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Reproductive biology and genetic structure in fragmented populations of the temperate mangrove Avicennia marina

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Reproductive biology and genetic structure in fragmented populations of the temperate mangrove *Avicennia marina*

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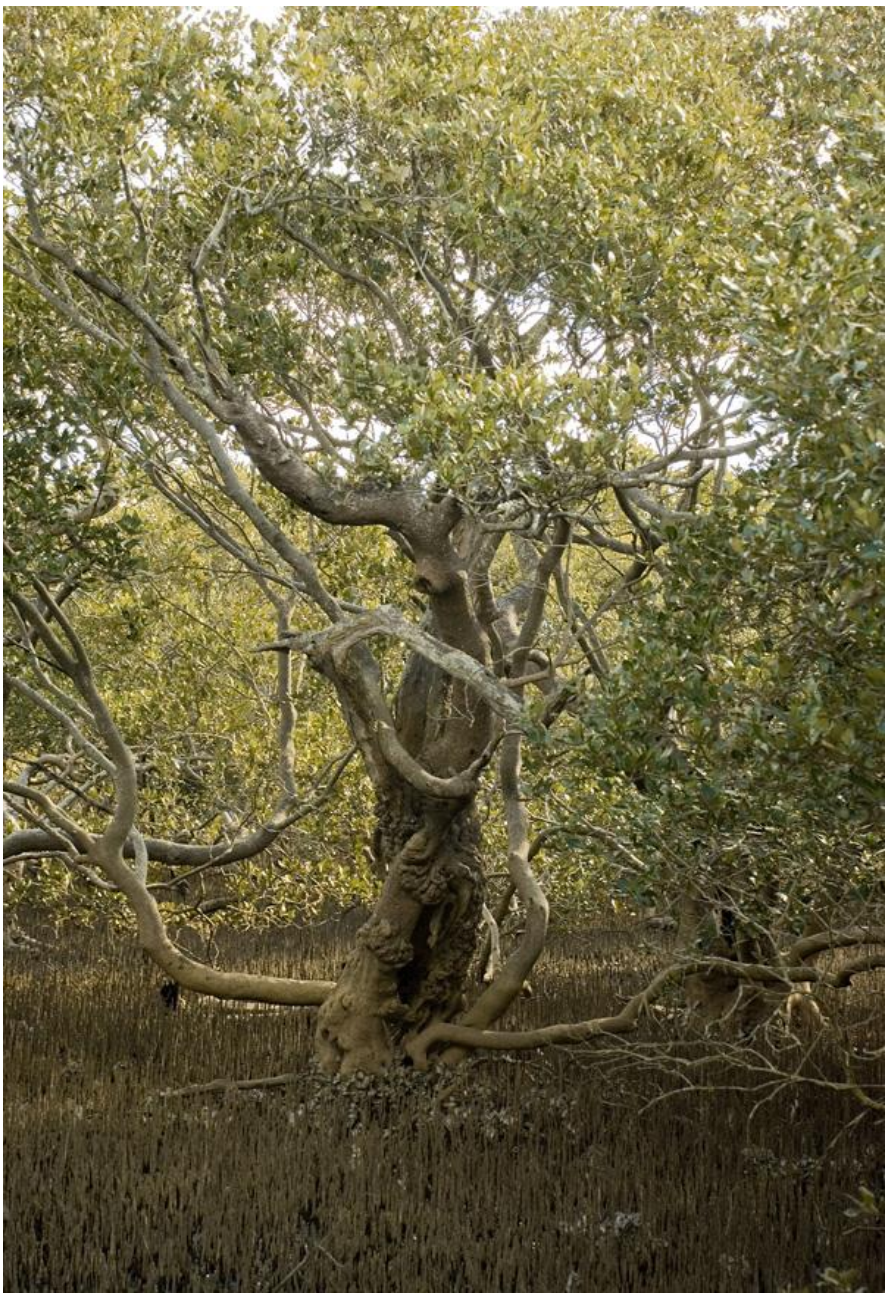
“It is unwise to be too sure of one’s own wisdom. It is healthy to be reminded that the strongest might weaken and the wisest might err.”

Mahatma Ghandi



Avicennia marina from the mangrove forest in Minnamurra, NSW, Australia

Certification

I, Tyge D. Hermansen, hereby declare that this thesis, submitted in fulfilment of the requirements of the award of Doctor of Philosophy, is wholly my work unless otherwise referenced or acknowledged, and none of the included chapters has been submitted to any other tertiary institution or accepted for the award of any other degree or diploma at any other tertiary institution.

Tyge Dahl Hermansen

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PUBLICATIONS

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Abbreviations

AFLP = Amplified Fragment Length Polymorphism

CPS = Corbicula pollen store

NSW = New South Wales of Australia

PCR = Polymerase Chain Reaction

SE = Standard error

SNK = Student-Newman Keuls

ABSTRACT

Land clearance and conversion have resulted in habitat loss and anthropogenic fragmentation (including reduction of stand size and increased isolation), which are some of the greatest threats to plant populations today. It is of immense importance that we uncover the mechanisms behind these threats (i.e. impact on pollen and seed vectors and ecological consequences of this, leading to higher levels of inbreeding and reduced production and quality of fruits), because ignoring them would expose many anthropogenically disturbed plant populations to the risk of extinction. I investigated the impacts of stand size on temperate anthropogenically fragmented populations of the mangrove *Avicennia marina* in three Sydney and Minnamurra estuaries on the coast of New South Wales (NSW) in Australia, near the southern limits of mangroves, where mangrove populations have suffered from anthropogenic fragmentation, dividing them into stands of varying size, from large stands of 10000 trees or more down to single isolated trees. My study investigated the impacts of stand size (in the present study comparisons were done between large stands of 1500-10000 trees, medium stands of 300-500 frees and small stands of up to ca. 100 trees) on pollination biology, mating systems and reproductive output. It also combined highly replicated surveys of pollinator activity and diversity and reproductive success, experimental tests of pollinator activity and the use of neutral DNA markers to estimate the effects of stand size on genetic diversity and mating systems.

Today it is thought that a range of generalized pollinators pollinate mangroves. To test this hypothesis in temperate populations of *A. marina* flower visiting insects were captured during multiple surveys of flower visitation and it was investigated which

species touched the stigma during foraging and which species carried pollen on body parts touching the stigma. Species that did were tested for pollen removal and deposition to establish their identity as pollinators.

I identified 38 species of insects visiting the flowers of *A. marina,* but only the exotic honeybee *Apis mellifera* was identified as an important pollinator species. *A. mellifera* was the only species that carried large numbers of pollen grains and foraged in a manner that permitted the transfer of pollen to floral stigmas. *A. mellifera* was also the dominant flower visitor and during experiments I demonstrated that it was effective in pollen removal and deposition. These results are in contrast to the existing hypothesis that mangroves are typically pollinated by a range of generalized pollinators. However, in carefully reviewing the literature on mangrove pollination I found that no earlier studies provide convincing evidence of the nature of species pollinating mangroves. I therefore emphasise the need for other studies that follow my suggested template. On studying the pollen loads on the body of *A. mellifera* I found that honeybees of the two investigated estuaries with pollen in their corbicula carried on average 1215 pollen grains on the body and 8813 in their corbiculae, while honeybees without pollen grains in their corbiculae carried on average 1027 pollen grains on the body. On the body of individual honeybees on average 89%-95% of the pollen grains was from *A. marina,* demonstrating fidelity for the species on which they forage. My investigations of pollen removal revealed that only 4% pollen remains on anthers after one visit from a honeybee compared to flowers bagged to exclude pollinators. Finally, tests of pollen deposition for night pollination revealed that only on average 4% of the investigated floral stigmas carried pollen grains while day pollination revealed that on average 92% did, and honeybees deposited an average of ca. three pollen grains on floral stigmas

after one visit to a flower by a honeybee. These experiments suggest that honeybees have the potential to carry large amounts of *A. marina* pollen and be effective pollinators of *A. marina*, as they remove and carry pollen enough to ensure sufficient pollen deposition.

For terrestrial plants the abundance and foraging behaviour of pollinators has been shown to vary between large and small stands, which in turn can alter outcrossing rates and plant fitness dependent of the compatibility system of a plant. Such relations have never been investigated in mangroves. In the present study on fragmented populations of *A. marina*, I found this mangrove to be partially autogamous. I also tested a number of factors for effects of stand size. I found that on average the abundance of pollinators was 71%-84% lower, the duration of foraging bouts on individual plants 10%-30% longer, the visitation to floral shoots 9% higher (during foraging within adult trees), and the pollen deposition 61% lower in small stands. I found that on average fruit production was 41%-59% and 61%-69% lower per floral shoot or tree respectively and the production of floral shoots was 22%-37% lower, while fruit weight was ca. 19% lower. Finally, I investigated the consequences for recruitment of saplings. I found that the average density of propagules (fruit after fall) on the forest floor immediately after abscission from the trees was 69% lower, the density of newly established seedlings was 67% lower and the density of seedling surviving to 3 months was ca. 61% lower in small stands. These results show that the impact of stand size influences negatively on pollination biology (as performed by the exotic honeybee *Apis mellifera*) and reproductive output in temperate populations of the mangrove *A. marina*.

Avicennia marina in the Sydney region of Australia commonly comprise small stands with reduced pollinator services (as compared to pollinator services in large stands), which may potentially reduce the genetic diversity and change the mating systems of small stands as compared to large stands. Using four microsatellite markers I investigated the effects of stand size on the levels of genetic and genotypic diversity and I inferred the mating system that had generated the samples of adult plants within stands of *A. marina* of two anthropogenically disturbed estuaries. I also used progeny arrays to estimate the effect of stand size on multilocus outcrossing rates (t_m) and other parameters of the current mating systems. I did not detect any significant effect of stand size on levels of genetic diversity (N_a, N_e and H_e). Comparison of adult genotypes with expectations for random mating revealed an average inbreeding coefficient (F_{IS}) of 0.089 ± 0.03 indicating only a slight heterozygous deficit and allelic differentiation was low within ($F_{SE} = 0.021$, $P < 0.01$) and among ($F_{ET} = 0.055$, $P < 0.01$) the two estuaries, and I found no evidence of isolation by distance. These results indicate that the connectedness (as inferred from variation in adult genotypes) is relatively high and only a small impact of anthropogenic fragmentation was found among stands of the investigated system. My analysis of the progeny arrays however, suggest that while all stands display high levels of biparental inbreeding (t_m-t_s), the multilocus outcrossing rates (t_m) were on average significantly (ca. 28%) lower in small stands compared to large stands, which indicates a significant effect of stand size and may potentially reduce the fitness among plants of the small stands.

Conclusion

This study shows that the pollination system of anthropogenically fragmented temperate populations of the mangrove *A. marina* has been invaded by the exotic honeybee *Apis melliffera* that is the only effective pollinator identified within the investigated stands. It also indicates a significant effect of stand size on factors such as pollinator abundance and services and reproductive output in small stands of the investigated estuaries. The genetic data on adult trees revealed that regardless of size stands are strongly interconnected and have similar diversity while the current mating system revealed a significant effect of stand size on the level of multilocus outcrossing and high levels of biparental inbreeding in all stands regardless of size. As judged on the data revealed from pollination biology and reproductive output and genetic data on the current mating system, the results of this study suggest that the small stands are at a much higher risk for the exposure to ecological and genetic effects caused by reduced stand size than those of large stands. However, the genetic data on adult trees revealed a strong interconnection among stands of both estuaries, which could be due to that the stands being recently formed (formed within the last century). This could result in two alternative scenarios. The dispersal of pollen and propagules among stands regardless of size may be effective enough to oppose the negative effects of stand size, implying that all investigated stands would remain healthy regardless of the detrimental impacts of stand size affecting the small stands, or, future generations may over time experience significant genetic effects within small stands if small stands become progressively more inbred.

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This thesis is dedicated to the memory of my parents, Arne Hermansen and Meta Dahl Hermansen.

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Chapter 1: General introduction

1.1 Impacts of stand size on pollination biology and reproductive output of plant species

1.1.1 *Impacts of anthropogenic fragmentation*

Anthropogenic fragmentation can reduce continuous populations to smaller stands of varying size (for example, large medium and small stands as used in the present study), which may affect the fitness of plants in small stands by disrupting the diversity, abundance and foraging behaviour of pollinators (Aizen *et al*. 2002; Ghazoul 2005; Aguilar *et al*. 2006). This in turn may result in reduced pollen supply and pollen limitation (Aizen & Feinsinger 1994*a*; Sih & Baltus 1987; Cascante *et al*. 2002), or change the mating systems by increasing the transfer of self or near related pollen grains (Steffan-Dewenter & Tscharntke 1999). For terrestrial plants such conditions have been shown to promote higher levels of inbreeding (reviewed by Ghazoul 2005) and result in reduced production and quality of fruits and seedlings (Jennersten 1988; Aizen & Feinsinger 1994*b*; Ghazoul & McLeish 2001; Barbeta *et al*. 2011). Moreover, fragmentation and reduced dispersal of pollen or propagules should cause population differentiation via genetic drift although the extent of such differentiation will be determined by factors such as generation length, the presence of seed banks and the frequency of migration (e.g. Llorens *et al*. 2004).

1.1.2 *Introducing the four data chapters*

In this thesis I investigate the effects of stand size on pollination biology, reproductive output, genetic structure and mating systems of temperate anthropogenically fragmented

populations of the mangrove *Avicennia marina* in southeast Australia (Fig. 1.1-1.3). In this first chapter I present a literature review as a general introduction to concepts and aspects of the research presented in the next chapters. In chapter 2 the pollination biology of temperate *A. marina* is investigated, and I reveal the suite of flower visitors and through experimentation and detailed observation determine the set of true pollinators and their foraging behaviour. In chapter 3 a preliminary study on the effects of stand size on the pollinator abundance, behaviour and services, as compared to the effect of stand size on the fruit production is presented. This preliminary study helped me to refine the approach for a comprehensive and thorough study of the effect of stand size on pollinator visitation and behaviour and consequent effects of stand size on the quality and production of fruits and the production and survival of seedlings, which is presented in chapter 4. In chapter 5 a study on genetic variation across four

Fig. 1.1. Trees of the mangrove *Avicennia marina* are flanking the waterfront of the mangrove forest in Minnamurra of New South Wales, Australia.

Fig. 1.2. The landward edge of the mangrove forest in Minnamurra of New South Wales, Australia, flanked by saltmarsh and terrestrial trees.

Fig. 1.3. The landward edge of the mangrove forest in Minnamurra of New South Wales, Australia, showing the mangrove *Avicennia marina* flanked by smaller trees of the mangrove *Aegiceras corniculatum*.

microsatellite loci that determine the effect of stand size on genetic diversity and population differentiation in adult plants, and levels of outcrossing and biparental inbreeding in progenies is present. Finally, chapter 6 is a general discussion on the outcome and perspective of the research presented in chapter 2-5 where I discuss and compare my results with results of other studies.

1.1.3 *Disruption of ecological stability in small stands*

The relationship between the abundance and diversity of pollen and seed dispersers and stand size, and the ecological stability and stand size, has been investigated during the last decades (reviewed by Bierregaard *et al*. 1992). For example, Klein (1989) investigated the species diversity of dung and carrion beetles (that both function as pollinators and seed dispersers; e.g. Beath 1996; Vulinec 2002) in one and 10 hectare stands isolated by more than 350 m from a continuous forest, and found a lower species diversity of beetles that resulted in lower decomposition of dung in the small stands. This indicates that small stands are restricted in the ability to attract pollinators and seed dispersers and that ecological processes are less effective in small stands. Such impacts have been revealed in several studies (Reviewed by Aizen *et al*. 2002, Ghazoul 2005, Aguilar *et al*. 2006 and Collinge 2009), inferring reduced ecological stability in small stands (e.g. reviewed by Bierregaard *et al*. 1992). This may have a negative effect on the reproductive output of flowering plants (Aguilar *et al*. 2006). Some theoretical models focusing on biological dynamics have revealed strong influences of patch size on viability and local extinction of populations (Jacquemyn *et al*. 2002; Bailey 2007; Hadley & Betts 2011), indicating a tendency of disruption of ecological processes in small stands of anthropogenically fragmented plants, with in some cases fatal

consequences (Aizen *et al*. 2002). For example, using a patch occupancy model to analyze population dynamics in fragmented landscape by linking genetic and demographic methods, Dornier & Cheptou (2013) emphasized the importance of external seed supply in spatio-temporal dynamics of fragmented landscapes. The conclusion may be that a biological system (for example a plant population) in dynamic equilibrium has a better change of keeping sufficient gene flow and genetic connectivity to keep a high degree of population fitness and ensure a successful reproduction, than a biological system not in dynamic equilibrium (for example stands of anthropogenically fragmented populations affected by reduced stand size).

1.1.4 *Outline of the background knowledge of the data chapters*

In the subsequent sections I will briefly outline the existing knowledge on the topics described in each of the four data chapters of this thesis, and present them in the same order as described above, equivalent to the order of which the four data chapters are presented below. First I will discuss pollination biology as a basis to understand the research presented in chapter 2.

1.1.5 *Influence of stand size on vector mediated gene flow*

For plants, connectivity among stands or populations must involve the dispersal of pollen or seed, and the level of gene flow (or connectivity) within individual plant species may reflect the range of vectors that disperse the pollen and seed. During the process of anthropogenic population fragmentation, the different pollen and seed vectors may respond quite differently to the rapid change from continuous plant populations into smaller stands, and the flow of genes among the resulting smaller

stands may be disrupted and connectivity may be lost due to genetic isolation driven by reduced services and changed foraging behaviour of different animal vectors (Aguilar *et al*. 2006; Collinge 2009).

1.1.6 *Specialization vs. generalization in plant-pollinator associations*

In fragmented plant populations, pollen limitation can reduce the pollination success of individual plants, leading to a reduced fecundity (Bierzychudek 1981; Burd 1994; Larson & Barrett 2000; Ghazoul 2005). Such influence can be mediated by a change in pollinator specialisation in small stands as compared to large stands of fragmented plant populations (Ghazoul 2005). Especially if a plant species is dependent on few specialized pollinators, reduced pollinator diversity could result in pollination failure because loss of one species could lead to scarcity in pollinator availability (Aizen *et al*. 2002). Such dependency may have a higher impact on small stands of fragmented plant populations because pollinator diversity and abundance is likely to be reduced in these (Aizen *et al*. 2002; Aguilar *et al*. 2006). However, if a plant is dependent of generalized rather than specialized pollinators, the risk of loss of pollinators is reduced, because generalized pollinators are both attracted from other plant species and from the surrounding plant environments (Fernandes 1999; Ings *et al*. 2009), which may be an advantage for small stands of fragmented plant populations because they could more easily retain or attract their pollinators.

Indeed, plants dependent on generalized pollinators may share their pollinators with other plants (Schemske 1981; Thomson 1982; Collinge 2009; Ings *et al*. 2009; Landry 2013), which may have a negative effect (Bell *et al*. 2005; Kandori *et al*. 2009; Landry 2013) or a positive effect (Rathcke 1988; Moeller 2004) on the reproduction of

plant populations. Nevertheless, some plant populations that share pollinators have been found to share pollinators without individually losing important pollinator services (Raine *et al*. 2007; Baldock *et al*. 2011). Those plant populations that suffer from negative effects on the reproduction may be more sensitive to the effects of stand size than the others because the reproduction of fruits and seedlings may be disrupted (Aguilar *et al*. 2006).

1.1.7 *Introducing chapter 3 and 4*

The fact that successful reproduction can be disturbed if pollinator availability is disrupted leads to the research presented in chapters 3 and 4, where pollinator availability, pollinator services and reproductive output is investigated and the impact of stand size on these factors are compared. This topic will be discussed in the proceeding sections.

1.1.8 *Self-compatibility vs. self-incompatibility in flowering plants*

Self-incompatibility might be a better reproductive tactic than self-compatibility in fragmented habitat (Oakley *et al*. 2007). Obligate self-incompatible plants may involve high vulnerability to impacts of stand size for factors such as pollinator diversity, abundance, services and foraging behaviour because only outbreed pollen can pollinate such plants successfully (e.g. Aizen *et al*. 2002; Wilcock & Neiland 2002; Aizen & Feinsinger 2003), while self-compatible plants may be vulnerable to pressures from inbreeding caused by selfing. Deciding which is the most advantageous of these strategies may depend on the individual plant species under investigation. However, Aizen *et al*. (2002) and Ghazoul (2005) found that the Allee effect was similar (did not

vary significantly) in self-compatible and self-incompatible plants, and concluded that it is not possible to generalize on plant species' sensitivity to fragmentation (in the present study to the effect of stand size) solely based on the compatibility system. Further, Aizen *et al*. (2002), Ghazoul (2005) and Aguilar *et al*. (2006) found that the impacts of fragmentation (in the present study of the effect of stand size) in general were significantly greater in small stands as compared to large stands, and in extreme cases small stands may be prone to extinction (Murcia 1996; Jules & Rathcke 1999; Aizen *et al*. 2002; Jacquemyn *et al*. 2002; Bailey 2007), which make many anthropogenically fragmented plant populations important targets for conservation (e.g. Fahrig & Merriam 1994). However, the mangrove *Avicennia marina* (which is subject of the present study) might be a self-compatible plant (Clarke & Myerscough 1991), but because of its naturally fragmented distribution its mating and dispersal systems may have evolved to cope with eventually negative effects of self-compatibility as higher levels of inbreeding or selfing and disrupted reproductive output in small stands of anthropogenically fragmented populations (e.g. Aizen *et al*. 2002).

1.1.9 *Effects of stand size on reproductive output*

Reduced stand size has been shown to greatly reduce the reproductive output of terrestrial plant populations (e.g. Burd 1994; Waser *et al*. 1996; Larson & Barrett 2000; Becker *et al*. 2011). For example, Kwak *et al*. (1998) found a complete absence of seed production in small and isolated temperate stands of the Black Rampion (*Phyteuma nigrum*), and Oostermeijer *et al*. (1992) found loss of seed set in small temperate populations of the Marsh Gentian (*Gentiana pneumonanthe*). However, the effect of stand size varies among species and can be more or less pronounced. For example, in

the study of Mavraganis & Eckert (2001) on the plant *Aquilegia canadensis* from Ontario in Canada, outcrossing occurred twice as frequently in large populations as in small populations where inbreeding depression was extremely strong. The study by Aizen & Feinsinger (1994*b*) investigated 16 subtropical tree species in Argentina (of which 10 species were self-incompatible) and identified significant or non-significant reductions of 73%-81% in the number of pollen tubes per style, fruit set and seed set. In another study by Klank *et al*. (2010) on the globeflower *Trollius europaeus*, reproductive output was reduced with increasing stand size, while pollinator abundance was independent of plant population size, but at local, (within flower) level, pollinator abundance was inversely correlated with local flower density of *T. europaeus*. These studies show that responses to variation in stand size can range from not significant to strong and significant and may depend on the environmental requirements and the pollination strategy of the individual populations.

1.1.10 *Introducing chapter 5*

Finally the genetic response of the effects of stand size on the ecologically processes described in chapters 2-4 will be discussed below, where I will explain how mating systems are inferred in the present study and introduce the concepts of gene flow and connectivity, and relate them to anthropogenically fragmented plant populations.

1.1.11 *Estimating past and current mating systems*

In closed populations of sexually reproducing organisms mating systems are often inferred either by determining adult single locus genotype frequencies and comparing these with expectations for Hardy-Weinberg equilibria or by judging the fit of the

genotypes of seed (progeny arrays) from a sample of maternal plants to expectations for random mating within each stand (e.g. England *et al*. 2001; Hedrick 2011). Mating systems have been inferred by both approaches in many terrestrial studies (e.g. Butcher *et al*. 2005; Breed *et al*. 2012). Because the genotypic structure of adult populations may reflect other historical processes (e.g. colonisation from one or more sources) and because mating systems may change with pollinator disruption, it may be convenient to use both approaches in studies of molecular ecology on anthropogenically fragmented populations. In the present study, mating systems have been inferred by this approach by comparing the level of inbreeding of the adult mating systems with the multilocus outcrossing of the progenies mating systems of stands of anthropogenically fragmented populations of *A. marina* in temperate southeast Australia (chapter 5).

1.1.12 *Gene flow, demography and environmental factors are strongly connected*

Studies of gene flow in natural populations indicate that genetic structure of many species may reflect past demographic events rather than gene flow (Slatkin 1989). Other researchers think that demographic and environmental factors may be as important as reduced gene flow and genetic isolation in reducing the viability of populations (e.g. Schemske *et al*. 1994; Holsinger 2000). However, it is inevitable that these factors are strongly connected (Aizen *et al*. 2002; Aguilar *et al*. 2006). For example, gene flow to small stands of anthropogenically fragmented populations is restricted by lower diversity and abundance of pollinators due to reduced movements of pollinators towards the small stands (Collinge 2009). This again may result in disruption of the reproductive output and in the worst scenario lead to extinction of small stands (e.g. Aizen *et al*. 2002). In the present study I investigated the gene flow among stands of

anthropogenically fragmented populations of the temperate *A. marina* and found a high connectivity among stands, similar to the findings by Homer (2009) who investigated the distance of gene flow, and revealed a high connectivity among stands of the northern rivers of New South Wales in Australia.

1.1.13 *Genetic factors*

Susceptibility to fragmentation is highly species specific, and depends in part on historical stand sizes, dispersal efficiency and historical genetic variation (Galbusera *et al*. 2004; Luoy *et al*. 2007; Dixo *et al*. 2009). Plant species that typically have smaller stand sizes are often less genetically diverse (Frankham 1996), and may be more vulnerable to loss of genes (Collinge 2009). Similar effects however, can be found in small stands of other (more common) species (e.g. Peakall & Lindenmayer 2006).

As landscapes around the globe become increasingly fragmented, it will be essential for conservation of plant species to understand the effects of reduced stand size on gene flow, especially to small stands of anthropogenically fragmented plant populations (Murphy *et al*. 2010; Storfer *et al*. 2010). Pollen and seed vectors visiting the small stands may have changed the foraging behavior and the diversity and abundance of these may be reduced, which may negatively affect the gene flow (Collinge 2009). In small stands of anthropogenically fragmented populations reduced gene flow may cause low genetic diversity, genetic drift and isolation, and higher risks of inbreeding (Fahrig 2003; Johansson *et al*. 2007; Dixo *et al*. 2009). Reduced genetic diversity can also be due to lower levels of outbreeding because the males available for breeding are near related (Charlesworth & Charlesworth 1987). Factors like this may result in the development of genetically diverged populations or sub-populations, and in

such populations the reproductive output may be disrupted by reduced quality and production of seed and seedlings, and in some species lead to inbreeding depression (Charlesworth & Charlesworth 1987; Aizen *et al*. 2002; Llorens *et al*. 2004). Stands affected by such factors have a lower evolutionary potential and higher risk of extinction (Avise *et al*. 1987; Reed & Frankham 2003; Dixo *et al*. 2009).

A plant species resistance to factors that influence genetic drift, such as loss of pollinators and variation in reproduction among individual plants (Lande & Barrowclough 1987), may be important for the ability to resist the pressures of reduced stand size. Further, neutral genetic variation can be lost and mildly deleterious alleles can be fixed in small stands of anthropogenically fragmented populations, which may lead to lower fitness and viability. The speed of this process depends on the effective population size (called N_e) (Kalinowski & Waples 2002), which is dependent of overlapping generations and variation in reproduction among individuals (Lande & Barrowclough 1987).

1.2 Reproductive biology and gene flow in mangroves

Because fragmentation has not been investigated in mangroves before I introduced the literature on fragmentation of terrestrial forest in the sections above and related the different topics to the relevant data chapters. In the proceeding sections I will introduce these topics on mangroves and present the relevant mangrove literature although this does not include the effects of fragmentation.

1.2.1 *Pollination biology*

Species observed to visit the flowers of mangroves are insects, birds and mammals (Revieved by Kathiresan & Bingham 2001). However, not much is known about the pollination biology of mangroves. Nevertheless, seventeen out of 25 studies on flower visitation in mangroves conclude that the insects investigated are pollinators without giving any convincing proof. These papers have been discussed in a mini review in chapter 2. Therefore, it is not possible to make any general conclusion about the suite of pollinators that visit and pollinate the flowers of mangroves. However, a PhD thesis by Homer (2009) investigated the pollination biology and population structure of *A. marina* populations in subtropical northern New South Wales. She identified a range of flower visitors, and found pollen on the body of a spider, a ladybird beetle, and the exotic honeybee *Apis mellifera*. She concluded that *A. mellifera* was a pollinator of *A. marina*, but did not provide evidence which shows that *A. mellifera* deposited pollen on *A. marina* flowers. Therefore, this study does not give any further knowledge on the suit of pollinators.

1.2.2 *Factors influencing reproductive output*

Some mangroves, such as *Ceriops decandra* (Raju *et al*. 2006) are self-incompatible whereas others such as *Aegiceras corniculatum* (Pandit & Choudhury 2001) are self-compatible, although it is not possible to generalize on plant species sensitivity to fragmentation solely based on the compatibility system (Aizen *et al*. 2002; Ghazoul 2005). The degree of inbreeding in a plant species (including mangroves) may be dependent of the foraging behaviour of pollinators. For example, honeybees are known generally to forage within few trees for longer periods of time (e.g. chapter 3 and

Whelan *et al*. 2009). Such behaviour may increase the possibility of inbreeding or selfing in a plant population. Selfing however, will not occur in obligate outbreeders.

1.2.3 *Inbreeding in mangroves*

Inbreeding (breeding between near relatives) is measured by F_{IS} (the average deviation from Hardy-Weinberg proportions in subpopulations). Wright describes F_{IS} as 'the average over all subdivisions of the correlation between uniting gametes relative to those of their own subdivision'. In his 1951 Nature publication Wright defines this as the fixation index or the inbreeding coefficient, *F* (Wright 1951; Wright 1965). Inbreeding has been investigated in a number of mangrove studies with a great variation in F_{IS} values (Xiao-Yong *et al*. 1996; Ge & Sun 1999; Maguire *et al*. 2000*b*; Dodd *et al*. 2002; Nunez-Ferfan *et al*. 2002; Giang *et al*. 2003; Arnaud-Haond *et al*. 2006; Giang *et al*. 2006; Landry & Rathcke 2007; Geng *et al*. 2008; Deng *et al*. 2008; Kahrood *et al*. 2008; Nettel *et al*. 2008*a*). However, the variation in F_{IS} among these studies may be explained by variation among the different primer types used (izozymes, dominant markers and microsatellites) and spatial variation.

1.2.4 *Genetic variation in mangroves*

In mangroves, genetic variation (or the degree of genetic differentiation) among populations or stands, measured by F_{ST} or G_{ST} (Huang 1994; Sun *et al*. 1998; Maguire *et al*. 2000*b*; Ge & Sun 2001; Takeuchi *et al*. 2001; Dodd *et al*. 2002; Nunez-Ferfan *et al*. 2002; Giang *et al*. 2003; Jian *et al*. 2004; Li & Chen 2004; Castillo-Cardenas *et al*. 2005; Ge *et al*. 2005; Tan *et al*. 2005; Arnaud-Haond *et al*. 2006; Su *et al*. 2006; Arbelaez-Cortis *et al*. 2007; Nettel & Dodd 2007; Chen *et al*. 2008; Deng *et al*. 2008;

Kahrood *et al*. 2008) varies greatly, indicating variation among other the different primer types used (izozymes, dominant markers and microsatellites) and/or spatial variation. This variation mirrors a high level of natural fragmentation among the populations due to their estuarine nature. F_{ST} and G_{ST} are measures of relative variation among populations or stands and by using a hierarchical approach, as I do in the present study, the genetic subdivision within the system that is tested can be revealed, where F_{ST} is the correlation between random gametes within subdivisions, relative to gametes of the total population (Wright 1965).

1.3 Structural configuration of the investigated system

To get a better understanding of the investigated system, I will explain the history of development and the structural configuration of the inverstigated estuaries, including the different levels of fragmentation and the invironmental conditions in the estuaries, and I will explain the ecological consequences of this.

1.3.1 *Development history and conditions of the estuaries and stands*

Mangroves are plants situated in river systems (estuaries) along the coastlines of tropical and subtropical (and in Australia temperate) regions (e.g. Duke 2006). Specialization to the life in estuaries includes adaptation to three levels of natural fragmentation, namely between estuaries, between river-branches within estuaries (e.g. West *et al*. 1985), and finer scale fragmentation due to patchiness of suitable habitat. The levels of isolation caused by natural fragmentation may result in decline of pollinator diversity and abundance, and therefore also in pollinator services, which may result in pollen limitation and influence negatively on the reproductive output (Aguilar

et al. 2006). Besides this, mangroves have adapted to the specific conditions of the intertidal zone where water levels vary according to the tidal amplitude (which is up to ca. 2 m in the investigated estuaries), and because of the ingoing seawater and the muddy sediments capability to bind salt, the salt level of the mangrove environment can be high (Hogarth 1999). As mangrove trees use much energy on keeping the physiological salt level minimal and secrete exceeding salt in the plant, the fecundity of individual mangrove trees may suffer, which may also result in reduced reproductive output (Hogarth 1999).

Besides this, during the last century or more, mangroves have suffered from anthropogenic fragmentation dividing mangrove populations into stands of varying size, from large stands of 10000 trees or more down to single isolated trees (e.g. West *et al*. 1985). This development leave the small stands (in the present study up to ca. 100 trees) with the disadvantages of being small and isolated according to the land that has been cleared (e.g. Collinge 2009), which may again have a negative influence on the density of pollinators, and in many terrestrial forest trees result in disruption of reproductive output (Aizen *et al*. 2002; Ghazoul 2005; Aguilar *et al*. 2006).

In the two Sydney estuaries investigated in the present study, besides the small stands created by anthropogenic fragmentation of existing mangrove forests, new stands of varying size have developed along the coastlines during the last century (contributing to the fragmented mangrove matrix) on mudflats formed by sediment from runoff of cleared forest areas (Thorogood 1985; McLoughlin 2000; Dunstan 1990). The stands of the two Sydney estuaries I investigated in chapter 4 and 5 were developed on mudflats during the last century (Thorogood 1985; McLoughlin 2000; Dunstan 1990 – and personally investigation of aerial photos). Of the stands I investigated in chapter 2 and

3, the development of the large stand in Minnamurra happened both through anthropogenic fragmentation and establishment on mudflat while the small stand was created through anthropogenic fragmentation only. The stand of Salt Pan Creak in Sydney was created through anthropogenic fragmentation while the small Sydney stand was developed on mudflat (Dunstan 1990 – and personally investigation of aerial photos). Further, the stands of the two Sydney estuaries have most possible been developed by settlement of a mix of propagules from the existing stands of the estuaries, and propagules have been dispersed by the powerful tidal currents that are able to transport propagules up to 20 km of the estuary (Clarke 1993; Minchinton 2006). This is equivalent to the distance between the two Sydney estuaries (see Table 5.1), making exchange of propagules possible.

1.3.2 *About the subsequent data chapters*

The subsequent data chapters (chapters 2-5) will be presented as manuscripts prepared for publication and consequently each include an abstract, and introductions and discussion will include some slight repetition although I believe this makes the chapters easier to follow.

Chapter 2: Identification of flower visitors and pollinators of temperate populations of *Avicennia marina*

This chapter is in press in the journal Estuaries and Coasts and has been slightly modified for the thesis.

Tyge D. Hermansen, David R. Britton, David J. Ayre and Todd E. Minchinton (2013) Identifying the real pollinators? Exotic honeybees are the dominant flower visitors and only effective pollinators of *Avicennia marina* in Australian temperate mangroves. DOI: 10.1007/s12237-013-9711-3.

2.1 Abstract

The literature suggests that in the tropics mangroves are typically pollinated by a range of generalist pollinators, whereas in temperate populations pollination biology is largely unstudied. I predicted that for the mangrove *Avicennia marina* in temperate southeast Australia, pollinator diversity would be low and its pollination system modified by the exotic honeybee *Apis mellifera*. Multi-year surveys and experiments were used to test these hypotheses by determining the identity and frequency of flower visitors, quantifying pollinator foraging behaviour, determining the species composition of pollen loads, and demonstrating pollen removal and deposition. I identified 38 species that visited flowers, but only *A. mellifera* was a significant pollinator. It was the only species to carry large amounts of pollen and forage in a manner permitting transfer of pollen to stigmas. Moreover, *A. mellifera* was the numerically dominant flower visitor and was effective in both pollen removal and deposition. This study demonstrates the importance of distinguishing flower visitors from pollinators and emphasizes the surprisingly widespread influence of the exotic *A. mellifera*. Finally, my study and a

worldwide review of the literature on the pollination of mangroves reveal that the pollination biology of other mangrove systems requires similar scrutiny.

2.2 Introduction

For mangroves, like terrestrial flowering plants, selection favours associations with particular pollinators or suites of pollinators that lead to effective pollination and production of high quality propagules (e.g. Ren *et al*. 2005). Nevertheless, like many terrestrial species within temperate forests, the ephemeral nature of the pollinator associations displayed by tropical mangroves may of necessity favour flexibility in floral morphology (e.g. Tomlinson 1986). Indeed mangroves typically have vast geographic ranges (Duke 2006) with individual populations potentially exposed to diverse suites of tropical insect and vertebrate visitors (e.g. Kathiresan & Bingham 2001). Moreover, their water-borne propagules provide a capacity for gene flow that may oppose local and regional specialisation of mangrove-pollinator associations. These propagules also allow the long distance colonisation of new habitat, with populations often founded by one or a few colonists, implying that they must develop opportunistic associations with smaller subsets of potential pollinators, and may self-fertilize (Baker 1955; Van Kleunen *et al*. 2008).

