, birds and invertebrates), by measuring seed weight, germination rates (T50), percent germination, seedling height after 14 days since the emergence of the cotyledon and time to emergence of the cotyledon.

Important Findings: Apis mellifera was the only apparent insect pollinator and the most frequent flower visitor, while the open treatment inflorescences were also frequently visited by avian pollinators, primarily honeyeater species. The foraging behaviour of honeybees and honeyeaters showed striking differences that potentially affect patterns of pollen transfer. Honeybees made significantly greater proportions of within cf. among plant movements and only 30% (n=48) of honeybees foraged for pollen (nectar foragers carried no pollen) whilst all birds were observed to contact both stigmas and anthers when foraging for nectar. Despite these fundamental differences in behaviour, there was little effect of treatment on seed set or quality. Our data show that while honeybees appear to alter patterns of pollen transfer within B. ericifolia populations, they do not impact reproductive rates or performance of early life-stages.

Disciplines
Medicine and Health Sciences | Social and Behavioral Sciences

Do introduced honeybees affect seed set and seed quality in a plant adapted for bird pollination?

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Abstract

Aims

Worldwide, evidence suggests that exotic pollinators can disrupt plant mating patterns. However, few studies have determined if pollination by the honeybee *Apis mellifera* (the world's most widely introduced pollinator) reduces offspring quality when compared with pollination by native birds. The Australian Proteaceae provide

an excellent opportunity to test the impact of honeybees in pollination systems that are adapted to birds and non-flying mammals.

Methods

We compared the frequency of flower visitation and foraging behaviour of birds and insects within seven populations of *Banksia ericifolia*. *Banksia ericifolia* is hermaphroditic and has large nectar-rich, orange inflorescences typical of bird and mammal pollinated species. For a subset of the study populations, we compared the quality of seed produced via an exclusion treatment (that only allowed invertebrates to access flowers) with an open-pollination treatment (potentially visited by mammals, birds and invertebrates), by measuring seed weight, germination rates (T_{50}), percent germination, seedling height after 14 days since the emergence of the cotyledon and time to emergence of the cotyledon.

Important Findings

Apis mellifera was the only apparent insect pollinator and the most frequent flower visitor, while the open treatment inflorescences were also frequently visited by avian pollinators, primarily honeyeater species. The foraging behaviour of honeybees and honeyeaters showed striking differences that potentially affect patterns of pollen transfer. Honeybees made significantly greater proportions of within *cf.* among plant movements and only 30% (n=48) of honeybees foraged for pollen (nectar foragers carried no pollen) whilst all birds were observed to contact both stigmas and anthers when foraging for nectar. Despite these fundamental differences in behaviour, there was little effect of treatment on seed set or quality. Our data show that while honeybees appear to alter patterns of pollen transfer within *B. ericifolia* populations, they do not impact reproductive rates or performance of early life-stages.

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Introduction

The European honeybee (*Apis mellifera*) has successfully invaded ecosystems worldwide and many studies have found evidence of consequent competition between honeybees and native pollinators (e.g. Kato *et al.* 1999; Hansen *et al.* 2002; Thomson 2004). However, relatively few studies have investigated whether seedling fitness is altered by consequent changes to patterns of pollen dispersal (Paton1993). Taken collectively, these papers suggest that when larger native vertebrate pollinators are excluded, seed production or rates of outcrossing may be decreased (e.g. Ramsey 1988; Richardson *et al.* 2000; England *et al.* 2001; Celebrezze and Paton 2004) but to our knowledge none has investigated the effects of *A. mellifera* on the quality of seeds produced.

The Proteaceae is a cosmopolitan family, represented across South America, Africa and Australasia (Johnson and Briggs 1975). The greatest diversity of species occurs in South Africa and Australia, and vertebrate-pollination is a feature of many genera (Collins and Rebelo 1987; Myerscough *et al.* 2001; Johnson and Pauw 2014). In the temperate regions of Australia, pollination systems have evolved without social bees (Michener 1979). Thus in this region, the flora is often dominated by species such as those within the Proteaceae, that have evolved with birds and mammals as their primary pollinators. Currently, however, the relatively recently introduced *A. mellifera* (present for ~200 years) (Doull 1973) is the numerically dominant insect pollinator of many plant species (Gilpin *et al.* 2014; Hermansen *et al.*

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2014) including many that would previously have been expected to be almost exclusively pollinated by birds (e.g. Whelan et al 2009).

The impact of honeybees is especially evident in the pollinator assemblages of the Proteaceae (Ramsey 1988; Vaughton 1992; Richardson *et al.* 2000; Celebrezze and Paton 2004; Whelan *et al.* 2009). This family features large, showy, nectar-rich inflorescences that are typically considered adapted to pollination by both birds and marsupials (Ayre and Whelan 1989) but are also highly attractive to both nectar and pollen-foraging honeybees (Paton and Turner 1985; Myerscough *et al.* 2001). Honeybees have been reported to be effective pollinators of a range of Proteaceae (e.g. Vaughton 1992; Whelan *et al.* 2009) but frequently, in foraging for both nectar and pollen, they are considered to deplete the resources available to native pollinators (Vaughton 1996) and are typically observed to make more foraging movements within plants or among sets of near neighbours than is the case for native birds or insects (Richardson *et al.* 2000; Celebrezze 2002; Rymer *et al.* 2005; Whelan *et al.* 2009).

