A remarkable uniformity in the densities of feral honey bee (Apis mellifera) colonies in South Eastern Australia

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Abstract It is often assumed that the density of feral honey bee colonies in Australia is sufficient to provide adequate pollination services to the many agricultural crops that require pollination. In contrast, there is concern that the density of feral colonies is sufficiently high to have inimical effects on Australian biota. For both these reasons it is desirable to have robust estimates of the density of feral honey bee colonies in Australian landscapes. In this study we mated 4-5 queens with wild drones at disturbed and undisturbed sites in three of the major ecosystems in Victoria Australia, and examined the paternities of worker offspring to estimate the density of feral colonies within mating range of the test queens. We show that the density of feral colonies differs little with land use (cleared or uncleared), and is similar across the state. Our data suggest that the density of feral colonies is probably insufficient to provide adequate pollination of agricultural crops and that neither land use nor local climate variation is a major factor determining density. Finally, our data suggest that the mating range sampled by test queens is significantly greater than previously assumed.

Key words: Feral bees, Pollination, Land use, *Apis mellifera*, Linked microsatellites
**Introduction**

Ecosystem services can be defined as ‘benefits to human welfare provided by organisms interacting in ecosystems’ (Klein et al. 2007). Pollination of crop plants by wild invertebrates is often cited as a classic example of an ecosystem service (Allen-Wardell et al. 1998). About 1/3 of the food that we eat is derived from insect pollinated crops, or pastures that are wholly or partially dependent on pollinators, most of which are wild (Klein et al. 2007). The value of insect pollination is estimated to be 9.5% of total world food production or up to $A 445 billion (Gallai et al. 2009). Pollinators are also keystone species in natural ecosystems, and decline in pollinator abundance may have subtle or major effects on plant communities (Dick 2001).

Recent declines in populations of wild and managed honey bees in Europe and North America have led to warnings of an impending ‘pollination crisis’, in which crop yields and quality would be seriously reduced, and natural ecosystems further stressed (Biesmeijer et al. 2006; Oldroyd 2007; Garibaldi et al. 2013). Concerns about the potential for such a crisis have led to calls for action to avert the perceived decline of pollinator abundance under the auspices of the International Convention on Biological Diversity (Ghazoul 2005).

The majority of crop pollination in Australia is performed by the European honey bee *Apis mellifera* (Cunningham et al. 2002). The value of crops pollinated by bees has been estimated to be as high as $A 1.6 billion (Gordon & Davis 2003). It is currently assumed that for most Australian agroecosystems there are sufficient numbers of native and introduced pollinators to provide adequate pollination (Cunningham et al. 2002). However a combination of pesticide use and land clearing, which reduces nest site availability of feral honey bees and floral resources for native pollinators, may mean that there are now insufficient pollinators available in some agricultural systems (Cunningham et al. 2002; Schellhorn et al. 2008). In 2002, the small hive beetle *Aethina tumida* was first confirmed in beehives New South Wales, and has since spread to Queensland and Victoria causing significant damage (Leemon 2012). Furthermore, Australia is particularly exposed to a future pollinator crisis because of the likely invasion of a parasitic mite, *Varroa destructor* (Cunningham et al. 2002). This mite has spread worldwide, with the single exception of Australia. In all temperate countries where *V. destructor* has established, wild populations of *A. mellifera* have been decimated, particularly in Europe and the United States (Ghazoul 2005). It is almost inevitable that *Varroa* will arrive in Australia, most likely via an illegal importation of queen bees (Cunningham et al. 2002). When this happens the estimated losses...
to agriculture are $A 21-50 million per annum (Cook et al. 2007). Another possible threat to Australian honey bees is the enigmatic Colony Collapse Disorder that has caused significant losses in some parts of the United States and Europe (Oldroyd 2007).

In contrast to agricultural ecosystems where the presence of exotic pollinators like honey bees is regarded as being desirable, in natural systems feral honey bees may have negative impacts (reviewed in Goulson 2003). Potential negative impacts include competition for nest sites (Saunders et al. 1982; Oldroyd et al. 1994; Wood & Wallis 1998), pollination of invasive plant species (Butz Huryn & Moller 1995; Richardson et al. 2000; Goulson & Derwent 2004; Simpson et al. 2005), competition with native pollinators (Gross 2001) and poor pollination of some native plants (Taylor & Whelan 1988; Vaughton 1996; Paton 1997; Gross & Mackay 1998; Goulson 2003).