In an often cited book, Tomlinson (1986) predicted, largely on the basis of observations of flower visiting species (Devay 1975; Start & Marshal 1976; Tomlinson 1979; Primark *et al*. 1981) and floral morphology, that tropical mangrove species were typically pollinated by many generalist pollinators, including insects and birds, and in a few cases bats. Nevertheless, on the basis of a critical examination of the literature I

argue that, while there are many accounts of potential pollinators visiting mangrove flowers and trees, the pollination biology of mangroves is poorly known.

I searched for all papers that identify animal species that land or climb on mangroves (visitors in the most general sense) using BIOSIS (from 1920 to 2012), Biological Abstracts (from 1974 to 2012), and ISI Web of Science (from 1965 to 2012), and I carried out a detailed examination of available earlier publications. This search revealed a total of 50 publications reporting mangrove visitors, but only 25 of these publications investigated flower visitation, and none provided sufficient critical evidence to establish that flower visitors act as pollinators (Table 2.1). Seventeen of these 25 publications specifically concluded that observed flower visitors were pollinators but without supporting evidence. A general study of the reproductive biology of the mangrove tree *Avicennia marina* (see Fig. 2.1) within the temperate Sydney region (Clarke & Myerscough 1991) reported a diverse array of flower visitors (from nine families), including the exotic honeybee *Apis mellifera*, but did not quantify their relative abundance, frequency of flower visitation, or foraging behaviour. Other Australian studies did not focus on pollination and only mentioned one or two species of insect visitors. None of the publications reviewed provide compelling evidence that the species listed are pollinators, and only a few of these are exhaustive lists of flower visitors (Table 2.1).

Distributions of mangrove species in Australia extend from the tropics into relatively high latitude temperate estuaries (Duke 2006). At high latitudes mangroves may be expected to encounter a different suite and reduced diversity of flower visitors because the composition and diversity of such species are generally reduced with latitude (MacArthur, 1972; Rhode 1992; Gaston & Williams 1996). Moreover, species

richness of mangroves is reduced from 41 in the tropics to one (*Avicennia marina*) at the southern range limit in Victoria (Duke 2006), which also may cause reduced pollinator richness (Kwak *et al*. 1998).

If the assumption that Australian tropical mangroves support a diverse suite of pollinators were correct, it should still seem likely that temperate populations would support a less diverse assemblage (e.g. Vicens & Bosch 2000a, b). Moreover, in temperate Australia the pollination systems of many terrestrial species of trees and shrubs that were historically visited by a range of indigenous generalist pollinators are now dominated by *A. mellifera*, which in some cases has altered plant mating systems (England *et al*. 2001; Whelan *et al*. 2009) and caused the competitive displacement of native pollinators (Paton 1993, 1996).

In order to determine the significance of flower visitors as pollinators it is vital to determine not only the abundance and diversity of these species but also: (i) whether the foraging behaviour of the visitors allow them to transport pollen between flowers on the same and different plants of the same species (e.g. Free & Durrant 1966; Free & Williams 1972; Fenster *et al*. 2004); (ii) the degree of host plant fidelity of these species, because generalist flower visitors that visit many plant species may transfer large quantities of foreign pollen grains that have negative impacts through clogging of stigmas and reducing the pollination efficiency (e.g. Shore & Barrett 1984; Fenster *et al*. 2004); and (iii) whether each visiting species effectively removes pollen and deposits it on conspecific stigmas (e.g. Olsen 1997; Fenster *et al*. 2004).

Here, for the first time, I characterise the pollinator association of the mangrove *Avicennia marina* in two temperate estuaries of the Sydney region of Australia, and determine the relative importance of the European honeybee *Apis mellifera* as an

invader of this pollination system. I use a range of essential approaches to determine: (1) the diversity of flower visitors, (2) the set of flower visitors that are potential pollinators (as judged by their ability to touch the stigma and the presence of pollen on their bodies), (3) the abundance of pollinators versus other flower visitors, (4) host plant fidelity of pollinators (as judged by the mix of pollen types on their bodies), and (5) the effectiveness of the available pollinators in removing and depositing pollen. Because pollinator associations may vary in space and time, I test for generality by examining patterns in two estuaries and across the flowering seasons of three years.

Fig. 2.1. Trees of *Avicennia marina* (the grey mangrove) with their greyish and crooked boles.

Table 2.1. A mini-review of species recorded visiting mangrove flowers in studies assessing flower visitation. Factors investigated to determine if flower visitors are pollinators or potential pollinators are listed in the table. The table shows whether (i) authors concluded that flower visiting species are pollinators, or (ii) potential pollinators. One publication (Hill 1992), does not refer to pollination, but mentions flower visitation. The number of species is listed under "Nr", where parentheses indicate a tentative identification. Factors investigated and conclusions are indicated with "Y". If only some of the species mentioned in a publication have been investigated for a specific treatment this is marked as "Y*". Orders without specification of the number of species are marked under "Nr" with an "x". Queensland is listed as Qld., deposition is listed as depos., observation is listed as obs. and potential is listed as pot. The 25 studies listed in this table are sorted by mangrove species in alphabetical order

						Factors investigated							**Conclusion**		
Mangrove	Flower visitors	Order	Nr	Location	Latitude	Species diversity	Density/ Visitation	Carry pollen	Foraging behaviour	Pollen removal	Pollen depos.	Night obs.	Polli-nators	Pot. pol-linators	Publi-cation
Acanthus ilicifolius	Bees, wasps	Hymenoptera	3	Andra Pradesh, India	16°55'N	Y	—	—	Y	—	—	—	Y	—	s)
	Birds	Passeriformes	2												
Acanthus ilicifolius	Birds	Passeriformes	1	Mid Qld. Australia	19°4'S	—	—	—	—	—	—	—	Y	—	t)
Aegiceras corniculatum	Butterflies, moths	Lepidoptera	16	East coast of India	20°30'N-20°50'N	Y	Y	—	Y	—	—	Y	Y	—	m)
	Bees, wasps	Hymenoptera	9												
	Flies	Diptera	2												
	Beetles	Coleoptera	1												
	Birds	Passeriformes	1												
Aegiceras corniculatum	Butterflies	Lepidoptera	2	Sth Qld. Australia	27°35'S	—	Y	—	—	—	—	—	—	—	q)
Aegiceras corniculatum	Bees	Hymenoptera	2	Andra Pradesh, India	16°55'N	Y	—	—	Y	—	—	—	Y	—	s)
Aegiceras corniculatum	Butterflies	Lepidoptera	10	Qld. Australia	—	—	—	—	—	—	—	—	—	Y	z)
Avicennia alba	Bees	Hymenoptera	1	Malaysia	5°40'N-102°43'E	—	—	Y	—	—	—	—	Y	—	c)

Avicennia alba	Bees Flies Butterflies	Hymenoptera Diptera Lepidoptera	3 2 2	Andra Pradesh, India	16°30'N-17°00'N	Y	Y	Y	Y	—	—	—	Y	—	e)
Avicennia germinans	Bees, wasps Flies Butterflies	Hymenoptera Diptera Lepidoptera	8 3 5	Sth Florida, US	—	Y	Y	—	—	—	—	—	Y	—	b)
Avicennia germinans	Bees, wasps Flies	Hymenoptera Diptera	5 1	Sth-west Caribbean	12◦28'N-72◦29 N	Y	Y	Y	Y	—	—	—	Y	—	f)
Avicennia germinans	Bees, wasps, ants Flies Butterflies, moths Birds	Hymenoptera Diptera Lepidoptera Passeriformes	17 3 11 1	Bahamas	—	Y	Y	—	—	—	—	—	Y	—	n)
Avicennia marina	Bees, wasps Flies Butterflies	Hymenoptera Diptera Lepidoptera	2 4 1	Andra Pradesh, India	16°30'N-17°00'N	Y	Y	Y	Y	—	—	—	Y	—	e)
Avicennia marina	Butterflies	Lepidoptera	2	Sth Qld. Australia	27°35'S	—	Y	—	—	—	—	—	—	—	q)
Avicennia marina	Wasps, ants Bugs Flies Bee flies Beetles Moths	Hymenoptera Hemiptera Diptera Diptera Coleoptera Lepidoptera	x x x x x x	Sth-east Australia	35°00'S-35°02'S	—	—	—	—	—	—	—	—	Y	r)
Avicennia officinalis	Bees, wasps Flies Butterflies	Hymenoptera Diptera Lepidoptera	4 4 5	Andra Pradesh, India	16°30'N-17°00'N	Y	Y	Y	Y	—	—	—	Y	—	e)
Avicennia officinalis	Bees Flies	Hymenoptera Diptera	1 2	Andra Pradesh,	16°55'N	Y	—	—	Y	—	—	—	Y	—	s)

				India											
Avicennia schaueriana	Bees, wasps	Hymenoptera	10	Nth-east Brazil	7°40′S-34°50′W	Y	Y	—	Y	—	—	—	Y	—	a)
	Flies	Diptera	12												
	Butterflies	Lepidoptera	2												
Avicennia spp	Birds	Passeriformes	4	Selangor, Malaysia	—	Y	Y	—	Y	—	—	—	Y	—	o)
Bruguiera exaristata	Birds	Passeriformes	2	Nth Qld. Australia	—	Y*	—	—	—	—	—	—	—	Y	l)
Bruguiera exaristata	Birds	Passeriformes	1	Nth Qld. Australia	—	—	—	Y	Y	—	—	—	Y	—	p)
Bruguiera exaristata	Birds	Passeriformes	2	Mid Qld. Australia	—	—	—	—	Y*	—	—	—	—	Y	u)
Bruguiera gymnorrhiza	Birds	Passeriformes	2	Selangor, Malaysia	—	—	—	Y	Y	—	—	—	Y	—	p)
Bruguiera gymnorrhiza	Birds	Passeriformes	2	Mid Qld. Australia	—	—	—	—	Y*	—	—	—	—	Y	u)
Bruguiera gymnorrhiza	Bees Birds	Hymenoptera Passeriformes	x x	Durban, South Africa	—	—	—	—	Y*	—	—	—	—	Y	y)
Bruguiera hainesii	Birds	Passeriformes	2	Selangor, Malaysia	—	—	—	Y	Y	—	—	—	Y	—	p)
Bruguiera parviflora	Butterflies	Lepidoptera	x	Mid Qld. Australia	—	—	—	—	Y*	—	—	—	—	Y	u)
Bruguiera sexangula	Birds	Passeriformes	2	Selangor, Malaysia	—	—	—	Y	Y	—	—	—	Y	—	p)
Bruguiera spp	Birds	Passeriformes	4	Selangor, Malaysia	—	Y	Y	—	Y	—	—	—	Y	—	o)
Caesalpinia Nuga	Bees, ants	Hymenoptera	5	Andra Pradesh, India	16°55'N	Y	—	—	Y	—	—	—	Y	—	s)
Ceriops decandra	Bees, wasps	Hymenoptera	2	Andra Pradesh, India	—	Y	Y	Y	Y	—	—	—	Y	—	i)
Ceriops decandra	Bees, wasps	Hymenoptera	2	Andra Pradesh, India	—	—	—	Y	Y	—	—	—	Y	—	j)
Ceriops targel	Bees	Hymenoptera	2	Andra	—	Y	Y	Y	Y	—	—	—	Y	—	i)

	Flies	Diptera	1	Pradesh, India											
Ceriops tagel	Moths	Lepidoptera	x	Mid Qld. Australia	—	—	—	—	Y*	—	—	—	—	Y	u)
Lumnitzera littorea	Bees, wasps	Hymenoptera	x	Nth Qld. Australia	—	—	—	—	—	—	—	—	—	Y	v)
Laguncularia racemosa	Bees, wasps	Hymenoptera	10	Sth Florida, US	—	Y	Y	—	—	—	—	—	Y	—	b)
	Flies	Diptera													
	Butterflies, moths	Lepidoptera	7 6												
	Beetles	Coleoptera	1												
Laguncularia racemosa	Bees, wasps	Hymenoptera	23	Florida, US	25°00'N-28°00'N	Y	Y	—	Y	—	—	—	Y	—	d)
	Flies	Diptera	10												
	Butterflies, moths	Lepidoptera	5												
	Beetles	Coleoptera	4												
Laguncularia racemosa	Bies, wasps	Hymenoptera	2	Sth-west Caribbean	12◦28'N-72◦29 N	Y	Y	Y	Y	—	—	—	Y	—	f)
	Flies	Diptera	7												
	Butterflies	Lepidoptera	1												
Laguncularia racemosa	Bees, wasps	Hymenoptera	13	Florida, US	—	Y	Y	—	—	—	—	—	Y	—	g)
	Flies	Diptera	7												
	Butterflies, moths	Lepidoptera	4												
	Beetles	Coleoptera	2												
Laguncularia racemosa	Bees, wasps	Hymenoptera	(2)	Bahamas	—	Y	Y	—	—	—	—	—	Y	—	n)
	Moths	Lepidoptera	2												
	Birds	Passeriformes	1												
Laguncularia racemosa	Flies	Diptera	5	Florida, US/ Bahamas	—	Y	Y	—	—	—	—	—	—	Y	k)
	Butterflies, moths	Lepidoptera	15												

	Beetles	Coleoptera	20												
	Birds	Passeriformes	1												
Lumnitzera racemosa	Bees	Hymenoptera	1	Malaysia	5°40'N-102°43'E	—	—	Y	—	—	—	—	Y	—	c)
Lumnitzera racemosa	Bees, wasps	Hymenoptera	6	Andra Pradesh, India	16°55'N	Y	—	—	Y	—	—	—	Y	—	s)
Lumnitzera racemosa	Bees, wasps Butterflies, moths	Hymenoptera Lepidoptera	x x	Nth Qld. Australia	—	—	—	—	—	—	—	—	—	Y	v)
Rhizophora annamalayana	Bees, wasps	Hymenoptera	10	Sth-east India	11°29′N-79°47′E	Y	Y	Y	Y	—	—	—	Y	—	h)
	Flies	Diptera	4												
	Butterflies	Lepidoptera	1												
	Spiders	Araneae	2												
Rhizophora apiculata	Bees, wasps	Hymenoptera	8	Sth-east India	11°29′N-79°47′E	Y	Y	Y	Y	—	—	—	Y	—	h)
	Flies	Diptera	4												
	Butterflies	Lepidoptera	1												
	Spiders	Araneae	2												
Rhizophora apiculata	Bees	Hymenoptera	1	Malaysia	5°40'N-102°43'E	—	—	Y	—	—	—	—	Y	—	c)
Rhizophora mangle	Bees, wasps	Hymenoptera	2	Sth-west Caribbean	12◦28'N-72◦29 N	Y	Y	Y	Y	—	—	—	Y	—	f)
	Flies	Diptera	1												
Rhizophora mucronata	Bees, wasps	Hymenoptera	8	Sth-east India	11°29′N-79°47′E	Y	Y	Y	Y	—	—	—	Y	—	h)
	Flies	Diptera	5												
	Butterflies	Lepidoptera	1												
	Spiders	Araneae	2												
Rhizophora spp	Birds	Passeriformes	4	Selangor, Malaysia	—	Y	Y	—	Y	—	—	—	Y	—	o)
Rhizophora spp	Bats	Chiroptera	2	West Malaysia	—	—	—	Y*	Y	Y*	—	Y*	Y	—	x)

Sonneratia alba	Birds	Passeriformes	4	Selangor, Malaysia	—	Y	Y	—	Y	—	—	—	Y	—	o)
Sonneratia alba	Hawk-moths	Lepidoptera	1	Mid Qld. Australia	19°40'S	—	—	—	—	—	—	—	Y	—	t)
Sonneratia caseolaris	Bees	Hymenoptera	1	Malaysia	5°40'N-102°43'E	—	—	Y	—	—	—	—	Y	—	c)
Sonneratia caseolaris	Butterflies, moths	Lepidoptera	17	East coast of India	20°30'N-20°50'N	Y	Y	—	Y	—	—	Y	Y	—	m)
	Bees, wasps	Hymenoptera	7												
	Flies	Diptera	3												
	Birds	Passeriformes	5												
	Rodents	Rodentia	2												
	Primates	Primates	1												
Sonneratia ovata	Bees	Hymenoptera	1	Malaysia	5°40'N-102°43'E	—	—	Y	—	—	—	—	Y	—	c)
Soneratia spp	Bats	Chiroptera	3	West Malaysia	—	—	—	Y*	Y	Y*	—	Y*	Y	—	x)
Suregada multiflora	Bees	Hymenoptera	1	Malaysia	5°40'N-102°43'E	—	—	Y	—	—	—	—	Y	—	c)

Publications

a) De Lima Nadia *et al*. (2013)
b) Landry (2013)
c) Azmi *et al*. (2012)
d) Landry & Rathcke (2012)
e) Raju *et al.* (2012)
f) Sánchez-Núnez & Mancera-Pineda (2012)
g) Landry (2011)
h) Seetharaman & Kandasamy (2011)
i) Raju & Karyamsetty (2008)
j) Raju *et al.* (2006)
k) Landry *et al.* (2005)
l) Noske (2003)
m) Pandit & Choudhury (2001)
n) Rathcke *et al.* (2001)
o) Noske (1995)
p) Noske (1993)
q) Hill (1992)
r) Clarke & Myerscough (1991)
s) Aluri (1990)
t) Primark *et al.* (1981)
u) Tomlinson *et al.* (1979)
v) Tomlinson *et al.* (1978)
x) Start & Marshall (1976)
y) Davey (1975)
z) Illidge (1925)

2.3 Materials and methods

2.3.1 *Study locations*

I studied visitation by potential pollinators of *A. marina* in mangrove forests at two locations: Salt Pan Creek on the Georges River in Sydney (33°56'47" S; 151°2'26" E) and Minnamurra River near Kiama Downs (34°38'15" S; 150°50'49" E), approximately 100 km south of Sydney, New South Wales, Australia (Fig. 2.2). These stands were at least twice as long as broad (West *et al*. 1985; personally investigations of air photos). Flowering was initiated ca. two weeks later within stands on the Minnamurra River than on the Georges River during the 2009 and 2010 flowering seasons (personal observations). Duke (1990) reported that timing in flowering and fruiting of *A. marina* in Australia varies considerably from tropical northern sites to temperate southern sites. The forest at Salt Pan Creek is in a highly urbanized landscape with both a highway and public pedestrian pathways bisecting it, whereas the forest at Minnamurra is in a partially agricultural landscape and surrounded by houses. Within both locations the forests are dominated by *A. marina* (Acanthaceae), with the smaller mangrove *Aegiceras corniculatum* (Myrsinaceae) on the landward side of *A. marina*. Both forests extend landward into salt marshes and are bordered by the dominant salt marsh plant *Sarcocornia quinqueflora* (Amaranthaceae), various flowering plants from gardens and small patches of terrestrial forest. Plants located in close proximity to *A. marina* are flowering simultaneously with *A. marina* at this latitude (including *A. corniculatum and S. quinqueflora*), and thus may attract visitors to the flowers of *A. marina* and potentially increase the abundance and diversity of flower visitors to the mangrove.

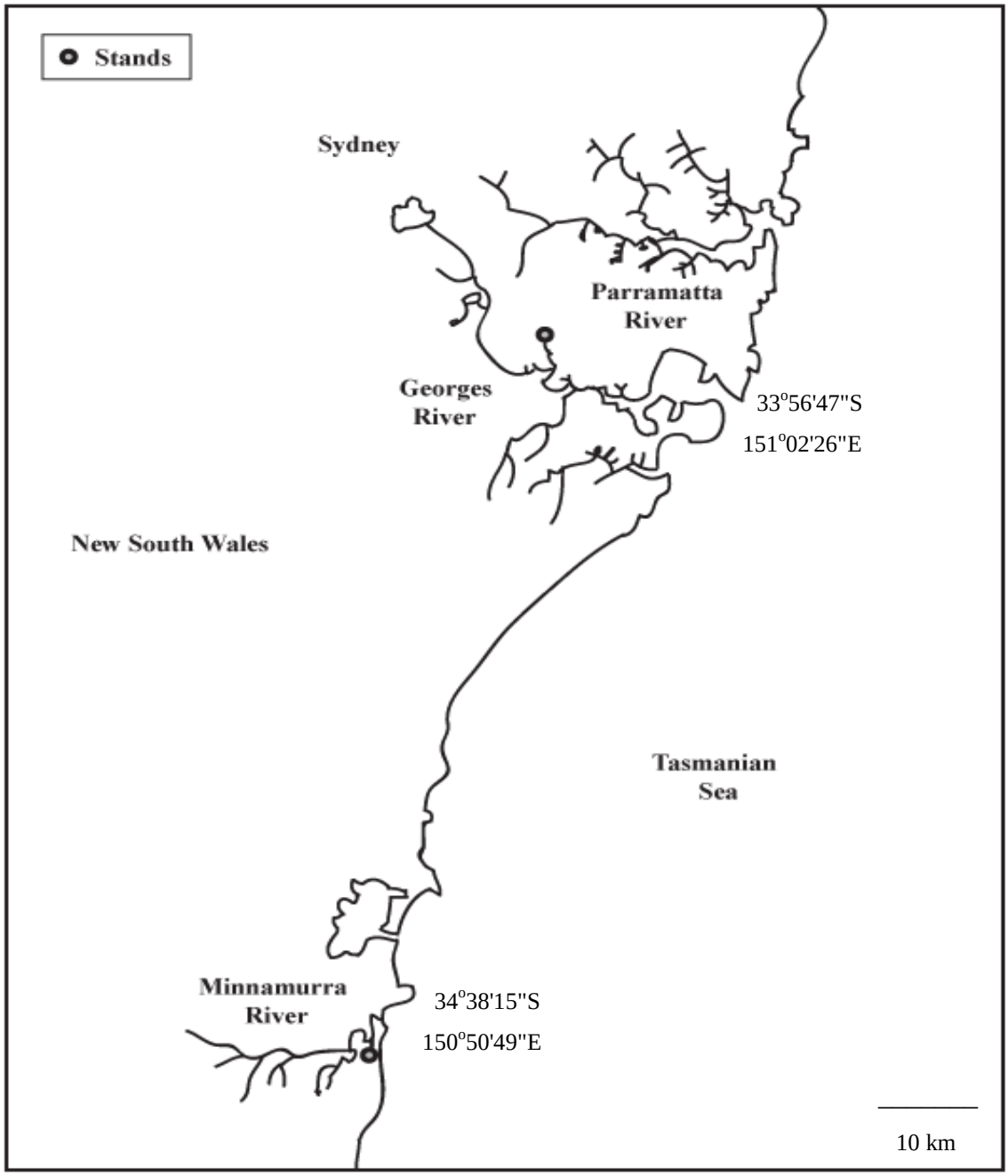


Fig. 2.2. Map showing the Parramatta River Georges River and Minnamurra River. The two locations studied are indicated.

All investigations were confined to the landward edge of the mangrove forests, where *A. marina* occurs adjacent to *A. corniculatum* and borders saltmarsh. All observations were done on trees of *A. marina* of intermediate height (5-10m), with approximately 200

floral shoots per m^2 (a density near maximum during the investigated flowering seasons). Investigations were done during the flowering periods of *A. marina*, which was from mid-January to mid-March in 2009, 2010 and 2011. Sampling was done when the sun was shining, and temperatures in the shade during sampling were between 17.6 and 32.3^{o}C in Sydney and between 13.5 and 26.7^{o}C in Minnamurra.

2.3.2 *The mangrove* Avicennia marina

In Australia the range of *A. marina* extends from tropical regions into the temperate southeast Australia, with a southern range limit in Victoria (Duke 2006). The inflorescence is organized as a compound syme (a branched inflorescence: Simpson 2006) where the hermaphroditic flowers are arranged in clusters of three to 14 flower buds and two to seven clusters of flowers develop from a floral shoot (see Clarke & Myerscough 1991), the inflorescence is organized as a compound syme. The flowers, each with four ovules, produce small amounts of nectar with little scent (containing fatty acids, terpenoids, benzenoids and some unknown components: Azuma *et al*. 2002) and are tiny (5 mm x 5 mm) and yellow. Mature stigmas are 1.5-2.0 mm in length and the four anthers are adnate to the petals at approximately the same height as the stigmatic surface (Clarke & Myerscough 1991). At the locations studied, *A. marina* flowers mainly from mid January to mid March and propagules develop for several months before they mature in October and November and may be dispersed by water (Duke 1990). The process from initiation of flower buds to abscission of fruit is completed within a year (Duke 1990; Clarke & Myerscough 1991).

2.3.3 *Identification of flower visitors*

To determine the identity of flower visitors and their potential to act as pollinators, flower visitors were captured within both forests and stored for further investigation. Diurnal and nocturnal sampling of flower visitors was spread throughout the flowering seasons of 2009 and 2010. At each location, diurnal sampling of flower visitors was done during 10 days of each of the two sampling year (in total 40 days). At five of the 10 days three sampling sessions of 2 h and at the other five days two sampling sessions of 2 h were performed (in total 200 hours). During the 10 sampling days performed at each location at each year, the 2 h sampling sessions were performed so they extended from eight am to six pm five times. All the 40 days used for sampling were evenly scattered over the flowering seasons without any overlap between dates. Within each 2 h session, sampling positions were changed every 15 min and the new position was separated from the old by 25-250 m. Nocturnal sampling was done at each location from sunset to sunrise on three occasions: mid February 2009, early March 2010 and end of January 2011. Observations were made at different sites separated by approximately 20 m, and site positions were changed every 15 min throughout the night. Flower visitors were observed by switching on a halogen flashlight for five seconds every minute throughout the night, giving a total of 600 observations (of five seconds) each night.

Because it would be difficult to keep track of the total number of flower visitors and also watch for new species and investigate their foraging behavior, I captured and examined five individuals of common species from each estuary (in total 10 of each flower visiting species). I investigate in more details the abundance of pollinators and

flower visitors in a separate section (see section 2.3.4 ‘Abundance of pollinators and flower visitors’ below).

During diurnal and nocturnal sampling, insects visiting the flowers of *A. marina* were captured for identification with a fine mesh insect net of polyorganza fabric and killed immediately with a freeze spray (© Dick Smith Freeze Spray). This spray was used because it killed insects instantly and preserved pollen loads on individual body parts. All captured insects were then stored at –20^{o}C and identified to species level with help of staff from the Australian Museum in Sydney.

2.3.4 *Abundance of pollinators and flower visitors*

To determine the abundance of pollinators and flower visitors visiting *A. marina*, I sampled during two 2 h intervals: midday (12-2 pm) and evening (5-7 pm) (time is adjusted according to daylight saving). The timing of these 2 h intervals was used because other surveys showed that the abundance of honeybees was considerably higher at midday than in the evening (chapter 3). During each 2 h interval I counted the numbers of flower visitors, honeybees or other insect species that I observed for one hour at each of two sites within each forest. This was done at each location (Sydney and Minnamurra) once during the flowering season of 2009 and twice during the flowering seasons of 2010 and 2011. The site of observation was changed every hour to a new, randomly chosen position along the landward edge of the mangrove forest, in total giving two randomly chosen and independent samples within each 2 h interval.

During each 2 h interval the number of insects visiting 10 m^2 of tree surface area (4.0 m wide by 2.5 m high, and measured from the lowest point of the canopy, approximately 0.25 m above the ground, to a height of 2.75 m) was counted. As the

canopy of trees along the forest edges often overlaps, each 10 m^2 area of canopy covered at least two trees. Visits were observed from the time when an insect first made contact with any flower within the 10 m^2 area until it left the area, making sure not to count it more than once.

Three-factor analysis of variance (ANOVA) was used to test for the effect of species of flower visitors (honeybees or other insects, considered a fixed factor), time interval (midday or evening, considered a fixed factor) and location (Sydney or Minnamurra, considered a fixed factor) on the abundance of flower visitors. As patterns were the same across the five sampling times (i.e. once in 2009, twice in 2010 and 2011), these were combined for analysis; however, to provide a good overview date for the five sampling times presented (Fig. 2.*3a-e*). Student-Newman Keuls (SNK) multiple comparisons tests were used to resolve differences among means following ANOVA.

Table 2.2. Species visiting flowers of *A. marina* at Sydney and Minnamurra in the flowering seasons of 2009 and 2010. Numbers listed under 'Sydney' and 'Minnamurra' show the number of individuals of each species that were captured at each estuary and examined for pollen loads under a stereomicroscope at 60x magnification. For common species, five individuals were examined from each estuary. *Apis mellifera* is marked with an "*" indicating that higher numbers of this species were captured and examined. "Di" indicates diurnal species and "No" are nocturnal species. "Nr Sy" and "Nr Mi" is number in Sydney and Minnamurra. (table on next page)

Order	Family	Species	Insect	Nr Sy	Nr Mi	Diurnal/ Nocturnal	Carrying pollen
Coleoptera	Cantharidae	*Chauliognathus lugubris*	Beetle	3	4	Di	No
Coleoptera	Cantharidae	Unidentified species	Beetle	2	0	Di	No
Coleoptera	Cerambycidae	*Aridaeus thoracicus*	Beetle	0	3	Di	No
Coleoptera	Coccinellidae	*Hippodamia variegata*	Beetle	1	0	Di	No
Coleoptera	Melyridae	*Carphurus* sp.	Beetle	0	2	Di	Yes
Coleoptera	Scarabaeidae	*Polystigma punctatum*	Beetle	0	2	Di	No
Diptera	Asilidae	*Cerdistus* sp.	Fly	0	3	Di	No
Diptera	Calliphoridae	*Chrysomya* sp. 1	Fly	5	5	Di	No
Diptera	Calliphoridae	*Chrysomya* sp. 2	Fly	0	4	Di	No
Diptera	Ephydridae	Un-identified species	Fly	5	5	Di	No
Diptera	Muscidae	*Musca vetustissima*	Fly	1	4	Di	No
Diptera	Muscidae	*Pygophora* sp.	Fly	1	0	Di	No
Diptera	Syrphidae	*Eristalis sp* 1	Fly	2	4	Di	No
Diptera	Syrphidae	*Eristalis sp* 2	Fly	3	5	Di	No
Diptera	Stratiomyidae	*Odontomyia* sp.	Fly	1	0	Di	No
Diptera	Sarcophagidae	*Sarcophaga aurifrons*	Fly	4	5	Di	No
Diptera	Sarcophagidae	Unidentified species	Fly	1	0	Di	No
Hemiptera	Rhopalidae	*Leptocoris* sp.	Bug	0	1	Di	No
Hymenoptera	Formicidae	*Iridomyrmex* sp.	Ant	5	5	Di	Yes
Hymenoptera	Formicidae	*Iridomyrmex* sp. 2	Ant	5	5	Di/No	No
Hymenoptera	Apidae	*Apis mellifera**	Bee	5	5	Di	Yes
Hymenoptera	Halictidae	*Lipotriches excellens*	Bee	2	0	Di	No
Hymenoptera	Scoliidae	*Scolia* sp. 1	Wasp	2	5	Di	No
Hymenoptera	Sphecidae	*Sphex bilobatus*	Wasp	0	1	Di	No
Hymenoptera	Sphecidae	*Sphex fumipennis*	Wasp	0	1	Di	No
Hymenoptera	Sphecidae	*Spex* sp.	Wasp	0	2	Di	No
Hymenoptera	Vespidae	*Bidentodynerus bicolor*	Wasp	2	2	Di	No
Hymenoptera	Vespidae	*Epiodynerus tamarinus*	Wasp	2	0	Di	No
Hymenoptera	Vespidae	*Paralastor* sp.	Wasp	3	2	Di	No
Hymenoptera	Vespidae	*Polistes humilis*	Wasp	5	5	Di	No
Hymenoptera	Vespidae	*Polistes* sp. 1	Wasp	0	2	Di	No
Hymenoptera	Vespidae	*Ropalidae plebiana*	Wasp	2	0	Di	No
Hymenoptera	Vespidae	*Scolia* sp. 2	Wasp	1	0	Di	No
Lepidoptera	Lycaenidae	*Theclinesthes sulpitius*	Butterfly	0	1	Di	No
Lepidoptera	Lycaenidae	*Zizina labradus*	Butterfly	1	0	Di	No
Lepidoptera	Arctiidae	*Amatia* sp.	Moth	3	4	Di	No
Lepidoptera	Noctuidae	*Noctua* sp.	Moth	4	3	No	No
Lepidoptera	Pyralidae	*Nephopteryx melanostyle*	Moth	2	3	Di	No

2.3.5 *Identification of pollinators*

To reveal if species visiting *A. marina* touch the anthers and stigmas of flowers, the foraging behaviour of flower visitors was observed in each forest during the flowering seasons of 2009, 2010 and 2011. During the flowering seasons the foraging behaviour of 500 insects (200 during each of 2009 and 2010 and 100 during 2011) was carefully observed with a 40x magnification lens when they visited the flowers. Sampling was done during each of five 2 h sampling sessions (see *Identification of flower visitors* above), and I recorded the body parts that contacted the anthers and the stigma. All insects captured (Table 2.2) were carefully examined for pollen loads on all parts of the body (head, mandibles, glossa, legs, pretarsus, thorax, abdomen and wings) under a stereomicroscope at 60x magnification, and the number of pollen grains was counted on each body part. Only those insects contacting the stigma and found to carry pollen were considered to be pollinators.

2.3.6 *Honeybees as pollen carriers*

Investigations were made during the flowering season of 2009 to measure the potential of honeybees as pollen carriers. Using the sampling design described above (see section 2.3.3 'Identification of flower visitors'), sampling was done three times (on different days) and five honeybees were captured during each 2 h sampling session, giving 15 honeybees per time interval (five honeybees at each day during 8-10 am, 10-12 am, 12-2 pm, 2-4 pm and 4-6 pm), and in total 75 honeybees for all five time intervals, in each mangrove forest. On each honeybee, the number of pollen grains was counted on all body parts (see *Identification of pollinators* above). The number of pollen grains in the corbicula (Michener 1999) of individual honeybees was estimated as follows. A Nikon

D300 camera was used to photograph each corbicula through the ocular of the stereomicroscope. On these photos the length, width and depth of each corbicula pollen store and of 25 pollen grains from each pollen store were measured by computer using imageJ software (Abramoff *et al*. 2004). These measures were used to estimate the volume of each pollen store and the average volume of the 25 pollen grains from each pollen store. The estimated number of pollen grains in each corbicula pollen store was calculated by dividing the volume of each pollen store by the average volume of the 25 pollen grains.

2.3.7 *Composition of pollen*

To test the fidelity of honeybees as pollinators (i.e. to determine whether they foraged and captured pollen from a single plant species), I assessed the species of pollen grains carried on the body and in the corbicula of honeybees from Sydney and Minnamurra. Pollen grains from the body (100 grains) and the corbicula (1000 grains) of 60 honeybees foraging on *A. marina* were collected during the flowering seasons of 2009, 2010 and 2011 (five honeybees with and five without a corbicula pollen store at each estuary in each year, giving a total of 30 honeybees with and 30 without a corbicula pollen store). These were examined under an Olympus BHA compound microscope and photographed with a Nikon D300 SLR camera. To estimate the fidelity of foraging honeybees on other flowering plants surrounding *A. marina* at the two estuaries, 30 honeybees foraging on the salt marsh plant *Sarcocornia quinqueflora* (five honeybees collected from each estuary in 2009, 2010 and 2011), and 40 honeybees foraging on the mangrove *Aegiceras corniculatum* (20 honeybees with and 20 without corbicula pollen

stores, sampled as five honeybees from each estuary in 2009 and 2010 only) were likewise examined under the compound microscope.

To identify sources of pollen grains from the corbicula and body, pollen from the body of insects was transferred to a glass microscope slide using a small cube of agarose gel fastened to the tip of a dissection needle. Pollen was then viewed under the compound microscope at 120x magnification, and the number of pollen grains of each species was counted and compared with pollen grains sampled from the anthers of *A. marina* flowers. The percentage of pollen grains from the plant species upon which they were foraging when captured was calculated.

2.3.8 *Pollen removal and deposition*

Three experiments were used to determine if honeybees removed or deposited pollen on the flowers of *Avicennia marina*. In the first experiment I determined the proportion of pollen that was removed from anthers of flowers by honeybees during one visit. To do this I compared the amount of pollen on the anthers of flowers using two treatments where flowers (floral shoots) were either continuously bagged, or bagged after which each individual flower was exposed to a single honeybee visit. The effect of pollen removal was assessed by comparing the pollen remaining on the anthers of flowers receiving a single visit from a honeybee to pollen remaining on the anthers of continuously bagged flowers. Insects were excluded by bags (25 cm x 15 cm) constructed of polyorganza fabric lined with rigid nylon mesh to distend the bags and isolate the flowers from the inner surface of the fabric. The bags were placed over the tip of *A. marina* branches with floral shoots and secured with plastic coated wire.

In the second experiment I determined the amount of pollen deposited by honeybees on stigmas during the day. Flowers used for this experiment were bagged to ensure that anthers were loaded with pollen and pollinators had not visited stigmas. To test for pollen deposition, bags were removed for the duration of a single honeybee visit to individual flowers under close observation, and all stigmas displayed no pollen grains prior to visitation. In a second treatment I verified that flowers bagged continuously during the experimental period did not display any pollen on stigmatic surfaces. In the first and the second experiments sampling was performed on five different days during the flowering season of 2011 in both forests. On each of these five days, there were ten flowers from each of three trees (separated by at least 20 m) for each treatment (i.e. 150 flowers per treatment).