The effect of different patterns of pollen transfer will almost certainly vary with each species' level of self-compatibility, the quantity and quality of pollen transferred and underlying spatial genetic variation. Evidence from a number of studies shows that self-compatibility and realised mating systems can vary sharply within and among species of Proteaceae (Carthew *et al.* 1988; Ayre *et al.* 1994; Hoebee and Young 2001; Llorens 2004). Moreover, seedling fitness can be affected by pollen dispersal distance, in some but not all cases, where pollen has been experimentally transferred within and among populations (e.g. Heliyanto *et al.* 2005; Holmes *et al.* 2008; Forrest *et al.* 2011; O'Brien *et al.* 2013). However, the consequences of honeybee *cf.* vertebrate pollination for both outcrossing rates and offspring fitness

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have been largely ignored and no study has tested for changes in seed quality as a consequence of honeybee pollination. England *et al.* (2001) demonstrated that for *Grevillea macleayana*, pollination by honeybees in a vertebrate exclusion experiment produced a small but significant decrease in outcrossing rates. Vaughton (1996) found decreased seed set (50%) in inflorescences of the same species when birds were excluded compared to inflorescences where both birds and honeybees had access, while similar vertebrate exclusion experiments by Paton and Turner (1985) and Vaughton (1992) detected no clear effect on seed production in *Banksia ericifolia* or *Banksia spinulosa* respectively. However, none of these studies comment on seed or seedling quality. Studies of vertebrate pollinator-adapted *Protea* in Africa have reported reduced seed set following experimental exclusion of vertebrates (Wiens *et al.* 1983; Hargreaves *et al.* 2004) but it is unclear whether this simply reflects decreased pollen transfer.

In this study we tested the prediction that vertebrate exclusion and consequent pollination by honeybees would reduce seed set and seedling vigour for the vertebrate pollinator-adapted *B. ericifolia* (Carpenter 1978) which is also known to be frequently visited by honeybees (Paton and Turner 1985). We focused on three questions: (1) What proportion of inflorescence visits are made by honeybees as compared with birds and mammals? (2) Are honeybees more likely than vertebrates to transfer self or outcross pollen? (3) Is there a difference in seed production, seed weight and seedling vigour between seeds produced when vertebrate pollinators are excluded and those produced under open-pollination?

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Materials and Methods

Study area and study species

The study was conducted in seven sites, at four locations, six within National Parks: Royal - two sites, (34°09'06.7"S 151°03'34.0"E); Dharawal - two sites, (34°14'30.5"S 150°50'27.2"E) and Budderoo - two sites (34°38'45.3"S 150°41'58.2"E); and one on private land in Helensburgh - (34°10'28.9"S, 150°58'39.2"E), all located south of Sydney, New South Wales, Australia. Sites were similarly sized (~ 6 ha) and chosen due to their similarity in density and size of *B. ericifolia* plants. *Banksia ericifolia* is a shrub or small tree which produces inflorescence spikes in Autumn/Winter each year. Inflorescences range in length from ~10-25 cm, are red-orange in colour and produce copious amounts of nectar and pollen (Lloyd *et al.* 2002). Flowers open sequentially and the inflorescences produce nectar over two to three weeks (Lloyd *et al.* 2002). After nectar production had ceased and all flowers began to brown, we judged the inflorescence to be senescent. Fertilised seed are retained within woody follicles and form an infructescence or cone. The number of seeds per infructescence is limited by the space available for seed development (George 1984). The winged seeds are released after fire stimulates opening of follicles. The mating system of *B. ericifolia* is partially self-compatible (Goldingay *et al.* 1991a; Carthew *et al.* 1996).

Exclusion experiment

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In order to compare the frequency of visitation and the pattern of foraging behaviour by vertebrates and insects on inflorescences of *B. ericifolia* and to test the subsequent effectiveness of insects as pollen vectors, we randomly allocated 15 *B. ericifolia* plants at each of seven study sites (Royal site 1 and 2, Dharawal site 1 and 2, Budderoo site 1 and 2 and Helensburgh) to one of three pollination treatments. The treatments were; a spontaneous autogamy treatment, an open-pollination and a vertebrate exclusion treatment. For the latter two treatments we selected all inflorescences on which the process of flower opening was clearly about to begin, providing 10 to 11 inflorescences per plant. We removed a small number of inflorescences that had open flowers from most plants ensuring that each treatment featured an identical number of similarly mature inflorescences. Older inflorescences with senescent flowers or early stage immature inflorescences were not removed.