The extent of positive effects of feral honey bees in agricultural ecosystems, and the potential negative effects in natural ecosystems are density dependent. Generally, a density of 100-200 colonies km\(^{-2}\) is required for effective pollination of fruit crops (Free 1970). Only the highest density of feral honey bees ever recorded globally (150 colonies km\(^{-2}\) Oldroyd et al. 1997) comes close to this. More typically feral colonies have a density of \(1 \times 10^5\) to \(1 \times 10^3\) colonies km\(^{-2}\) (reviewed in Ratnieks et al. 1991).

For several reasons it would be useful to have good estimates of the densities of feral honey bee colonies in different habitats in Australia. In agricultural settings, we need to know if the density of feral bee colonies is sufficient to provide adequate pollination. In conservation areas we would like to know if the density of feral colonies is sufficient to be of concern. A third reason is that should an incursion of \textit{Varroa destructor} or other exotic parasite or pathogen occur in Australia it will be necessary to determine whether location and destruction of feral nests is a feasible method of containing the outbreak (Stevenson et al. 2005; Taylor et al. 2007).

Directly counting the number of honey bee colonies in the environment over broad scales is not often feasible because colonies are cryptic and difficult to locate (Oldroyd et al. 1997). Here we implement a new indirect method of estimating colony density based on microsatellite analysis of workers (Moritz et al. 2007; Shaibi et al. 2008; Jaffé et al. 2009; Arundel et al. 2012). This method provides a measure of the relative spatial and temporal density of bee colonies without having to locate them.

The principle behind the technique is conceptually simple. A diploid queen produces haploid males by asexual parthenogenesis (Winston 1987; Oldroyd & Wongsiri 2006). Thus,
at any one marker locus, her sons will carry one of her two alleles. If we select multiple marker loci that are tightly linked, then recombination events between the loci will be rare or absent. Thus each queen in a population will produce just two haplotypes of this combination of loci in offspring males due to linkage. If the loci chosen are highly polymorphic, then each queen in a population is likely to carry unique haplotypes (Shaibi et al. 2008). Thus if we randomly sample the males of a population, genotype them at a set of linked markers, count the number of unique haplotypes, and divide the answer by two, this provides an estimate of the number of queens (colonies) contributing males to the sample. If the sampling is done during the breeding season (in Australia September to March), then virtually all colonies will be producing males and thus be represented in the male pool of potential mates.

But how to efficiently sample the males? Honey bee drones congregate in mating leks known as drone congregation areas (DCAs), which have a limited spatial extent and tend to persist in the same location from year to year (Loper et al. 1992). It is possible to lure males to a drone trap using the sex pheromone 9-oxy-2-decenioic acid (Williams 1987). As the pheromone is effective at attracting drones only within an area roughly 100m in diameter (Winston 1987; Brockmann et al. 2006), the trap needs to be located at the DCA itself. The difficulty of this approach is that it can be hard to find a DCA in the survey area and inclement weather can prevent deployment of the trap. An efficient alternative is to allow virgin queens to fly to the (unknown) DCAs where they mate with a random selection of males. Honey bee queens mate on the wing with 6-30 males (Palmer & Oldroyd 2000). Thus if four colonies, each with a virgin queen, are placed at a site, the queens will collectively mate with 24-120 males. Thus the worker progeny of several queens mated at a site provides a sample of the haplotypes present in DCAs within the mating flight range of the queens and drones. Males have a maximum flight distance of about 7 km (Ruttner & Ruttner 1972; Koeniger et al. 2005). While the maximum observed mating distance between colonies is 15 km, more than 50% of matings occur within 2.5 km, and 90% within 7.5 km (Jensen et al. 2005).

Based on the assumption that all colonies in flight range send, on average, the same number of drones to a DCA, and that the combined flight range of queens and drones is 15 km (Jensen et al. 2005), the number of unique colonies contributing gametes to the brood samples, can be used to infer the density of colonies in flight range of the test queens (Moritz et al. 2007; Jaffé et al. 2010; Arundel et al. 2012). The estimate can be corrected for non-sampling error, by fitting the frequency distribution of colonies in the sample to the Poisson
distribution (Baudry et al. 1998), or using sampling distributions generated by agent based
models (Arundel et al. 2012). The method cannot sample weak colonies that are not
producing drones, but these colonies are likely to die, and are therefore not ecologically
significant. Similarly, the method will not sample nascent colonies which are yet to produce
drones, and thus the estimates obtained from the data are conservative.