In the third experiment I tested for nocturnal pollen deposition. To do this I compared the amount of pollen on the stigmas of flowers from floral shoots of which a third were bagged until exposed to flower visitors during the night, a third were exposed to flower visitors during the day and a third were continuously bagged. To reveal pollen deposition in the third experiment the same sampling design was used as in experiments one and two. In the third experiment, however, the bags were removed from the flowers at sunset, and the experiment was terminated at sunrise, and during the day the bags were removed from the flowers at sunrise, and the experiment was terminated at sunset.

After termination of experiments one, two and three, floral shoots were carefully and gently harvested with scissors (to prevent remaining pollen from anthers to accidently deposit on stigma), after which they were transported to the lab in small plastic boxes to prevent them from being creased during transport, and the number of

pollen grains on stigmas of individual flowers was counted under a stereomicroscope at 60x magnification.

For each experiment, independent, two-tailed t-tests were done comparing flowers open to pollen removal or deposition to those continuously covered with bags and not open to pollen removal or deposition. Data from each of the five independent days and from Sydney and Minnamurra were pooled for each t-test (except for t-tests where these two locations were compared).

2.4 Results

2.4.1 *Identification of flower visitors*

I found a diverse assemblage of species visiting the flowers of the mangrove *A. marina*. Overall, 38 species of insects were observed, with 27 and 28 species detected in the forests of Sydney and Minnamurra, respectively (see Table 2.2). Of these, 17 species were common to both forests. Flower visiting species included ants (Formicidae), flies (Muscidae, Calliphoridae, Stratiomyidae, Syrphidae, Sarcophagidae), moths (Noctuidae, Pyralidae, Arctiidae), wasps (Scoliidae, Sphecidae, Vespidae), beetles (Scarabaeidae, Coccinellidae), bugs (Rhopalidae), bees (Halictidae) and the European honeybee *A. mellifera* (Apidae) (Table 2.2). The only nocturnal species observed was a noctuid moth and an ant, *Iridomyrmex* sp., that was also diurnal, and both species were common to both forests.

2.4.2 *Abundance of pollinators and flower visitors*

Honeybees dominated as flower visitors during both 2 h intervals (midday and evening). At both estuaries, during all five sampling days (one in 2009 and two in each of 2010

and 2011) during both 2 h intervals, the number of honeybees visiting the flowers of *A. marina* was significantly higher (interaction between visitors and time interval: F=9.21; *P*=0.003; $df_{1,72}$) than the total number of other insects during the midday, but not during the evening (Fig. 2.3; Table 2.3; SNK tests). In addition, during all five sessions, the number of honeybees was significantly higher during midday than evening, whereas the number of other insects did not vary significantly between these two time intervals (Fig. 2.3; Table 2.3; SNK tests).

Table 2.3. Three factor ANOVA on the abundance of the two categories of visitors (honeybees and other insects) visiting the flowers of *A. marina* in Sydney and Minnamurra. Each sampling period were conducted during two independent time intervals, midday (12-2 pm) and evening (5-7 pm), at each of the two estuaries during three flowering seasons (2009, 2010 and 2011) (see Fig. 2.3). Location (L) and visitors (V) and time interval (T) were all fixed factors.

Source	d.f.	MS	F	*P*
L	1	23.52	3.01	0.0872
V	1	39.97	5.11	0.0268
T	1	137.93	17.63	0.0001
LxV	1	2.87	0.37	0.5464
LxT	1	1.11	0.14	0.7076
VxT	1	72.06	9.21	0.0033*
LxVxT	1	2.95	0.38	0.5413
Res	72	7.82		
Transformation	Sqrt(X+1)			
Cochran's test	NS			
SNK	Honeybees (H): M>E	Other insects (O): M=E		
	Midday (M): H>O	Evening (E): H=O		

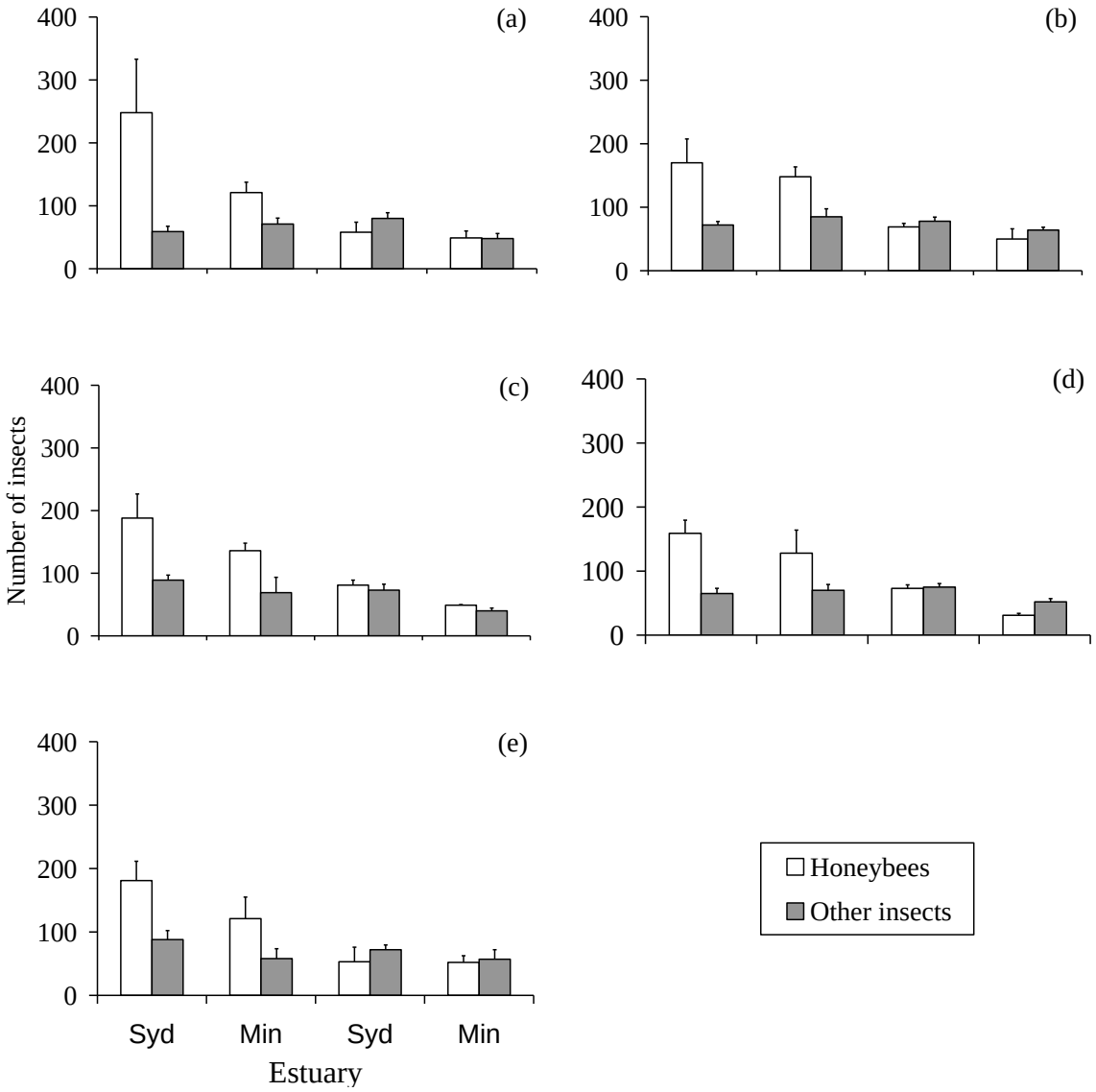


Fig. 2.3. Mean (± SE) of the abundance of flower visiting insect species (honeybees and other insects) across five independent sampling days and carried out at each of two time intervals (midday and evening) during (a) 2009, (b,c) 2010 and (d,e) 2011, at the mangrove forests in Sydney and Minnamurra respectively. Syd = Sydney, Min = Minnamurra.

2.4.3 *Identification of pollinators*

Only three of the visiting insect species carried pollen of *A. marina* on their bodies: the European honeybee *A. mellifera*, the flower beetle *Carphurus* sp. (only at Minnamurra)

and the ant *Iridomyrmex* sp. Honeybees were by far the most frequent of all the visiting insect species (Fig. 2.3) and individuals were directly observed for more than 150 hours during the flowering seasons of 2009-2011. When honeybees moved between flowers within a floral shoot, they often touched the anthers and honeybees collected pollen or nectar, they often stroked the inside of the flower with their front limbs and touched the anthers and stigma (Fig. 2.4*a*). When the honeybees collected pollen or nectar they frequently touched the anthers and stigma with their front and hind limbs, pretarsus, mandibles and glossa (Fig. 2.4*b*,*c*). The ants of *Iridomyrmex* sp. and flower beetles of *Carphurus* sp. were small enough to crawl inside individual flowers. The two flower beetles observed were about 8 mm long and touched the anthers at least with their front limbs, mandibles and head and the stigma with their head. One of these carried 157 pollen grains on its head and mandibles while the other carried no pollen on its head and mandibles. The two flower beetles carried 11 and 19 pollen grains on the abdomen, and from 1 to 9 pollen grains on the remaining body parts. Each of the 5 ants observed with pollen carried fewer than 10 pollen grains, and observations (for 1 hour of 27 ants) revealed that they touched the anthers of *A. marina* flowers with their mandibles and occasionally with the limbs, but not the stigma.

Most of the other visiting insect species positioned themselves outside the flower, with only their glossa in the nectar, though a few small species, such as the introduced ladybird species *Hippodamia variegata* and an ephydrid fly were positioned in the flower during visitation. These species were not pollen collectors and were not found to have behaviour that resulted in body parts touching the anthers or stigma.

Fig. 2.4. Foraging behaviour of honeybees to flowers of *A. marina*. (a) Honeybee grooming a flower with its front limbs before collection of the reward (pollen or nectar). (b) Honeybee in a flower with its hind legs while collecting nectar from another flower. (c) Honeybee touching the stigma of a flower with the pretarsus of its right hind leg.

2.4.4 *Honeybees as pollen carriers*

Honeybees foraging on *A. marina* carried pollen on all body parts, including those that contacted the anthers and stigma of flowers. In Sydney, honeybees with pollen in the corbicula carried on average 1358 (SE=145) pollen grains on the body and 7968 (SE=2779) in the corbicula, and those in Minnamurra carried on average 1072 (SE=85) pollen grains on the body and 9657 (SE=3235) in the corbicula, showing that honeybees can remove large amounts of pollen from the anthers and store them in the corbicula. Honeybees without pollen grains in the corbicula carried on average 1085 (SE=109) pollen grains on the body in Sydney and 968 (SE=82) in Minnamurra (Table 2.4).

Table 2.4. Comparison of the mean (± SE) number of pollen grains on body parts of honeybees foraging on *A. marina*, with (+) or without (–) Corbicula Pollen Store (CPS) from Sydney (Syd) and Minnamurra (Min). "(n)" shows the number of honeybees.

	Mandible-glossa	Front pre-tarsus	Hind Pre-tarsus	Front limbs	Hind limbs (excluding corbicula)	Total	Corbicula	(n)
Syd								
– CPS	23 (3.42)	4 (0.82)	5 (1.17)	43 (5.34)	177 (21.24)	1085 (106.75)		30
+ CPS	17 (2.25)	7 (1.26)	6 (1.08)	52 (5.10)	253 (31.80)	1358 (149.92)	7968 (1716)	45
Min								
– CPS	14 (2.37)	3 (0.96)	5 (0.64)	64 (6.31)	271 (20.75)	968 (79.99)		32
+ CPS	13 (1.73)	6 (1.49)	4 (0.53)	50 (3.30)	329 (25.09)	1072 (82.27)	9657 (2018)	43

Fig. 2.5. Pollen store in the corbicula of the hind limbs of honeybees foraging on (a) *A. marina*, (b) *S. quinqueflora* and (c) *A. corniculatum*. The pollen store of the corbicula is compared to pollen displayed on the anther of a flower (shown by arrows) to show that the pollen colour is matching.

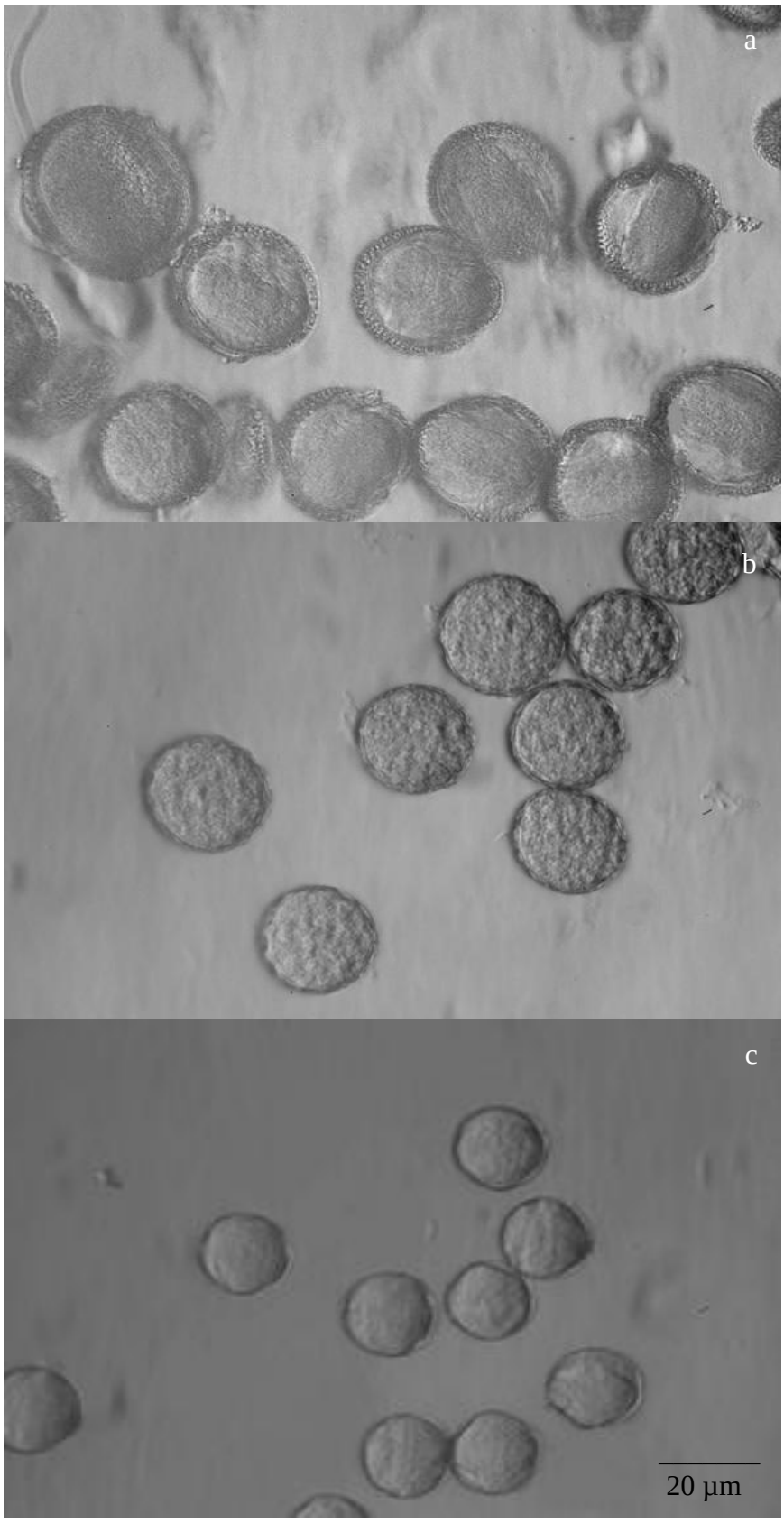


Fig. 2.6. Pollen grains from the corbicula of the hind limbs of honeybees foraging on (a) *A. marina*, (b) *A. corniculatum*, and (c) *S. quinqueflora* respectively, photographed with a Nikon D300 camera at 120x magnification through an Olympus compound microscope.

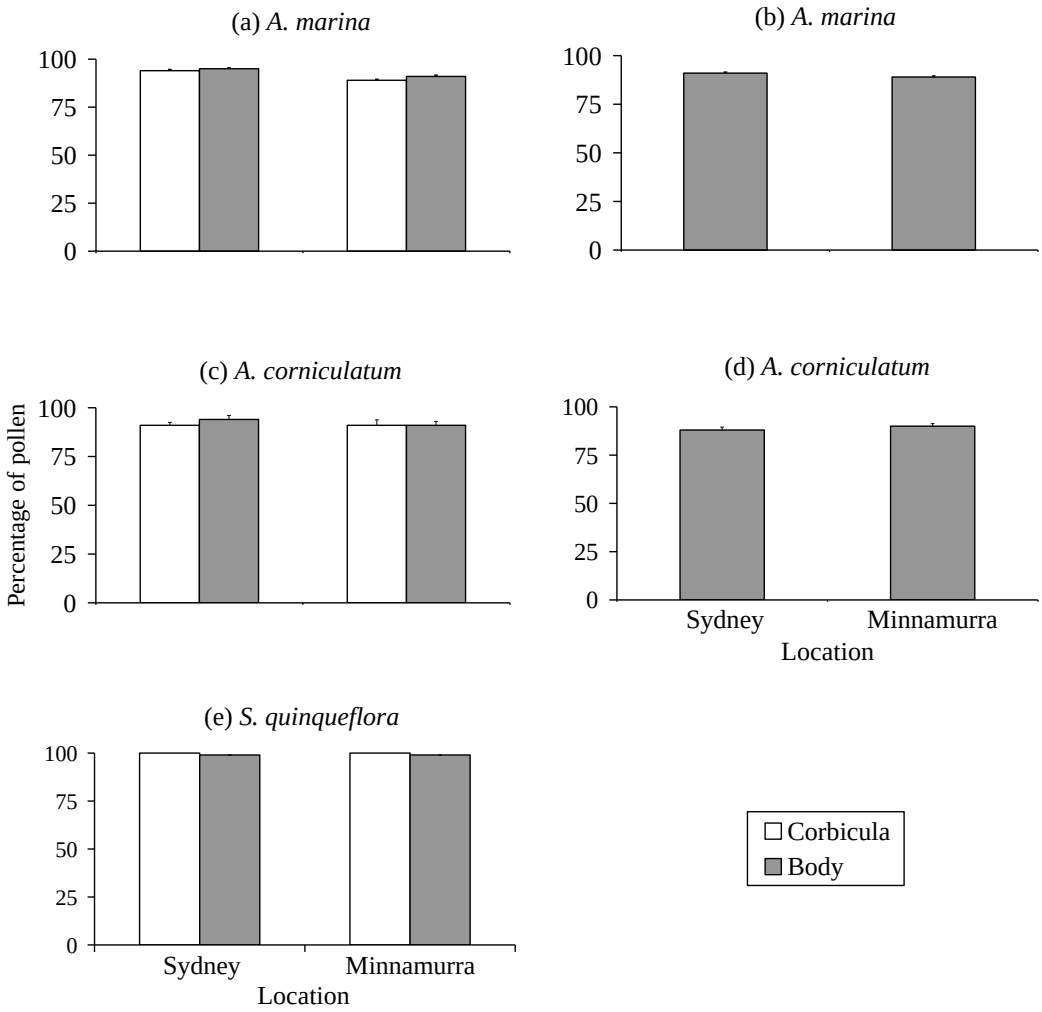


Fig. 2.7. Mean (± SE) percentage of pollen from the plant upon which bees were foraging for: (a) honeybees with pollen in the corbicula and (b) without pollen in the corbicula, foraging on *A. marina*; (c) honeybees with pollen in the corbicula and (d) without pollen in the corbicula, foraging on *A. corniculatum*; (e) honeybees with pollen in the corbicula foraging on *S. quinqueflora*. Sampling was done in Sydney and Minnamurra in 2009, 2010, and 2011 (except 2009 and 2010 only for *A. corniculatum*).

2.4.5 *Composition of pollen*

The honeybees captured while foraging on *A. marina*, *S. quinqueflora* and *A. corniculatum* carried almost exclusively the pollen of those plant species, both on

the body and in the corbicula (Fig. 2.5-2.7). Honeybees foraging on *A. marina* carried on average 89%-95% *A. marina* pollen, those foraging on *A. corniculatum* carried on average 88%-94% *A. corniculatum* pollen, and those foraging on *S. quinqueflora* carried on average more than 99% *S. quinqueflora* pollen (Fig. 2.7). These results clearly demonstrate that honeybees are relatively faithful to their target plant species. Foreign pollen grains from two plant species were found on honeybees foraging on *A. marina*, of which one was identified to be from *A. corniculatum*. Only foreign pollen grains from one unidentified species were found on honeybees foraging on *S. quinqueflora*, and three unidentified species of pollen were found on honeybees foraging on *A. corniculatum*.

2.4.6 *Pollen removal and deposition*

My investigation of removal of pollen by honeybees revealed that the average number of pollen grains remaining on anthers after one visit from a honeybee was only 4% of the number present on the anthers of flowers bagged to exclude pollinators (i.e. an average of 65 (SE=4.5) versus 1762 (SE=103.9) pollen grains). This demonstrates that honeybees remove pollen very effectively (Fig. 2.8 & 2.9). There was a significant difference in the number of pollen grains on floral shoots that were continuously bagged and those visited once by a honeybee ($t_{1,598} = 15.04$; $P < 0.001$) and between floral shoots from Sydney and Minnamurra only visited once ($t_{1,298} = 2.77$; $P = 0.006$).

The diurnal pollen deposition experiments revealed that honeybees deposit an average of 2.8 and 3.0 pollen grains after one visit to a flower in Sydney and Minnamurra, respectively (Fig. 2.10), and control flowers (bagged and without visits from honeybees) had no pollen grains on their stigmas. Moreover, the level of pollen deposition by honeybees was similar at the two locations.

Diurnal pollen deposition was more important than nocturnal pollen deposition. Pollen deposition experiments at night revealed that only 4% of stigmas carried any pollen (between one and seven pollen grains) compared to 92% of stigmas with an average of approximately eight pollen grains per stigma (Fig. 2.10). Indeed, pollen deposition between day and night was substantially different ($t_{1,598} = 9.10$; $P < 0.001$), and these differences were similar at forests in Sydney and Minnamurra. None of the flowers from continuously bagged floral shoots carried any pollen on stigmas and the difference between day/night experiments and continuously bagged experiments were significant ($t_{1,598} = 9.26$; $P < 0.001$ and $t_{1,598} = 3.24$; $P = 0.001$, respectively).

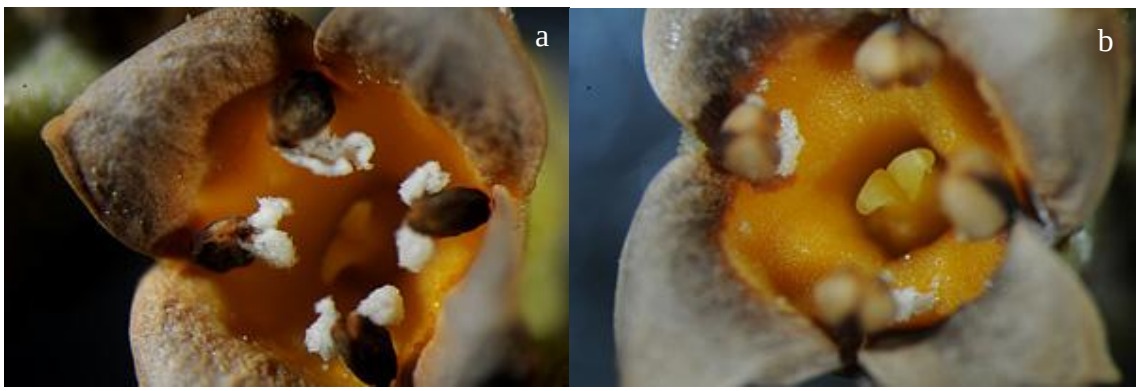


Fig. 2.8. (a) An *A. marina* flower that was bagged and therefore not was visited by any insects, with developed pollen clumps displayed on the anthers (flowers carried up to approximately 12000 pollen grains). (b) An *A. marina* flower after 1 visit by a honeybee. Most of the pollen has been removed although a small number of pollen grains can be seen to have fallen from two of the anthers.

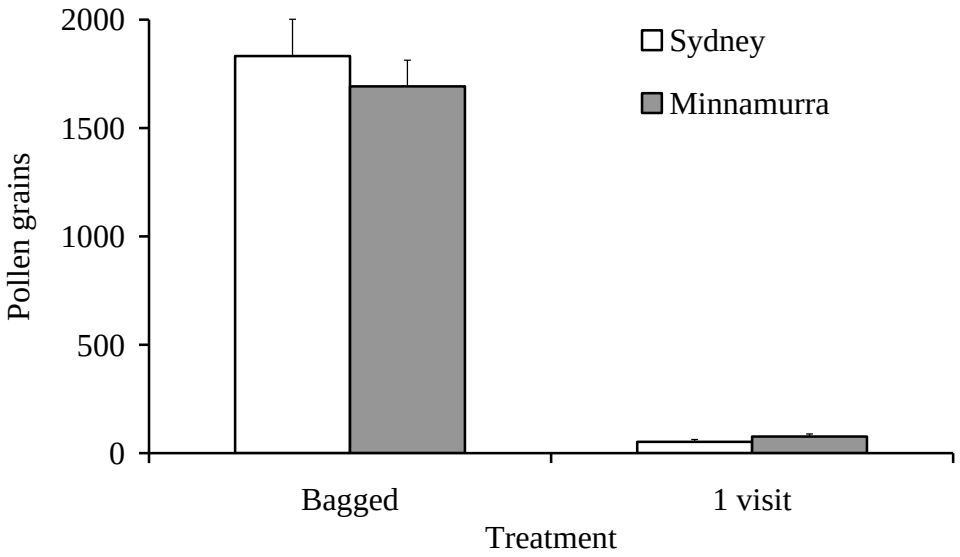


Fig. 2.9. Mean (± SE) of the number of pollen grains removed by honeybees from anthers of *A. marina* flowers after one visit, compared to bagged (untouched) flowers. Honeybees remove significant amounts of pollen during one visit.

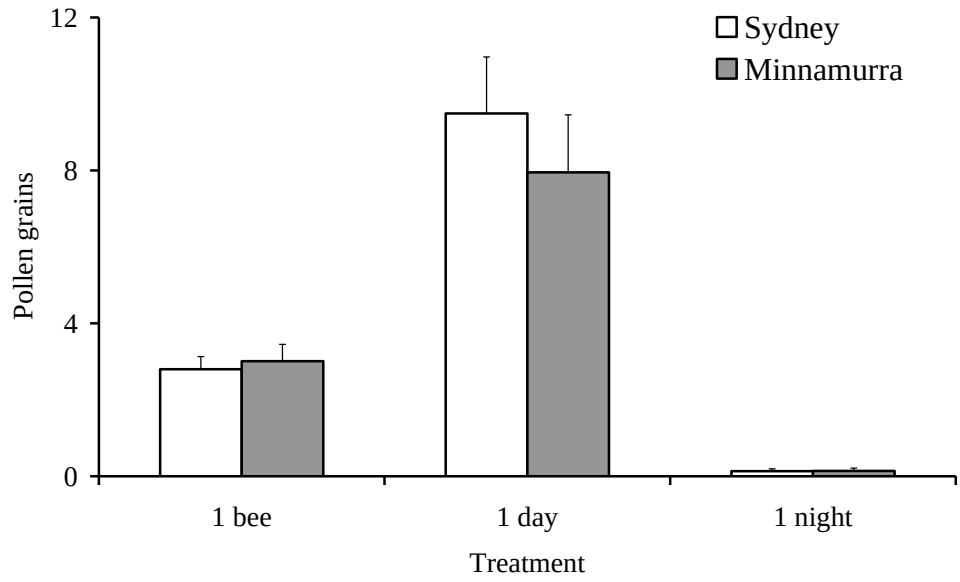


Fig. 2.10. Mean (± SE) of the number of pollen grains deposited after one visit by a honeybee and during one day and one night respectively.

2.5 Discussion

This study demonstrates a large discrepancy between the list of species that could be considered likely pollinators, based on flower visitation, and the real set of pollinators of the mangrove species *A. marina* in temperate forests of central New South Wales (NSW), Australia. In apparent contrast to my prediction that temperate mangrove forests would display a low diversity of pollinator species, I detected 38 visiting insect species. Homer (2009) similarly, identified 48 species of insect flower visitors in the subtropical northern NSW (Coffs Harbour, Clarence River and Richmond River: 435-600 km north of Sydney). Moreover, I found that the exotic honeybee *Apis mellifera* was the most abundant flower visitor of *A. marina* (which is again supported by Homer (2009), who found *A. mellifera* to be one of the five most common flower visitors, with the four other species being spiders and ants), and also the only significant pollinator, which is similar to results of a previous study in a Sydney estuary on the mangrove *Aegiceras corniculatum* (Hermansen, unpublished data). Here, as for an increasing number of ecosystems beyond the native range of *A. mellifera* (Butz Huryn 1995; Paton 1993, 1996), these data imply that this bee has displaced one or more native pollinators and in this case appears to be effective in transferring *A. marina* pollen, as judged by both pollen removal and deposition.

2.5.1 *Flower visitors and pollinators*

In order to understand the pollination biology of any plant species it is crucial to distinguish between species that visit flowers to collect nectar or pollen and those that by virtue of their behaviour, anatomy and abundance are able to function as effective pollinators. My surveys of patterns of flower visitation revealed that the mangrove *A.*

marina, in the Sydney region of New South Wales, is visited by a broad suite of insect species that includes ants, wasps, bees, beetles, flies, butterflies, moths and bugs, which is in accordance with the findings by Clarke & Myerscough (1991), but the flowers are not visited by birds or mammals as it is the case for many mangrove species in the tropics (see Table 2.1). I discovered that of 38 insect species that visited flowers, many were in low abundance and only three foraged in a manner that caused pollen to be carried on their bodies. Only two of these, the flower beetle of *Carphurus* sp. and the exotic honeybee *A. mellifera*, made contact with stigmatic surfaces, and hence could be considered potentially important pollinators. Most surprisingly, only *A. mellifera* met each of the requirements of an important pollinator of *A. marina*. *Apis mellifera* carried out between 45% and 72% of all flower visits, carried large numbers of pollen grains and made frequent contact with both anthers and stigmas. Although honeybees currently appear to out compete native pollinators in the Sydney region, this does not necessarily mean that the original pollinators are absent; they may be suppressed as it was the case at Santa Cruz Island in California (Wenner & Thorp 1993). A bagging experiment of Clarke & Myerscough (1991) indicated the existence of a repressed native pollinator in temperate *A. marina*. By excluding honeybees but not other insects they attained a certain fruit set. This could be explained by early pollination (at Salt Pan Creek ca. 20% of pollen grains deposited by honeybees) taking place before honeybees reach the source after flower initiation (unpublished data). *Avicennia marina* in the temperate Sydney area could regain its native pollinators if the honeybee populations were to diminish, as is currently the case in Europe and North America (Higes *et al*. 2009; vanEngelsdorp *et al*. 2009; Johnson 2010).

Estimates of the diversity of flower visitors will almost inevitably underestimate the contribution of rare species. Moreover, I had expected that the identity of both flower visitors and pollinators might vary between estuaries as it has been shown for flowers of terrestrial species (e.g. Cosacov *et al*. 2008). However, because I carried out over 300 hours of diurnal and nocturnal observation spread across two seasons, and in each of two estuaries, this leaves little doubt that at least in the years 2009 and 2010 there were essentially no obvious native pollinators of *A. marina* along the landward sides of the relatively large urban and suburban mangrove forests within the Sydney and Minnamurra region. Nevertheless, because as is the case for terrestrial plants, the set of insect species reaching mangrove populations may vary with population size and isolation (Kwak *et al*. 1998), and the surrounding matrix of vegetation (Burkle & Alarcón 2010). I therefore acknowledge that it will also be valuable to compare my data with surveys of small population fragments and especially those to both the north and south that are surrounded by predominantly native vegetation.

Importantly I was able to use experimental approaches to confirm that *A. mellifera* not only removes the majority of pollen from the anthers of *A. marina* but it also deposits pure *A. marina* pollen onto stigmas. This experimental demonstration of pollen transfer is a critical step in determining the effectiveness of flower visitors as pollinators that has not been previously carried out for mangroves (see Table 2.1). Indeed, honeybees have been shown to be efficient pollinators for a wide range of plant species in different environments and ecosystems (e.g. Free & Williams 1972; Young *et al*. 2007). Most of the insect species I observed and captured were large, collected only nectar, and did not touch the anthers and stigma of the *A. marina* flowers. Of the smaller insects, besides the flower beetle *Carphurus* sp. and the ant *Iridomyrmex* sp., only the ladybird beetle

Hippodamia variegata (that did not carry any pollen) touched the stigma. Only one ladybird was observed and captured, so if this species is a pollinator it may not be of importance. These findings show that *A. mellifera* was the only effective pollinator identified in my study, carried out along the landward side of the forests. It may be reasonable, however, to conclude that this is the case for the investigated mangrove forests in general.

Although honeybees in my study were overwhelmingly the numerically dominant pollinators in both Sydney and Minnamurra it is unclear why honeybees were more common in the highly urbanised Sydney estuary. This variation in bee abundance may simply reflect regional variation in numbers of managed hives. Data of Beekeepers Registration System of Australia (updated to 2009), retrieved from the Sydney City Council, and shows that in the areas around the Georges River there are 76 registered farmers of honeybees (*Apis mellifera*) with 564 honeybee hives (Sutherland and Bankstown shires), while in the shire of Kiama where the Minnamurra River is located there are 7 registered honeybee farmers with only 22 hives.

2.5.2 *Fidelity and pollen transfer*

In my study, honeybees removed on average 96% of pollen grains and deposited three pollen grains on the stigma of *A. marina* flowers during a flower visit. During the day an average of seven pollen grains was observed to accumulate, compared to approximately 11 pollen grains on average deposited on stigmas of open flowers (unpublished data) that retained an open corolla for 2-5 days (Clarke & Myerscough 1991). Similarly Clarke & Myerscough (1991) counted nine pollen grains on average on the stigmas of open flowers. I conclude that *A. mellifera* is a surprisingly effective pollinator of temperate *A.*

marina within the area of Sydney, where it seems to have displaced the unknown native pollinator of *A. marina*. Similar displacement of native pollinators by *A. mellifera* has been observed for vast numbers of terrestrial plants in Australia and worldwide (Butz Huryn 1995; Paton 1993, 1996).

2.5.3 *Conclusion*

I found that the European honeybee *A. mellifera* is now the dominant pollinator of *A. marina* in this region, with at least two probable consequences. Results suggest that *A. mellifera* has displaced an unknown set of native pollinators (Butz Huryn 1995; Paton 1993, 1996). By this it may have altered the pattern of pollen dispersal for *A. marina*. It is difficult to predict whether *A. mellifera* is having beneficial or detrimental effects on the reproductive biology and fitness of *A. marina*, which is also the case for other terrestrial systems that have been invaded by this species (e.g. Whelan *et al*. 2009; Caraballo-Ortiz & Santiago-Valentín 2011). This is a complex issue because there are rarely good estimates of pollen dispersal, or optimal or realised mating systems (Richardson *et al*. 2000) in the absence of *A. mellifera.* Moreover, any detrimental effects on reproductive fitness will inevitably be difficult to discern in long-lived perennials. My study and observations of many populations of *A. marina* on the New South Wales coast suggest that *A. mellifera* is not having a major detrimental impact on *A. marina*, at least in terms of fruit production because viable propagules are abundant, however, it is unclear if *A. marina's* fitness is reduced by elevated inbreeding particularly within small stands.

Chapter 3: Effects of stand size on pollinator services and fruit set in temperate stands of the mangrove *Avicennia marina*

3.1 Abstract

Populations of the mangrove *Avicennia marina* within estuaries in the Sydney region exist as stands of varying size, ranging from singular plants to extensive forests. I have shown previously that *A. marina* attracts a diversity of flower visitors but its only significant pollinator is the exotic honeybee *Apis mellifera*. However, nothing is known about the movements of *A. mellifera* between mangrove stands separated either by water or urban habitat matrix. I hypothesised that, as observed in many terrestrial forests, small stands (<100 plants) would experience lower pollinator densities and altered pollinator behaviour and services (visitation and pollen deposition) promoting higher levels of self-pollination or inbreeding (or biparental inbreeding) and in consequence would display reduced fruit production as compared with large stands (>10000 plants). My detailed surveys within pairs of large and small stands in two estuaries support these predictions with pollinator abundance on average reduced significantly by 84% and pollen deposition reduced significantly by 61% in small as compared to large stands. Moreover in small stands the duration of foraging by *A. mellifera* was 10% and 12% longer on individual floral shoots and trees respectively. Fruit production was on average reduced by 61% and 57% in small stands as compared to large stands when calculated per tree or per floral shoot respectively. Taken together, my data strongly suggest that while the honeybee *A. mellifera* is currently vital to the reproductive success of *A. marina*, its performance as a pollinator of plants in small stands is reduced, with reduced fruit production reflecting a combination of pollen limitation and elevated self pollen transfer.