Plants allocated to the autogamy treatment each had five tagged inflorescences. These inflorescences were then bagged using a hard plastic inner layer of coarse mesh (Gutter Guard™) covered with a fine organza cloth to exclude all potential flower visitors. We included an autogamy treatment in order to determine whether pollen vectors were necessary, but because there was no seed set within the autogamy treatment at any of the seven sites, these results were excluded from later analysis. Plants allocated to the open pollinated and vertebrate-exclusion treatments each had 10 to 11 inflorescences tagged or caged per plant with identical numbers (10 or 11) of inflorescences allocated to each of these two treatments at each site. Plants allocated to the vertebrate-exclusion treatment were completely covered in netting with a 25 mm x 25 mm aperture which was small enough to ensure no bird or mammal could enter but large enough to allow easy access by

honeybees. Trees allocated to the vertebrate-exclusion treatment were also fitted

with a plastic guard around their trunk to prevent small mammal access.

Diurnal flower visitor surveys

To determine and compare the assemblage and behaviour of bird and insect flower visitors as well as compare insect visitors to open and vertebrate-excluded treatments, surveys were undertaken during the peak diurnal foraging time of these species within the peak of the flowering season at each study site (May to August). Through preliminary observations, we found that no honeybees visited *B. ericifolia* before 1000 or after 1500, most likely due to air temperatures being low (always below 13°C) (Abou-Shaara 2014). Bird visitors were observed to visit plants primarily early in the morning (before 1000) or later in the afternoon (after 1430), with far fewer visits outside these observation times. As such, bird surveys were undertaken between either 0630 and 1000 or 1430 and 1800, and insect visitor surveys between 1000 and 1500.

Birds were surveyed on all trees in the open-pollination treatment for a total of seven days at each site, spread throughout the peak flowering period. All inflorescences on each plant were observed simultaneously for 10 minutes from a distance of more than 20 m to minimise disturbance. Insect visitor surveys were carried out on all plants within the open-pollination and vertebrate exclusion treatments on the same seven days as bird observations. The specific time of observation for each plant was chosen at random to avoid any temporal bias. Honeybee movements and behaviour were recorded for 10 minutes on both the open-pollination and vertebrate-excluded treatment plants (see below), with each plant simultaneously observed by two observers to ensure that visits to all inflorescences could be recorded. For both bird and insect visitors we recorded the length of time spent on each inflorescence on the study plant, as well as the number of inflorescences visited within the study plant, and the number of cases where the

visitor flew to an inflorescence on a neighbouring plant or alternatively left the observation area. Each flower visitor was observed to determine whether it was foraging in a manner that would facilitate pollen transfer among inflorescences. Subsequently, it was noted that all birds foraged in a manner that would lead to pollen transfer, whilst honeybees were split into those foraging for nectar (no contact made with pollen presenters) and those foraging for pollen (pollen sequestered within their corbiculae) (Thorp 2000) which frequently contacted both pollen presenters and the stigmatic region.

Nocturnal flower visitor surveys

To determine whether *B. ericifolia* received nocturnal flower visitors we first undertook direct observations at night using torches at each site on all of the open-pollination treatment plants for three nights (spread throughout the flowering season), and failed to detect any nocturnal visitors. Subsequently, we deployed a set of four infrared cameras (Faunatech) to conduct observations, at each site on each of three days, spread throughout the flowering season. Cameras were set with infrared trips that triggered the filming of two minute digital video sequences. Preliminary work showed that they were able to detect both nocturnal vertebrate and invertebrate visitors. In contrast to the diurnal surveys, observations were possible for only a subset of the target inflorescences on each of the open-pollination treatment plants (typically 2 to 3), with the number limited by the field of view of the cameras.

Seed weight

After inflorescences had been pollinated, bags and bird netting were removed (approximately one month after bagging) and seeds left to develop. All infructescences from the study plants were harvested once they reached maturity. In the laboratory, infructescences were then subjected to a heat treatment of 200°C for 20 minutes to open follicles and allow seeds to be extracted. The seed wing, septum and false seed were separated and the seed subsequently weighed to the nearest 0.1 mg. Thirty seeds per plant were randomly selected and weighed. In two cases where 30 were not produced, all available seeds were weighed (n = 12 for the vertebrate exclusion treatment and 28 for the open pollination treatment). Seed weight data for all plants from within the same site and treatment groups were pooled to compare among treatments.

Seed germination trials

Seeds from five plants within each treatment (vertebrate excluded and open pollination) at four sites were used to assess the effects of different pollinator types on germinability (Helensburgh, Royal site 1, Dharawal site 1 and Budderoo site 1). From each plant 75 seeds were randomly selected, giving a total of 375 seeds per treatment at each site. In order to discern the number of germinable seeds and the rate of germination, seeds were placed in petri dishes on moistened filter paper, sealed and then placed in an incubator on a 12 hour light/dark and 25°C/ 18°C temperature cycle to simulate mean day/night summer temperatures of the region (Ooi *et al.* 2014). Dishes were checked every two days for a total of 25 days, and germination scored based on emergence of the radicle. At the end of the trial period, any seeds that failed to germinate were tested for viability using tweezers to discern

the hardness of the seed, followed by a cut test. Any soft or mouldy seeds as determined from the cut test (Ooi *et al.* 2004) were discarded and scored as inviable. Total germination at the end of the trial was then calculated as the percentage of viable seeds. The time to reach 50% germination (T_{50}) was also calculated by plotting cumulative germination against time and fitting either a linear or quadratic model to the data, and solving the equation for x = 0.5.