Arundel et al. (2013) pointed out that the method of Baudry et al. (1998) does not take
into account the fact that drones prefer DCAs that are nearby their colony over those that are
further away (Koeniger et al. 2005), the heterogeneous distribution of DCAs (Loper et al.
1987; 1992) and colonies (Oldroyd et al. 1995; Oldroyd et al. 1997; Baum et al. 2005) in the
environment and variability in the number of mating flights and matings by queens (Woyke
1964). Arundel et al. (2012) used agent-based modelling to produce sampling distributions of
the number of colonies likely to be genetically represented in the broods of test queens.
These sampling distributions can be used to infer the range of possible feral colony densities
from the results of a genetic analysis, and are likely to be more realistic than when variability
in mating behaviour and DCA distributions are ignored (Arundel et al. 2012).

In this study we mated a total of 29 queens at three locations in the state of Victoria,
Australia (Table 1). The locations encompassed three of the major ecosystems in Victoria. 1)
The sub-alpine wet sclerophyll forest between 600 and 1300 m above sea level. This forest
type is dominated by mountain ash (Eucalyptus regnans), and is irregularly exploited by
beekeepers for species such as Messmate (E. obliqua). The region provides important habitat
for over 40 species of native arboreal vertebrates (Lumsden et al. 1991), that may compete
with honey bees for nest sites (Lindenmayer et al. 2009). 2) The central box-ironbark forest.
This region is between 150-600 m above sea level, and is characterized by a sparse canopy of
box, iron bark and smooth-barked eucalypts. The ecosystem is an important honey producing
region from species such as yellow box (E. melliodora), grey box (E. microcarpa) and
mugga iron bark (E. sideroxylon). 3) The mallee. This low-rainfall region is dominated by
short (5-8 m) multi-stemmed eucalypts. It is a major honey producing region from species
such as dumosa mallee (E. dumosa), yellow mallee (E. costata) and white mallee (E.
gracilis). Along water courses beekeepers exploit species such as black box (E. largiflorens)
and river gum (E. camaldulensis).

At each location we established two mating apiaries, one in a disturbed habitat and
one in as undisturbed habitat as possible. Our goal was to determine if feral honey bee
colonies are present at similar densities throughout Victoria, to examine the effect of land use on feral nest density.

**Materials and Methods**

*Establishment of mating nuclei*

Mating nuclei (n = 38) were established in early October 2009 in Castlemaine, Victoria. To create a nucleus, three frames containing brood and one frame containing honey were removed from a strong colony. All bees were removed from the frames, and the frames returned to the colony in a box placed above a queen excluder on top of the source colony. Workers moved up onto the brood frames through the queen excluder, but the larger queen and drones could not.

After 24 hours the four frames, now densely covered exclusively with workers, were transferred to a standard nucleus hive. During this transfer, any drone pupae were destroyed, and the nucleus colony was furnished with a queen cell that was about to emerge.

The nuclei were then transported to the seven sites (Table 1) where they were left for 21-30 days. During this time the queens (hereafter ‘test queens’) emerged, mated, laid eggs and produced a first batch of brood. Some test queens failed to emerge from their cells – in most of these cases the nucleus colonies successfully reared a queen from their own female brood. We treated the offspring of these self-raised queens in the same way as the artificially-reared test queens. Worker brood were successfully reared from 29 of the test queens. Brood (n = ~200 pupae per queen) were harvested and stored in 100% ethanol.

*Location of the sites*

Sites were paired to reflect disturbed and undisturbed habitat within the same landscapes (Table 1). In the mallee region, we placed colonies within Wyperfeld National Park, more than 4 km from the park boundary. Commercial beekeeping has been excluded from the park for over 40 years, yet it has very high (up to 160 colonies km\(^{-2}\)) densities of feral bee colonies in the red gum - black box forests that line Outlet Creek (Oldroyd et al. 1997). The site chosen for the mating, at Black Flat, was repeatedly surveyed for the presence of feral colonies for the period 1993-1995 by Oldroyd *et al.* (1995; 1997). A matched, disturbed site was selected 5 km outside the park towards the township of Yaapeet. Here the predominant mallee vegetation has been cleared to make way for wheat, barley, oats and canola cropping. Remnant mallee vegetation remains along fence lines and road verges.

Within the box-ironbark region of central Victoria we established two sites (Puckapunyal North and South) 2 km apart within the 400 km\(^2\) Puckapunyal army training
base, and a third site, Puckapunyal East, 12 km north east of Puckapunyal North and South. Because of military training exercises on the base, there has been no logging or beekeeping activity there since the 1970s. We established adjacent sites at Puckapunyal so that we could replicate our sampling (Table 1) and determine if queens mating 2 km apart would mate with drones from the same colonies. We established a matching disturbed site within the box-ironbark region at Dookie Agricultural College. At this site only remnant tree cover remains. Principle agricultural activities include cattle grazing and mixed cropping.