3.2 Introduction

In terrestrial forests habitat fragmentation can have profound effects on pollinator behaviour and services provided to trees because pollinator diversity, abundance and visitation and the deposition of pollen are reduced, important pollinators are lost and the patterns of pollen transfer are altered (Jennersten 1988; Aizen & Feinsinger 1994a; Ghazoul 2005; Aguilar 2006). This can result in negative consequences for plant reproduction (Murcia 1996; Aizen 1998). For example, pollen supply, quality or diversity can be limited by reduced flower visitation (Aizen & Feinsinger 1994*a*; Sih & Baltus 1987; Cascante *et al.* 2002), the resultant mating system is altered by changed foraging patterns of pollinators (Steffan-Dewenter & Tscharntke 1999), or numbers and genetic diversity of mates are reduced. Separately or together these changes can lead to higher levels of self-pollination and inbreeding (Ghazoul 2005) and reduced fruit production (Jennersten 1988; Aizen & Feinsinger 1994*b*; Ghazoul & McLeish 2001).

In the present study I aimed to investigate the effects of stand size on the abundance of pollinators and pollinator services (visitation and pollen deposition) and resultant fruit production, by comparing two large and two small stands of *Avicennia marina* from estuaries at Sydney and Minnamurra. In earlier investigations observations of the foraging behaviour of all the species identified as visitors of the flowers of the mangrove *A. marina* in the Sydney region revealed that only the honeybee *Apis mellifera* foraged in a manner allowing transport of pollen to the stigma of *A. marina* flowers and, of only two species foraging for pollen *A. mellifera*, was the only species carrying sufficient pollen to effect successful pollination (chapter 2). The foraging behaviour of *A. mellifera* typically involves pollen transfer within plants or among near neighbours (Paton 1993; Whelan *et al.* 2009) and therefore is expected to produce high rates of self-

pollination or biparental inbreeding within individual stands. Importantly using a population genetic approach I have shown that large and small stands display similarly high levels of biparental inbreeding and similar levels of allelic and genotypic diversity, but within small stands rates of outcrossing are significantly lower (see Chapter 5). Therefore, I anticipated that the effects of stand size on temperate *A. marina* forests might parallel those observed in terrestrial systems where altered pollinator abundance, diversity and behaviour lead to reduced fruit production (Ghazoul 2005).

Typically *A. mellifera* is present in urban and bushland areas on the landward margins of my study sites where it is a dominant pollinator (see also Lomov *et al.* 2010). Little is known about the tendency of *A. mellifera* to fly across estuarine waters on foraging bouts (a foraging bout is defined as starting when a honeybee leaves the hive, and ending when returning to the hive; e.g. Lihoreau *et al.* 2012). The habitat matrix, which is a combination of open water and wetland and terrestrial vegetation and urban and suburban development, surrounding *A. marina* populations could either exacerbate or reduce any effects of stand size. However, social bees such as honeybees seem to be less sensitive to changes in matrix within urban areas than other bees (Stefan-Dewenter *et al.* 2002), indicating that it is easier for honeybees to adapt to new environments.

Based on the predictions that small stands would experience reduced pollinator density, altered foraging behaviour and reduced pollen deposition resulting in reduced fruit production, I specifically ask weather do small A. marina stands experience: (1) an alteration in the duration of honeybees foraging within floral shoots and trees and altered patterns of foraging behaviour? (2) A reduction in the number of honeybee visits? (3) Decreased pollen deposition? And (4) Decreased fruit set?

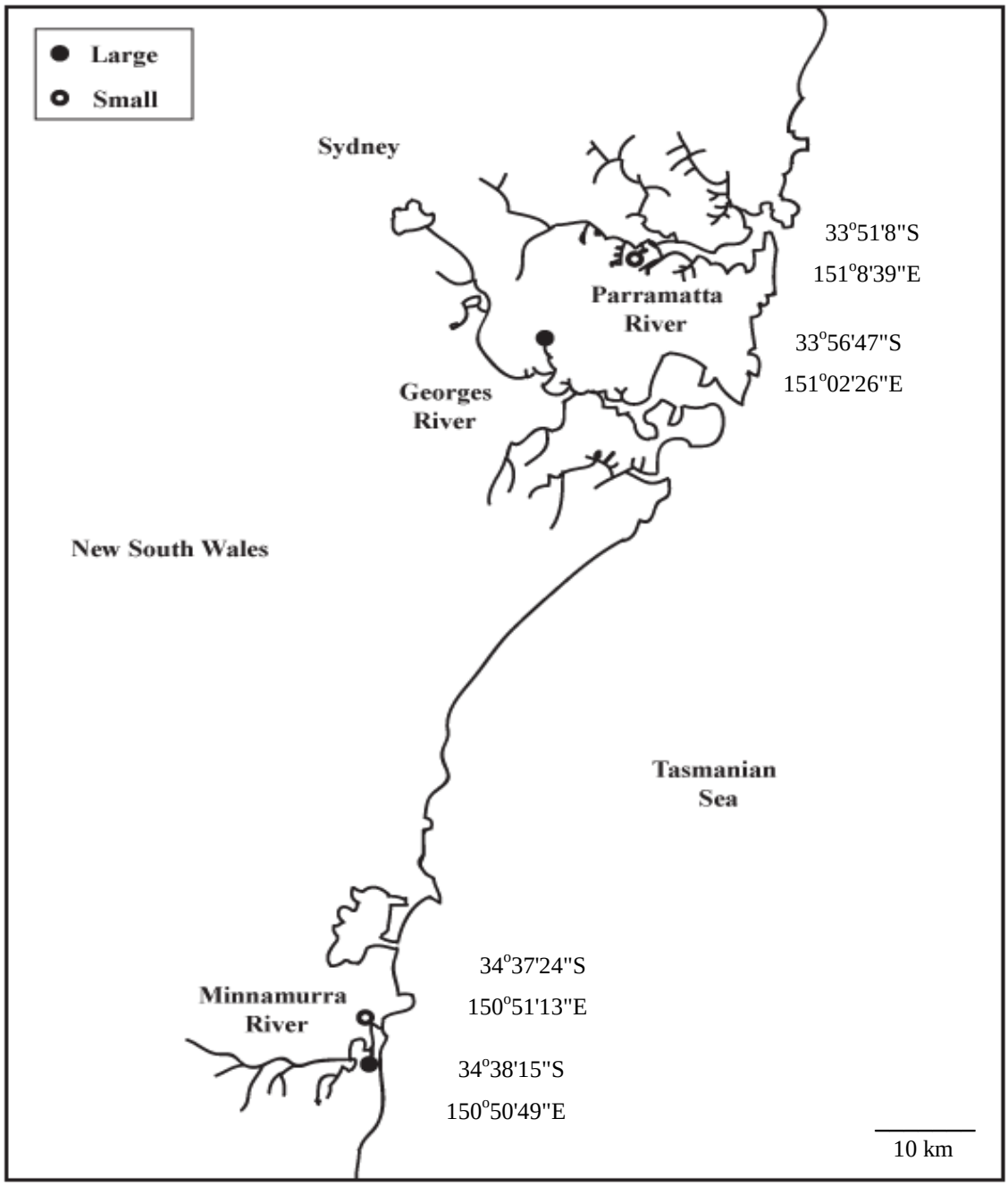


Fig. 3.1. Map of Parramatta River, Georges River and Minnamurra River and shows the location of the two large and small stands.

3.3 Materials and Methods

3.3.1 *Study sites*

The study was carried out in mangrove forests dominated by *A. marina* within two locations of the Sydney and Minnamurra regions, New South Wales, Australia. I selected one large (ca. 10000 trees) and one small (ca. 100 trees) stand within each of the Sydney and Minnamurra regions (Fig. 3.1). All stands were at least twice as long as broad with the small stand of Sydney being the most elongated stand, four to five times as long as broad (West *et al*. 1985; personal investigation of aerial photos). In Sydney, the large stand was located at Salt Pan Creek (33^{o}56'47" S; 151^{o}2'26" E), which forms a branch on the northern side of the Georges River, and the small stand was located at Five Dock Bay (33^{o}51'8" S; 151^{o}8'39" E) on the southern bank of the Parramatta River. In Minnamurra, the large stand was selected at Kiama Downs (34^{o}38'15" S; 150^{o}50'49" E) and the small stand near the Minnamurra River entrance (34^{o}37'24" S; 150^{o}51'13" E), and both located within the Minnamurra River reserves.

The large stand in Sydney is within an urbanized landscape with both a highway and public pedestrian pathways bisecting it, whereas the large stand in Minnamurra is within an agricultural landscape and surrounded by houses on its landward edge. Houses and open grassland border the small stand at Sydney and Minnamurra. The large stands are dominated by *A. marina*, with the smaller mangrove *Aegiceras corniculatum* occurring on their landward edge. Both large stands extend landward into salt marshes and are bordered by the dominant salt marsh chenopod *Sarcocornia quinqueflora*. Also various flowering plants from urban and suburban gardens and small patches of terrestrial forest located in close proximity to the mangroves flower simultaneously with *A. marina* at this latitude. The small stands are exclusively *A. marina* (not bordered by saltmarsh)

and adjacent habitat includes various flowering plants from gardens and grassy areas (lawns and parks) that flower simultaneously with *A. marina*.

Investigations of the large stands were confined to the landward edge of the stands, whereas for small stands I used both the landward edge and sides. The small stands were flanked by mudflat making them free assessable for honeybees from the landward edge during low tide. The sides were chosen to get a higher number of observation sites from small stands. Observations were done on *A. marina* trees of intermediate height (5-10 m), with approximately 200 floral shoots per m^2 (a density near maximum during the investigated flowering seasons). Investigations were conducted from mid to late summer (mid January to mid March) of the flowering seasons of 2009 and 2010 and on sunny days with temperatures in the shade between 16.4 and 33.8^{o}C in Sydney and between 13.9 and 28.2^{o}C in Minnamurra. The study was confined to days of sunny weather because preliminary observations on cloudy days revealed substantially lower and highly variable abundances of honeybees (Hermansen, unpublished data).

3.3.2 *Flowering, pollination and fruit production of* A. marina

Avicennia marina is a hermaphroditic species with yellow flowers organized in clusters (Tomlinson 1986). These clusters are further organized into floral shoots. Flowers are small (≈5 mm tall and ≈5 mm wide), each with a stigma of 1.5-2.0 mm in length, and four anthers are anchored on the petals at a height approximately level with the stigmatic surface (Duke 1990, 2006; Clarke & Myerscough, 1991). In the Sydney and Minnamurra regions *A. marina* flowers from mid January to mid April. Individual flowers are open for 2-5 days and a flower cluster has open flowers for 2-4 weeks. A flower can produce up to 16000 pollen grains and four ovules (Duke 1990; Clarke & Myerscough 1991). In a

study at Jervis Bay, Australia, ca. 50 km south of Minnamurra, between 4% and 41% of open-pollinated flower buds from individual trees produced fruit (Clarke & Myerscough 1991).

3.3.3 *The abundance of flower visiting honeybees*

The effect of stand size on the abundance of honeybees visiting flowers of *A. marina* was tested during the *A. marina* flowering season of 2009. To measure the abundance of honeybees during the day, the numbers of honeybees visiting 10 m^2 areas of canopy (4.0 m wide by 2.5 m high, and measured from the lowest point of the canopy, approximately 0.25 m above the ground, to a height of 2.75 m) were counted during each of seven, 2 h intervals (with each interval done on different days) covering the period from sunrise to sunset (6 am-8 pm). Counts were made at two different sites within each stand during the first and second hour of each 2 h interval. In each case bees were counted every 10 minutes, giving 6 counts per hour. Within the large stands the two sites were separated by 100-150 m and in the small stands they were separated by 20-30 m (a distance proportional to stand size). As the canopies of trees often overlap, each 10 m^2 area of canopy covered at least two trees and the honeybees could move freely between these trees. Finally, all honeybees observed in the 10 m^2 areas on which abundance was measured were also observed to visit the flowers of *A. marina* within these areas during the observation period.

3.3.4 *Foraging of honeybees within floral shoots and trees*

To compare the duration of foraging by honeybees within individual floral shoots and trees in the large and small stands during the flowering season of 2009, we quantified the

foraging behaviour of (i) 200 honeybees on individual floral shoots within each stand (i.e. 200 independent observations per stand), and (ii) 55 honeybees on individual trees within each stand (i.e. 55 independent observations per stand). The duration of foraging within individual floral shoots or trees was measured using a stopwatch and observations were spread evenly across the seven, 2 h sampling intervals as described above (see *The abundance of flower visiting honeybees* subsection). Further, to determine the number of movements between floral shoots we observed 280 honeybees that were foraging on floral shoots of a single tree or a pair of neighbouring trees with overlapping canopies within small and large stands, respectively.

3.3.5 *Pollen deposition on stigmas of A. marina flowers*

To test the effect of stand size on pollen deposition during the flowering seasons of 2009 and 2010, a total of 150 flowers per year were harvested from each of the two large and two small stands (i.e. a total of 1200 flowers). In each year for each stand, fifty randomly chosen flowers (10 from each of five randomly chosen trees) were harvested on each of three days at three weeks intervals across the flowering season. The number of pollen grains per stigma was counted under a stereomicroscope (60x magnification) where it was possible to count them directly on the stigma (*in situ*). Pollen grains from the stigma of 50 flowers from each stand were captured on the tip of a needle and added to a drop of water prior to identification and photographed using an Olympus BHA 1.2 X dissection microscope at 120x magnification and a Nikon D300 camera.

3.3.6 *Production of floral shoots and fruit of* A. marina

I tested the effect of stand size on the production of floral shoots and fruit during the flowering and fruiting season of 2009 by counting all shoots and fruit on 50 trees within the two large stands and 17 and 19 trees within the two small stands. Trees were chosen because their canopies were distinct allowing thorough counts. However, because branches from other trees visually covered a part of the canopy in some cases, only a third of the canopy was counted and the result was multiplied by three. In each case the number of floral shoots was first counted in the beginning of February when all shoots had formed, and fruit were counted at the beginning of October when they become mature and before they abscise from the trees.

3.3.7 *Statistical analysis*

To assess the effect of stand size on the number of honeybees visiting the floral shoots of *A. marina* a three factor analysis was used, where factors were location (Sydney or Minnamurra; L - random), stand size (Large or Small; S - fixed) and time interval (6-8 am, 8-10 am, 10-12 am, 12-2 pm, 2-4 pm, 4-6 pm or 6-8 pm, T - fixed). To assess the effects of stand size on the foraging duration of individual floral shoots or trees, pollen deposition and fruit production a two-factor analysis was used (Table 3.3-3.7), where factors were location (Sydney or Minnamurra; L - random) and Stand size (Large or Small; S - fixed). Data were appropriately pooled and/or transformed with Sqrt(X+1) or Ln(X+1) to normalise data and reduce variance heterogeneity. All data used for ANOVA analyses were balanced and analysed by the statistical software WinGmav5. A two-tailed paired t-test was used to determine differences in the number of insect species (i.e. species richness) visiting large versus small stands.

3.4 Results

3.4.1 *The abundance of flower visiting honeybees*

The number of honeybees (*A. mellifera*) visiting *A. marina* flowers varied throughout the day in a similar manner within both large stands and small stand of Minnamurra and within the large stand of Sydney, with the abundance increasing steadily from six am to a peak at approximately noon (12-2 pm), followed by a steady decline until eight pm (Fig. 3.2*a*,*b*). In contrast there was no discernable peak of honeybee abundance within the small stand in Sydney where the plants of *A. marina* received fewer overall visits.

The greatest abundance of honeybees was observed within the large stand in Sydney at midday (12-2 pm), where the numbers were almost 20 orders of magnitude greater than in the small stand in Sydney (on average 78 honeybees in the large and 4 in the small stand during midday) (Fig. 3.2*a*). At Minnamurra the corresponding difference was 43% (on average 21 honeybees in the large and 12 in the small stand during midday) (Fig. 3.2*b*). Overall I detected an average of 49.5 ± 0.8 (mean ± SE) honeybees at the large and 8.0 ± 0.2 at the small stands (on average ca. 6x higher abundance in large stands). The effect of time intervals ($F=99.02$; $P<0.001$, $df_{6, 308}$), the interaction effect of location and stand size ($F=40.76$; $P<0.001$, $df_{1, 308}$), and the interaction effect of location, stand size and time interval ($F=5.31$; $P<0.001$, $df_{6, 308}$) were all significant (Fig. 3.2; Table 3.1).

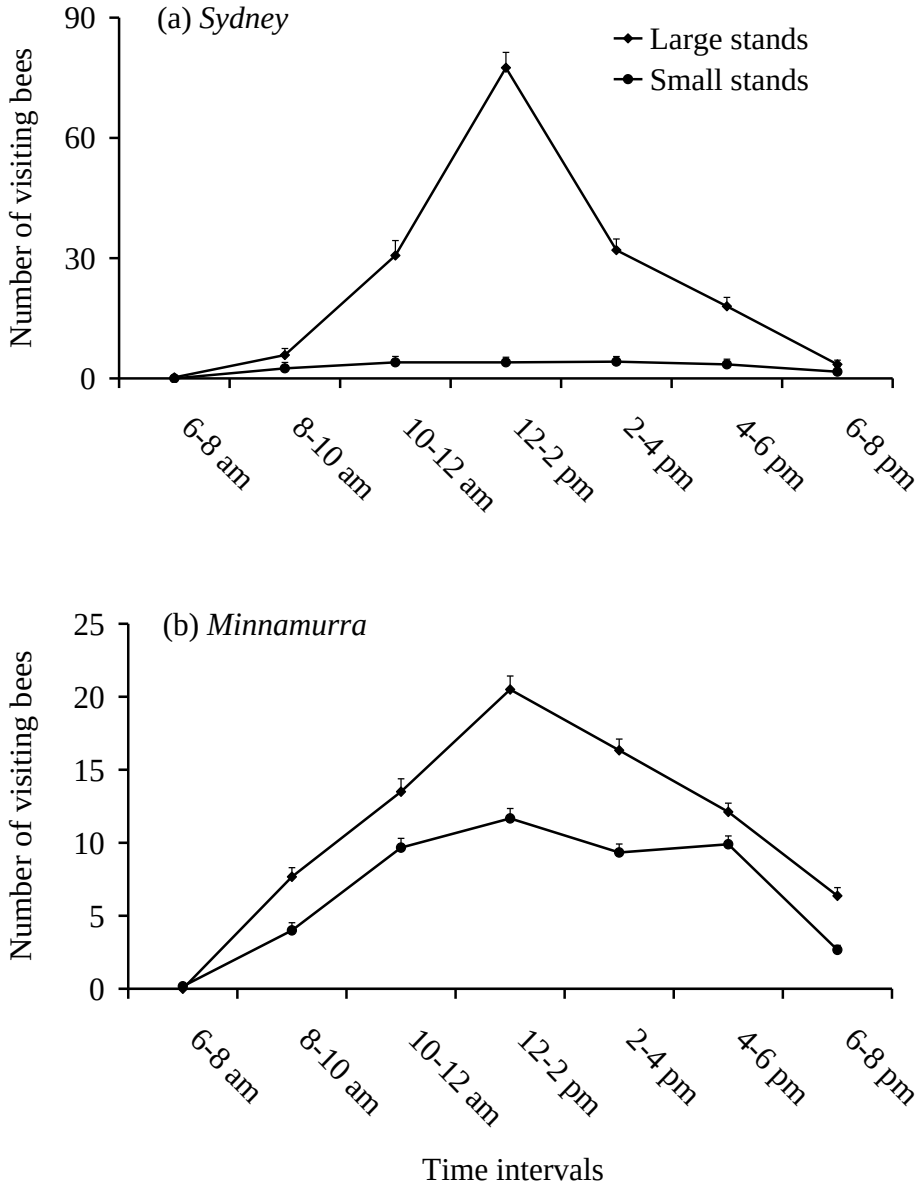


Fig. 3.2. Mean (± SE) number of honeybees visiting *A. marina* within large and small stands of *A. marina* in (a) Sydney and (b) Minnamurra. Visitation to eight flower clusters was measured by 12 counts of honeybees, during each of seven 2 h intervals spread across the daylight hours during the flowering season of 2009.

Table 3.1. Three factor ANOVA on the abundance of honeybees visiting 10 m^2 of *A. marina* canopy during seven independent 2 h sampling periods in each of the large and small stand in Sydney and Minnamurra. The seven sampling periods of each stand were conducted during seven time intervals, 6-8 am, 8-10 am, 10-12 am, 12-2 pm, 2-4 pm, 4-6 pm and 6-8 pm. During each sampling period 12 counts were done in 10 min intervals. Results are estimates of mean square (MS), F-ratio (F) and probability levels (*P*) of analysis of variance. Location (L) was treated as a random factor while stand size (S) and time interval (T) were fixed factors.

Source	d.f.	MS	F	*P*
L	1	0.42	1.47	0.226
S	1	41.64	3.59	0.309
T	6	27.73	99.02	0.000*
LxS	1	11.59	40.76	0.000*
LxT	6	0.28	0.99	0.435
SxT	6	3.21	2.13	0.190
LxSxT	6	1.51	5.31	0.000*
Res	308	0.29		

Transformation = Ln(X+1)
Cochran's test = NS
SNK = Sydney (Syd): Ls>Ss Minnamurra (Min): Ls>Ss
Large stand (Ls): Syd>Min Small stand (Ss): Syd>Min

3.4.2 *The duration of foraging and frequency of movements*

The tests of foraging behaviour performed to assess the potential of pollinators to increase the level of biparental inbreeding or selfing revealed a not significant variation in the duration of foraging on individual floral shoots between stand sizes (F=3.65; *P*=0.057, $df_{1, 797}$) and the interaction effect was not significant (F=0.13; *P*=0.722, $df_{1, 797}$), although the variation between locations was significant (F=70.60; *P*<0.001, $df_{1, 797}$) (Table 3.2). Nevertheless, the average duration of foraging on floral shoots was 15 ± 0.3 sec (mean ± SE) (Fig. 3.3*a*).

Table 3.2. Two factor ANOVA on the duration of foraging on floral shoots within trees from a large and a small stand from Sydney and Minnamurra respectively, from the flowering seasons of 2009. Results are estimates of mean square (MS), F-ratio (F) and probability levels (*P*) of analysis of variance. Location (L) is a random factor while stand size (S) is a fixed factor.

Source	d.f.	MS	F	*P*
L	1	21.11	70.60	0.000*
S	1	1.09	3.65	0.057
LxS	1	0.04	0.13	0.722
Res	796	0.30		
Pooled	797	0.30		

Transformation = Ln(X+1)
Cochran's test = NS
SNK for L: Georges>Parramatta

*To increase the power of the test, the estimate of MS used in the denominator of the F-ratio is a pooled estimate from the MS of the LxS interaction and the residual, and the effect LxS was tested with 1 and 797 d.f. (see Winer, *et al*. 1991 for pooling procedure)

Table 3.3. Two factor ANOVA on the duration of foraging on individual trees from a large and a small stand from Sydney and Minnamurra respectively, from the flowering seasons of 2009. Results are estimates of mean square (MS), F-ratio (F) and probability levels (*P*) of analysis of variance. Location (L) is a random factor while stand size (S) is a fixed factor.

Source	d.f.	MS	F	*P*
L	1	4.86	4.49	0.035*
S	1	0.02	2.05	0.388
LxS	1	0.01	0.01	0.931
Res	216	1.08		

Transformation = Ln(X+1)
Cochran's test = NS
SNK for L: Georges>Parramatta

Within trees the variation between stand sizes (F=2.05; P=0.388, $df_{1, 216}$) and the interaction effect (F=0.01; P=0.931, $df_{1, 797}$) were not significant, while the variation

between locations was significant (F=4.49; P=0.035, $df_{1, 797}$) (Table 3.3). The average duration of foraging on trees was 506 ± 21 sec. Foraging were on average 10% and 12% longer on floral shoots and trees respectively, in small stands as compared to large stands (Fig. 3.3b).

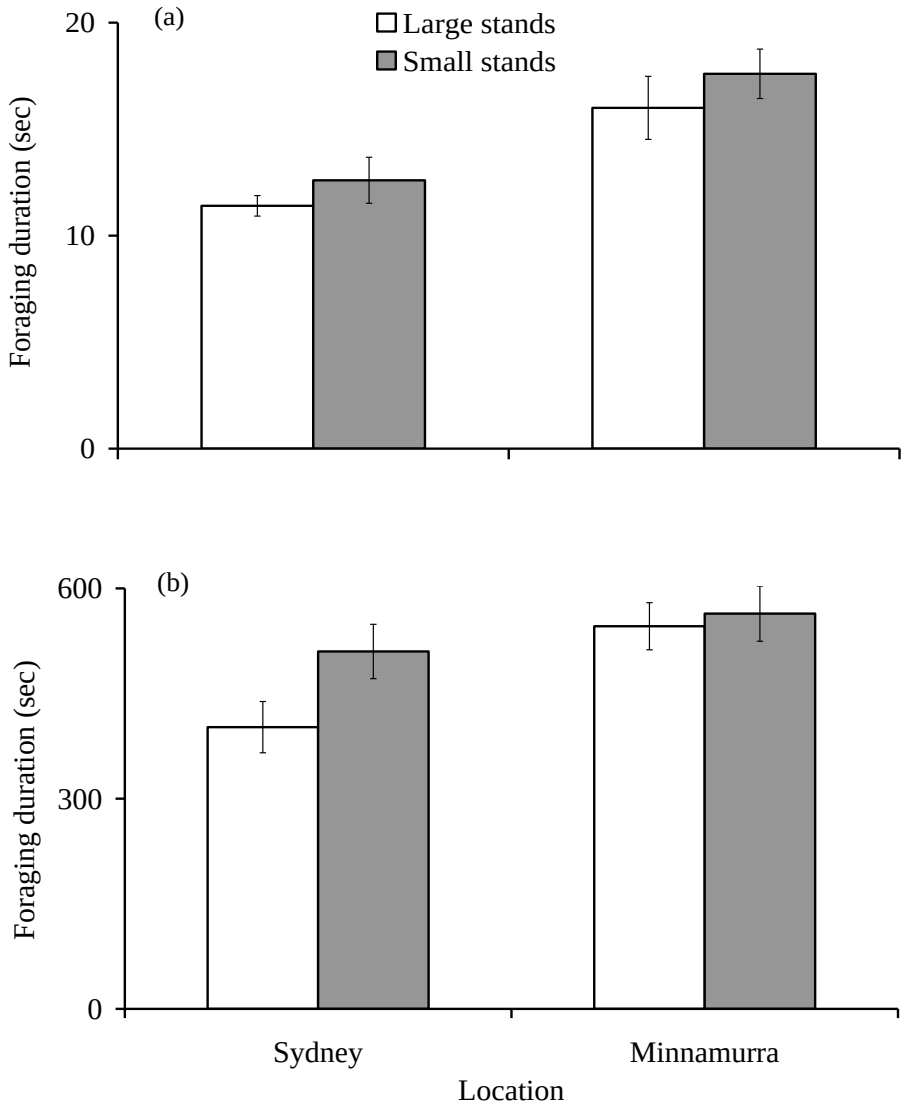


Fig. 3.3. Mean (± SE) duration of honeybee foraging within (a) floral shoots and (b) trees, from large and small stands at each of two locations (Sydney and Minnamurra) during the flowering season of 2009.

Among 88 honeybees foraging on individual trees I recorded almost identical mean numbers of movements between floral shoots with 30 ± 2 movements (mean ± SE) in the large and 33 ± 2 movements in the small stands, with a difference of 9% between large and small stands. The effect of stand size (F=0.19; P=0.736, $df_{1, 84}$), the effect of location (F=0.07; P=0.794, $df_{1, 84}$) and the interaction effect (F=3.91; P=0.051, $df_{1, 84}$) were all not significant (Table 3.4).

Table 3.4. Two factor ANOVA on the number of floral shoots visited during foraging bouts within trees of a large and a small stand from Sydney and Minnamurra respectively, from the flowering seasons of 2009. Results are estimates of mean square (MS), F-ratio (F) and probability levels (*P*) of analysis of variance. Location (L) is a random factor while stand size (S) is a fixed factor.

Source	d.f.	MS	F	*P*
L	1	16.41	0.07	0.794
S	1	180.41	0.19	0.736
LxS	1	929.50	3.91	0.051
Res	84	237.93		
Transformation = none				
Cochran's test = NS				

Table 3.5. Two factor ANOVA on the number of floral shoots visited during foraging bouts among trees of a large and a small stand from Sydney and Minnamurra respectively, from the flowering seasons of 2009. Results are estimates of mean square (MS), F-ratio (F) and probability levels (*P*) of analysis of variance. Location (L) is a random factor while stand size (S) is a fixed factor.

Source	d.f.	MS	F	*P*
L	1	100.63	0.17	0.677
S	1	245.26	0.31	0.677
LxS	1	796.26	1.37	0.243
Res	188	579.34		
Transformation = none				
Cochran's test = NS				

Among 192 honeybees foraging between floral shoots across trees next to each other, the number of movements was also almost identical with 52 ± 2 movements (mean ± SE) in the large and 54 ± 2 movements in the small stands with a difference of 4% between large and small stands. The effect of stand size (F=0.31; P=0.677, $df_{1, 188}$), the effect of location (F=0.17; P=0.677, $df_{1, 188}$) and the interaction effect (F=1.37; P=0.243, $df_{1, 188}$) were not significant (Table 3.5). However, at the end of these measured foraging events honeybees within small stands were significantly more likely to visit other neighbouring trees when continuing their foraging bout at a new place (92 of 140 observations in small stands) *cf.* (57 of 140 observations in large stands) (χ^2 = 5.8; df_1; P<0.016), while those that disappeared in the horizon either flew to more distant trees or home.

3.4.3 *Pollen deposition on stigma of* A. marina *flowers*

The average number of pollen grains (mean ± SE) deposited on the stigmas of *A. marina* flowers was greater in the large (11.8 ± 0.7 pollen grains and 9.9 ± 0.6 pollen grains) than in the small stands (2.9 ± 0.4 pollen grains and 5.5 ± 0.6 pollen grains) in Sydney and Minnamurra, respectively, giving a difference of 75% between large and small stands in Sydney and 44% in Minnamurra (Fig. 3.4). The effect of stand size was not significant (F=8.80; P=0.207, $df_{1, 1196}$) while the effect of location (F=9.60; P=0.002, $df_{1, 1196}$) and the interaction effect (F=31.91; P<0.001, $df_{1, 1196}$) were significant (Table 3.6). Within the large stands only 7% of 600 (300 from each stand) stigmas examined did not carry any pollen grains, as compared to an average of 19% of 600 stigmas in small stands.

Table 3.6. Two factor ANOVA on the deposition of pollen on floral stigmas of *A. marina* flowers from a large and a small stand of Sydney and Minnamurra respectively, from the flowering seasons of 2009 and 2010. Results are estimates of mean square (MS), F-ratio (F) and probability levels (*P*) of analysis of variance. Location (L) is a random factor while stand size (S) is a fixed factor.

Source	d.f.	MS	F	*P*
L	1	8.55	9.60	0.002*
S	1	249.87	8.80	0.207
LxS	1	28.41	31.91	0.000*
Res	1196	0.89		

Transformation = Ln(X+1)
Cochran's test = $P < 0.05$
SNK = Sydney (Syd): Ls>Ss Minnamurra (Min): Ls>Ss
Large stand (Ls): Syd~Min Small stand (Ss): Syd>Min

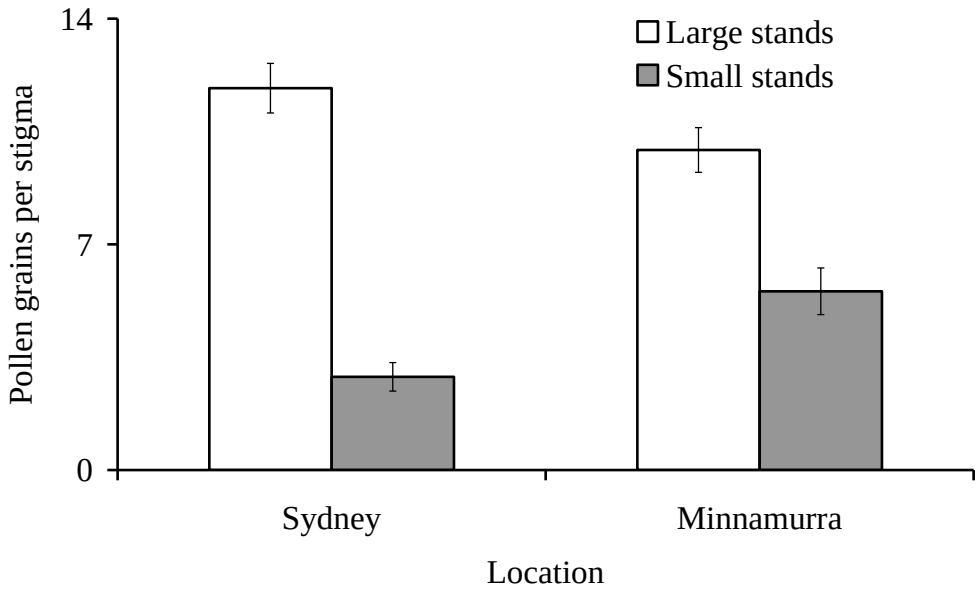


Fig. 3.4. Mean (± SE) number of deposited pollen grains on stigmas of *A. marina* flowers within large and small stands in Sydney and Minnamurra. A total of the 150 stigmas were harvested from each of the four stands during the flowering seasons of 2009 and 2010.

Nevertheless pollinator fidelity did not vary with stand size, with on average 47 of the sets of 50 flowers examined per stand displaying only *A. marina* pollen grains and on average only 6% of all pollen grains examined was from other species, which was significantly lower compared to the number of *A. marina* pollen grains ($\chi^2 = 19.1$; df_1; $P<0.001$).

3.4.4 *Production of floral shoots and fruit of* A. marina

Trees within small stands were on average strikingly less fecund than trees within large stands. The average number of fruit produced per floral shoot (Fig. 3.5*a*) was 0.19 ± 0.04 (mean ± SE) in the small stand and 0.65 ± 0.16 in the large stand of Sydney (in small stand 29% of that in large stand) and 0.42 ± 0.09 in the small stand and 0.78 ± 0.12 in the large stand of Minnamurra (in small stand 54% of that in large stand), and the variation between small and large stands was significant (F=10.69; P=0.002, $df_{1, 65}$) while the variation between locations (F=3.10; P=0.083, $df_{1, 65}$) and the interaction effect (F=0.41; P=0.526, $df_{1, 65}$) were not significant (Fig. 3.5*a*; Table 3.7). The average number of fruit produced per tree (Fig. 3.5*b*) was 162 ± 39 (mean ± SE) in the small stand and 734 ± 169 in the large stand of Sydney (in small stand 22% of that in large stand) and it was 416 ± 106 in the small stand and 741 ± 138 in the large stand of Minnamurra (in small stand 56% of that in large stand). However, neither the variation between small and large stands (F=6.97; P=0.230, $df_{1, 64}$), the variation between locations (F=0.69; P=0.410, $df_{1, 64}$) or the interaction effect (F=1.63; P=0.207, $df_{1, 64}$) were significant (Table 3.8).

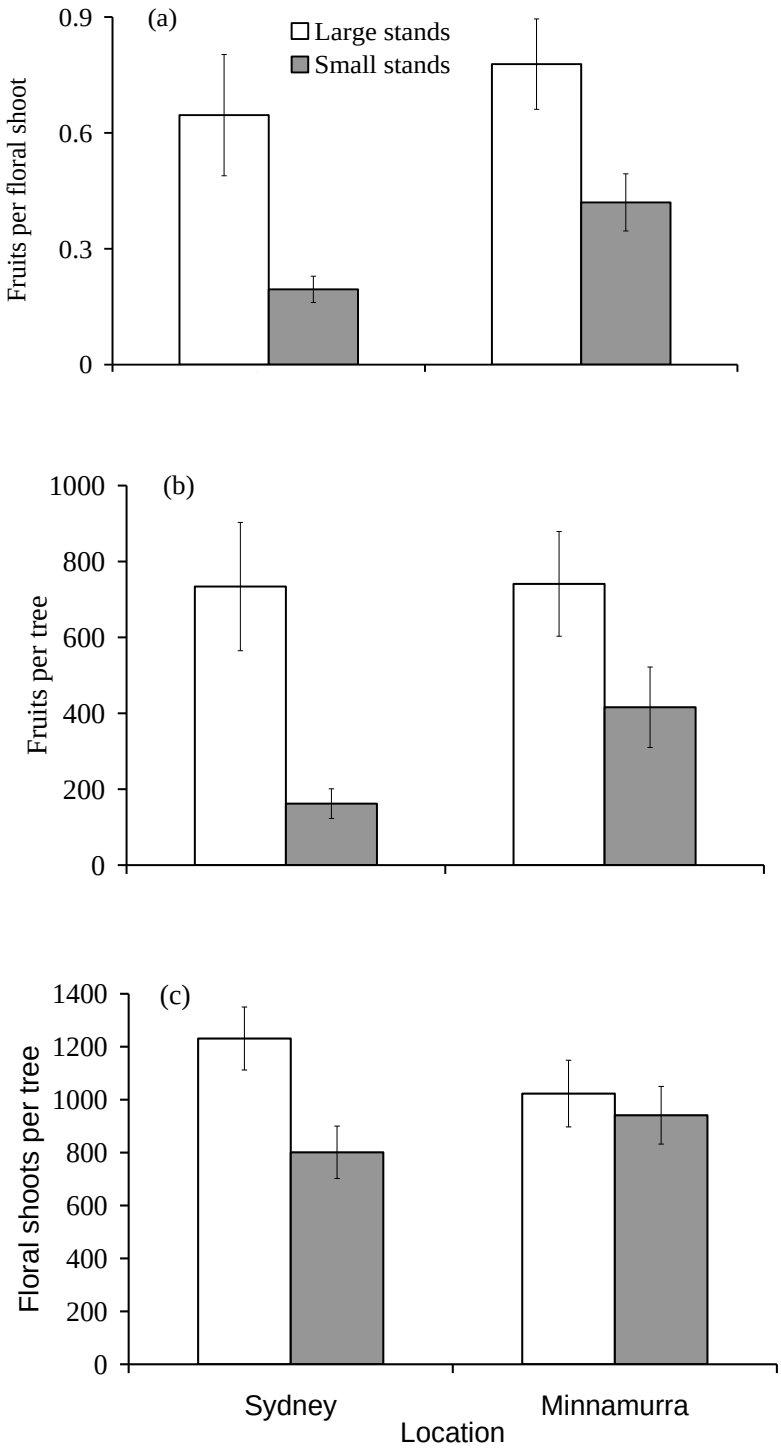


Fig. 3.5. Mean (± SE) number of (a) fruit produced per floral shoot, (b) fruit produced per tree, and (c) floral shoots produced per tree by *A. marina* from 50 trees of each of the two large stands and 17 and 19 trees from the small stands in Sydney and Minnamurra, respectively.