Growth rate of seedlings and emergence of the cotyledons

In order to measure seedling growth rates, seeds from each of four sites (Helensburgh, Royal site 2, Dharawal site 1 and Dharawal site 2) were used to assess the effects of different pollinator types on the growth rate of seedlings and the time till emergence of the cotyledon. Twenty seeds in total from each of the two treatments at each site were randomly selected. The twenty seeds were then divided into two groups of ten, with one group from each treatment sown in each of two pots to account for potential pot effects. The timing of emergence of the cotyledon was recorded and seedling height was compared two weeks after germination.

Data analysis

Pollinator observation data were analysed using t- tests and chi square tests. To test for significant effects of site and treatment for all other experiments, we used Generalized Linear Models or ANOVA. Seed set was analysed using a 2-factor GLM with quasi-Poisson distribution and log-link function, to account for overdispersion of the data. Seedling height data were normally distributed and were therefore analysed using a GLM with a Gaussian distribution. Time to emergence of the cotyledons was analysed using a GLM with a Poisson distribution with a log-link

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function. Seed viability and germination data were analysed using a 2-factor GLM with binomial distribution and logit link function. Seed weight data fitted the assumptions of normality and homogeneity of variances and were analysed using a 2-factor ANOVA. Results are presented as means $\pm$ 1 SE unless otherwise noted.

Results

Identification and frequency of diurnal flower visitors

The most common flower visitor observed on open inflorescences of *B. ericifolia* was the European honeybee *A. mellifera* (n =146) (both nectar and pollen gatherers) which was observed to visit all of the study plants and 344 study inflorescences. Honeybees were found to make similar numbers of visits to that of all other flower visitors combined, with little variation among sites (range = 1 to 3 plants and range = 1 to 7 inflorescences visited per site). The only other insect visitors that we observed were ants (*Formicidae* species) (n = 31) and flies that appeared to be *Muscidae* species (n = 8). The diversity and number of insects visiting the vertebrate-exclusion treatment was almost identical, with no additional species recorded.

Open inflorescences were frequently visited by bird species with 97% of observed plants visited by avian pollinators (seven honeyeater species). During the study period, birds were observed to make 161 visits to study plants and made 339 visits to study inflorescences. The bird species observed were; New Holland Honeyeater (*Phylidonyris novaehollandiae*) (n = 21), Silvereye (*Zosterops lateralis*) (n = 82), Eastern Spinebill (*Acanthorhynchus tenuirostris*) (n = 2), Whistler (*Pachycephala* sp) (n = 5), Brush Wattlebird (*Anthochaera chrysoptera*) (n = 9), Superb Blue Wren (*Malurus cyaneus*) (n = 7) and Yellow Faced Honeyeater

(*Lichenostomus chrysops*) (n = 9). Total numbers of bird visits varied across sites, ranging from 8 to 61 and 8 to 178 for plants and inflorescences respectively.

Identification and frequency of nocturnal flower visitors

During a total of 144 hours of observations at each site in which an average of 57 ± 8.5 newly opened inflorescences were observed, we detected no nocturnal flower visitors at any of the study sites.

Effect of treatment on the frequency of flower visitation by insects and the foraging behaviour of pollinators

Apis mellifera was by far the most frequent invertebrate visitor and the only one foraging in a manner likely to affect pollination (but only when pollen gathering – see below). Broadly similar visitation rates were made by *A. mellifera* (both nectar and pollen gatherers) to plants with vertebrate exclusion (15.6 ± 5.7) and to the open treatment (20.9 ± 4.4) ($t_{(6)} = 1.25, p = 0.25$) (Table 1). Moreover, the average number of honeybees foraging for pollen and hence acting as pollinators also did not vary significantly among treatments (vertebrate-exclusion treatment, 4 ± 0.90, open treatment 6.86 ± 1.71) ($t_{(6)} = 1.32, p = 0.23$). Across all seven sites, foraging individuals of *A. mellifera* (both nectar and pollen gatherers combined) made similar numbers of within plant movements among inflorescences irrespective of treatment (vertebrate-exclusion 2.2 ± 0.1, n = 93; open treatment 1.6 ± 0.1, n = 75; $t_{(6)} = 0.24$, $p = 0.81$).

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Bird species

During 40.8 hrs of observations conducted on bird species, 0.55 birds per 10 minute observation period were recorded foraging on *B. ericifolia* (n = 35). Birds on average made 1.9 ± 0.1 intra-plant movements (Table 1).