The remaining two sites were established in wet sclerophyll alpine forest in south east Victoria. One site near the township of Marysville had been heavily burned during the widespread bushfires of January 2009, and we classified this site as disturbed. At the Marysville site, all trees and ground flora had been severely burnt or destroyed by the fire, severely limiting the forage available for bees. The other site, near Eildon, was largely unaffected by the 2009 bushfires. At this site, approximately 20% of the vegetation within 5 km of the site was burnt in the 2009 bushfires, and the remainder had not been burnt in the last 10 years. The extent of the 2009 bushfires can be viewed online at http://nremap-sc.nre.vic.gov.au/MapShare.v2/imf.jsp?site=forestexplorer (Department of Environment and Primary Industries 2013).

DNA extraction and genotyping

DNA was extracted from 48 progeny from each queen. A single hind leg from each pupa was used to extract DNA using a high-salt precipitation method (Aljanabi & Martinez 1997). Each pupa was then genotyped at two sets of linked microsatellites (Shaibi et al. 2008): UN351, HB-SEX-01, HB-SEX-02 and HB-SEX-03 (hereafter ‘LG-3’), and HB-THE-01, HB-THE-02, HB-THE-03 and HB-THE-04 (hereafter ‘LG-13’). PCRs were performed using the conditions given in Shaibi et al. (2008). PCR products were fluorescently labelled and run on an Applied Biosystems 3130xl Genetic Analyzer. Genotyping was performed using Genemapper software (Applied Biosystems). Measures of allelic richness and expected heterozygosity were determined for each site using GENEPOP (Raymond & Rousset 1995) based on the inferred paternal allele of each genotyped worker.

Analysis

A test queen carries two haplotypes at both LG-3 and at LG-13 (assuming that she is not homozygous at all loci in either linkage group), and each of her worker offspring inherits one of the queen’s two haplotypes for each linkage group. Identifying the two haplotypes that are represented in all of the worker progeny of a test queen allows the identification of the test
queen’s haplotypes at LG-3 and LG-13. The paternal haplotype for each worker, derived from the feral drone with which the test queen mated, is then determined by subtraction of the maternal haplotype (Oldroyd et al. 1996; Oldroyd & Wongsiri 2006).

A queen heterozygous at LG-3 and LG-13 produces haploid drones with one of four genotypes (i.e. the four possible combinations of her two pairs of haplotypes). Thus the number of wild colonies present at a location can be estimated by dividing the number of unique paternal genotypes observed in the worker progeny at a site by four. However it can happen that the number of haplotypes observed in one linkage group significantly exceeds the number observed for the other linkage group. When this occurs it is appropriate to take the linkage group with the maximum number of drone haplotypes and divide this number by two as an improved estimate of the number of colonies present.

Having determined a range for the number of feral colonies represented in the broods of test queens we used the agent based modelling approach of Arundel et al. (2012) to estimate the number of colonies per square kilometre, based on likely assumptions about the flight range of queens (8 km) and drones (7 km) and mean mating frequency (n = 14). This method produces a lower estimate of feral colony numbers than does the traditional method where the area sampled by the test queens is assumed to be 4.5 km² (Jaffé et al. 2010). To allow comparison of the two kinds of estimates and with previous work, we have estimated feral colony density using both the agent based modelling approach and by simply dividing the number of unique feral colonies represented in broods by 4.5.

**Non detection error**

The genetic technique used here makes the simplifying assumption that all males present in a test queen’s colony are sampled without error (Arundel et al. 2012). This assumption is close to reality, provided that the number of worker bees genotyped from each test queen progeny is large. The probability of not sampling a patriline (i.e. at least one worker daughter of each male in a mating) is given by \((1-p)^n\) where \(p\) is the proportion of the progeny sired by a male, and \(n\) is the sample size (Foster et al. 1999). Thus if a male is the father of 5% of progeny, the probability of not sampling his progeny in a sample of 48 is 0.0085. Therefore non-sampling of a patriline in a particular test queen brood is a negligible source of error in this study.