Table 3.7. Two factor ANOVA on the fruit production per floral shoot of each of two large and two small stands in Sydney and Minnamurra respectively. Results are estimates of mean square (MS), F-ratio (F) and probability levels (*P*) of analysis of variance. Location is a random factor while visitors and time interval are fixed factors. Location (L) is a random factor while stand size (S) is a fixed factor.

Source	d.f.	MS	F	*P*
L	1	0.22	3.10	0.083
S	1	0.74	10. 69	0.002*
LxS	1	0.03	0.41	0.526
Res	64	0.07		
Pooled	65	0.07		

Transformation = Ln(X+1)
Cochran's test = NS
SNK for S: Large>Small

*To increase the power of the test, the estimate of MS used in the denominator of the F-ratio is a pooled estimate from the MS of the LxS interaction and the residual, and then the effect LxS was tested with 1 and 65 d.f. (see Winer et al. 1991 for pooling procedure)

Table 3.8. Two factor ANOVA on the fruit production per tree of each of two large and two small stands in Sydney and Minnamurra respectively. Results are estimates of mean square (MS), F-ratio (F) and probability levels (*P*) of analysis of variance. Location is a random factor while visitors and time interval are fixed factors. Location (L) is a random factor while stand size (S) is a fixed factor.

Source	d.f.	MS	F	*P*
L	1	180044.13	0.69	0.410
S	1	2976581.31	6.97	0.230
LxS	1	426761.31	1.63	0.207
Res	64	262017.45		

Transformation = None
Cochran's Test = NS

Table 3.9. Two factor ANOVA on the production of floral shoots per tree of each of two large and two small stands in Sydney and Minnamurra respectively. Results are estimates of mean square (MS), F-ratio (F) and probability levels (*P*) of analysis of variance. Location is a random factor while visitors and time interval are fixed factors. Location (L) is a random factor while stand size (S) is a fixed factor.

Source	d.f.	MS	F	*P*
L	1	1720.06	0.01	0.938
S	1	897460.94	2.28	0.372
LxS	1	393680.53	1.45	0.233
Res	64	270979.42		
Transformation = None				
Cochran's Test = NS				

The average number of floral shoots produced per tree (Fig. 3.5*c*) was 801 ± 99 (mean ± SE) in the small stand and 1231 ± 119 in the large stand of Sydney (in small stand 65% of that in large stand) and 941 ± 109 inthe small stand and 1023 ± 126 in the large stand of Minnamurra (in small stand 92% of that in large stand), and the variation between small and large stands (F=2.28; *P*=0.372, $df_{1, 64}$), between locations (F=0.01; *P*=0.938, $df_{1, 64}$) and the interaction effect (F=1.45; *P*=0.233, $df_{1, 64}$) were not significant (Table 3.9).

3.5 Discussion

I predicted that the diversity of flower visiting species, and the abundance and services (i.e. visitation and pollen deposition) of the exotic honeybee *Apis mellifera* would be significantly reduced in small compared to large stands. As far as I know such fragmentation effects have never been investigated in mangroves and, therefore, comparisons of my results can only be done with investigations of fragmented terrestrial plant populations. Indeed, my predictions are in accordance with data from terrestrial forests where a frequent effect of fragmentation is reduced pollinator diversity, abundances and services and changed foraging behaviour (Jennersten 1988; Aizen & Feinsinger 1994a Aguirre & Dirzo 2008). Moreover, I also expected that altered pollinator services would increase levels of inbreeding within small stands and reduce reproductive success, as it has been shown to be the case in many terrestrial forest plants (Murcia 1996; Aizen 1998; Aguilar *et al.* 2006), and overall my study revealed that, as for terrestrial forest plants (Ghazoul 2005; Aguilar *et al.* 2006), small stands receive fewer honeybees and poorer pollinator services, and display reduced reproductive success when compared to large stands. My observations of pollinator activity suggest that this reflects a combination of significantly reduced pollen deposition and changed foraging behaviour by *A. mellifera* within small stands, which is congruent with results from terrestrial forest plants (Bierzychudek 1981; Burd 1994). I found that *A. marina* attracted a diverse set of flower visitors (as predicted by Tomlinson 1986 for mangroves in general), but surprisingly, the exotic honeybee *A. mellifera* was the only effective pollinator (Chapter 2). These results emphasize the importance of *A. mellifera* for the reproduction of temperate *A. marina*. Similarly, exotic *A. mellifera* is also important for the reproduction of many terrestrial species in

Australia (e.g. Paton 1993, 1996; Whelan *et al.* 2009; Lomov *et al.* 2010; Gross *et al.* 2010) and in other continents (e.g. Krend & Murphy 2003; Neves & Viana 2011; Dupont *et al.* 2004; Taha & Bayoumi 2009, Cayuela *et al.* 2011).

3.5.1 *Reduced pollinator abundance and pollen deposition*

My results show that the abundance of honeybees is significantly reduced in small as compared to large stands, and small stands display correspondingly lower levels of pollen. This matches observations for many terrestrial plants, which show evidence of reduced pollinator abundance and pollen limitation in small stands (Bradshaw & Marquet 2003; Ward & Johnson 2005; Aguilar 2006). In the present study, the deposition of pollen was much greater in large than small stands suggesting that trees within small stands are more likely to be pollen limited which is also the case in terrestrial forest plants (Bierzychudek 1981; Burd 1994). On average 9-11 pollen grains were deposited on stigma of flowers taken from the two large stands, which is similar to values reported by data from stands of temperate *A. marina* in the area of Sydney by Clarke and Myerscough (1991) who reported an average of 9 pollen grains per stigma, while on average 3-5 pollen grains were deposited on stigma of flowers from the two small stands. Moreover, within small stands a much higher proportion of stigmas displayed no pollen. As far I know these two studies are the only studies reporting data on pollen deposition in mangroves. These results indicate a greater risk for pollen limitation within the small stands and the deposition of pollen followed the same pattern as the abundance of honeybees and the fruit production. This is in congruence with results from terrestrial plant populations (Ghazoul 2005; Ward & Johnson 2005), suggesting that fruit set is highly dependent of services from the exotic honeybee *A.*

mellifera and that these factors suffers from significant impact of stand size on the small stands.

3.5.2 *Altered foraging behaviour may lead to increased inbreeding*

The present study suggests that within both large and small stands honeybees disperse *A. marina* pollen grains among the flowers from floral shoots of individual trees or across neighbouring trees, although a range of measures suggest that their foraging within small stands may produce higher levels of self pollen transfer than within large stands (i.e. the duration of foraging within floral shoots and trees were approximately 10% and 12% higher in small stands than large stands). Such effects may increase the level of inbreeding (Spigler *et al.* 2009; Borrell 2012) as I also found in small stands using genetic data (chapter 5). In the present study movements between floral shoots within trees were increased (but not significantly) by 9% while movements across trees were increased (also not significantly) by 4% in small stands while two third of the individual foraging events were done across near neighbouring trees (where the canopies were overlapping between two trees). These results suggest a high level of biparental inbreeding in stands independent of size, which also is in accordance with my genetic survey of progeny arrays (chapter 5). Finally, when honeybees ended foraging and left a tree, on average 41% of these bees in the large and 66% in the small stands (a difference of 38%) flew to a nearby site of the same stand and started foraging again. In this case the difference between large and small stands was statistically significant although the effect this would have on mating patterns or fitness is unclear. The outcome must be dependent on whether honeybees that end a foraging event and then leave the area visit another stand or return to the hive, which gives three possible

outcomes; First, if honeybees in general leave a tree of a certain small stand and fly to another tree of the same small stand, then this would result in higher levels of inbreeding in small stands. Second, if honeybees in general arrive from another stand to a certain small stand, then they would deliver outcross pollen to this particular small stand and potentially increase the level of outcrossing in small stands. Third, if honeybees in general end a foraging bout and go home to the hive, then it would result in a higher level of outcrossing in small stands. Because honeybees when they arrived to a tree often had large pollen stores on their corbicula (see chapter 2), it is likely that they may have visited trees located at different sites during a foraging bout, and thus the first possible outcome is supported by my genetic survey on progeny arrays of temperate *A. marina* (chapter 5) where the multilocus outcrossing rates (t_m) were significantly reduced in small stands, suggesting a significant effect of stand size on inbreeding. This explanation is also congruent with theory and results from terrestrial forest plants where a frequent effect of fragmentation (including the effect of stand size and isolation by distance) is reduced genetic diversity and higher levels of inbreeding in small rather than large stands (Ghazoul 2005; Aguilar 2006; Collinge 2009). However, these results, together with the significant lower pollinator abundance and pollen deposition also found in small stands of this study, may explain the great difference in reproductive output displayed by adult plants and reflect the effect of stand size on small stands. This is similar to results from terrestrial plants where such effect may reduce the number and quality of offspring as a consequence of increased inbreeding (Colling *et al.* 2003; Murcia 1996; Aizen 1998).

3.5.3 *Reduced fruit set is promoted by reduced pollinator density and pollen deposition*

In my study production of fruit was expected to be significantly higher in large stands. My data shows that this was the case for production of fruit per floral shoot, while production of fruit per tree was only considerably higher. Overall these findings parallel the outcome of many terrestrial studies (Murcia 1996; Aizen 1998; Cunningham 2000) and suggest that reduced reproductive output may be driven by inferior pollinator services (Rathcke & Jules 1993; Murcia 1996; Kearns & Inouye 1997). I am mindful, however, that small stands may also differ in other respects including greater edge to area ratios, habitat quality and other factors that may impact directly on reproductive output such as the diversity of available mates and the density of floral shoots (Kwak *et al.* 1998; Bradshaw & Marquet 2003; Collinge 2009). In the present study the number of floral shoots per tree was not significantly lower in small stands although small stands did more poorly both in terms of total fruit production per tree and in terms of % fruit set measured within floral shoots, which is comparable to terrestrial data (e.g. Kwak *et al.* 1998). Taken together with the other results of this study, this at least indicates a distinct effect of stand size on the fruit production, which is promoted by significantly reduced abundance of pollinators (exotic honeybee *A. mellifera*) and pollen deposition. This is in accordance with the theory from terrestrial forests (Aizen *et al.* 2002; Bradshaw & Marquet 2003; Ghazoul 2005; Aguilar *et al.* 2006; Collinge 2009).

3.5.4 *Conclusion*

The present study revealed that the exotic honeybee *A. mellifera* (the only important pollinator of temperate *A. marina*) was less common and had altered foraging behaviour within small stands (as compared to large stands) of temperate *A. marina*. Within small

stands honeybees increased the number of within-tree movements and the duration of foraging within floral shoots and trees, leading to an increase in the deposition of self-pollen (pollen from the same plant) within the small stands. As temperate *A. marina* is self-compatible (geitonogamous) this may lead to increased inbreeding (or biparental inbreeding) within the small stands. Together with a significant reduction in pollen deposition this may affect reproductive output negatively within the small stands. In the present study the fruit set was considerable reduced in the small stands, displaying a similar pattern as for the abundance of honeybees and the pollen deposition.

Chapter 4: Reduced pollinator density leads to reduced fruit set and quality in small stands of temperate *Avicennia marina*

4.1 Abstract

Within Sydney estuaries populations of the self-compatible mangrove *Avicennia marina* are highly fragmented and stand size varies widely. *A. marina* in this region attracts a variety of potential pollinators but is only pollinated by the exotic honeybee *Apis mellifera*. The foraging behaviour of *A. mellifera* within and among mangrove stands is unknown but I predicted that, as for terrestrial plants, honeybee abundance and foraging behaviour would vary with stand size. Moreover I predicted that these altered pollinator services in small stands would result in reduced outcrossing and increased selfing through autogamy or geitonogamy, with consequent reduction of fruit production, fruit quality and seedling density. My investigations within three large, three medium and three small stands in each of two estuaries (Parramatta and Georges River) matched my expectations. I found that *A. marina* was partially autogamous. I also found that pollinator density of medium stands on Parramatta River was on average 57% and on Georges River was 55% of that in large stands, and in small stands it was 29% of that in large stands on both estuaries. Fruit production (measured as fruit per tree) was also affected by stand size. In medium stands it was on average 68% and 73% and in small stands it was 33% and 29% of that in large stands of Parramatta and Georges River, respectively. Further, I found that the weight of propagules in medium stands of Parramatta River was on average 84% and of Georges River was 80% of that in large stands while in small stands it was 80% and 82% of that in large stands. After fall the density of propagules (immediatly after abscission) in medium stands was on average

62% and 55% of that in large stands and in small stands it was 37% and 25% of that in large stands of Parramatta and Georges River, respectively. Further, in medium stands of Parramatta River the density of newly established seedlings was on average 72% and on Georges River it was 61% and of that in large stands, while it was 33% of that in large stands in the small stands of both estuaries. Finally, the density of seedlings surviving to three months in medium stands was on average 65% and 73% of that in large stands and in small stands was 39% and 40% of that in large stands of Parramatta and Georges River, respectively. These results suggest that the density of pollinators and the quality and production of fruit and seedlings display a similar pattern with values reduced considerable from large to medium and from medium to small stands (although fruit weight of medium and small stands was significantly 18% and 19% lower than the fruit weight of large stands), suggesting a considerable effect of stand size on small stands of the investigated estuaries.

4.2 Introduction

In addition to having a naturally fragmented distribution (Tomlinson 1986; Duke 2006; West *et al*. 1985), mangroves in many regions, in common with many terrestrial plant species, experience anthropogenic fragmentation (Chafer 1998; Obade *et al*. 2004; reviewed by Rogers 2004), resulting in stands ranging from thousands of trees to isolated individuals (West *et al*. 1985). Mangrove populations may therefore experience severe impacts of reduced stand size on their reproductive biology similar to those shown by terrestrial plant species (Aizen *et al*. 2002; Ghazoul 2005; Aguilar et al. 2006; Collinge 2009). These impacts may include reduced pollinator diversity and abundance (Ghazoul 2005) and alteration of the foraging behaviour of pollinators (Steffan-Dewenter & Tscharntke 1999) in small stands, leading to fewer flower visits and limited supply of pollen (Aizen and Feinsinger 1994*a*; Sih & Baltus 1987; Cascante *et al*. 2002), which may lead to pollen limitation in small stands (e.g. Becker *et al*. 2011). Consequently levels of inbreeding may be elevated, resulting in reduced fecundity (Bierzychudek 1981; Burd 1994; Larson & Barrett 2000; Ghazoul 2005) and reduced fruit quality and production (Jennersten 1988; Aizen & Feinsinger 1994b; Ghazoul & McLeish 2001; Ghazoul 2005). Small stand size may therefore be associated with the production of fewer and less fit seedlings (e.g. Barbeta *et al*. 2011).

For plants within fragmented terrestrial habitats Oakley *et al*. (2007) argued that self-incompatibility provides a large advantage to plants in small stands by reducing levels of inbreeding. However, many terrestrial plants use self-compatibility as a strategy to guard against the effects of pollen limitation (Aizen *et al*. 2002). A preliminary study by Clarke & Myerscough (1991) predicted that temperate *A. marina* of the Sydney region is geitonogamous (self-compatible but needs pollinators for

pollination) and experimentally found that it is only partially autogamous (can pollinate itself without pollinators), while my investigations using genetic analyses of progeny arrays (chapter 5) show that temperate *A. marina* is self-compatible. However, the effects of stand size may vary greatly depending on the plant's level of self-compatibility (because self-compatibility favours inbreeding while self-incompatibility favours outcrossing) and the extent to which populations are pollen or seed limited. Nevertheless, due to limited change in foraging behaviour by pollinators in small as compared to large stands in an earlier study (chapter 3), autogamous fertilization may not vary with rates of visitation between stands of varying sizes.

The exotic honeybee *Apis mellifera* was the only effective pollinator of *A. marina* in the Sydney region (chapter 2). Typically *A. mellifera* is present in urban and bushland areas of terrestrial populations on the landward margins of my study sites where it is a dominant pollinator (Lomov *et al*. 2010). Yet, little is known about the habits of *A. mellifera* when it crosses the waters of estuaries (no matter the distance) on foraging bouts, which could either increase or reduce the effects of stand size on the abundance of honeybees. However, *A. mellifera* is known to be a generalist pollinator (Paton 1996) albeit with a high level of faithfulness to the plant species it is currently foraging on (e.g. Free & Williams 1972). *A. mellifera* is known generally to forage in a manner allowing transport of pollen between flowers on a single tree or a set of trees located within close proximity (Paton 1993; Whelan *et al*. 2009), which may potentially produce high levels of self-pollination or biparental inbreeding in self-compatible stands, and this effect may be exacerbated in small stands because pollinators change their foraging behaviour in these as compared to large stands (chapter 3), which may provide more self pollen transfer.

Because temperate *A. marina* is self-compatible (Clarke & Myerscough 1991) and pollinator abundance and pollen deposition is significantly reduced in small stands (chapter 3), I expect a profound effect of stand size on the production and quality of fruit and seedlings. Therefore I specifically asked: 1) Is temperate *A. marina* autogamous? 2) Is the density of *A. mellifera* lower in small compared to medium and large stands? 3) Does reduced stand size alter pollinator behaviour? 4) Does propagule production and quality vary with stand size? 5) Does recruitment of propagules and seedlings vary with stand size?

4.3 Materials and methods

4.3.1 *Study sites*

This study was performed within two estuaries in Sydney, the Parramatta River and Georges River estuaries, separated by 20 km over land. These estuaries contain a large number of mangrove stands of various sizes that occur within a matrix that includes stretches of water and terrestrial habitat with urban gardens and small patches of terrestrial forest both containing species that flower simultaneously with *A. marina*.

To perform this study nine stands of adult *A. marina* trees (three of >1500 trees, three of 300-500 trees and three of < 50 trees) were selected within each estuary (Fig. 4.1). Most stands were at least twice as long as broad, however, the small and intermediate stand of Oyster Bay of Georges River (see Fig. 4.1) were also elongated, but more than half as broad as long. Along the Parramatta River the mangrove vegetation only consists of *A. marina* plants. Along the Georges River however, the vegetation in large and medium stands is dominated by *A. marina* but flanked by the smaller mangrove *Aegiceras corniculatum*, in some cases on the landward and in others on the seaward sides

(Pickthall *et al*. 2004). *A. corniculatum* is absent from small stands in both estuaries with a single exception. The small stand of Oyster Bay on the Georges River includes two trees of *A. corniculatum*.

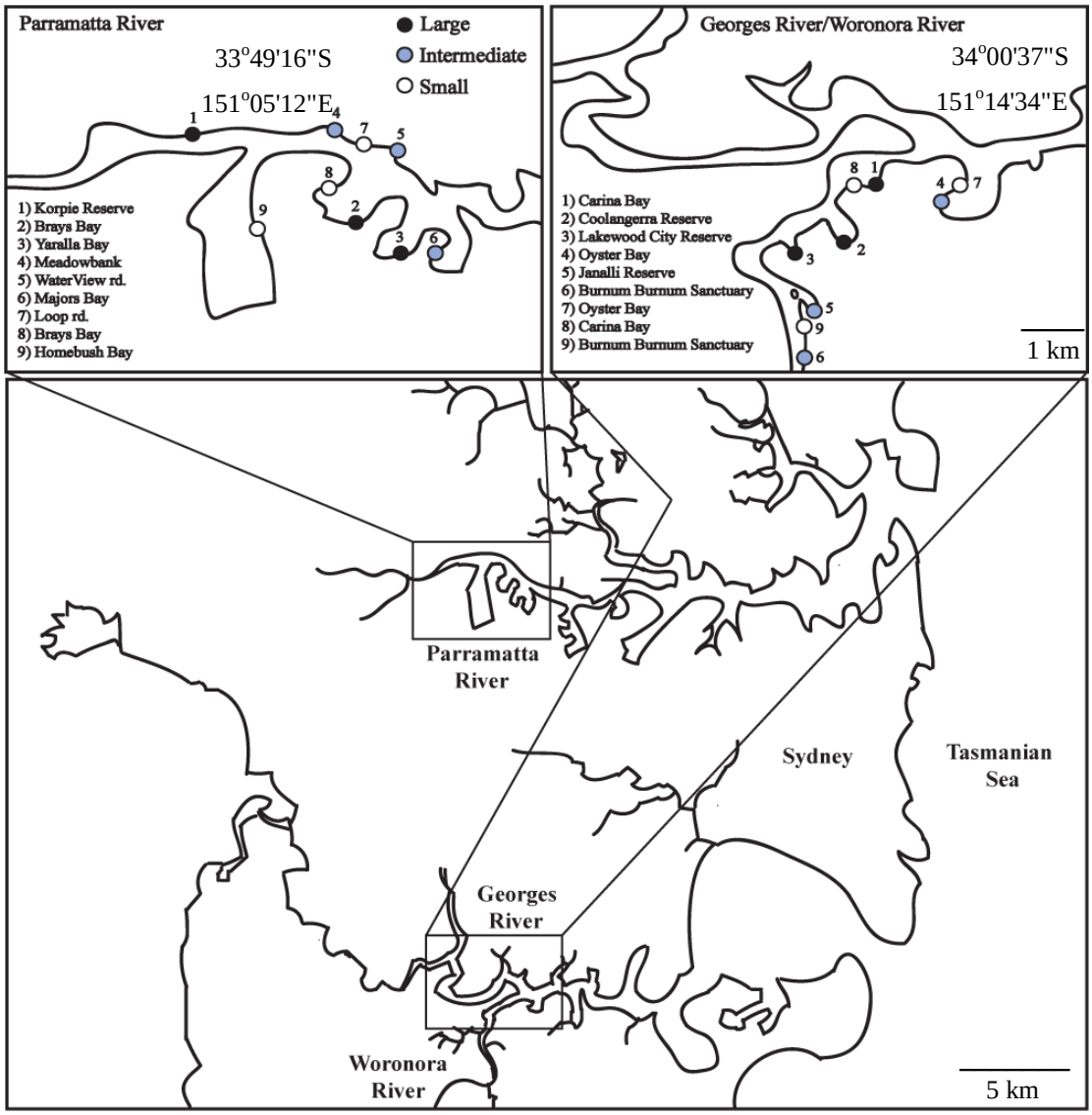


Fig. 4.1. Map of the Parramatta and Georges River catchments in Sydney, showing the location and indicating the size of the investigated stands.

Fieldwork was carried out along the landward edge of the stands, as this had the most accessible canopies. However, within the small stands investigations were also carried

out along the sides, which were bordered by mudflat. This was done to achieve a stretch with suitable canopy within individual small stands comparable to those of the large and medium stands. Selected trees were 5-10 m high. The flowering season of *A. marina* extends from mid January 2010 to mid-March, and seedling counts were made from mid-January to end of February 2011. Investigations of pollinator activity were done in sunny weather from mid-January to mid-March 2010 in temperatures ranging between 19.3^{o}C and 28.7^{o}C.

4.3.2 *The mangrove* A. marina

A. marina is a hermaphroditic mangrove species with yellow androgynous flowers each carrying four anthers and four ovules (Tomlinson 1986; Clarke & Myerscough 1991), and it is believed to be dependent on animals for pollination (Tomlinson 1986). Flowers are developed from flower clusters extending from floral shoots (Clarke & Myerscough 1991). Flowers are ca. 5 mm high and broad, each with the mature stigma 1.5-2.0 mm in high and four anthers anchored on the petals approximately at the height where the stigma ends (Clarke & Myerscough 1991). In the Sydney region *A. marina* flowers from mid January to mid March and mature fruit (propagules) develop during several months and drop from the trees from October to December (Duke 1990; Clarke & Myerscough 1991), after which they develop into seedlings. *A. marina* produces cryptoviviparous propagules covered by a buoyant pericarp, which may disperse via flotation (Saenger 2003).

4.3.3 *Tests for autogamy in* A. marina

I tested the ability of *A. marina* plants in large and small stands to reproduce via autogamy during the flowering and fruiting season of 2010 and 2011. Ten trees were selected from each stand (if available). However, in most small stands the number of trees available was lower (either because not enough trees carried floral shoots, or because the number of floral shoots available for bagging on a particular tree was insufficient). During 2010 only six trees were selected from one of the small stands of Parramatta River, and six and three trees were selected from two of the small stands of Georges River. During 2011 five trees were selected from the same small stand of Parramatta River and four and three trees were selected from the same two small stands of Georges River. For these investigations three treatments were used: 1) three floral shoots were randomly selected and bagged on each tree (this was done on a total of 258 plants to test if fruit were produced when pollinators were excluded), 2) open pollination where three floral shoots were randomly selected and marked on each three (this was done on in total 258 plants as a control to test for fruit production from open flowers), and 3) a partially open bag was supplied to a randomly chosen floral shoot on each three (this was done on a total of 86 plants as a control to check for bag effects on fruit set and development when a floral shoot was bagged but still open for pollination).

4.3.4 *Density of honeybees*

To investigate the effect of stand size on the density of honeybees I used video cameras to record the flower visitation to 1 m^2 of canopy (recorded one to three meter above the ground) on six trees from each of nine mangrove stands (see *Study sites* above) from each of two estuaries (the Parramatta and Georges Rivers). Each tree was recorded for

30 min on different days during the flowering season of 2010. Videos were recorded between 11 am and 3 pm where the abundance of honeybees were significantly higher than at other times of the day (chapter 3). For each video recording the number of honeybees visiting the targeted quadrat of canopy during individual recordings was counted. Every time a honeybee entered the recorded area it was counted as a new arrival (so it is possible that individual honeybees were counted more than once). Surveyed trees were selected randomly from trees bearing floral shoots.

4.3.5 *Duration of foraging on floral shoots by honeybees*

I used the video sampling design described above (see *Density of honeybees*) to test the effect of stand size on the average duration of foraging on floral shoots by 30 honeybee from each of six individual stands of each of three sizes (large, medium and small) during the 2010 flowering season. During each video recording the duration of five individual visits to each of five randomly selected floral shoots was measured by stopwatch (if a particular floral shoot was visited less than five times during the 30 min of recording, additional measurements were made on the other four floral shoots from that particular video recording). The average duration of the foraging on five individual floral shoots from each of the six video clips was calculated for each individual stand. The duration of a visit to a floral shoot was measured from the time a honeybee arrived on a particular floral shoot to the time of its departure.

4.3.6 *Production of floral shoots and propagules*

I tested the hypothesis that the number of floral shoots per tree was inversely related to stand size. I gathered these data during the season of 2010 and used the same sampling

design as described above (see *Density of honeybees*) although in these surveys the number of trees, again selected at random from those with floral shoots within each stand was 20 (if available). Again, in most small stands less than 20 trees carried floral shoots. Eight, 17 and 14 trees were selected from small stands of Parramatta River and 10, 20 and nine trees were selected from small stands of Georges River. Within each stand I counted the number of floral shoots present at the beginning of February where all floral shoots had developed flowers. The number of mature propagules was counted on the same trees in the beginning of October immediately before fall of the first propagules. Because branches from other trees visually covered a part of the canopy in some cases, only the half of the canopy was counted and the result was multiplied with two.

4.3.7 *The quality of propagules*

To test the effect of stand size on the weight (the weight of a propagule is an indication of the size of the cotyledons, which contain the nutrients for growth of the new developed siblings: e.g. Tomlinson 1986) of propagules during November and December 2010 I used the same sampling design as described above (see *Production of floral shoots and propagules*). From each stand 25 propagules (with pericarp) were randomly collected immediately after they fell from the trees. All collected propagules were weighed on a precision scale (to three decimal places) and the average weight was measured for each stand.

4.3.8 *Density of fallen propagules and the resultant seedlings*

To determine the effect of stand size on the density of fallen propagules on the forest floor within each individual stand and the resultant successfully settled seedlings, I determined both the number of propagules per m^2 immediately after the period where fall of propagules peaked (and the density of propagules on the forest floor was highest), the number of newly developed seedlings per m^2, and the number of seedlings per m^2 surviving three months after the last census. These surveys were performed during the season of 2010, and propagules were counted at the end of November, the number of new settled seedlings at the end of December, and the number of surviving seedlings at the end of March. For each of the three surveys fifty 1 m^2 quadrats were haphazardly placed in each individual stand.

4.3.9 *Statistical analyses*

Two factor ANOVAs were performed to test for significant effects of stand size on described variables. Factors were Location (Lo = random factor) and Stand size (Ss = fixed factor). When necessary data were transformed to Sqrt(X+1) or Ln(X+1). All data sets used for analyses were balanced and analysed by the statistical software WinGmav5.

4.4 Results

4.4.1 *Tests for autogamy*

There was evidence of partial autogamy in temperate *A. marina*. During 2010 five fruit (three from large and two from small stands) were produced by autogamy from five different trees. These fruit only reached a length of 8-12 mm after which they died before abscission. During 2011 six fruit were produced by autogamy from five different trees (two from large and four from small stands) reaching a length of 9-14 mm after which they died. In total this gave five autogamous fruit produced by trees of large stands and six produced by trees of small stands. Of the control treatments partially open bags and unbagged floral shoots produced from zero to eight propagules per floral shoot. On average 3 ± 0.34 and 1 ± 0.21 (mean ± SE) propagules was produced from partially bagged floral shoots of large and small stands respectively. On average 3 ± 0.46 and 2 ± 0.29 propagules (of 561 propagules of large stands and 370 of small stands) were produced from unbagged floral shoots of large and small stands respectively, suggesting that the investigated trees are fecund.

4.4.2 *Density of honeybees*

I found that within the videotaped quadrats of canopy the density of honeybees was far greater on Parramatta River than Georges River. Between the two estuaries the difference were almost double for all stand sizes. The effect of location was significant ($F=10.27$; $P=0.002$; $df_{1, 102}$) (Fig. 4.2; Table 4.1). I also found that small stands had the lowest densities of honeybees while large stands had the highest density of honeybees in both estuaries. Also the effect of stand size on the density of honeybees was significant ($F=57.15$; $P=0.017$; $df_{2, 102}$) but the interaction effect was not significant ($F=0.19$;

P=0.825; $df_{2, 102}$) (Fig. 4.2; Table 4.1). On Parramatta River the average number of honeybees per m^2 in large stands was 28 ± 4 (mean ± SE), in medium stands was 16 ± 2 (which was 57% of that in large stands) and in small stands was 8 ± 1 (which was 29% of that in large stands). On Georges River the average number of honeybees per m^2 in large stands was 13 ± 2, in medium stands was 7 ± 1 (which was 55% of that in large stands) and in small stands was 4 ± 0.6 (which was 29% of that in large stands) (Fig. 4.2).

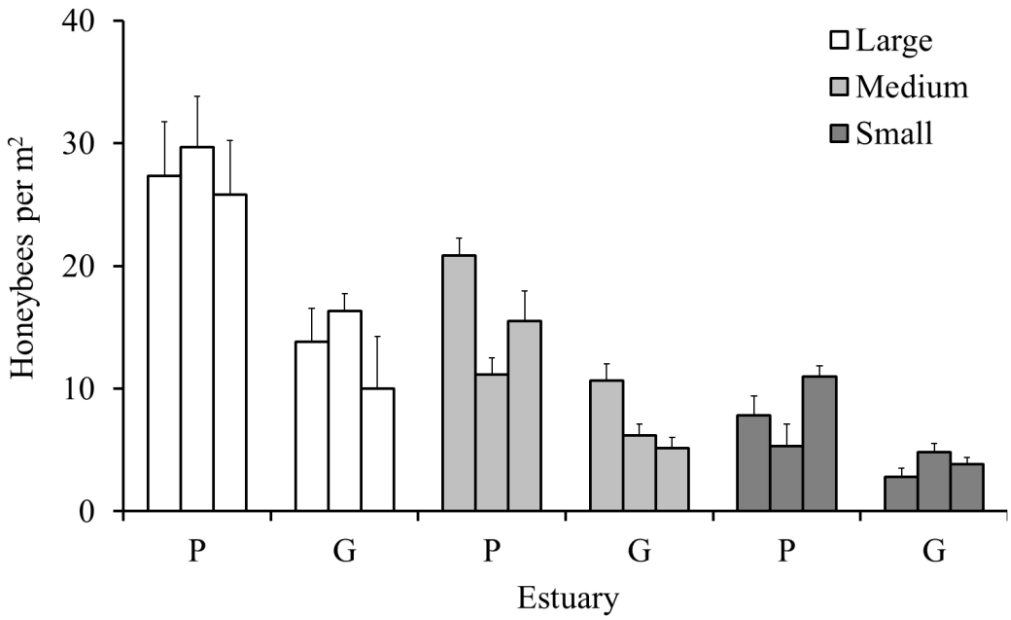


Fig. 4.2. Mean (± SE) density of honeybees, measured as the number of honeybees visiting 1 m^2 of canopy during six 30 min video recordings (each representing an individual tree), from each of nine stands of three different sizes from each of two estuaries (the Parramatta (P) and Georges (G) River's) during the season of 2010.

Table 4.1. ANOVA of the density of honeybees per m^2 of canopy counted on six half hour video recordings from three large three medium and three small stands from each of Parramatta River and Georges River, during the flowering season of 2010. Results are estimates of mean square (MS), F-ratio (F) and probability levels (*P*) of analysis of variance. Location (L) was treated as a random factor while Stand size (S) was treated as a fixed factor.

Source	DF	MS	F	*P*
L	1	8.864	10.27	0.002*
S	2	9.507	57.15	0.017*
LxS	2	0.166	0.19	0.825
Res	102	0.863		

Transformation = Ln(X+1)
Cochran's test = NS
SNK for L: Parramatta (P)>Georges (G)
for S: Large>medium>Small

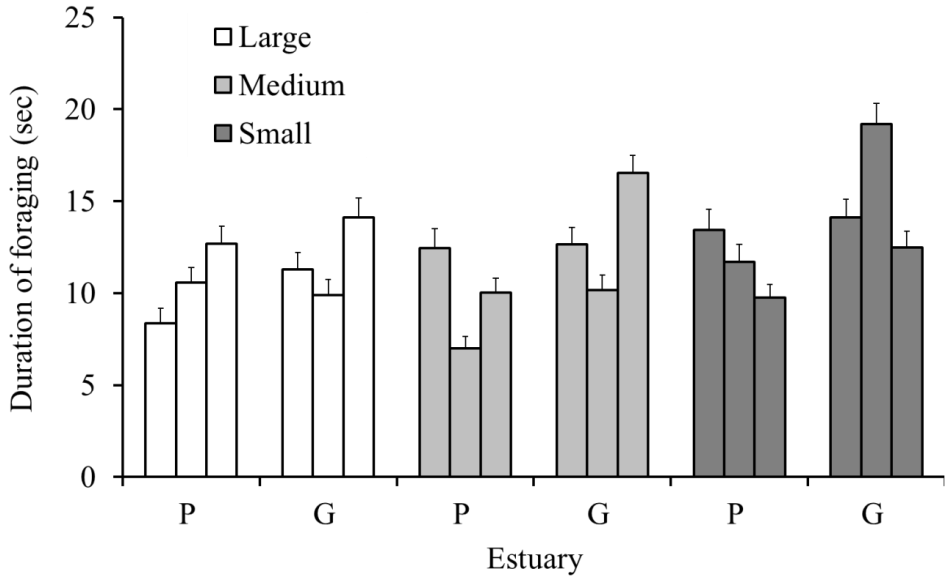


Fig. 4.3. Mean (± SE) of the duration of foraging on floral shoots by honeybees, measured as the average of foraging on each of five floral shoots from each of six video recordings (each recording representing an individual tree) within each of three large, medium and small stands within each of two estuaries (the Parramatta (P) and Georges (G) River's), during the season of 2010.

4.4.3 *Duration of foraging* on floral shoots *by honeybees*

The duration of foraging on floral shoots by honeybees was lowest at the large stands and highest at the small stands both on Parramatta River and Georges River, but on Parramatta River the duration of foraging on floral shoots within the medium stands was lower than within the large stands. In this case the effect of stand size on the duration of foraging on floral shoots (F=3.19; *P*=0.239; $df_{2, 534}$) and the interaction effect (F=2.69; *P*=0.069; $df_{2, 534}$) were not significant, while the effect of location was significant (F=31.42; *P*<0.001; $df_{1, 534}$) (Fig. 4.3; Table 4. 2). On Parramatta River the average foraging duration was 10.6 ± 0.5 sec (mean ± SE) in large stands and 9.8 ± 0.5 sec in medium stands (which was 93% of that in large stands), while it was 11.6 ± 0.6 sec in small stands (which was 10% higher than in large stands) (Fig. 4.3). On Georges River the average duration of foraging on floral shoots by honeybees was 11.8 ± 0.6 sec in large stands and 13.1 ± 0.6 sec in medium stands

Table 4.2. ANOVA of the duration of foraging by honeybees on five floral shoots per m^2 canopy measured on six half hour video recordings from three large three medium and three small stands from each of Parramatta River and Georges River, during the flowering season of 2010. Results are estimates of mean square (MS), F-ratio (F) and probability levels (*P*) of analysis of variance. Location (L) was treated as a random factor while Stand size (S) was treated as a fixed factor.