Comparison of foraging behaviour of birds and honeybees

Overall we observed more honeybees (both nectar and pollen gatherers) visiting inflorescences (146) than birds (135) in the open-pollination treatment. However, birds were clearly more common pollinators than honeybees. All 135 birds that we observed foraged in a manner in which it was likely that they contacted the pollen presenter (which surrounds the stigma) while only 48 honeybees foraged for pollen and in a manner likely to affect pollination. Nectar foraging honeybees avoided contacting reproductive parts by collecting nectar at the base of the flowers. Ants were never observed touching pollen presenters and both ants and flies were only observed gathering nectar. As a result, it is likely that *A. mellifera* is the only observed invertebrate pollinator as both ants and flies did not have a foraging behaviour likely to induce pollination. Moreover, slightly more birds made intra-plant movements among inflorescences than pollen collecting honeybees (75 vs. 36) within the open treatment. These honeybees, however, moved 10% more frequently among inflorescences within plants than birds ($t_{(6)} = 2.76$, $p = 0.03$). Importantly, the movements of birds would be expected to produce more outcrossing as they displayed a significantly greater proportion of inter-plant movements (26 of 135 birds observed moved among plants *cf.* 4 of 48 for pollen collecting bees) ($\chi^2_{(1)} = 4.23$, $p = 0.039$). The time spent foraging on inflorescences differed between nectar and pollen

collecting honeybees and birds. Nectar gathering honeybees on average spent 104 seconds ± 11.5, pollen gathering honeybees spent 54 seconds ± 6.5, compared to birds that spent 5 seconds ± 0.2.

Effect of treatment on seed set and seed weight

We found no consistent relationship for effects of treatment on the percentage of inflorescences that set seed (Fig. 1). The GLM analysis revealed no significant interaction between site and treatment ($\chi^2_{(6)} = 6.08$, $p = 0.41$) and no main effects. Our experiment also revealed no consistent effect of vertebrate exclusion on the number of seeds set (Fig 2). On average the open pollinated inflorescences produced more seed at five of seven sites but this difference was significant only at Budderoo site 1. GLM analysis revealed no significant interaction between treatment and site ($\chi^2_{(6)} = 151$, $p = 0.48$).

Mean (n = 30) seed weight did not vary markedly with site or treatment (range 21.5-27.2 mg across all sites for both the vertebrate-exclusion and open treatments). The interaction between site and treatment was significant ($F_{7, 430} = 10.102$, $p =$ <0.0001) with the seeds in the open treatment significantly heavier than those in the vertebrate-exclusion treatment at the Helensburgh site (Fig.3). They were also heavier than seeds from all other sites.

Effect of treatment on germination

Across all sites and treatments, seed viability (93% - 99.5%) and germinability (98.2% -100%) were high and there was no significant effect of site or treatment on either (viability $\chi^2_{(7)} = 4.356$, $p = 0.738$; germinability $\chi^2_{(7)} = 3.89$, $p = 0.792$). For germination rate, the mean time taken to reach 50% germination (T_{50}) ranged from 8

$\pm$ 0.5 days at Budderoo site 1 for both treatments to 12 $\pm$1 days for the open treatment at Royal site 1 (Fig. 4). Site had a significant effect on T_{50} ($F_{3,101}$ = 15.17, p<0.0001) but there was no significant difference in T_{50} between treatments at each site.

Effect of treatment on growth rate of seedlings and emergence of the cotyledon

GLM analysis revealed that there were no significant differences between treatments or sites for seedling growth. Average height in the open treatment was 25.9mm $\pm$ 1.6 (n = 39) compared to 24.6mm $\pm$ 1.01 (n = 46) for the vertebrate-exclusion treatment. There was also no significant effect of site or treatment on the numbers of days to emergence of the cotyledons (open treatment, 26.1 $\pm$ 0.5 (n = 31); vertebrate-exclusion treatment, 26.4 $\pm$ 0.4 (n = 29)).

Discussion

Plants that are considered to be adapted to vertebrate pollination are now increasingly visited by the invasive pollinator *A. mellifera* (Paton and Turner 1985; Vaughton 1992; Hansen *et al*. 2002). Nevertheless the consequences of this phenomenon are poorly understood (Traveset and Richardson 2006). Our findings support those of other studies that have found that the foraging behaviour of birds and honeybees differ in regard to length of foraging bouts, exploitation of floral rewards (Hansen *et al*. 2002) and, importantly, the proportion of intra and inter-plant movements (Paton 1993). Our data also support earlier studies showing that honeybees make fewer inter-plant movements and more intra-plant movements as compared to avian pollinators (Richardson *et al*. 2000; Whelan *et al*. 2009). The

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difference in foraging behaviour of bird and honeybee pollinators might be expected to influence plant fitness by reducing seed set and altering the genotypic composition of seed produced. Most significantly, our study, which is the first to experimentally evaluate these predictions by using a vertebrate exclusion experiment, found no clear evidence that either seed set or seed quality were reduced when inflorescences were pollinated by honeybees.