Non-detection error also arises from the possibility that two unrelated males share the same haplotypes by chance rather than because they are brothers (Foster et al. 1999). This probability is the frequency of each haplotype in the population. Here we have used two
groups of three linked microsatellite that are highly polyallelic. If the loci used for genotyping were unlinked, the probability that two random males in a population would share the same haplotype by chance would be \( \prod q_i \), where \( q_i \) is the frequency of the allele shared by the two random males at the \( i \)th locus. Because the loci used here are linked, we cannot legitimately use \( \prod q_i \) to estimate this probability, but must use the frequency of haplotypes in the sampled population (Jaffé et al. 2010). The frequency of each multi-haplotype genotype in the population is \( (q_{\text{Thee}})(q_{\text{Sex}}) \), the probability that two random males will share the same multi-haplotype genotype. We have calculated this probability for every multi-haplotype male genotype observed in our data, and compute the average non-detection error per fathering male as the average of these probabilities per site (Jaffé et al. 2010).

**Results**

Measures of allelic diversity and heterozygosity are given in Table 2. The minimum number of feral colonies represented in the progeny of test queens is given in Table 3. Treating Puckapunyal North and South as the same site, the mean minimum number of feral colonies represented in the progeny of test queens per site (i.e. the number of unique feral queens that contributed drones to the matings) was 18.0 ± 2.5 s.e. We performed two-way ANOVAs of the random effects of land type (disturbed vs undisturbed) and ecosystem (mallee, box-iron bark, alpine) on the minimum number of feral colonies represented in the brood. Levene’s test of equality of variances between treatment groups showed that the data satisfied this assumption of ANOVA \( (P > 0.05) \). There was no significant interaction between land type and ecosystem, so we report an ANOVA of main effects only. Land type had no significant effect on the number of colonies detected \( (F_{1,3} = 1.2, P = 0.36) \). Disturbed sites had a mean of 17.7 feral colonies represented in broods, whereas undisturbed sites had 19.5 feral colonies. Ecosystem had a significant effect \( (F_{2,3} = 17.0, P = 0.02) \), with the mallee region \( (x = 27.0) \) having a higher density of feral nests than the other two \( (x = 14.15) \).

Remarkably, the number of feral colonies represented in the progeny of queens that mated at Eildon (unburnt) and Marysville (burnt) was numerically similar (Table 3). There was no significant difference \( (P > 0.05) \) in the number of feral colonies detected between the two mountain ash sites combined \( (x = 14.0) \) and the four box-ironbark sites combined \( (x = 14.3) \).
The Wyperfeld and Yaapeet sites were separated by a distance of 15 km. Between the ten test queens from the two sites (five at each site), the brood shared eight drone genotypes—that is brood from four of the Wyperfeld queens shared both LG-3 and LG-13 paternal haplotypes with brood from one of three Yaapeet queens. These data suggest that drones from a single colony mated with queens from colonies that were 15 km apart.

Puckapunyal North and South were 2 km apart. Broods shared one drone genotype between the two sites. In addition, one LG-3 haplotype and two LG-13 haplotypes were common to brood from both sites. For this site pair, the results are consistent with drones from six feral colonies being represented in broods at both Puckapunyal North and South.

The sum of the minimum number of feral colonies identified in broods from Puckapunyal North and South (3 and 11, respectively—Table 3) is not identical to the minimum number of feral colonies calculated when the genotypes from both sites are combined (12, Table 3). This is because the patrilines from Puckapunyal North and South that share identical drone haplotypes are considered to have originated from a single queen when the sites are combined, but from separate queens when the sites are considered separately. If Puckapunyal North and South are considered a single mating site, the number of feral colonies inferred across all sites ranged from 11 to 29 (Table 3).

Based on the agent-based modelling approach of Arundel et al. (2012) the likely minimum density of feral colonies is about 0.1 km$^{-2}$ at the box ironbark and mountain ash sites, and about 0.2 km$^{-2}$ at the mallee sites (Table 3). Based on the ‘area sampled’ approach of Jaffé et al. (2010) the estimates of feral colony density range from 2.7 colonies km$^{-2}$ at the combined Puckapunyal North and South sites, to 6.4 colonies km$^{-2}$ at Wyperfeld (Table 3).

**Discussion**

The number of unique drone haplotypes represented in test queen progenies shows that feral honey bee colonies are present throughout Victoria, in both disturbed sites and in areas where the original tree cover is still present. We found no evidence for relative differences in feral colony densities between disturbed and undisturbed sites. Even at Marysville, where the vegetation had been recently destroyed by fire, feral colonies are present in densities similar to the unburned mountain ash site at Eildon and to the box-ironbark sites at Puckapunyal.