Source	d.f.	MS	F	*P*
L	1	962.669	31.42	0.000
S	2	262.639	3.19	0.239
LxS	2	82.302	2.69	0.069
Res	534	30.637		

Transformation = none
Cochran's test = NS
SNK for L: Georges>Parramatta

(which was 11% higher than in large stands), while it was 15.3 ± 0.6 sec in small stands (which was 30% higher than in large stands).

Table 4.3. ANOVA of the production of fruit per floral shoot counted on 20 trees from each of three large three medium and three small stands from each of Parramatta River and Georges River, during the fruiting season of 2010. Results are estimates of mean square (MS), F-ratio (F) and probability levels (*P*) of analysis of variance. Location (L) was treated as a random factor while Stand size (S) was treated as a fixed factor.

Source	d.f.	MS	F	*P*
L	1	0.0739	1.76	0.1862
S	2	1.2485	14.73	0.0636
LxS	2	0.0847	2.02	0.1353
Res	228	0.042		
Transformation = Ln(X+1)				
Cochran's test = NS				

4.4.4 *Production of floral shoots and propagules*

The fecundity of trees was highest in large stands and lowest in small stands of both estuaries and trees within small stands were on average strikingly less fecund than trees within large and medium stands. Stands of Parramatta River were more fecund than those of Georges River in most cases and I found great differences in fecundity among individual stands (including stands of same size). In this case of fruit produced per floral shoot the effect of stand size (F=14.73; *P*=0.064; $df_{2, 228}$), the effect of location (F=1.76; *P*=0.186; $df_{1, 228}$) and the interaction effect (F=2.02; *P*=0.135; $df_{2, 228}$) were not significant (Fig. 4.4*a*; Table 4.3). On Parramatta River the average number of fruit produced per floral shoot (Fig. 4.4*a*) in large stands was 1.05 ± 0.04 (mean ± SE) and in medium stands was 0.91 ± 0.03 (which was 87% of that in large stands) while it was 0.67 ± 0.04 in small stands (which was 64% of that in large stands). On Georges River

the average number of fruit produced per floral shoot (Fig. 4.4*a*) in large stands was 0.98 ± 0.05 and in medium stands was 1.07 ± 0.02 (which was 9% higher than that of large stands) while it was 0.54 ± 0.04 in small stands (which was 55% of that in large stands). In the case of fruits produced per tree the effect of stand size (F=55.24; P=0.018; $df_{2, 228}$) and the effect of location (F=22.83; P<0.001; $df_{1, 228}$) were significant while the interaction effect was not (F=0.33; P=0.721; $df_{2, 228}$) (Fig. 4.4*b*; Table 4.4). On Parramatta River the average number of fruit produced per tree (Fig. 4.4*b*) in large stands was 1094 ± 78 (mean ± SE) and in medium stands was 745 ± 62 (which was 68% of that in large stands) while it was 345 ± 34 in small stands (which was 33% of that in large stands). On Georges River the average number of fruit produced per tree (Fig. 4.4*b*) in large stands was 609 ± 76 and in medium stands was 449 ± 85 (which was 73% of that in large stands) while it was 176 ± 33 in small stands (which was 29% of that in large stands).

Table 4.4. ANOVA of the production of fruit per tree counted on 20 trees from each of three large three medium and three small stands from each of Parramatta River and Georges River, during the fruiting season of 2010. Results are estimates of mean square (MS), F-ratio (F) and probability levels (P) of analysis of variance. Location (L) was treated as a random factor while Stand size (S) was treated as a fixed factor.

Source	d.f.	MS	F	*P*
L	1	43.643	22.83	0.000
S	2	34.558	55.24	0.018*
LxS	2	0.626	0.33	0.721
Res	228	1.912		

Transformation = Ln(X+1)
Cochran's test = NS
SNK for L: Parramatta>Georges
for S: Large=Imedium>Small

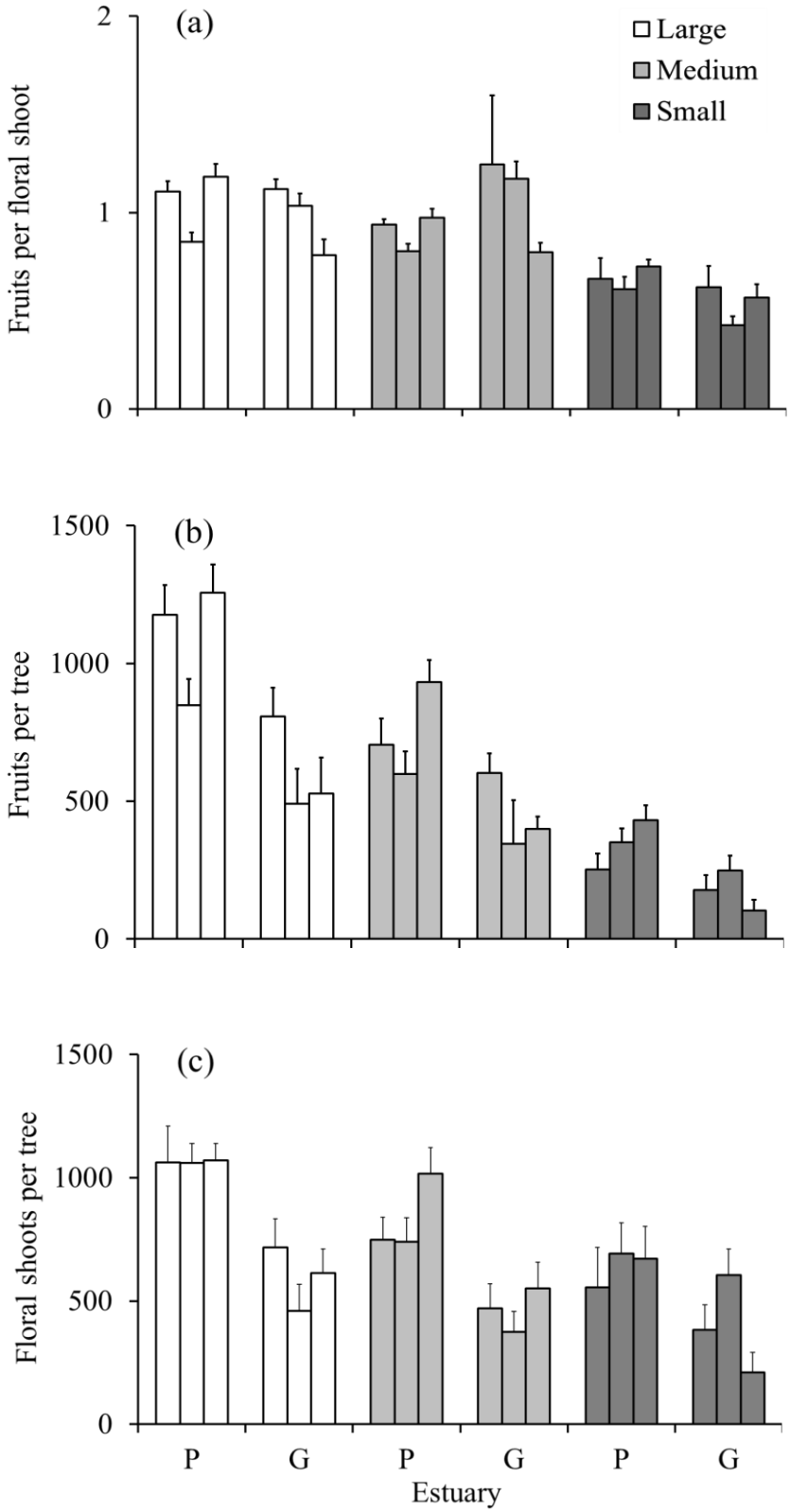


Fig. 4.4. Mean (± SE) number of (a) floral shoots per tree, (b) fruit per floral shoot, and (c) fruit per tree, for 20 trees (if available) within each of three large, medium and small stands within each of two estuaries (the Parramatta (P) and Georges (G) River's) during the season of 2010.

Table 4.5. ANOVA of the production of floral shoots per tree counted on 20 trees from each of three large three medium and three small stands from each of Parramatta River and Georges River, during the flowering season of 2010. Results are estimates of mean square (MS), F-ratio (F) and probability levels (*P*) of analysis of variance. Location (L) was treated as a random factor while Stand size (S) was treated as a fixed factor.

Source	d.f.	MS	F	*P*
L	1	5710234.684	27.15	0.000
S	2	1396745.115	2.49	0.287
LxS	2	561127.594	2.67	0.072
Res	228	210343.752		
Transformation = none Cochran's test = NS SNK for L: Parramatta>Georges				

In the case of floral shoots produced per tree the effect of stand size (F=2.49; *P*=0. 287; $df_{2.\ 228}$) and the interaction effect (F=2.67; *P*=0.072; $df_{2,\ 228}$) were not significant while the effect of location was significant (F=27.15; *P*<0.001; $df_{1,\ 228}$) (Fig. 4.4*c*; Table 4.5). On Parramatta River the average number of floral shoots produced per tree (Fig. 4.4*c*) in large stands was 1064 ± 66 (mean ± SE) and in medium stands was 835 ± 75 (which was 78% of that in large stands) while it was 640 ± 75 in small stands (which was 60% of that in large stands). On Georges River the average number of floral shoots produced per tree (Fig. 4.4*c*) in large stands was 597 ± 76 and in medium stands was 465 ± 73 (which was 78% of that in large stands) while it was 399 ± 68 in small stands (which was 67% of that in large stands).

4.4.5 *The quality of propagules*

The weight of propagules from medium and small stands was lower than the weight of propagules from large stands of both estuaries (Fig. 4.5). The effect of stand size on the

weight of propagules was not significant (F=7.46; *P*=0.118; $df_{2, 2994}$) while the effect of location (F=179.29; *P*<0.001; $df_{1, 2994}$) and the interaction effect (F=10.41; *P*<0.001; $df_{2, 2994}$) were significant (Fig. 4.5; Table 4.6). On Parramatta River the average propagule weight of large stands was 7.77 ± 0.11 g (mean ± SE) and of medium stands was 6.52 ± 0.12 g (which was 84% of that in large stands), while it was 6.19 ± 0.12 g in small stands (which was 80% of that in large stands). On Georges River the average propagule weight in large stands was 6.30 ± 0.10 g and of medium stands was 5.05 ± 0.08 g (which was 80% of that in large stands), while it was 5.15 ± 0.07 g in small stands (which was 82% of that in large stands).

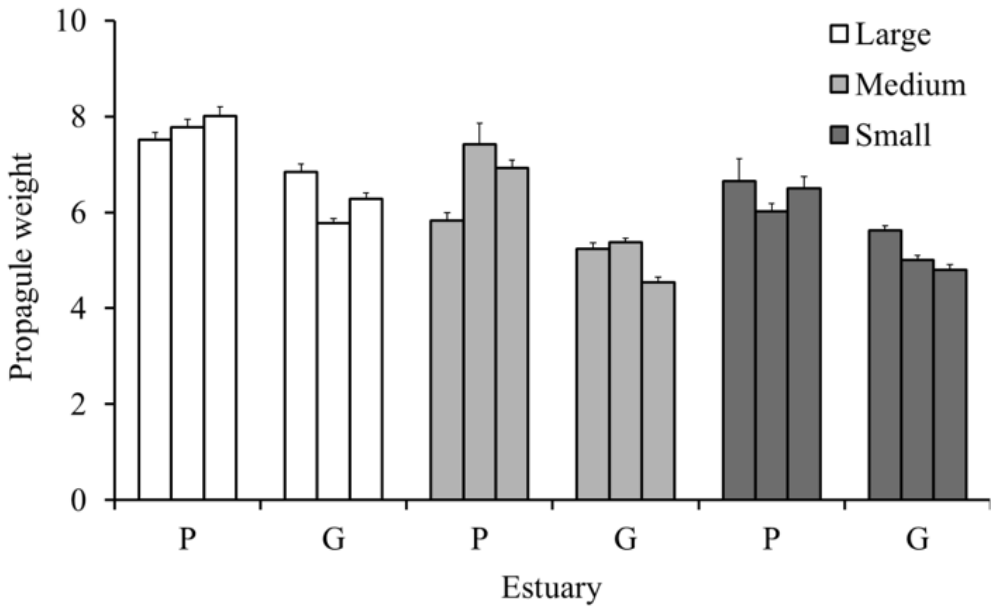


Fig. 4.5. Mean (± SE) of the weight (g) of 25 propagules from each of 10 trees from each of three large, medium and small stands within each of two estuaries (the Parramatta (P) and Georges (G) River's), measured as the average of the 250 propagules collected at each stand during the 2010 season.

Table 4.6. ANOVA of the propagule weight of 25 propagules from each of 10 trees from each of three large three medium and three small stands from each of Parramatta River and Georges River, collected during the fruiting season of 2010. Results are estimates of mean square (MS), F-ratio (F) and probability levels (*P*) of analysis of variance. Location (L) was treated as a random factor while Stand size (S) was treated as a fixed factor.

Source	d.f.	MS	F	*P*
L	1	923.57	179.29	0.000*
S	2	400.18	7.46	0.118
LxS	2	53.64	10.41	0.000*
Res	2994	5.15		

Transform = None
Cochran's test = P < 0.01
SNK: Parramatta (P)>Georges (G)
For P & G: Large>Medium=Small

4.4.6 *Density of propagules and the resultant seedlings*

Georges River had considerably more propagules and seedlings per m^2 than Parramatta River, and small stands displayed considerably fewer fallen propagules and seedlings per m^2 than medium and large stands at both estuaries. In the case of the number of propagules per m^2 the effect of stand size ($F=20.71$; 0.046; $df_{2, 894}$) and the effect of location ($F=45.04$; $P<0.001$; $df_{1, 894}$) were significant while the interaction effect was not ($F=1.58$; $P=0.207$; $df_{2, 2994}$) (Fig. 4.6*a*; Table 4.7). On Parramatta River the average number of propagules per m^2 in large stands was 42.1 ± 4 (mean ± SE) and in medium stands was 26.2 ± 3 (which was 62% of that in large stands), while it was 15.7 ± 2 in small stands (which was 37% of that in large stands) (Fig. 4.6*a*). On Georges River the average number of propagules per m^2 in large stands was 22.3 ± 3 and in medium stands was 12.3 ± 2 (which was 55% of that in large stands), while it was 5.5 ± 1 in small stands (which was 25% of that in large stands) (Fig. 4.6*a*).

Table 4.7. ANOVA of the number of propagules per m^2 of forest floor counted after fruit fall of 2010 as 50 randomly distributed samples of each of three large three medium and three small stands from each of Parramatta River and Georges River. Results are estimates of mean square (MS), F-ratio (F) and probability levels (*P*) of analysis of variance. Location (L) was treated as a random factor while Stand size (S) was treated as a fixed factor.

Source	d.f.	MS	F	*P*
L	1	48077.871	45.04	0.000
S	2	34912.043	20.71	0.046*
LxS	2	1685.574	1.58	0.207
Res	894	1067.3576		
Transformation = none				
Cochran's test = P<0.01				
SNK for L: Parramatta>Georges				
for S: Large=Medium=Small & Large>Small				

In the case of the number of newly settled seedlings per m^2 the effect of stand size was significant (F=19.89; *P*=0.048; $df_{2, 894}$), and so was the effect of location (F=77.76; *P*<0.001; $df_{1, 894}$) and the interaction effect (F=4.36; *P*=0.013; $df_{2, 894}$) (Fig. 4.6*b*; Table 4.8). On Parramatta River the average number of newly settled seedlings per m^2 in large stands was 6.7 ± 0.4 (mean ± SE), in medium stands was 4.8 ± 0.3 (which was 72% of that in large stands) and in small stands was 2.2 ± 0.9 (which was 33% of that in large stands) (Fig. 4.6*b*). On Georges River the average number of newly settled seedlings per m^2 in large stands was 3.8 ± 0.3, in medium stands was 2.4 ± 0.2 (which was 61% of that in large stands) and in small stands was 1.3 ± 0.1 (which was 33% of that in large stands) (Fig. 4.6*b*).

Table 4.8. ANOVA of the number of seedlings per m^2 counted after fruit fall of 2010 as 50 randomly distributed samples from each of three large three medium and three small stands from each of Parramatta River and Georges River. Results are estimates of mean square (MS), F-ratio (F) and probability levels (*P*) of analysis of variance. Location (L) was treated as a random factor while Stand size (S) was treated as a fixed factor.

Source	d.f.	MS	F	*P*
L	1	41.839	77.76	0.000*
S	2	46.689	19.89	0.048*
LxS	2	2.347	4.36	0.013*
Res	894	0.538		

Transformation = Ln(X+1)
Cochran's test = NS
SNK: Parramatta (P)>Georges (G)
For P & G: Large>Medium>Small

Also in the case of the number of seedlings per m^2 surviving for three months the effect of stand size (F=21.02; *P*=0.045; $df_{2, 894}$) and the effect of location (F=23.22; *P*<0.001; $df_{1, 894}$) were significant, but the interaction effect was not (F=1.81; *P*=0.164; $df_{2, 894}$) (Fig. 4.6*c*; Table 4.9). On Parramatta River the number of seedlings per m^2 surviving for three months in large stands was 2.3 ± 0.2 (mean ± SE), in medium stands was 1.5 ± 0.1 (which was 65% of that in large stands) and in small stands was 0.9 ± 0.1 (which was 39% of that in large stands) (Fig. 4.6c). On Georges River the number of seedlings per m^2 surviving for three months in large stands was 1.5 ± 0.1, in medium stands was 1.1 ± 0.1 (which was 73% of that in large stands) and in small stands was 0.6 ± 0.1 (which was 40% of that in large stands) (Fig. 4.6*c*). My results show that the average number of new established seedlings makes up 14% in large stands and 12% in small stands of the average number of fallen propagules. Furthermore, the average number of seedlings surviving after three months makes up 36% in large stands and 44% in small stands of the average number of new established seedlings.

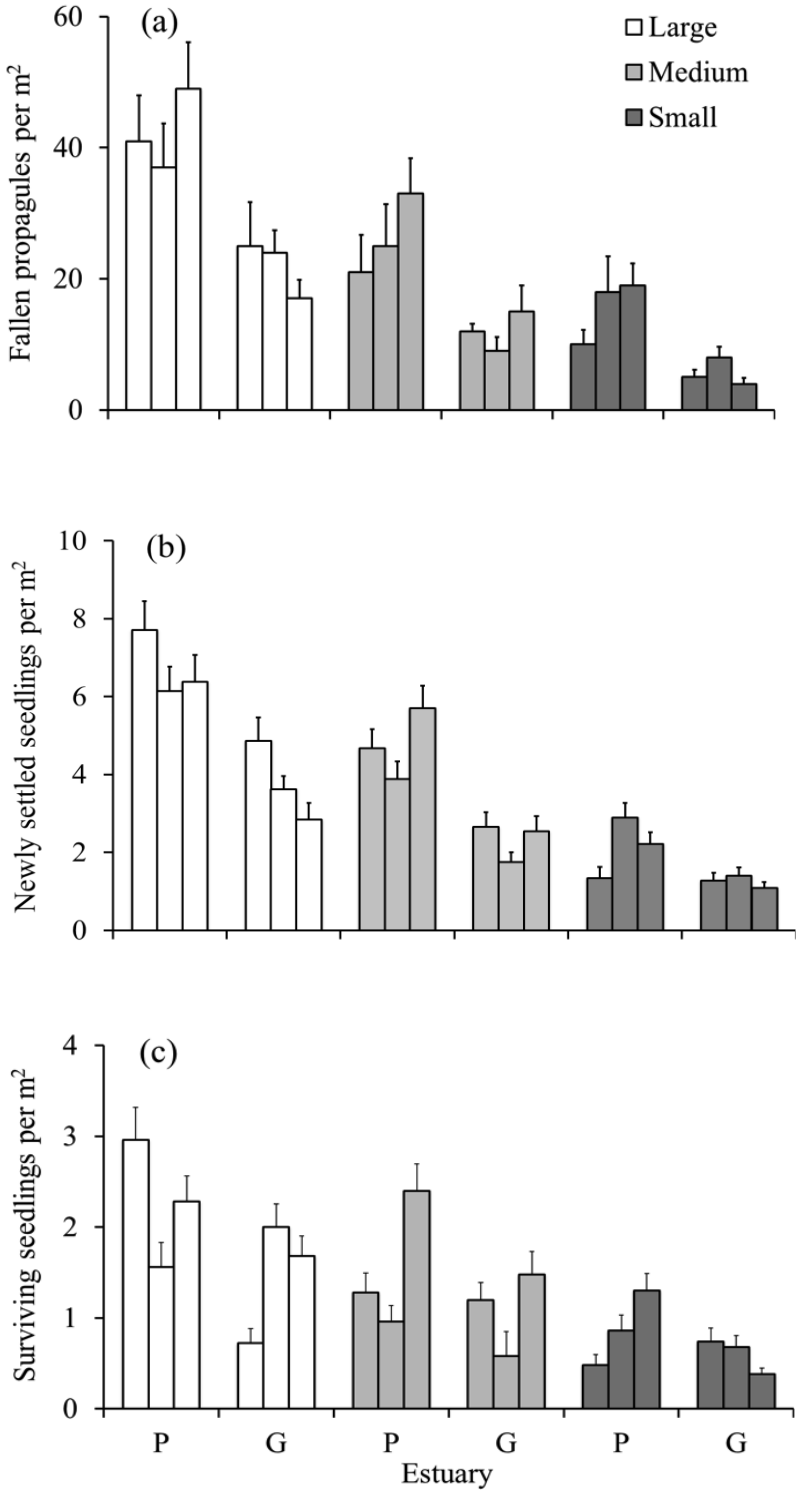


Fig. 4.6. Mean (± SE) of (a) the number of fallen propagules, (b) the number of newly settled seedlings, and (c) the number of seedlings surviving for three months, all measured within each of three large, medium and small stands within each of two estuaries (the Parramatta (P) and Georges (G) Rivers) during the season of 2010. For each individual stand all three factors were measured as the average of 50 randomly chosen samples of 1 m^2 of the sediment surface.

Table 4.9. ANOVA of the number of seedlings per m^2 counted after three months 2011 as 50 randomly distributed samples from each of three large three medium and three small stands from each of Parramatta River and Georges River. Results are estimates of mean square (MS), F-ratio (F) and probability levels (*P*) of analysis of variance. Location (L) was treated as a random factor while Stand size (S) was treated as a fixed factor.

Source	d.f.	MS	F	*P*
L	1	57.760	23.22	0.000
S	2	94.648	21.02	0.045*
LxS	2	4.503	1.81	0.164
Res	894	2.487		

Transformation = none
Cochran's test = P<0.01
SNK for L: Parramatta>Georges
for S: Large=Medium=Small & Large>Small

4.5 Discussion

Overall my study revealed that temperate *A. marina* was partially autogamous (but fruit died before maturing) which is in accordance with the results of Clarke & Myerscough (1991). Further, I found that small stands received significantly fewer honeybees and the duration of foraging on floral shoots showed a considerable (but not significant) variation among stand sizes (10% difference at Parramatta River and 30% difference at Georges River between large and small stands) indicating that effective pollen limitation or lower mate choice may also influence in small stands, resulting in reduced production and quality of fruit and seedlings in small stands as compared with large stands. These results are in accordance with my predictions and congruent to results of terrestrial forests (Aizen *et al*. 2002; Ghazoul 2005; Aguilar *et al*. 2005; Collinge 2009; Barbeta *et al*. 2011). My results imply that *A. mellifera* is important for the reproduction

of temperate *A. marina*, which is also the case for many terrestrial plants in Australia (e.g. Paton 1993, 1996; Whelan *et al*. 2009; Lomov *et al*. 2010; Gross *et al*. 2010) and in other countries (e.g. Krend & Murphy 2003; Neves & Viana 2011; Dupont *et al*. 2004; Taha & Bayoumi 2009, Cayuela *et al*. 2011).

4.5.1 *Temperate* A. marina *is partially autogamous*

If fragmented plant populations compensate for lower pollinator density by being self-compatible, this may increase the level of inbreeding in individual stands, resulting in the production of potentially lower quality of fruit (Aizen *et al*. 2002) but ensuring at least some reproduction or higher levels of fruit set than would occur by reliance on outcrossing. My results showed that temperate *A. marina* was partially autogamous, which is supported by results from another study (Clarke & Myerscough 1991). However, while Clarke & Myerscough (1991) found that a few bagged flowers could set healthy fruit, I found that all fruit died before maturing. This discrepancy cannot be resolved without further investigation, but it may reflect variation in tolerance of selfing among sites or simply temporal variation in resource availability. Further, with a potential capability of selfing (Clarke & Myerscough 1991) and the exotic honeybee *A. mellifera* as only important pollinator today (chapter 2) combined with a considerable (on average 10%-30%) increase of the duration of foraging on floral shoots in small stands as compared to large stands, it was not surprising to find a significantly lower level of outcrossing in small stands (see chapter 5), which may have caused the significantly lower propagule set and quality in small stands, a response that has also been found in some terrestrial plant species (Ghazoul 2005).

4.5.2 *Reduced pollinator density in small stands*

My study revealed that the density of honeybees is significantly lower in medium and small stands as compared to large stands, as it is also the case in many terrestrial plant populations (Aizen & Feinsinger 1994*ab*; Ghazoul 2005). My earlier results show that this can lead to a reduction in the deposition of pollen in small stands (chapter 3). In small stands within fragmented terrestrial forest populations reduced pollinator densities result in pollen limitation (Ghazoul 2005; Ward & Johnson 2005). Pollen limitation together with mate choice, pollinator behaviour (pollinators forage extensively within compared to among plants) and the degree of self-compatibility are factors leading to higher levels of inbreeding (or biparental inbreeding) and reduced fruit quality, which may lead to lower fruit set and quality in terrestrial plant populations (Aizen *et al*. 2001; Ghazoul 2005; Ward & Johnson 2005). This was also the case in the present study in temperate stands of *A. marina* where a significantly lower level of outcrossing was found in small stands (as compared to large stands) as judged after genetic tests of plant siblings (chapter 5), which may have resulted in the significantly lower production of propagules in small stands and in reduced fruit quality which may have caused a significant reduction of propagule size in small stands.

4.5.3 *Changed foraging behaviour may result in increased inbreeding*

I found the duration of foraging on floral shoots by honeybees to be 10%-12% longer in small stands as compared to large stands, but this difference was not significant (chapter 3). The duration of foraging on floral shoots in small stands of the present study was 10% longer on Parramatta River and 30% longer on Georges River (which was not significant either). These results may not be enough to explain the low level of

outcrossing found in small stands of my genetic survey (chapter 5). However, other factors that may effects the mating systems is effective pollen limitation, fewer potential mates available and significantly more within than among plant movements in small stands rather than a real difference in the amount of self vs. outcross pollen transferred. One or more of these effects in combination may cause the lower levels of outbreeding in small stands. Results like this is also found in terrestrial forest plants (Ghazoul 2005; Aguilar *et al*. 2006). Together with terrestrial studies showing that honeybees forage among trees within near proximity of each other (Paton 1993; Whelan *et al*. 2009) my findings suggest that honeybees may have the potential to increase the level of biparental inbreeding within individual stands too.

4.5.4 *Reduced propagule and flower production in small stands*

In the present study the production of propagules per tree and per floral shoot was significantly lower in small than large stands, which is also the case in many terrestrial plant populations (Cunningham 2000; Ghazoul 2005; Aguilar *et al*. 2006). An effect like this may be caused by the lower pollinator abundance and reduced pollinator services (see also chapter 3) I found within the small stands, resulting in lower levels of outcrossing (chapter 5). Effects like this is also found in terrestrial plants where lower pollinator abundance may lead to pollen limitation, resulting in lower fruit production (Bierzychudek 1981; Burd 1994; Larson & Barrett 2000; Ghazoul 2005; Aguilar *et al*. 2006). My tests of the production of floral shoots per tree revealed a not significant reduction. However, it was considerable with fewer flowers developed in small stands. This is in accordance with results from terrestrial plant populations (Klank *et al*. 2010), and shows that reduced stand size in temperate *A. marina* may lead to limitations in

flower production. Such effect may have been involved in the reduction of pollinator density and fruit production in the present study.

4.5.5 *Reduced fruit quality in small stands*

Many terrestrial forests plants produce smaller fruit in small stands as compared to large stands (Aguilar *et al*. 2006). Like this my results revealed a significant reduction of the propagule weight in small stands, potentially reducing the competitive qualities of these propagules compared to those from large stands because they will grow slower and support the seedling with less nutrients (smaller fruit means less stored nutrients: Tomlinson 1986). This may be caused by the significantly reduced pollinator density in small stands of the present study equivalent to that found in terrestrial forest plants resulting in pollen limitation in small stands (Aizen *et al*. 2001; Ghazoul 2005; Aguilar *et al*. 2006), which may again reduce the potential for mate choice and increase the likelihood of inbreeding and result in inbreeding depression in small stands (Ghazoul 2005). Further, this may have an effect on newer *A. marina* stands developed (within the last century) on mudflats by recruitment of propagules from neighbouring stands of the same estuary, which is the way stands of the present study were created (Thorogood 1985; McLoughlin 2000; Dunstan 1990 – and personal investigation of aerial photos). Such stands may contain a mix of genes from several or most stands of the same estuary, ensuring a diverse and homogeneous pool of genes in these stands. However, because propagules from large stands are significantly larger and more abundant than those from small stands, genes from large stands may dominate stands developed in this way (including those investigated in the present study).

4.5.6 *Reduced production of propagules and seedlings in small stands*

My results show that the density of propagules within the individual stands after fall of propagules was significantly lower within small stands, suggesting a generally low production of propagules from trees of small stands as compared to large stands. Such effect has been shown to be a common effect of fragmentation in terrestrial forest plants (Ghazoul 2005; Aguilar *et al*. 2006). Furthermore, the density of new established seedlings and seedlings surviving after three months were also significantly lower in small stands in the present study. My results show that the average number of new established seedlings only makes up ca. 13% of the average number of fallen propagules (no matter of the stand size). Furthermore, the average number of seedlings surviving after three months only makes up 36-44% of the average number of new established seedlings. This indicates that there may be a great reduction in the number of propagules germinating and developing into small plants and there may be a significant effect of stand size on the development of seedlings within the small stands.

4.5.7 *Conclusion*

In summary, I found that the density of honeybees (the only effective pollinators of *A. marina* within this system) was significantly lower in small as compared to large stands, and the production of propagules and seedlings displayed a similar pattern of reduction to the density of honeybees. This suggests that the production of propagules and seedlings is dependent of pollinator services from the exotic honeybee *Apis mellifera*. Overall the present study reveals a significant effect of stand size on the fruit production and quality in small stands.

Chapter 5: Small urban stands of the mangrove *Avicennia marina* are genetically diverse but experience elevated inbreeding

5.1 Abstract

Anthropogenic impacts contribute to the fragmentation of mangrove forests in urban estuaries, and in the Sydney region of Australia *Avicennia marina* is commonly found in small stands of <50 adult trees that have altered pollinator services. I tested the predictions that, despite the potential of propagules for long distance dispersal by water and honeybees to move pollen over long distances, fragmentation causes small stands of *A. marina* to display reduced genetic diversity and elevated rates of inbreeding. Using four variable microsatellite markers I quantified the levels of genetic diversity and genotypic diversity present within samples of 20 adults taken from sets of three large (>1500 trees), medium (300-500 trees) and small (<50 trees) stands within each of two urbanised estuaries. I used these markers to estimate multilocus outcrossing rates (t_m) using progeny arrays for sets of five adults within each of the large and small stands. I detected no significant effect of stand size on levels of single-locus genetic diversity (as judged by H_e and Allelic richness), but low though significant (Lower 99% CI is pocitive; see Table 5.5) levels of allelic differentiation within (F_{SE} = 0.021, P = 0.01) and among (F_{ET} = 0.055, P = 0.01) estuaries with no evidence of isolation by distance using either Euclidean distances or distances measured along the shoreline. Taken together the genetic makeup of the adult populations implies that they are relatively well interconnected and suggests little impact of habitat fragmentation. In partial contrast my analysis of progeny

arrays reveals that while all stands display high levels of biparental inbreeding, t_m was significantly lower in small (0.55 ± 0.043) *cf.* large (0.75 ± 0.071) stands, suggesting that plants within small stands may display reduced fitness.

5.2 Introduction

Mangroves are increasingly subject to anthropogenic disturbance including fragmentation into small stands and, as observed in terrestrial forests, plants within small stands may suffer reduced fitness through reduction of genetic diversity, increased levels of inbreeding and reduced fruit production (Reed & Frankham 2003; Ghazoul 2005). Predicting the impact of fragmentation on mangrove forests is difficult, however, because mangrove populations also have naturally fragmented distributions (Duke 2006), poorly documented pollination systems (chapter 2) and water borne dispersal of propagules that may maintain connectivity among stands (Duke *et al*. 1998; Minchenton 2006). Moreover, within terrestrial forests a common effect of fragmentation is a reduction in pollinator diversity and abundance, with consequent alteration of pollinator services, which can drive decreases in genetic diversity and increased inbreeding (Aizen 2002; Ghazoul 2005; Aguilar 2006). The existing literature on mangrove pollination might suggest that small mangrove stands would have a similar fate, but my recent work (Chapter 2) indicates that little is known about the natural pollinators of mangroves and that in southeastern Australia the pollination of the dominant mangrove species *Avicennia marina* appears entirely dependent on services provided by the exotic honeybee *Apis mellifera* (Chapter 2).

Genetic diversity is essential for the continuity of a plant species because it provides the opportunity to adapt to the local biotic and abiotic environmental conditions

through adaptation in the genetic composition, enabling a plant species to cope with conditions and changes in the local environment (Caliskan 2012). If genetic diversity is reduced then it could (in the worst case scenario) over time result in extinction of small populations of anthropogenically fragmented populations (Reviewen by Aizen *et al*. 2002).

Wright (1943) developed a model for the effective size of a set of populations in an island model. In this model each population contributes equally to the pool of migrants and subdivision increases the effective population size (N_e) for the whole population over that of panmixia (random mating within a population with breeding individuals showing no tendency to choose a partner with any particular traits), which may enhance regional biodiversity by providing opportunities for local adaptations (Quinn & Robinson 1987). However, in highly subdivided habitats (as those of anthropogenically fragmented plant populations) the average patch size is small and local biodiversity may be low with consequently negative effects of stand size on fragmented plant populations (Reviewed by Quinn & Robinson 1987).

Previous studies have focused on inferences made from comparisons of adult genotype frequencies with expectation for Hardy-Weinberg equilibria. Using this approach deficits of hetetrozygotes imply some level of inbreeding while excesses would imply preferential outcrossing. In mangroves the majority of studies have found from high levels of inbreeding to moderate levels of outcrossing (F_{IS} = -251-663 worldwide and -0.080-337 for Australian populations) (e.g. Maguire et al. 2000*b*; Arnaud-Haond et al. 2006), showing a variability that may reflect variation in the scale of sampling. This approach is limited because it assumes that each stand is closed to migration of seed and pollen and that genotype frequencies are at stable equilibrium (Hedrick 2011), which is

unlikely given the typically dynamic nature of mangrove populations. The alternative is to compare the genotypes of adults and their progeny to infer the current pattern of mating (e.g. Butcher *et al*. 2005). This approach also assumes that populations are closed to immigration of pollen but does not assume that populations are in stable equilibria. Because the genotypic structure of adult populations may reflect other historical processes (e.g. colonisation from one or more sources) and mating systems may change with pollinator disruption (e.g. Thompson 1999; Aizen *et al*. 2002), I studied both adult plants and their progeny in the present study.

Only two studies have focused on adult *A. marina* populations in the Sydney region, and these investigated variation in isozyme and allozyme frequencies with geographical distances (Melville & Burchett 2002; Melville *et al*. 2004). The first study found no significant differences in allele frequency among neighbourhoods within each estuary while significant inter-estuarine differences in allele frequencies were found in 20 of 22 isozyme loci indicating a marked divergence in allele frequencies among the estuaries (Melville & Burchett 2002). The other study found significant variation in allozyme frequencies among sampling sites within each of the investigated estuaries (Melville *et al*. 2004). However, while I used hierarchical *F* statistics to estimate differentiation in the present study (allowing easy comparison of the magnitude of population differentiation), both of the two studies mentioned (Melville & Burchett, 2002; Melville *et al*. 2004) did not include formal tests of the magnitude of differentiation. Thus, it does not allow for direct comparison of results. However, Homer's (2009) study of the genetic differentiation of populations in northern New South Wales (NSW) provides a relevant comparison. Using microsatellite markers and an analysis of eleven stands separated by up to 165 km, Homer found a differentiation very

similar to my findings, but over a larger spatial scale. She revealed an overall F_{ST} of 0.06, and concluded that, although there was evidence of significant isolation by distance, dispersal of propagules was sufficient to maintain connections among even quite widely separated stands.