Flower visitation and foraging behaviour

As might be expected for a 'vertebrate-adapted' species, birds were the most common and presumably most effective pollinators of *B. ericifolia* due to the way they foraged on inflorescences, contacting the reproductive parts of the plant. Earlier studies of the pollination of Australian plants suggested that the importance of honeybees as pollinators is hard to evaluate and may frequently be overestimated because the foraging behaviour and morphology (body size relative to flower size and shape) of honeybees typically leads to them removing both nectar and pollen, without pollen transfer (Gross and Mackay 1998; Richardson *et al.* 2000). In *Grevillea macleayana* which shares a similar floral morphology to *B. ericifolia* it was found that nectar gathering honeybees were able to actively avoid touching the reproductive parts of the plant and therefore were thought to contribute less to the pollination than pollen gathering honeybees (Whelan *et al.* 2009). In this study, the majority of bees foraged only for nectar and this behaviour may make inflorescences less attractive to all other effective pollinators. When foraging for pollen on *B. ericifolia,* honeybees inevitably contact the stigma because, before flowers open, the pollen is deposited onto the stigmatic surface as a pollen presenter (Ayre and Whelan 1989). However, as is typical of foraging honeybees, most pollen is gleaned

from their bodies and deposited in corbiculae where it is not available for pollination (Hargreaves *et al.* 2009).

In all *B. ericifolia* populations examined in this study, honeybees were found to be the most common flower visiting species (although less numerous than the total set of flower visiting birds). However, this clearly overestimated their importance as pollinators as only 30% of honeybees were foraging for pollen. Nectar gathering honeybees that visited *B. ericifolia* avoided contacting pollen presenters by accessing flowers at their base and gathering nectar that ran down the core of the inflorescence. Similarly, Paton (1993) found that for the South Australian *Callistemon regulosus,* honeybees harvesting nectar only contacted the stigma in 4.4% of 8000 visits compared to pollen harvesting honeybees which contacted the stigma in 16.7% of 1649 visits. In contrast, nectar-foraging birds contacted the stigma more than 50% of the time.

The contribution of non-flying mammals to pollination in our open-pollination treatment is difficult to assess. Although earlier work identifies both birds and non-flying mammals, especially *Antechinus flavipes, Melomys burtoni a*nd *Rattus tunneyi* as pollinators of *B. ericifolia* (Hackett and Goldingay 2001), our study did not detect any inflorescence visitation by non-flying mammals or moths despite the use of both human observations and infrared cameras. While our failure to capture images of marsupial pollinators could reflect insufficient trapping effort (e.g. Goldingay *et al.* 1991b), it is likely that their local densities are low (*M. burtoni* and *R. tunneyi* do not occur in the study area) and hence they would not significantly influence pollination of the inflorescences in our study.

For many plant species, pollinator effectiveness will be determined by both the quantity and quality of pollen transferred and this will in turn vary with the degree of self-compatibility and the spatial genetic structure of the plant populations (Burley and Willson 1983; Waser 1993; Holmes *et al.* 2008). For *B. ericifolia*, birds and honeybees were observed to make a majority of intra-plant movements, with both likely to transfer pollen within and among inflorescences on each plant visited. This pattern of self-pollen transfer is likely to produce less seed set than among plant movements since this species is at least partially self-incompatible (Carthew *et al*. 1996). However, in common with observations for many other Australian Proteaceae, the foraging behaviour of birds and honeybees differed, with birds making greater numbers of inter-plant movements and hence expected to transfer more outcross pollen (England *et al*. 2001; Whelan *et al.* 2009). Moreover birds are more likely than bees to move pollen among more distantly separated plants within populations or among neighbouring populations and hence may deliver more suitable pollen than honeybees.

There appear to be few, if any, comparable observations of the contrasting effects of bird and insect pollination in predominantly bird pollinated African Proteaceae, although Steenhuisen *et al*. (2012) report that for the largely insect pollinated and autogamous *Protea caffra,* outcrossing rates do not vary when vertebrates are excluded.

Effects of vertebrate exclusion on seed set and performance

The results of this study confirm that honeybees can be effective pollinators of *B. ericifolia* as has been reported for a range of other Proteaceae (Vaughton 1992; Richardson *et al.* 2000; Whelan *et al.* 2009). For six of seven sites, similar levels of

infructescence production and seed set were produced on open pollinated plants that received similar visitation by birds and honeybees, and on vertebrate-excluded plants that were almost exclusively visited by honeybees. The one exception was found at Buderoo site 1, where open treatments produced greater seed set than exclusion treatments. This was likely to be a result of comparatively few honeybees present at this site.