Our agent-based modelling (Figure 2, Arundel et al. 2012) shows that there is a log-linear relationship between feral colony densities and the count of unique colonies with which a queen mates. Consequently, at lower densities ($\leq 1$ colony km$^{-2}$), three or more test queens will detect order of magnitude differences in the underlying feral colony density. The number
of feral colonies detected in our experiment is in all cases consistent with these lower feral colony densities. Based on Figure 1, the observed number of feral colonies represented in broods at Wyperfeld (29; Table 3) is compatible with a minimum feral colony density of 0.2 colonies km\(^{-2}\). A mid-range estimate of feral colony density of 0.9 colonies km\(^{-2}\) was obtained by using Figure 1 to infer the density from a count (of 51) halfway between the minimum (29) and maximum (73). Importantly, Arundel et al. (2012) showed that the spatial distribution of feral colonies and drone congregation areas has no effect on the number of feral colonies represented in test queen broods. Rather, the only important factor affecting the number of feral colonies represented in test queen broods is the variable of interest: the density of feral colonies. This finding is unsurprising when we consider that the mating system of the honey bee evolved to maximise intra-colonial genetic diversity (Crozier & Page 1985; Palmer & Oldroyd 2000; Crozier & Fjerdingstad 2001; Oldroyd & Fewell 2007).

Given these caveats, we note that our agent-based estimates (c.a. 0.1 – 0.5 colonies km\(^{-2}\)) are an order of magnitude lower than those reported on the basis of field surveys in Botswana (4.2 colonies km\(^{-2}\) McNally & Schneider 1996), New York (4.2 Morse et al. 1990), Mexico (6 Ratnieks et al. 1991), Panama (4-7 Boreham & Roubik 1987) and Texas (12.5 Baum et al. 2005). More importantly, they are 2-3 orders of magnitude lower than field counts of feral colonies conducted from 1993 to 1995 in Wyperfeld National Park (Oldroyd et al. 1997). At the specific location of Black Flat where the queens for this study were mated, Oldroyd et al. (1997) found densities of 120 colonies km\(^{-2}\) in 1993, 100 in 1994, and 20 in 1995. Note, however, that these estimates based on physical searches refer to the narrow band of riparian woodland that surrounds Black Flat. At broader scales, the density of feral colonies is much lower, and probably more similar to that estimated here.

Using the simple device of dividing the minimum number of feral colonies represented in broods by 4.5 (Jaffé et al. 2010), the estimate of the density of feral colonies is considerably higher than the estimate obtained from Figure 1, and closer to estimates obtained internationally by the same method (Jaffé et al. 2010). However, our experiment has shown that dividing the count of feral colonies present in test queen brood by a sample area of either 4.5 km\(^{2}\) (Jaffé et al. 2010) or 10.0 km\(^{2}\) (Moritz et al. 2007) significantly overestimates the density by underestimating the true extent of the area being sampled.

Our results from the Wyperfeld and Yaapeet sites showed a significant overlap in the feral colonies detected by test sites separated at a distance of 15 km. This mating distance is consistent with findings of Jensen et al. (2005), and implies a minimum sampled area with a
radius of half this distance (7.5 km); that is, an area of 177 km². The riparian area surveyed by Oldroyd et al. (1997) is 7.7km from Yaapeet at its closest point. This same point is 8.3km from the Wyperfeld site. This suggests that colonies in this area were sampled by the test queens from both the Wyperfeld and Yaapeet sites. It further implies that four of the five test queens from Wyperfeld took one or more mating flights in a southerly direction, and that at least three of the four test queens from Yaapeet took one or mating flights in a north-westerly direction. This suggestion could potentially be confirmed by direct sampling of colonies in the area between the two sites.

Our study provides the first relative measures of feral bee densities across Australian ecosystems. We find that land use has no significant effect on the density of bees, but that there are significantly more feral colonies in the mallee than elsewhere. Our estimates are based on assumptions about mating flight distances supported by our experimental findings, and we thus believe our model provides a realistic range for the density of colonies at a site. If we make the assumption that our density estimates are accurate within an order of magnitude, what can we say about the environmental effects of feral colonies on Australian agricultural and natural ecosystems? First, our data suggest that the density of feral bees is probably insufficient to provide adequate pollination in a horticultural setting. Typical recommended stocking rates are 100-200 colonies km⁻² (e.g. Free 1970), whereas our estimates are < 10 colony km⁻². We suggest that without supplementation with domestic colonies, it is unlikely that any crops requiring insect pollination are adequately pollinated. Second, concerns about the impacts of feral honey bee colonies on natural ecosystems (Goulson 2003) are likely to be unfounded in most areas, because the density of feral colonies is quite low. While there may be some competition for forage, competition for nest sites with native fauna seems unlikely except in specific sites such as riparian woodland where the density of feral colonies can be very high.