In the present study I compare genetic variation at microsatellite loci within large, medium and small stands of *A. marina* in each of two neighbouring Sydney estuaries to test for effects of stand size on genetic diversity and to test for evidence of population differentiation and limited dispersal. I also use progeny arrays from plants within large and small stands to test for evidence of reduced levels of outcrossing and increased inbreeding and biparental inbreeding within small stands.

5.3 Materials and methods

5.3.1 *Species description, study sites and collection of samples*

Avicennia marina is one of only two species of mangroves growing in the Sydney region of New South Wales (NSW) in Australia (Duke 2006). My study focussed on stands of *A. marina* in two urban estuaries in Sydney, NSW, the lower parts of Parramatta and Georges (including Woronora River) River catchments, hereafter referred to as Parramatta River (33°50'0" S; 151°6'5" E) and Georges River (33°59'1" S; 151°2'8" E) (Fig. 5.1). At this latitude in these estuaries mangrove stands are typically naturally fragmented, reflecting the availability of suitable habitat (i.e. mudflats, the banks of creeks etc.) (West 1988; Duke 2006). In addition, a greater than 70 year history of urban sprawl including reclamation of saltmarsh for housing, industry, and park-land for recreation and new mangrove colonization of mudflats during the last century has increased the development of isolated mangrove stands in both estuaries (Thorogood

1985; Dunstan 1990; McLoughlin 2000; Adam 2002). I selected nine *A. marina* stands from each of two estuaries and classified each stand as small, medium or large, based on a survey of the number of adult trees contained in each stand (Table 5.1). Large stands (three from each estuary) were distributed over 15000 – 40000 m^2 and contained greater than 1500 adult trees. Medium stands (three from each estuary) were distributed over

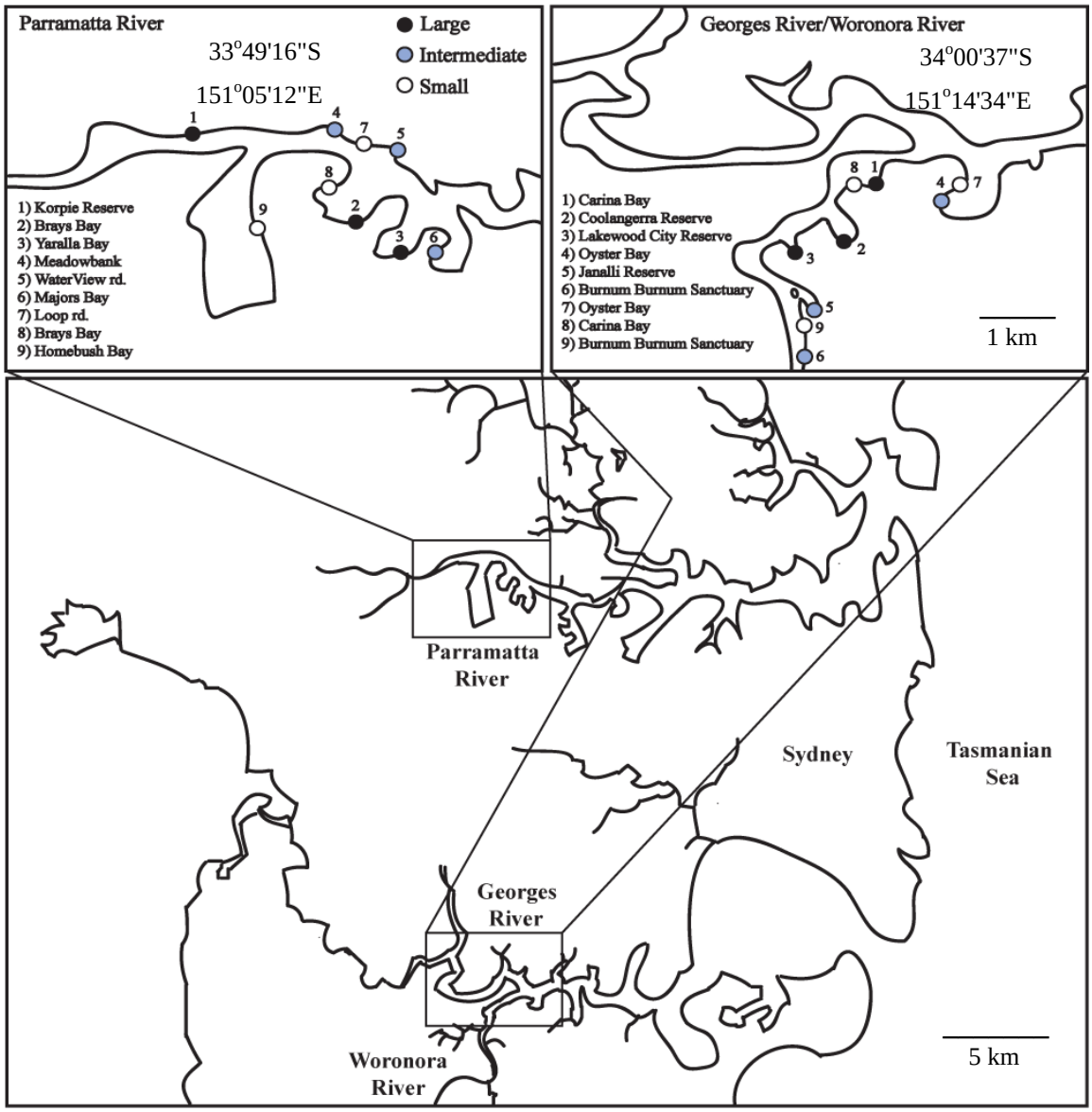


Fig. 5.1. Map of the two estuaries showing the location of stands at (a) Parramatta River and (b) Georges River, and (c) shows a map of Sydney and indicates the locations of (a) and (b).

5500 – 9000 m^2 and contained 300-500 adult trees. Small stands (three from each estuary) were distributed over 600-1600 m^2 and contained fewer than 50 adult trees. Stands were isolated from conspecific stands by from ca. 100 m and up to ca. 4 km by Euclidean distance and ca. 7 km by water (Appendix 5.1: end of this chapter – shows distances between all stands). Shape of these stands is described in section 4.3.1 Page 86.

Stands were chosen so they were as comparable as possible in terms of their size and condition and history. I collected leaf material (for subsequent genetic analysis) from each of 20 adult trees per stand (n = 180 per estuary). In large and medium stands these trees were selected in groups of 6-7 trees (one group in each end of the stand and one in the middle in a line along the coastline), while trees were randomly selected in small stands. Within each estuary I also collected five fruit for mating system analysis from each of five trees (randomly selected among trees carrying fruits) in each of the three large and the three small stands. All samples were stored at -80°C in the laboratory pending genetic analysis.

5.3.2 *DNA extraction and genotyping*

DNA was extracted from freeze-dried leaves (of adult trees) and shoots (after careful dissection from propagules) following Doyle and Doyle (1987). I scored genetic variation at four *A. marina* microsatellite loci described by Maguire *et al*. 2000*a* (Am32, Am49, Am81 and Am98) and later modified by Arnaud-Haond *et al*. (2006). Polymerase chain reactions (PCR) were carried out in 10-µL reaction-volumes. Reaction mixtures comprised 1 X PCR buffer supplied by the manufacturer (Applied Biosystems), 0.25 mM of each dNTP, 2 mM of $MgCl_2$, 0.25 mM of each forward and reverse primer, and 0.45 units of AmpliTaq Gold (Applied Biosystems), with the remainder of the reaction

cocktail made up of deionised H_2O and approximately 5 – 20ng of DNA template. Each forward primer was 5' labeled with one of four fluorescent dyes (6FAM, HEX, NED or PET). PCR was carried out on an Eppendorf Mastercycler (Eppendorf) gradient thermocycler under the following cycling conditions: 10 min at 95°C (denature) followed by 30 cycles of 30 sec at 94°C, 30 sec at either 50°C (anneal) (Am49, Am81 and Am98) or 55 °C (Am32), 30 s at 72°C (extension) followed by a final extension step of 72°C for 10 min. I included negative controls in each PCR reaction, and conducted multiple runs of randomly selected DNA templates to ensure repeatability of allele scoring. All four PCR products were combined in a single reaction cocktail containing formamide and LIZ size standard (Applied Biosystems) and denatured at 95°C for 3 min. Genotyping was performed on an ABI 3130 automated capillary DNA sequencer (Applied Biosystems). Assignment of allele size was achieved with the aid of GeneMapper (v3.7 Applied Biosystems) software, with allele sizing through comparison of electrophoretic migration of PCR products and LIZ DNA fragments of known size.

5.3.3 *Data analysis*

I used the computer software Micro-checker (van Oosterhout *et al*. 2004) to check for any incorrect interpretation of microsatellite electropherograms caused by miss-priming during PCR or for the existence of any non amplifying (null) alleles resulting from point mutations in the primer binding sites. The results of my analyses indicated that my data were apparently free of these potential sources of error (data not reported), and I report the results of all other analyses based on four microsatellite loci.

I used GENEPOP to test for linkage disequilibria among all pairs of loci in all populations to verify that loci assort independently and to test for departures from Hardy-

Weinberg equilibria within each population. This was done to test for evidence of inter-population variation in levels of outcrossing. All four microsatellite loci used were polymorphic for all stands (Appendix 5.2: end of this chapter), and I detected linkage disequilibrium in only three of 108 tests for each pairwise combination of loci within individual stands, and finally, I detected no linkage disequilibrium when analysing the entire data set (Appendix 5.3: end of this chapter). All loci were therefore considered independent and used in subsequent analyses. For each stand, and overall for each estuary, I used GENALEX v6.1 (Peakall & Smouse 2006) to calculate standard measures of genetic diversity, including the average number of alleles per locus (N_a), the average effective number of alleles per locus (N_e), and the average number of private alleles per locus (A_P), and to calculate expected heterozygosity (H_e). I also estimated the inbreeding coefficients, F_{IS}, of each stand using the program FSTAT (Goudet 2001). As I expected higher values in the small stands, I used analyses of variance (ANOVA) on the average values for each stand for each estuary to test for variation in levels of N_a, N_e, H_e and F_{IS} among different stand sizes. I used Weir & Cockerham's (1984) formulations of Wright's *F* statistics and the program TFPGA (Miller 1997) to determine how genetic variation was partitioned within and among estuaries. Standard errors were estimated by jackknifing across loci, and statistical significance of $F \pm SE$ was inferred when zero lay outside the 95% and 99% CI determined based on 1000 bootstrap replicate data sets. GENALEX v6.1 was used to reveal F_{ST} across the estuaries.

Because dispersal among mangrove stands may be restricted, but can either occur via floating within river channels (propagules) or by flight (dispersal of pollen by flying insects), I used a Mantel test to test for evidence of isolation by distance in two ways. Analyses were based upon matrices of geographic distance, measured as (i) the Euclidean

(as the crow flies) distance between stands, and (ii) the shortest path by water along the shoreline, and the genetic similarity index M of Slatkin (1989, 1993). Analyses were performed using the Isolation by Distance web service (http://ibdws.sdsu.edu/~ibdws/codominant.html) (Jensen *et al*. 2005).

2.3.4 *The realized mating system within large and small stands*

I estimated mating system parameters by comparison of maternal genotypes (for leaf samples) and those of newly produced progeny (i.e. propagules) under Ritland's mixed/correlated mating models (Ritland & Jain 1981; Ritland 1989, 1997) and the software package MLTR. Allele frequency estimates of potential pollen donors were included in the analysis and consisted of samples of 20 adult mangroves for each stand. I estimated single and multilocus-outcrossing rates (t_s and t_m) by maximum-likelihood using the expectation-maximization method, and I used the difference between the multi-locus and single-locus estimates of outcrossing (t_m-t_s) to characterise the level of biparental inbreeding. The correlation of outcrossed paternity or the proportion of full-sibs among outcrossed sibs (r_p) was used to estimate the number of likely pollen donors or male parents (estimated as $1/r_p$) that would result in the observed correlation. Standard errors were determined with 1000 bootstraps across progeny arrays, resampling within families. Values of t_m and t_m-t_s were tested by two factor ANOVA with the factors estuary Location (Parramatta or Georges River, as a random factor) and Stand size (large, medium or small, as a fixed factor).

5.4 Results

5.4.1 *Effects of stand size on genetic diversity*

Genetic diversity did not vary with stand size. I detected similar levels of diversity for small, medium and large stands in each of the two estuaries as judged by N_a, N_e, H_e and A_p. On average N_a (Fig. 5.2*a*) was 5.3 ± 0.4 (mean ± se) in large stands of Parramatta River and 5.1 ± 0.8 in larges stands of Georges River, it was 5.3 ± 0.2 in medium stands of Parramatta River and 4.8 ± 0.2 in medium stand of Georges River, and it was 5.3 ± 0.3 in small stands of Parramatta River and 4.9 ± 0.6 in small stands of Georges River.

Compared to large stands, N_a was the same among the three stand sizes on Parramatta River while it was 7% lower in medium stands and 3% lower in small stands of Georges River (Fig. 5.2*a*). The effect of stand size (F=6.33; P=0.136; $df_{2, 17}$), the effect of location (F=0.01; P=0.911; $df_{1, 17}$) and the interaction effect (F<0.01; P=0.996; $df_{2, 17}$) across estuaries were not significant for N_a (Table 5.1).

Table 5.1. Two factor ANOVA of the average number of alleles per locus (N_a) of three large three medium and three small stands of each of Parramatta River and Georges River. Results are estimates of mean square (MS), F-ratio (F) and probability levels (P) of analysis of variance. Location (L) is a random factor while stand size (S) is a fixed factor.

Source	d.f.	MS	F	*P*
L	1	0.125	0.01	0.911
S	2	0.264	6.33	0.136
LxS	2	0.042	0.00	0.996
Res	12	9.677		
Transformation = none				
Cochran's test = NS				

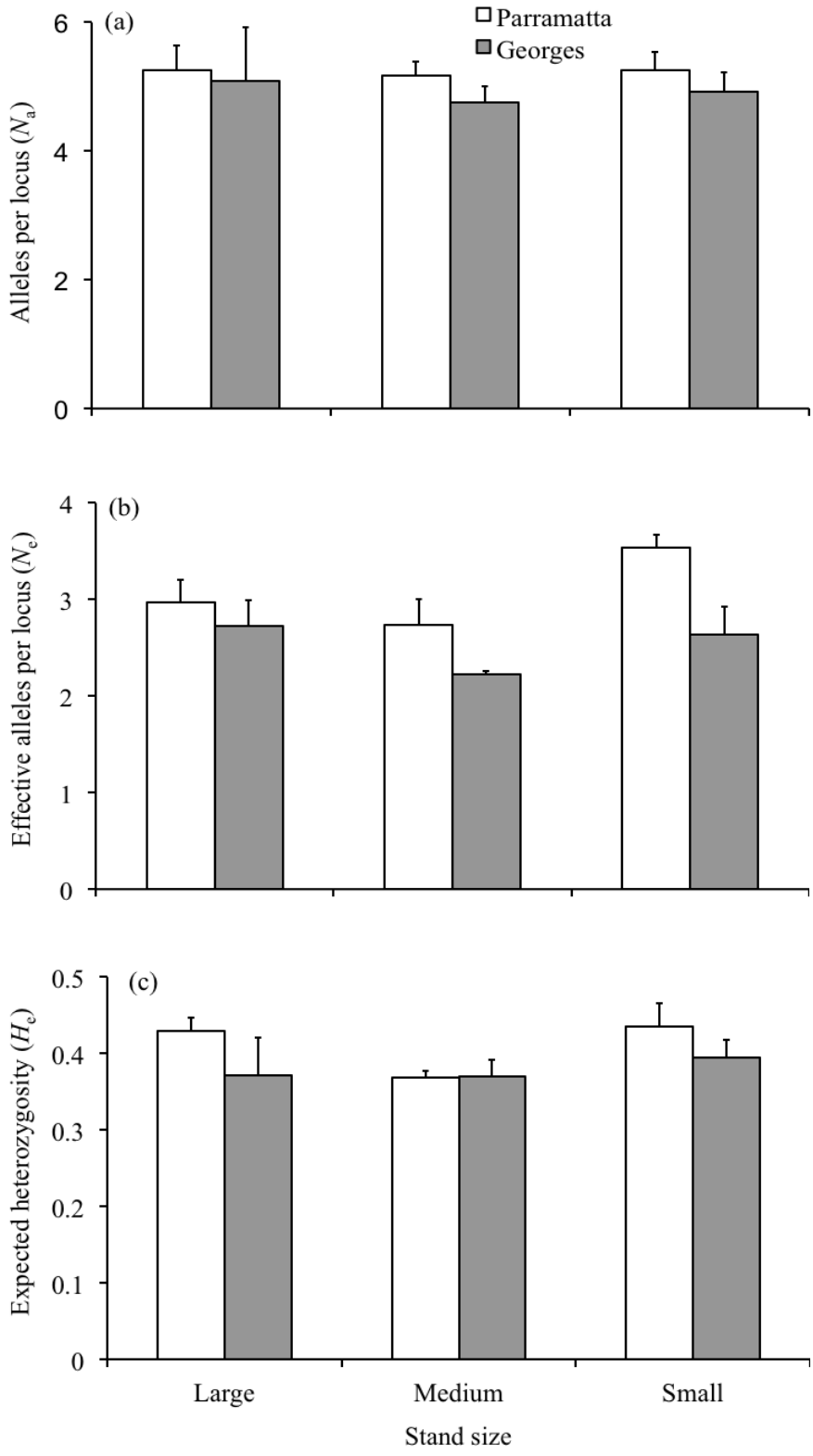


Fig. 5.2. Mean (± SE) of (a) N_a, the observed number of alleles, (b) N_e, the effective number of alleles and (c) H_e, the expected heterozygosity, all based on data from four microsatellite loci and n = 20 trees for each of 18 stands of the grey mangrove *A. marina*, with three stands of each of three sizes (large, medium and small) in each of Parramatta and Georges Rivers, classified as large, medium and small.

N_e (Fig. 5.2*b*) was 3.0 ± 0.3 in large stands of Parramatta River and 2.7 ± 0.2 in larges stands of Georges River, it was 2.7 ± 0.3 in medium stands of Parramatta River and 2.3 ± 0.1 in medium stand of Georges River, and it was 3.6 ± 0.1 in small stands of Parramatta River and 2.7 ± 0.2 in small stands of Georges River. Compared to large stands N_e was on average 9% lower in medium stands and 18% higher in small stands of Parramatta River while it was 17% lower in medium stands and 1% lower in small stands of Georges River (Fig. 5.2*b*). The effect of stand size (F=7.91; *P*=0.112; $df_{2, 17}$), the effect of location (F=0.09; *P*=0.775; $df_{1, 17}$) and the interaction effect (F=0.02; *P*=0.976; $df_{2, 17}$) across estuaries wwere not significant for N_e (Table 5.2).

Table 5.2. Two factor ANOVA of the average effective number of alleles per locus (N_e) of three large three medium and three small stands of each of Parramatta River and Georges River. Results are estimates of mean square (MS), F-ratio (F) and probability levels (*P*) of analysis of variance. Location (L) is a random factor while stand size (S) is a fixed factor.

Source	d.f.	MS	F	*P*
L	1	0.242	0.09	0.775
S	2	0.543	7.91	0.112
LxS	2	0.069	0.02	0.976
Res	12	2.816		
Transformation = none				
Cochran's test = NS				

H_e (Fig. 5.2*c*) was 0.43 ± 0.02 in large stands of Parramatta River and 0.37 ± 0.04 in larges stands of Georges River, it was 0.38 ± 0.01 in medium stands of Parramatta River and 0.37 ± 0.02 in medium stand of Georges River, and it was 0.44 ± 0.3 in small stands of Parramatta River and 0.41 ± 0.01 in small stands of Georges River. Compared to large stands N_e was on average 13% lower in medium stands and 1% higher in small stands

Table 5.3. Two factor ANOVA of the expected heterozygosity (H_e) of three large three medium and three small stands of each of Parramatta River and Georges River. Results are estimates of mean square (MS), F-ratio (F) and probability levels (*P*) of analysis of variance. Location (L) is a random factor while stand size (S) is a fixed factor.

Source	d.f.	MS	F	*P*
L	1	0.001	0.01	0.925
S	2	0.003	11.42	0.086
LxS	2	0.000	0.00	0.996
Res	12	0.054		
Transformation = none				
Cochran's test = NS				

of Parramatta River, while it was 1% higher in medium stands and 9% higher in small stands of Georges River (Fig 5.2*c*). The effect of stand size (F=11.42; *P*=0.086; $df_{2, 17}$), the effect of location (F=0.01; *P*=0.925; $df_{1, 17}$) and the interaction effect (F<0.01; *P*=0.996; $df_{2, 17}$) across estuaries were not significant for H_e (Table 5.3).

Table 5.4. Two factor ANOVA of the number of private alleles (A_p) of individual stands, of three large three medium and three small stands of each of Parramatta River and Georges River. Results are estimates of mean square (MS), F-ratio (F) and probability levels (*P*) of analysis of variance. Location (L) is a random factor while stand size (S) is a fixed factor.

Source	d.f.	MS	F	*P*
L	1	0.500	0.90	0.362
S	2	0.500	0.90	0.432
LxS	2	0.167	0.30	0.746
Res	12	0.556		
Transformation = none				
Cochran's test = NS				

On average A_p revealed 2.25 private alleles per locus (not shown), and the effect of stand sizes (F=0.90; P=0.432; $df_{2, 17}$), the effect of location (F=0.90; P=0.362; $df_{1, 17}$) and the interaction effect (F=0.30; P=0.746; $df_{2, 17}$) across estuaries were not significant for A_p (Table 5.4).

Table 5.5. Hierarchical *F*-statistics estimated for four microsatellite loci, and overall, for *A. marina* in two urban estuaries. Genetic variation between estuaries (F_{ET}) and among stands within estuaries (F_{SE}) and fixation index "inbreeding coefficients" (F_{IS}) of adult plants. Parramatta River is marked with "P" and Georges River is marked with "G", and "both" means both rivers. CI means Confidence Interval.

LOCUS 1		F_{ET}	F_{SE}	F_{IS}
Allele				
	1	0.005	-0.002	-0.026
	2	0.014	-0.004	-0.018
	3	0.000	0.000	0.000
	4	0.099	0.027	0.090
	5	0.049	0.022	0.018
	6	0.065	-0.006	-0.104
	7	0.000	0.000	0.000
	All	0.065	0.020	0.030
Locus 2				
Allele				
	1	0.004	0.002	-0.016
	2	-0.003	-0.003	0.000
	3	0.000	0.000	0.000
	4	0.000	0.000	0.000
	5	0.112	0.051	0.283
	6	0.000	0.000	0.000
	7	0.053	0.007	-0.013
	8	0.015	0.009	0.142
	9	0.106	0.078	0.179
	10	-0.004	-0.002	0.000
	11	-0.004	0.001	0.000
	12	0.103	0.031	0.200
	13	0.089	0.034	0.012
	14	0.017	0.000	0.102

	15	-0.008	0.001	0.286
	16	0.013	0.003	0.321
	17	-0.010	-0.002	0.105
	18	0.001	0.000	-0.012
	19	0.026	0.006	0.069
	20	0.020	-0.006	0.115
	21	0.008	-0.003	0.002
	22	0.045	0.041	0.009
	23	0.055	0.024	0.102
	24	0.035	0.006	0.023
	25	0.026	0.000	-0.027
	26	0.000	0.003	0.000
	27	0.053	0.000	-0.056
	28	0.000	0.000	0.000
	All	0.050	0.022	0.104
Locus 3				
Allele				
	1	0.000	0.003	0.000
	2	0.034	0.034	0.040
	3	0.030	0.020	0.111
	4	0.021	-0.001	0.441
	5	0.026	0.000	-0.027
	6	0.032	0.009	-0.033
	All	0.030	0.021	0.115
Locus 4				
Allele				
	1	0.069	0.017	0.104
	2	0.000	0.000	1.000
	3	0.075	0.020	0.085
	All	0.071	0.018	0.105
All loci		0.055	0.021	0.089
99 % C.I.				
Upper		0.069	0.022	0.115
Lower		0.030	0.019	0.041

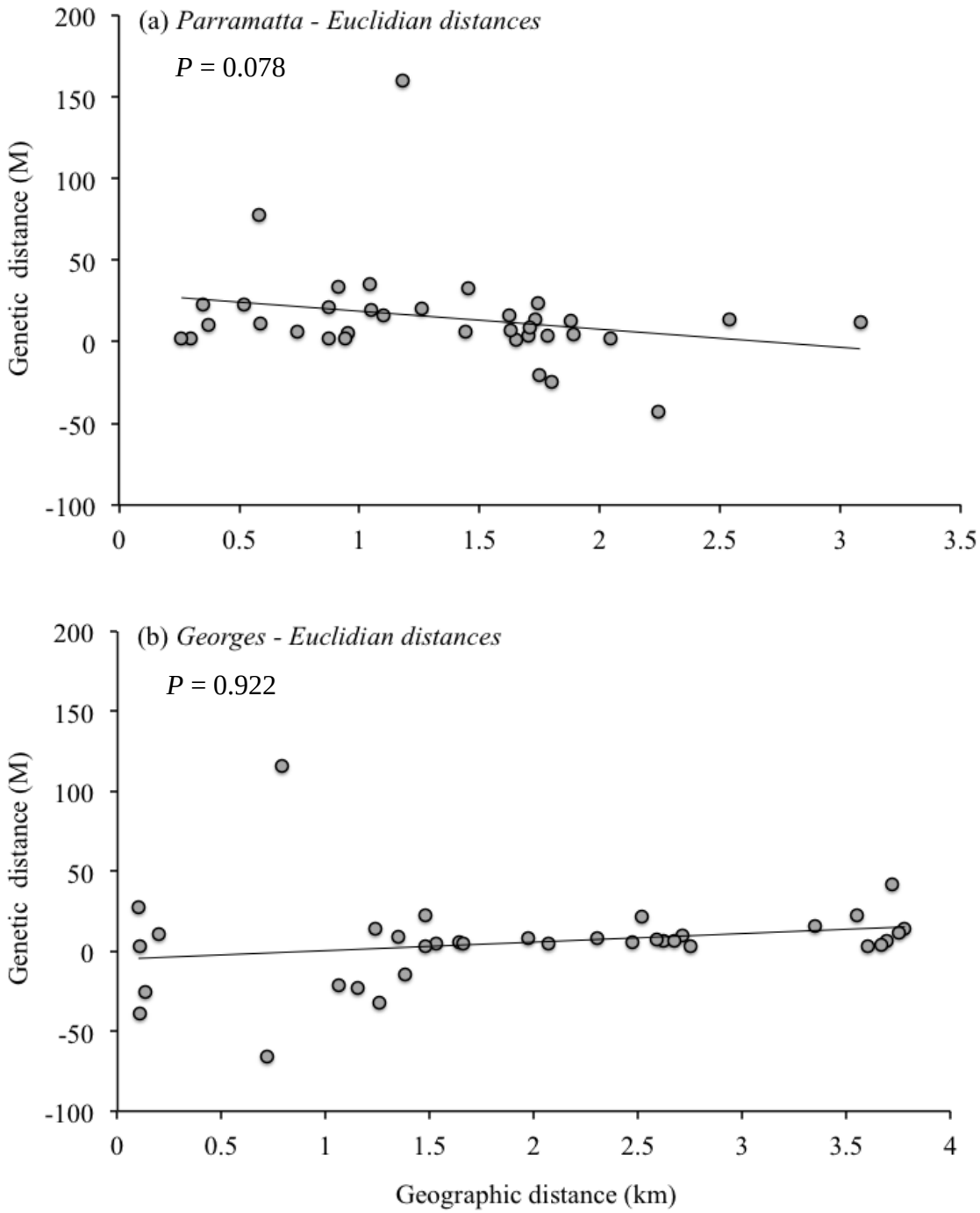
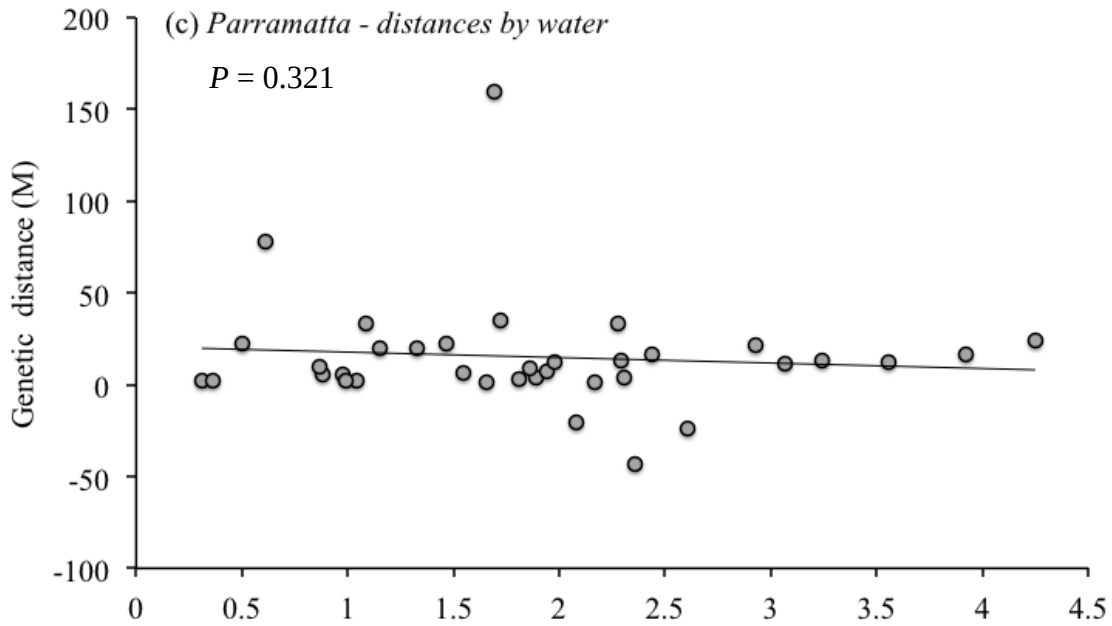


Fig. 5.3. Pattern of Isolation by Distance revealed by comparison of genetic distances and geographic distances. (a) and (b) show the pair wise Euclidean distance (X axis) vs. genetic distance (Y axis) for (a) the nine stands of Parramatta River and (b) the nine stands of Georges River. (c) and (d) show the pair wise distance by water (X axis) vs. genetic distance (Y axis) for (c) the nine stands of Parramatta River and (d) the nine stands of Georges River.


(c) Parramatta - distances by water
P = 0.321
Genetic distance (M)
200
150
100
50
0
-50
-100
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5

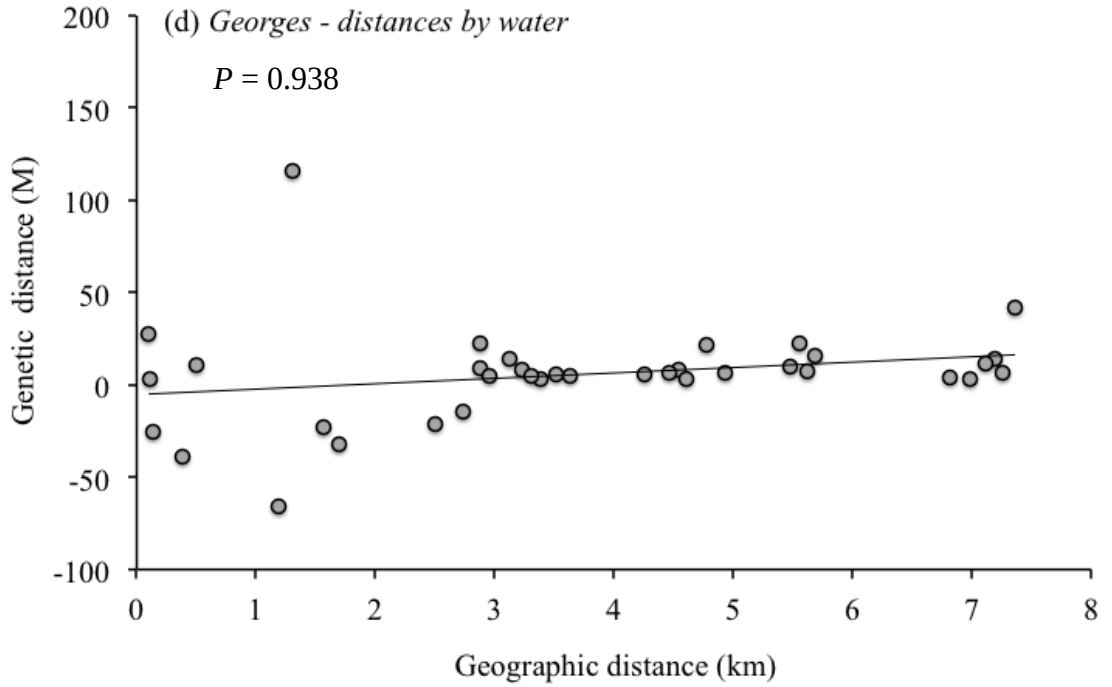

(d) Georges - distances by water
P = 0.938
Genetic distance (M)
200
150
100
50
0
-50
-100
0
1
2
3
4
5
6
7
8
Geographic distance (km)

Table 5.6. F_{IS} values of adult plants are shown for individual estuaries. F_{ST} is genetic variation among the 18 investigated estuaries.

Estuary	Stand Size	F_{IS} per stand
Parramatta	Large	0.079
		0.054
		0.082
	Medium	0.065
		0.111
		0.003
	Small	0.190
		0.085
		0.235
Georges	Large	-0.014
		0.140
		0.016
	Medium	-0.009
		0.150
		0.144
	Small	-0.055
		-0.002
		0.112
F_{ST}		0.050 ± 0.010

5.4.2 *Population Differentiation and isolation by distance*

The hierarchical survey of population differentiation revealed on average significant (P<0.01) but low variation between estuaries (F_{ET} = 0.055 ± 0.01) and among stands within estuaries (F_{SE} = 0.021 ± 0.002) (Table 5.5), and there was a high consistency among loci for both F_{ET} and F_{SE} (Table 5.5). F_{ST} among the 18 stands was 0.050 ± 0.01 (Table 5.6). The tests for isolation by distance described by Slatkin (1989, 1993) showed no significance of isolation by distance (*P*=0.078-0.938: see Fig. 5.3*a-d*). (Figure and Tanles above)

Table 5.7. Two factor ANOVA of the inbreeding coefficients (F_{IS}) of three large three medium and three small stands of each of Parramatta River and Georges River. Results are estimates of mean square (MS), F-ratio (F) and probability levels (*P*) of analysis of variance. Location (L) is a random factor while stand size (S) is a fixed factor.

Source	DF	MS	F	*P*
L	1	0.000	0.000	0.977
S	2	0.001	0.14	0.875
LxS	2	0.006	1.03	0.386
Res	12	0.006		
Transform = None				
Cochran's test = NS				

5.4.3 *Estimating genetic data of adult plants and siblings*

For individual adult stands, inbreeding coefficients (F_{IS}) ranged between -0.014 and 0.082 in large stands, between -0.009 and 0.144 in medium stands and between -0.002 and 0.235 in small stands, and the majority of F_{IS} values (14/18) were positive (Table 5.6). Compared to the average of the F_{IS} values of large stands, the average of the F_{IS} values of medium stands was 17% lower and the average of the F_{IS} values of small stands was 58%

higher on Parramatta River, while it was 51% higher in medium stands and 62% lower in small stands of Georges River (not shown in Table 5.6). The effect of stand size (F=0.14; P=0.875, $df_{2, 17}$), the effect of location (F<0.001; P=0.977, $df_{1, 17}$) and the interaction effect (F=1.03; P=0.386, $df_{2, 17}$) across the two estuaries were not significant for F_{IS} (Table 5.7). The overall F_{IS} value of the hierarchical F-statistics was 0.089 ± 0.03

Table 5.8. r_p = the correlation of paternity (fraction of siblings that share the same father). $1/r_p$ = the number of fathers. t_m-t_s = the level of biparental inbreeding. t_m = the level of multilocus outcrossing, in all cases from three large and three small stands from each of two estuaries P = Parramatta, G = Georges.