Importantly, across all seven sites, inflorescences assigned to an autogamy treatment did not set seed, demonstrating the need for a pollen vector. We detected low overall seed set, with many inflorescence setting no seed, which is consistent with other studies of *B. ericifolia* (Paton and Turner 1985; Carthew *et al.* 1996) and Proteaceae in general (Ayre and Whelan, 1989). This pattern has been used to argue that *Banksia* may display high levels of mate choice to compensate for variation in the quality of pollen transferred by different pollinators (Ayre and Whelan 1989; Goldingay and Carthew *et al.* 1988). Nevertheless, our finding that the vertebrate-excluded inflorescences did not produce fewer seeds is surprising since Carthew *et al.* (1996) provide experimental evidence that inflorescences given both self and outcross pollen predominantly set seed from outcross pollen.

Perhaps the most surprising outcome of our study, given the reduced pollen diversity expected within the vertebrate exclusion treatment, was that seed quality and early seedling performance were again little affected by treatment. Pollen transfer between neighbouring subpopulations has been shown to increase seedling performance in some other self-incompatible Proteaceae (Holmes *et al.* 2008; Forrest *et al.* 2011; but see Ayre and O'Brien 2013) and again we expected this to be facilitated by bird but not honeybee visitation. We detected similarly high levels of germination success and viability and similar time to germination, emergence from

the cotyledon and seedling growth. Overall the similar reproductive success and early performance of seed from plants visited only by honeybees, as compared with those visited by both birds and bees, implies that within most sites seed set is limited by resource availability (Ayre and Whelan 1989) rather than pollen quantity or quality.

Contrary to our expectations, we found no evidence that pollination by honeybees has a detrimental effect on the fitness of *B. ericifolia*. Our study is the first to test experimentally whether introduced honeybees are having an impact on seed or seedling fitness in a bird-adapted plant species. Without such studies, understanding the true impact of honeybees remains speculative. Although we acknowledge that the effects of our pollination treatment on seedling vigour may not be detectable until later stages of the life-history, we predict that most seeds set are outcrossed as observed for this species by Carthew *et al.* (1996). Further studies are needed to determine whether the effect of honeybee pollination appears equally benign when plants are pollen rather than resource limited, or for species that display higher levels of self-incompatibility.

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Figure legends

Fig. 1. The percentage (%) of inflorescences that set seed for the two treatments (open and vertebrate-exclusion) at each site.

Fig. 2. The mean number of seeds ($\pm$ 1 standard error) produced per plant in each of two treatments, open (■) and vertebrate-exclusion (□), at each of the seven study sites.

Fig. 3. The average seed weight (mg) ($\pm$ 1 standard error) for the open treatment (■) and vertebrate-exclusion treatment (□) at each site. The number of seeds weighed per treatment were: Buderroo S1 open n=150, vertebrate excluded n=94; Dharawal S1 open n=117, vertebrate excluded n= 143; Royal S1 open =118, vertebrate excluded n= 141; Helensburgh open n=90, vertebrate excluded n=128. Different letters above bars denote significant differences ($p > 0.05$; Tukey's HSD test).

Fig. 4. The mean T_{50} (the time required to reach 50% germination) in days ($\pm$ 1 standard error), for *Banksia ericifolia* seeds at four study sites, comparing the open (■) and vertebrate-excluded (□) treatments. Different letters above bars denote significant differences.

Table title

Table 1. Results from observations of flower visitation and behaviour for the open and vertebrate-excluded treatments. Data are means per 10 minute observation period (N = 7) ($\pm$ 1 standard error).

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Table 1 Observations of flower visitation and behaviour during 10 minute observation periods. Data are means $\pm$ SE