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Table 1: Locations of mating sites, listed from north to south. Land use is described in detail in the materials and methods. Nearest mating site indicates the next closest mating site. The distance between each mating site and its nearest neighbour is indicated in the rightmost column.

<table>
<thead>
<tr>
<th>Site Name</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Land use</th>
<th>Disturbed/ undisturbed</th>
<th>Nearest mating site</th>
<th>Distance between paired sites</th>
<th>Ecosystem</th>
</tr>
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<td>Wyperfeld</td>
<td>35°34'43.94&quot;S</td>
<td>142° 1'4.93&quot;E</td>
<td>National park</td>
<td>Undisturbed</td>
<td>Yaapeet</td>
<td>15 km</td>
<td>Mallee</td>
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<tr>
<td>Yaapeet</td>
<td>35°42'23.97&quot;S</td>
<td>142° 3'58.57&quot;E</td>
<td>Cereal and canola farming</td>
<td>Disturbed</td>
<td>Wyperfeld</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dookie</td>
<td>36°23'41.12&quot;S</td>
<td>145°43'56.17&quot;E</td>
<td>Mixed farming/grazing</td>
<td>Disturbed</td>
<td>Puckapunyal</td>
<td>85 km</td>
<td>Box-ironbark</td>
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<td>Puckapunyal</td>
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<td>144°59'56.16&quot;E</td>
<td>Military base</td>
<td>Undisturbed</td>
<td>Puckapunyal</td>
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</tr>
<tr>
<td>East</td>
<td>36°56'45.79&quot;S</td>
<td>144°53'33.01&quot;E</td>
<td>Military base</td>
<td>Undisturbed</td>
<td>Puckapunyal</td>
<td>South</td>
<td></td>
</tr>
<tr>
<td>North</td>
<td>36°57'29.24&quot;S</td>
<td>144°53'8.29&quot;E</td>
<td>Military base</td>
<td>Undisturbed</td>
<td>Puckapunyal</td>
<td>North</td>
<td></td>
</tr>
<tr>
<td>South</td>
<td>37°18'32.45&quot;S</td>
<td>145°55'25.83&quot;E</td>
<td>Forestry</td>
<td>Undisturbed</td>
<td>Marysville</td>
<td>30 km</td>
<td>Wet sclerophyll</td>
</tr>
<tr>
<td>Eildon</td>
<td>37°32'30.68&quot;S</td>
<td>145°44'45.61&quot;E</td>
<td>Forestry</td>
<td>Disturbed (burnt)</td>
<td>Eildon</td>
<td></td>
<td>alpine forest</td>
</tr>
</tbody>
</table>
Table 2: Measures of genetic variability in honey bee populations in Victoria, Australia. Data are based on the paternal allele of the sampled worker genotypes. Where the worker carried the same genotype as the queen both the paternal and maternal alleles were entered into the data set.

<table>
<thead>
<tr>
<th>Population</th>
<th>K</th>
<th>HBSex1</th>
<th>HBSex3</th>
<th>UN351</th>
<th>HBTh2</th>
<th>HBTh3</th>
<th>HBTh4</th>
<th>Ar</th>
<th>HE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wyperfeld</td>
<td>11</td>
<td>3</td>
<td>16</td>
<td>5</td>
<td>6</td>
<td>4</td>
<td></td>
<td>7.5</td>
<td>0.73</td>
</tr>
<tr>
<td>Yaapeet</td>
<td>7</td>
<td>3</td>
<td>18</td>
<td>5</td>
<td>7</td>
<td>3</td>
<td></td>
<td>7.2</td>
<td>0.72</td>
</tr>
<tr>
<td>Dookie</td>
<td>6</td>
<td>3</td>
<td>15</td>
<td>6</td>
<td>6</td>
<td>3</td>
<td></td>
<td>6.5</td>
<td>0.71</td>
</tr>
<tr>
<td>Puckapunyal</td>
<td>14</td>
<td>5</td>
<td>21</td>
<td>4</td>
<td>8</td>
<td>5</td>
<td></td>
<td>9.5</td>
<td>0.74</td>
</tr>
<tr>
<td>Eildon</td>
<td>5</td>
<td>3</td>
<td>16</td>
<td>5</td>
<td>7</td>
<td>4</td>
<td></td>
<td>6.6</td>
<td>0.72</td>
</tr>
<tr>
<td>Marysville</td>
<td>6</td>
<td>3</td>
<td>11</td>
<td>5</td>
<td>6</td>
<td>4</td>
<td></td>
<td>5.8</td>
<td>0.72</td>
</tr>
</tbody>
</table>

K, number of microsatellite alleles; Ar, average allelic richness; Average $H_E$ expected heterozygosity over all loci.
Table 3: Estimates of the number of feral honey bee colonies at 8 sites in Victoria, Australia. The number of unique drone haplotypes identified at each mating site is given for two linkage groups. The minimum number of feral colonies represented in broods at each site is then determined by halving the number of unique haplotypes at LG-13 or LG-3 (whichever is the greater). The density of feral colonies at the site is then estimated based on the agent based model of model of Arundel et al (2012).