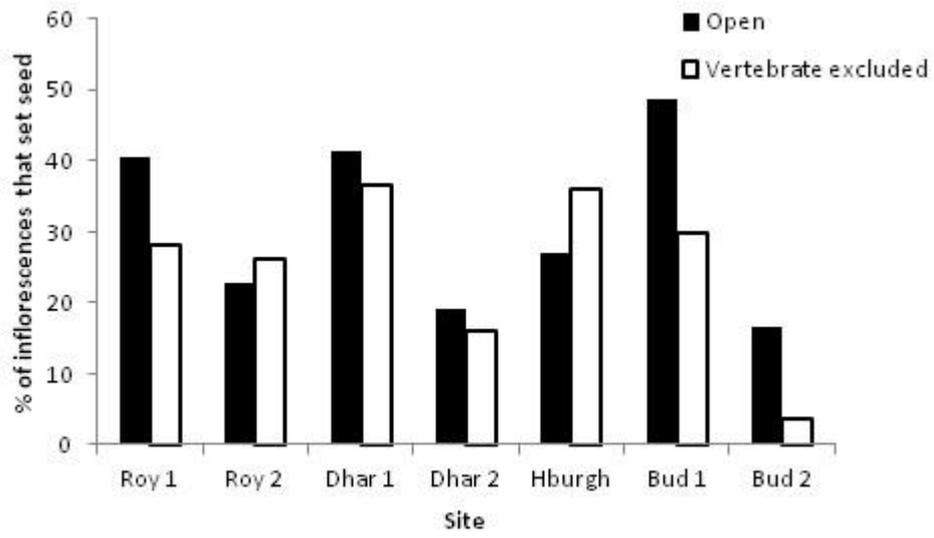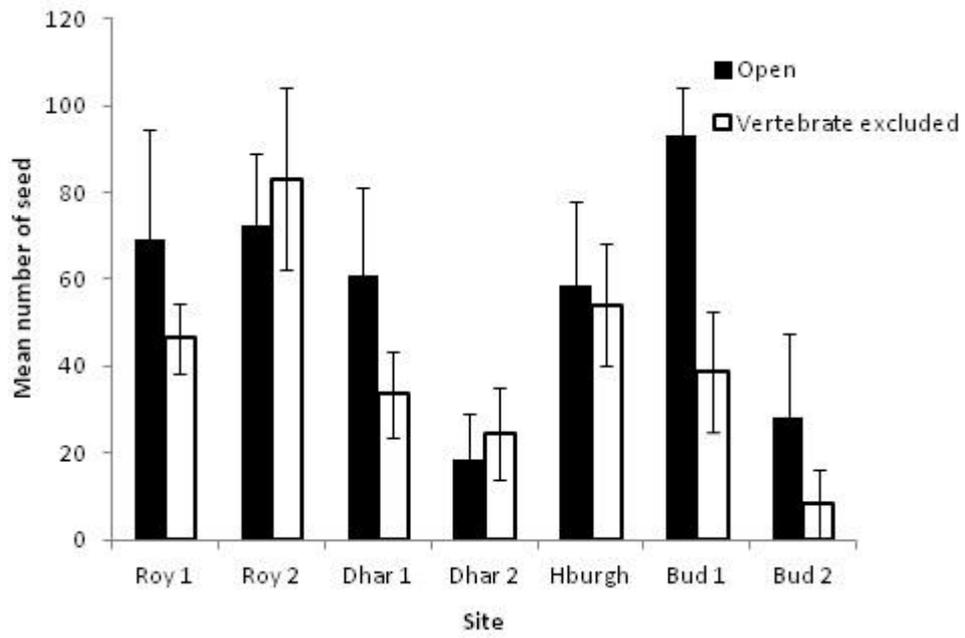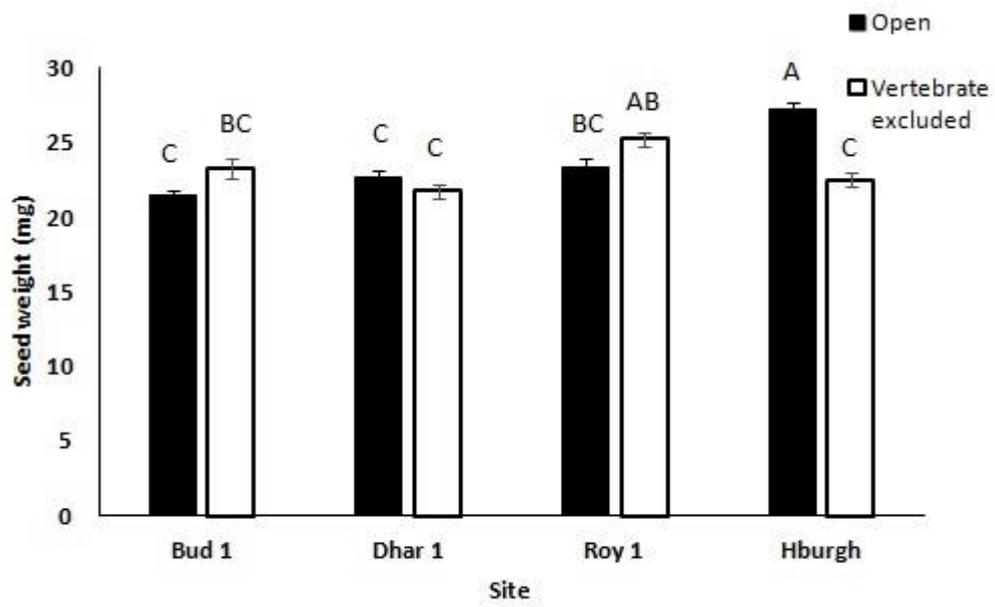| Comparison	Vertebrate exclusion	Open	Test
A. mellifera total visits per plant (nectar and pollen gathering)	15.6 $\pm$ 5.7	20.9 $\pm$ 4.4, n=146	$t_{(6)}$ = 1.25, p = 0.25
A. mellifera visits per plant (pollen gathering only)	4 $\pm$ 0.90	6.86 $\pm$ 1.71	$t_{(6)}$ = 1.32, p = 0.23
A. mellifera intra plant movements	2.2 $\pm$ 0.1, n = 93	1.6 $\pm$ 0.1, n = 75	$t_{(6)}$ = 0.24, p = 0.81
Bird visits per plant	N/A	19.3 $\pm$ 6, n=135	N/A
Bird intra plant movements	N/A	1.9 $\pm$ 0.1, n=109	N/A

Figure 1

Figure 2

Figure 3

Figure 4