<table>
<thead>
<tr>
<th>Site</th>
<th>Number of broods examined</th>
<th>Average mating number (± s.e.)</th>
<th>Unique Haplotypes Identified</th>
<th>Minimum number of feral colonies</th>
<th>Mid-range estimate of feral colonies</th>
<th>Maximum number of feral colonies</th>
<th>Approximate feral colony density km$^{-2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NDE$^a$</td>
<td>Total</td>
<td>LG-13</td>
<td>LG-3</td>
<td>Estimate based on minimum: Arundel$^b$</td>
</tr>
<tr>
<td>Wyperfeld</td>
<td>5</td>
<td>16.8 ± 0.6 4.8 × 10$^{-4}$</td>
<td>73</td>
<td>23</td>
<td>58</td>
<td>29</td>
<td>0.2</td>
</tr>
<tr>
<td>Yaapeet</td>
<td>5</td>
<td>17.2 ± 0.9 5.2 × 10$^{-4}$</td>
<td>68</td>
<td>28</td>
<td>50</td>
<td>25</td>
<td>0.15</td>
</tr>
<tr>
<td>Dookie</td>
<td>4</td>
<td>13.5 ± 1.8 1.8 × 10$^{-4}$</td>
<td>51</td>
<td>28</td>
<td>27</td>
<td>14</td>
<td>0.1</td>
</tr>
<tr>
<td>Puckapunyal East</td>
<td>4</td>
<td>14.5 ± 0.5 1.4 × 10$^{-4}$</td>
<td>58</td>
<td>22</td>
<td>35</td>
<td>18</td>
<td>0.15</td>
</tr>
<tr>
<td>Puckapunyal</td>
<td>2</td>
<td>14.5 ± 5.6 × 10$^{-4}$</td>
<td>26</td>
<td>12</td>
<td>21</td>
<td>11</td>
<td>-</td>
</tr>
<tr>
<td>Location</td>
<td>North</td>
<td>Puckapunyal North</td>
<td>South</td>
<td>Puckapunyal South</td>
<td>North + South</td>
<td>Eildon</td>
<td>Marysville</td>
</tr>
<tr>
<td>------------</td>
<td>-------</td>
<td>-------------------</td>
<td>-------</td>
<td>-------------------</td>
<td>---------------</td>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8.5</td>
<td>10^{-4}</td>
<td>6.3 \times 10^{-4}</td>
<td>12.3 ± 5.4</td>
<td>15.3 ± 1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>8 ± 0</td>
<td>7.2 \times 10^{-4}</td>
<td>8 4 6 3 5.5 8 - - - -</td>
<td>59 22 19 15 37 59 0.1 0.6 &gt; 10 3.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>12.3 ± 5.4</td>
<td>6.3 \times 10^{-4}</td>
<td>34 16 24 12 23 34 0.1 0.3 1.5 2.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>15.3 ± 1.3</td>
<td>1.7 \times 10^{-4}</td>
<td>52 20 25 13 32.5 52 &lt; 0.1 0.4 5 2.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>2.5 \times 10^{-4}</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Nondetection errors, calculated as in Jaffé et al. (2010).

\(^b\) Based on the agent model of Arundel et al (2012) and the number of test broods sampled, this is the mean density of feral colonies that is compatible with the number of feral colonies observed in broods (n = 100 simulations). See Figure 1.

\(^c\) Based on the assumption that queens sample the feral colony population in a 4.5 km\(^2\) area centred on the test apiary (Jaffé et al. 2010). This estimate is the minimum number of feral colonies represented in test queen broods divided by 4.5.
Figure 1: The relationship between feral colony density and the number of colonies represented in test queen broods assuming (a) 3, (b) 4 and (c) 5 test queens based on agent-based modeling (Arundel et al. 2012). The shaded grey area represents the region within 2 standard deviations of the mean (thus accounting for 95% of observations) for each of the densities for which simulations were performed. This region shows the range of actual densities associated with a predicted maximum likelihood density. The count of unique feral colonies with which these queens mated (Y axis) can be used to estimate the number of feral colonies per km$^2$ (X axis).
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