Estuary/Stand size	r_p	Fathers	t_m-t_s	t_m
Parramatta/large	0.904	1.106	0.516	0.885
	0.708	1.412	0.376	0.842
	0.950	1.053	0.461	0.842
Parramatta/small	0.999	1.001	0.357	0.601
	0.995	1.005	0.393	0.560
	0.999	1.001	0.447	0.721
Georges/large	0.992	1.008	0.322	0.600
	0.939	1.065	0.293	0.475
	0.999	1.001	0.525	0.885
Georges/small	0.995	1.005	0.311	0.444
	0.997	1.003	0.353	0.501
	0.969	1.032	0.299	0.445

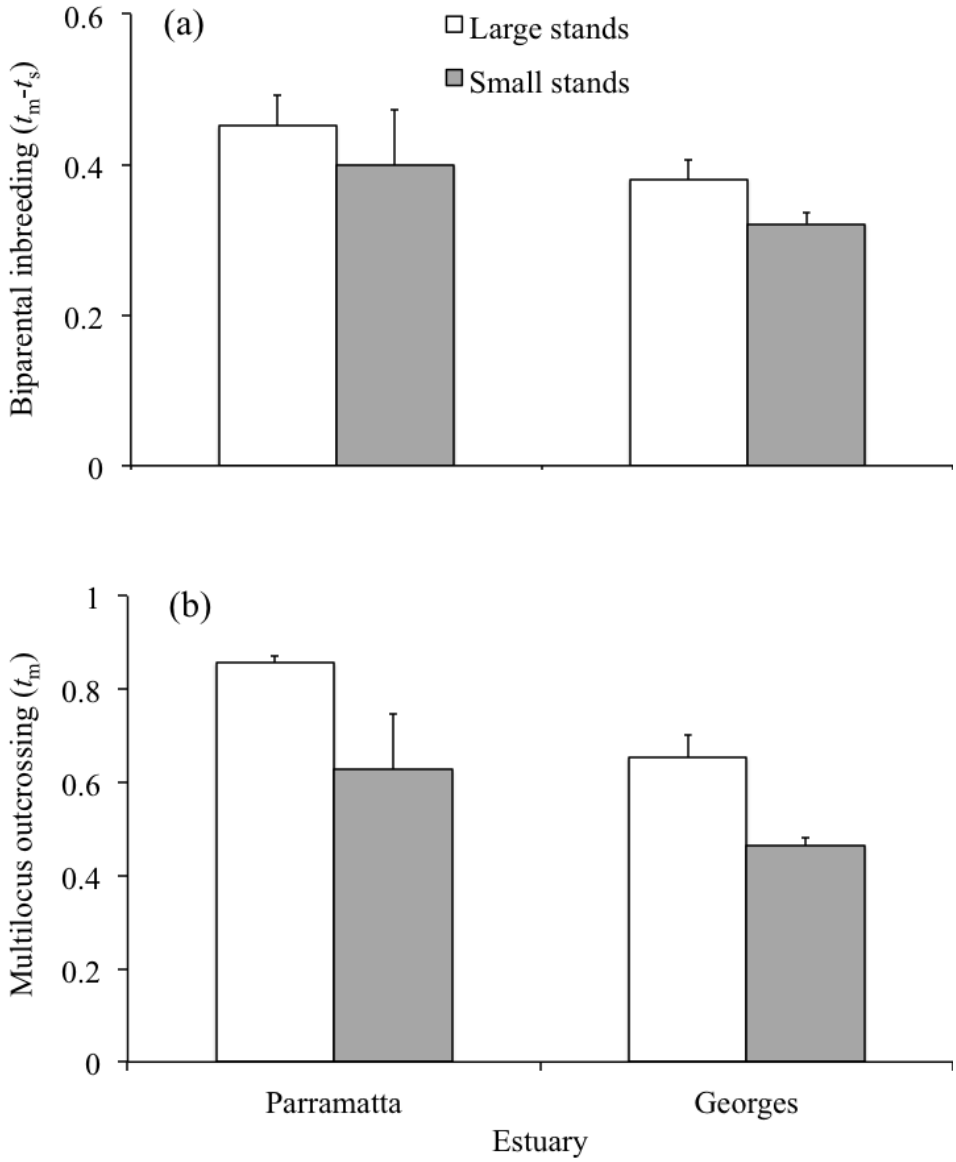


Fig. 5.4. Mean (± SE) of (a) t_m, the multilocus outcrossing rate and (b) t_m-t_s, the biparental inbreeding coefficient, in both cases measured by four microsatellite loci on 25 siblings from each of five trees from three large and three small stands from each of two estuaries.

(Table 5.6), suggesting that populations within these estuaries are slightly inbred. The variation of F_{IS} across the alleles was consistent among three alleles while F_{IS} was lower in the last allele (Table 5.5).

For individual stands, rates of biparental inbreeding (t_m-t_s) of outcrossed seed displayed values between 0.293 and 0.525 in large stands and between 0.299 and 0.447 in

small stands (Table 5.8). On Parramatta River the average value (of the three t_m-t_s values of small stands given in Table 5.8) was 12% lower than the average value of large stands (0.399 ± 0.03 vs. 0.451 ± 0.04), and on Georges River it was 16% lower (0.321 ± 0.02 vs. 0.380 ± 0.07) (Fig 5.4*a*). However, the effect of stand size (F=0.93; *P*=0.512; $df_{1, 11}$), the effect of location (F=0.06; *P*=0.808; $df_{1, 11}$) and the interaction effect (F=0.04; *P*=0.842; $df_{1, 11}$) across estuaries did not vary significantly for t_m-t_s (Table 5.9).

Table 5.9. Two factor ANOVA of biparental inbreeding (t_m-t_s) of three large three medium and three small stands of each of Parramatta River and Georges River. Results are estimates of mean square (MS), F-ratio (F) and probability levels (*P*) of analysis of variance. Location (L) is a random factor while stand size (S) is a fixed factor.

Source	d.f.	MS	F	*P*
L	1	0.004	0.06	0.808
S	1	0.002	0.93	0.512
LxS	1	0.002	0.04	0.842
Res	8	0.056		
Transform = None				
Cochran's Test = NS				

Further, for individual stands, rates of multilocus outcrossing (t_m) of outcrossed seed displayed values between 0.475 and 0.885 in large stands and between 0.444 and 0.721 in small stands (Table 5.8). On Parramatta River the average value (of the three t_m values of small stands given in table 5.8) was 27% lower than the average value of large stands (0.627 ± 0.05 vs. 0.856 ± 0.01), and on Georges River it was 29% lower (0.463 ± 0.02 vs. 0.653 ± 0.12) (Fig 5.4*b*). The effect of stand size across estuaries was significant (F=5516.44; *P*=0.009; $df_{1, 11}$), while the effect of location (F=0.22; *P*=0.650; $df_{1, 11}$) and the interaction effect (F<0.001; *P*=0.994; $df_{1, 11}$) were not (Fig. 5.4*b*; Table 5.10). Finally,

correlated paternity was high in all stands (r_p=0.708-0.999) (Table 5.8) and did not vary significantly with stand size (F=0.04; *P*=0.853; $df_{1, 11}$) or among locations (F=0.02; *P*=0.898; $df_{1, 11}$) and the interaction effect was not significant (F=0.03; *P*=0.876; $df_{1, 11}$) (Table 5.11). Using r_p values to estimate the number of sires per progeny array unsurprisingly yielded values between 1.001 and 1.412 (Table 5.8), indicating the participation of one to two fathers in generating each progeny array.

Table 5.10. Two factor ANOVA of multilocus outcrossing (t_m) of three large three medium and three small stands of each of Parramatta River and Georges River. Results are estimates of mean square (MS), F-ratio (F) and probability levels (*P*) of analysis of variance. Location (L) is a random factor while stand size (S) is a fixed factor.

Source	d.f.	MS	F	*P*
L	1	0.036	0.22	0.650
S	1	0.056	5516.44	0.009*
LxS	1	0.000	0.00	0.994
Res	8	0.163		
Transform = None				
Cochran's Test = NS				
SNK for S: Large>Small				

Table 5.11. Two factor ANOVA of correlated paternity (r_p) of three large three medium and three small stands of each of Parramatta River and Georges River. Results are estimates of mean square (MS), F-ratio (F) and probability levels (*P*) of analysis of variance. Location (L) is a random factor while stand size (S) is a fixed factor.

Source	DF	MS	F	*P*
L	1	0.005	0.02	0.898
S	1	0.011	0.04	0.853
LxS	1	0.008	0.03	0.876
Res	8	0.303		
Transform = None				
Cochran's Test = NS				

5.5 Discussion

Anthropogenic impacts in urban estuaries contribute to the fragmentation of mangroves (West *et al*. 1985), and in the region of Sydney in Australia *Avicennia marina* often comprises small stands (<50 adult trees) with changed pollinator abundance and services as compared to large stands (>1500 adult trees) (chapter 3 and 4). Based on this I predicted that, even with a disrupted pollination system that is dominated by *Apis mellifera* (chapter 2) and potential isolating effects of surrounding aquatic habitat, genetic diversity and outcrossing rates in temperate estuarine stands of *A. marina* like their terrestrial counterparts (Hall *et al*. 1998; Collinge 2009), would be inversely related to stand size. I also predicted that because the *A. marina* plants are self-compatible and surrounded by an aquatic matrix they might have experienced genetic sub division and isolation by distance.

This study, using variation at microsatellite loci, did not reveal any important effects of stand size on genetic diversity of adult trees. Genetic variation between estuaries (F_{ET}=0.055) and among stands (F_{SE}=0.021) was significant but low, and there was no significant effect of isolation by distance. The overall F_{IS} value of the hierarchical test was 0.089 suggesting that populations within these estuaries are only slightly inbred. My results on adult plants are similar to those of Melville and Burchett (2002) that found no important variation in allele frequency among neighbourhoods within estuaries. Further, the pattern of population differentiation and inferred dispersal in the present study is similar to that reported by Homer (2009), who found a high level of gene flow among 11 stands (scattered over 165 km in northern NSW) of a less urban mangrove population, with an average F_{ST} value of 0.065.

The analysis of progeny arrays revealed that current mating systems involve only moderate outcrossing and outcrossing rates (t_m) are significantly higher in large rather than small stands. They also involve a similar rate of biparental inbreeding (t_m-t_s) due to a similar number of fruit with near related parents in all stands.

My results imply that genotype frequencies of adult plants with similar moderate levels of inbreeding may have contributed to the development of all stands regardless of size, while analysis of the progeny arrays revealed that while all stands displayed evidence of high levels of biparental inbreeding (which may be caused by greater transfer of pollen from near related plants), large stands were more highly outcrossed than small stands (which may be caused by lower numbers of potential mates in small stands).

5.5.1 *Historic vs. contemporary fragmentation*

Although my data imply that the inter-stand dispersal of seed and/or pollen is sufficient to effectively homogenise allele frequencies within estuaries and ensure that levels of genetic diversity do not vary with stand size, this may reflect the dynamic nature and history of these populations. In the study by Llorens *et al*. (2004) on fragmented populations of the endangered shrub *Grevillea caleyi* of Sydney using Amplified Fragment Length Polymorphism (AFLP) markers genetic differentiation among ridges only contributed with 14% (P=0.062) while among populations within ridges contributed with 52% (P<0.001) of the total differentiation which is in contrast to my results, while genetic diversity did not vary within population size or with degree of isolation as in my study. Although ancient barriers to gene flow among nearby ridges were found in that study, fragmentation has yet to impact genetically upon the remaining populations as is the case in my study. A reasonable explanation for the maintenance of diversity in adult

trees of my study may be the same as in the study by Llorens *et al*. (2004), that since occurrence of fragmentation there may have been insufficient generations of *A. marina* trees within the stands to allow the loss of diversity.

As genetic drift depends on the number of generations a population remains small (Barrett & Kohn 1991), a consequence of sufficient generations within small stands of my study may together with the consequence of significantly lower rates of multilocus outcrossing in small stands (namely higher levels of inbreeding) be genetic isolation and drift. Such future prospects may make populations of temperate *A. marina* suitable for future management strategies

5.5.2 *Low genetic variation implies that stands are strongly connected*

In my study although I detected statistically significant variation in adult allele frequencies between estuaries and among stands within estuaries the variation was small and I did not detect any significant effect of stand size on genetic diversity and variation or any significant effect of isolation by distance. Our results suggest a high connectedness among stands in the adult data set, which is in accordance with results from terrestrial studies (Mathiasen *et al*. 2007; Leimu & Mutikainen 2005). The lack of differentiation of stands is likely explained by high levels of dispersal of pollen or propagules, which would act to oppose the effects of genetic drift (Lihoreau *et al*. 2012; Ribbands 1951). This level of connectedness likely reflects the fact that all stands were isolated by <900 m (Euclidean distances) or <1 km (following water courses) from the nearest neighbouring stand.

It is possible that honeybees foraging habits also may influence on the high level of connectedness among the investigated stands. Honeybees were introduced to Australia

in 1823 (The Western Mail 1823) and the investigated stands were developed during the last century, why it is likely that honeybees also were main pollinators of temperate *A. marina* when these stands were established. Pahl *et al*. (2011) found that honeybees could travel 11 km to a foraging destination during a foraging bout and Von Frisch (1967) reported that honeybees could travel 13.5 km on a foraging bout. The shortest distance between Parramatta and Georges River in Euclidean distance (Between Homebush Bay of Parramatta River and Salt Pan Creek of Georges River) is ca. 10.5 km, which makes it possible for honeybees to travel (and disperse pollen) between the two investigated estuaries, so honeybees might be involved in keeping a high level of gene flow among the stands. However, given the nature of the matrix, and the disturbed airflow associated with major roads it might be that there is little difference in pollen movement between Parramatta River and Georges River". Further, it might be that native insect pollinators were involved in pollen transfer among stands, of which many species disperse pollen over distances >1 km (Chifflet *et al*. 2011; Pasquet *et al*. 2008) (how pollinators react to the matrix in which mangroves are situated though is not known).

Effective dispersal of propagules may have been an important factor of gene flow among stands. Homer (2009) found that *A. marina* propagules sank after collection but refloated after 11 days, and kept floating for at least six weeks after that, making it possible for the propagules to disperse over long distances. Clarke (1993) showed that propagules dispersed effectively by up to 1 km and few by up to 10 km of a temperate estuary in Sydney, and Minchinton (2006) found that propagules dispersed up to 20 km along the coast of New South Wales (just south of Sydney) and stranded propagules were shown to be viable. This makes it possible that propagules disperse effectively over larger

distances within estuaries where the water currents are greater caused by tidal movements in and out of the estuaries.

The small distances between stands may have increased the possibility of effective dispersal of pollen and propagules in the investigated estuaries, resulting in increased gene flow among the stands, and the pool of genes within the individual stands may be combined by genes from various stands within the estuaries. Such cases are also known from terrestrial populations (e.g. Mathiasen *et al*. 2007), where small distances among stands tends to allow dispersal of genetic material among populations or stands and reduce genetic drift (Collinge 2009).

5.5.3 *Changing mating systems provides future avenues for management*

My examination of genotype frequencies of adult stands revealed significant but small deficits of heterozygotes (F_{IS}=0.089) with no significant effects of stand size. Given the low spatial variation the deficits of heterozygotes may be due to mixed mating with low levels of selfing or biparental inbreeding. Further, heterozygote deficit caused by a Wahlund effect (Wahlund 1928; Li 1955) are expected within a genetically heterogeneous population. The magnitude of the deficit should equal the variation in allele frequencies [i.e., (Sigma)((Sigma).sup.2) = (H.sub.e) – (H.sub.o), in which (H.sub.e) is the proportion of heterozygotes expected within a panmictic population, and (H.sub.o) is the average proportion of heterozygotes observed within collections; Li 1976]. In the present study the high levels of allelic variation could explain 22% ± 8% of the observed deficits of heterozygotes.

For the current mating system the multilocus outcrossing rate (t_m) was significantly lower (24%) in small stands (as compared to large stands), showing a

significant effect of stand size on the progeny arrays of the investigated system. This effect may be related to a significantly lower abundance and changed foraging behaviour by pollinators in small stands, from the only effective pollinator of temperate *A. marina* today, the exotic honeybee *Apis mellifera* (chapter 2), resulting in higher levels of inbreeding in small stands (Ghazoul 2005). In terrestrial plants such effects may result in reduced fruit quality (Aizen 2002; Ghazoul 2005; Aguilar 2006) as I have demonstrated for temperate *A. marina* in a previous study (chapter 4).

In all stands the correlation of paternity was extremely high (r_p = 0.708-0.999), suggesting that most progeny were produced from near related parents sharing the paternal and maternal genitors, and estimation of the number of fathers ($1/r_p$) was slightly higher than one in all cases (1.001-1.412), indicating the contribution of one to two fathers within each stand, which is congruent with data from terrestrial plants (Alves *et al*. 2003). Because of this and because of the geitonogamous self-compatible nature of temperate *A. marina* (Clarke & Myerscough 1991) and the way which honeybees move around during foraging bouts within small patches of trees of near vicinity of each other (Paton 1993; Whelan *et al*. 2009) within individual stands of temperate *A. marina*, it is not surprising that rates of biparental inbreeding (t_m-t_s) of progeny arrays were high in all stands with no effect of stand size. Such influences may affect the fruit production and quality negatively. However, because mangroves are naturally fragmented (West *et al*. 1985; Duke 2006) mangroves seem to have developed greater resistance against inbreeding than terrestrial trees, which may give them a greater potential to resist such impact.

A factor that may have influenced the adult data is the fact that the investigated stands of temperate *A. marina* have been formed by colonization on mudflats along the

riverbanks during the last century (Thorogood 1985; Dunstan 1990; McLoughlin 2000; and personal investigations of aerial photos). The significantly lower multilocus outcrossing rates in progeny arrays of small stands, may be caused by significantly reduced pollinator abundance and changed foraging behaviour by pollinators in small stands (chapter 3 and 4). This may again result in significantly lower fruit quality and production within small stands as compared to large stands (chapter 4). Such an effect is congruent with results from terrestrial forests (Aizen *et al*. 2001; Ghazoul 2005; Aguilar *et al*. 2006).

5.5.4 *Conclusion*

In the present study the data from adult plants showed no significant effects of stand size on levels of genetic diversity, differentiation and inbreeding, and no significant isolation by distance and low levels of inbreeding were detected among stand regardless of size. These results suggest a strong connectedness among stands with no important consequences of anthropogenic fragmentation on the adult mating systems, which may be caused by sufficient gene flow among stands regardless of size due to effective dispersal of pollen and propagules. The current mating systems however, showed high levels of biparental inbreeding within all stands which may be caused by pollen transfers within plants or among near neighbouring plants of stands regardless of size, while significantly reduced levels of multilocus outcrossing, also in the current mating systems of the small stands, may be due to the effect of localized foraging by honeybees (chapter 3) and the smaller number of available mates within the small stands. Our results indicate that gene flow among stands regardless of size serve to contain a high connectedness among the investigated stands, while a higher degree of localized foraging by pollinators within

small stands serve to significantly reduce the level of outcrossing in these. Which forces are strongest may be an aim for future research to reveal.

Appendix 5.1. Shows the Euclidean distances and the distances by water between stands of the same estuary and between stands of the two estuaries. P = Parramatta and G = Georges. The stands 1-9 are equivalent to the stands of same number in Fig. 5.1.

Euclidean distance (km)

	P-1	P-2	P-3	P-4	P-5	P-6	P-7	P-8	P-9	G-1	G-2	G-3	G-4	G-5	G-6	G-7	G-8
P-2	1.73																
P-3	2.54	0.52															
P-4	1.26	1.05	1.88														
P-5	1.89	0.74	1.44	0.58													
P-6	3.08	1.04	0.37	2.24	1.71												
P-7	1.65	0.87	1.70	0.30	0.26	2.04											
P-8	1.45	0.35	1.18	0.91	0.95	1.62	0.94										
P-9	1.63	0.59	1.10	1.75	1.80	1.74	1.78	0.87									
G-1	20.24	18.69	18.13	20.22	19.91	18.05	20.07	19.30	18.67								
G-2	20.87	19.41	18.87	20.94	20.66	18.81	20.81	20.01	19.35	1.24							
G-3	20.98	19.57	18.94	20.97	20.72	18.89	20.85	20.03	19.35	1.97	0.72						
G-4	20.41	18.76	18.16	20.28	19.93	18.04	20.10	19.37	18.79	1.53	2.59	3.35					
G-5	21.82	20.38	19.85	21.91	21.64	19.82	21.78	20.97	20.48	2.30	1.06	0.79	3.60				
G-6	22.25	20.83	20.30	22.36	22.09	20.25	22.23	21.42	20.83	2.62	1.48	1.26	3.72	0.20			
G.7	20.54	18.89	18.31	20.42	20.07	18.18	20.25	19.52	18.95	1.64	2.71	3.55	0.13	3.67	3.78		
G-8	20.06	18.49	17.93	20.01	19.71	17.85	19.88	19.10	18.47	0.11	1.35	2.07	1.48	2.47	2.75	1.66	
G-9	22.14	20.71	20.19	22.25	21.98	20.15	22.12	21.32	20.74	2.52	1.38	1.15	3.69	0.11	0.10	3.75	2.67

Distances by water (km)

	P-1	P-2	P-3	P-4	P-5	P-6	P-7	P-8	P-9	G-1	G-2	G-3	G-4	G-5	G-6	G-7	G-8
P-2	2.29																
P-3	3.24	1.47															
P-4	1.33	1.15	1.98														
P-5	1.89	0.88	1.55	0.61													
P-6	3.56	1.72	0.87	2.36	1.86												
P-7	1.66	1.04	1.81	0.31	0.36	2.17											
P-8	2.28	0.50	1.69	1.09	0.98	2.44	0.99										
P-9	1.94	3.07	3.92	2.08	2.61	4.25	2.31	2.93									
G-1	58.85	57.16	56.39	57.35	56.71	56.35	56.85	56.81	59.21								
G-2	60.89	59.18	58.38	59.33	58.69	58.33	58.84	58.79	61.21	3.13							
G-3	60.99	59.27	58.49	59.44	58.81	58.44	58.94	58.89	61.29	3.23	1.19						
G-4	57.83	56.06	55.26	56.22	55.59	55.22	55.74	55.70	58.07	3.63	5.62	5.69					
G-5	62.29	60.55	59.77	60.73	60.08	59.72	60.22	60.17	62.56	4.54	2.50	1.30	6.98				
G-6	62.69	60.80	60.17	61.14	60.49	60.12	60.61	60.57	62.96	4.93	2.88	1.69	7.36	0.50			
G.7	57.69	55.91	55.12	56.07	55.45	55.08	55.60	55.56	57.94	3.51	5.47	5.55	0.13	6.82	7.19		
G-8	58.70	56.99	56.19	57.16	56.52	56.15	56.64	56.61	59.01	0.11	2.88	2.96	3.38	4.25	4.61	3.31	
G-9	62.57	60.85	60.05	61.02	60.37	60.01	60.50	60.45	62.84	4.77	2.74	1.57	7.25	0.38	0.10	7.11	4.46

Appendix 5.2. Allele frequencies for each stand for each locus (Am32, Am49, Am81, Am98). Sample size for each fragment is indicated by "n". A = Allele. P refers to Parramatta River and G refers to Georges River. The individual stands are numbered 1-18.

	Large			Medium			Small			Large			Medium			Small		
A/n	P-1	P-2	P-3	P-4	P-5	P-6	P-7	P-8	P-9	G-10	G-11	G-12	G-13	G-14	G-15	G-16	G-17	G-18
Am32/n	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
149	0.100	0.025	0.025	0.025	0.025	0.025	0.000	0.000	0.000	0.025	0.025	0.000	0.000	0.025	0.000	0.050	0.000	0.025
151	0.000	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.000	0.000	0.000	0.000	0.000
153	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
155	0.075	0.075	0.150	0.225	0.325	0.125	0.500	0.125	0.350	0.025	0.125	0.200	0.225	0.100	0.175	0.125	0.000	0.100
157	0.825	0.800	0.750	0.750	0.625	0.800	0.500	0.850	0.600	0.950	0.775	0.800	0.775	0.825	0.825	0.825	0.725	0.825
159	0.000	0.050	0.075	0.000	0.025	0.025	0.000	0.025	0.025	0.000	0.050	0.000	0.000	0.050	0.000	0.000	0.275	0.050
161	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Am49/n	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
162	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.025	0.025	0.000	0.000	0.050	0.050	0.000	0.000	0.050	0.000	0.025
164	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.000	0.000	0.000	0.000
168	0.000	0.000	0.025	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
175	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.000	0.000	0.000	0.000	0.000
177	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.125	0.050	0.000	0.075	0.050	0.000	0.025	0.225	0.000
180	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
182	0.175	0.000	0.100	0.075	0.000	0.125	0.000	0.025	0.000	0.000	0.000	0.000	0.050	0.025	0.000	0.075	0.000	0.050
184	0.075	0.075	0.150	0.025	0.025	0.000	0.025	0.100	0.075	0.025	0.025	0.050	0.025	0.000	0.000	0.050	0.000	0.075
186	0.125	0.075	0.025	0.100	0.000	0.025	0.025	0.100	0.125	0.175	0.225	0.300	0.100	0.350	0.350	0.050	0.150	0.175
188	0.000	0.000	0.000	0.025	0.000	0.000	0.025	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.000	0.000	0.000
190	0.000	0.025	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.025	0.000	0.025	0.000	0.025
192	0.000	0.025	0.125	0.075	0.200	0.125	0.050	0.075	0.100	0.100	0.050	0.200	0.375	0.025	0.275	0.325	0.100	0.125
194	0.000	0.025	0.000	0.050	0.025	0.025	0.200	0.100	0.025	0.000	0.025	0.000	0.000	0.000	0.000	0.000	0.000	0.000
196	0.000	0.050	0.000	0.000	0.025	0.000	0.025	0.000	0.025	0.100	0.000	0.000	0.025	0.000	0.000	0.050	0.050	0.000
198	0.050	0.000	0.025	0.025	0.000	0.025	0.000	0.000	0.000	0.025	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.000

200	0.000	0.000	0.000	0.075	0.025	0.000	0.000	0.000	0.025	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.000
202	0.000	0.000	0.050	0.050	0.025	0.050	0.075	0.075	0.050	0.000	0.075	0.025	0.025	0.075	0.025	0.075	0.050	0.025
204	0.000	0.000	0.025	0.000	0.000	0.000	0.025	0.000	0.025	0.000	0.050	0.000	0.000	0.025	0.025	0.000	0.000	0.050
206	0.125	0.350	0.100	0.225	0.300	0.275	0.075	0.100	0.200	0.125	0.150	0.125	0.150	0.150	0.075	0.175	0.275	0.075
208	0.075	0.125	0.025	0.150	0.050	0.250	0.075	0.050	0.150	0.275	0.100	0.100	0.100	0.100	0.075	0.050	0.025	0.200
210	0.100	0.025	0.050	0.025	0.075	0.050	0.000	0.075	0.025	0.050	0.125	0.025	0.000	0.125	0.050	0.000	0.025	0.075
212	0.075	0.100	0.200	0.050	0.125	0.050	0.150	0.050	0.075	0.000	0.025	0.050	0.000	0.000	0.025	0.025	0.050	0.050
214	0.175	0.075	0.000	0.025	0.025	0.000	0.125	0.050	0.025	0.000	0.025	0.025	0.000	0.025	0.000	0.025	0.000	0.000
216	0.000	0.000	0.025	0.025	0.050	0.000	0.125	0.150	0.050	0.000	0.025	0.025	0.000	0.025	0.050	0.000	0.000	0.050
218	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.050	0.000
219	0.025	0.025	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
223	0.000	0.000	0.075	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
225	0.000	0.025	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Am81/n	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
129	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.025	0.000	0.000	0.000	0.000	0.000
133	0.050	0.025	0.000	0.000	0.050	0.025	0.000	0.025	0.025	0.025	0.075	0.100	0.050	0.050	0.050	0.100	0.175	0.125
137	0.900	0.875	0.925	0.950	0.950	0.975	0.950	0.975	0.975	0.975	0.775	0.900	0.925	0.925	0.925	0.900	0.825	0.825
139	0.000	0.100	0.075	0.000	0.000	0.000	0.050	0.000	0.000	0.000	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.050
148	0.050	0.000	0.000	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
153	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.075	0.000	0.000	0.025	0.025	0.000	0.000	0.000
Am98/n	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
206	0.875	0.775	0.750	0.925	0.950	0.850	0.525	0.775	0.875	0.875	0.900	0.925	0.775	0.975	0.800	0.850	0.900	0.950
209	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.050	0.000
212	0.125	0.225	0.250	0.075	0.050	0.150	0.475	0.225	0.125	0.125	0.100	0.075	0.225	0.025	0.200	0.150	0.050	0.050

Appendix 5.3. Shows the stage of linkage equilibrium between pairs of microsatellite locus within individual stands and pairs in disequilibrium are marked with asterisk; * indicates $P < 0.05$ and ** indicates $P < 0.01$. Pop 1-9 are stands of Parramatta River and Pop 10-18 are stands of Georges River. Pop 1-3 and 10-12 are large stands, Pop 4-6 and 13-15 are medium stands and Pop 7-9 and 16-18 are small stands.

Population	Locus A	Locus B	*P*-Value	S.E.
Pop 1	Locus 1	Locus 2	1.000	0.000
	Locus 1	Locus 3	0.089	0.003
	Locus 2	Locus 3	0.891	0.011
	Locus 1	Locus 4	0.454	0.006
	Locus 2	Locus 4	0.394	0.016
	Locus 3	Locus 4	0.621	0.008
Pop 2	Locus 1	Locus 2	0.775	0.027
	Locus 1	Locus 3	0.377	0.012
	Locus 2	Locus 3	0.953	0.007
	Locus 1	Locus 4	0.752	0.004
	Locus 2	Locus 4	1.000	0.000
	Locus 3	Locus 4	0.591	0.003
Pop 3	Locus 1	Locus 2	0.238	0.020
	Locus 1	Locus 3	0.355	0.005
	Locus 2	Locus 3	0.264	0.009
	Locus 1	Locus 4	0.540	0.007
	Locus 2	Locus 4	0.846	0.011
	Locus 3	Locus 4	0.295	0.003
Pop 4	Locus 1	Locus 2	0.524	0.017
	Locus 1	Locus 3	0.455	0.006
	Locus 2	Locus 3	1.000	0.000
	Locus 1	Locus 4	0.016*	0.001
	Locus 2	Locus 4	0.218	0.008
	Locus 3	Locus 4	1.000	0.000
Pop 5	Locus 1	Locus 2	0.979	0.005
	Locus 1	Locus 3	0.206	0.005
	Locus 2	Locus 3	0.506	0.010
	Locus 1	Locus 4	0.202	0.004
	Locus 2	Locus 4	0.474	0.010
	Locus 3	Locus 4	1.000	0.000
Pop 6	Locus 1	Locus 2	0.948	0.010
	Locus 1	Locus 3	1.000	0.000
	Locus 2	Locus 3	0.494	0.012

	Locus 1	Locus 4	0.063	0.002
	Locus 2	Locus 4	0.177	0.006
	Locus 3	Locus 4	0.302	0.002
Pop 7	Locus 1	Locus 2	0.385	0.013
	Locus 1	Locus 3	1.000	0.000
	Locus 2	Locus 3	1.000	0.000
	Locus 1	Locus 4	0.115	0.003
	Locus 2	Locus 4	0.001**	0.001
	Locus 3	Locus 4	0.299	0.002
Pop 8	Locus 1	Locus 2	0.531	0.021
	Locus 1	Locus 3	1.000	0.000
	Locus 2	Locus 3	0.902	0.007
	Locus 1	Locus 4	0.418	0.006
	Locus 2	Locus 4	1.000	0.000
	Locus 3	Locus 4	0.354	0.004
Pop 9	Locus 1	Locus 2	0.689	0.023
	Locus 1	Locus 3	1.000	0.000
	Locus 2	Locus 3	0.694	0.011
	Locus 1	Locus 4	0.655	0.008
	Locus 2	Locus 4	0.731	0.014
	Locus 3	Locus 4	1.000	0.000
Pop 10	Locus 1	Locus 2	0.587	0.015
	Locus 1	Locus 3	1.000	0.000
	Locus 2	Locus 3	0.456	0.009
	Locus 1	Locus 4	1.000	0.000
	Locus 2	Locus 4	0.925	0.004
	Locus 3	Locus 4	1.000	0.000
pop 11	Locus 1	Locus 2	1.000	0.000
	Locus 1	Locus 3	0.482	0.019
	Locus 2	Locus 3	0.383	0.025
	Locus 1	Locus 4	0.201	0.006
	Locus 2	Locus 4	0.759	0.008
	Locus 3	Locus 4	1.000	0.000
Pop 12	Locus 1	Locus 2	1.000	0.000
	Locus 1	Locus 3	0.115	0.002
	Locus 2	Locus 3	0.107	0.005
	Locus 1	Locus 4	0.049*	0.001
	Locus 2	Locus 4	0.961	0.003
	Locus 3	Locus 4	0.581	0.001
Pop 13	Locus 1	Locus 2	0.938	0.003
	Locus 1	Locus 3	0.191	0.003
	Locus 2	Locus 3	0.445	0.015
	Locus 1	Locus 4	0.806	0.002
	Locus 2	Locus 4	0.051	0.005

	Locus 3	Locus 4	1.000	0.000
Pop 14	Locus 1	Locus 2	0.319	0.027
	Locus 1	Locus 3	0.680	0.010
	Locus 2	Locus 3	0.630	0.018
	Locus 1	Locus 4	1.000	0.000
	Locus 2	Locus 4	0.550	0.012
	Locus 3	Locus 4	1.000	0.000
Pop 15	Locus 1	Locus 2	1.000	0.000
	Locus 1	Locus 3	1.000	0.000
	Locus 2	Locus 3	0.259	0.015
	Locus 1	Locus 4	0.752	0.003
	Locus 2	Locus 4	0.803	0.011
	Locus 3	Locus 4	0.591	0.005
Pop 16	Locus 1	Locus 2	0.686	0.012
	Locus 1	Locus 3	0.378	0.003
	Locus 2	Locus 3	0.750	0.007
	Locus 1	Locus 4	1.000	0.000
	Locus 2	Locus 4	0.853	0.005
	Locus 3	Locus 4	0.550	0.002
Pop 17	Locus 1	Locus 2	0.302	0.006
	Locus 1	Locus 3	0.372	0.003
	Locus 2	Locus 3	0.163	0.005
	Locus 1	Locus 4	0.075	0.002
	Locus 2	Locus 4	0.170	0.009
	Locus 3	Locus 4	1.000	0.000
Pop 18	Locus 1	Locus 2	0.488	0.031
	Locus 1	Locus 3	0.157	0.008
	Locus 2	Locus 3	0.585	0.018
	Locus 1	Locus 4	0.155	0.005
	Locus 2	Locus 4	0.695	0.012
	Locus 3	Locus 4	1.000	0.000

Chapter 6: General discussion

6.1 *Introduction*

The present study is the first investigation into effects of stand size on mangrove, and more generally the effects of stand size on self-compatible plants are infrequently represented in the literature (Hobbs & Yates 2003). Surprisingly the study highlighted the similarity of mangroves and terrestrial forests including the dependence of temperate Australian populations of the mangrove *A. marina* on the exotic honeybee *Apis mellifera*.

Within fragmented populations of terrestrial plants that are typically pollinated by a broad suite of both specialized and generalised pollinators (Olesen & Jordano 2002), empirical evidence reveals anthropogenic fragmentation with reduced stand size, impacting insect pollinated plant species through reductions of pollinator diversity, abundance and services and changed foraging behaviour (Aizen *et al*. 2002; Ghazoul 2005; Aguilar *et al*. 2006; Collinge 2009). Fragmentation may therefore raise the level of inbreeding in small stands, resulting in reduced reproductive output which may lead to reduced fruit quality (Burd 1994; Waser *et al*. 1996; Larson & Barrett 2000; Aguilar 2006; Becker *et al*. 2011). Mangroves however, that are typically thought to be pollinated by a broad suite of generalized pollinators (Tomlinson 1086), are naturally fragmented and may through evolution have become tolerant to levels of inbreeding higher than those observed for most populations of terrestrial plants (e.g. Maguire *et al*. 2000*b*; Arnaud-Haond *et al*. 2006 vs. Rossetto *et al*. 2004; Butcher *et al*. 2005).

Because the number of pollinators in general is lower in temperate than tropical regions (e.g. Mishra *et al*. 2004; Smith-Ramirez *et al*. 2005) I expected that in the temperate, anthropogenically fragmented populations of *A. marina* on the southeast

coast of Australia I would find a lower number of generalized pollinator species (e.g. as Smith-Ramirez *et al*. 2005, that identified 2-60 and on average 20 pollinator species of each of 26 temperate plant species) pollinating the flowers of *A. marina*. I also expected to reveal reduced levels of multilocus outcrossing in small stands due to reduced pollinator diversity and abundance and changed foraging behaviour within the current mating systems of the small stands. I expected this to result in increased levels of inbreeding and reduced fruit quality and production in small stands, and I expected to find a significant differentiation among stands of the investigated system as it would be expected among stands of anthropogenic fragmented terrestrial plant populations, although I expected a considerable higher level of inbreeding in the mangroves caused by the naturally level of fragmentation in these (e.g. Duke 2006).

Surprisingly, I discovered that the level of biparental inbreeding of the current mating system was high in all stands regardless of size (chapter 5). This is most easily explained by the consistent intra-plant foraging behaviour of the exotic *A. mellifera*, which I found to be the only effective pollinator of temperate *A. marina* (chapter 2). The rate of multilocus outcrossing however was, however, significantly reduced in small stands as compared to large stands (chapter 5) which seems likely to reflect the reduced abundance and services of *A. mellifera* (chapter 3). Moreover, small stands displayed significantly reduced production and quality of fruit and seedlings (chapter 3 & 4). The level of inbreeding was significant but low in the adult mating systems and the inferred connectedness among stands of the investigated estuaries was high (chapter 5). The high connectedness among stands may be due to an insufficient number of generations to change the genetic structure of adult plants within individual stands of the investigated system (Reviewed by Loveless and Hamrick 1084).

6.2 *Why is this study unique?*

The real strength of the present study is the approach of combining well replicated surveys of pollinator activity and well replicated surveys testing the ecological consequences of the effect of stand size on pollination biology and reproductive output with well replicated genetic surveys. During the present study I investigated the effects of stand size on genetic variation and diversity of stands within estuaries and found the differentiation to be low (chapter 5), which appears to contrast with most other genetic studies on *A. marina* that have typically detected intermediate to high levels of population differentiation (Duke *et al*. 1998; Maguire *et al*. 2000*b*; Giang *et al*. 2003; Arnaud-Haond *et al*. 2006; Kahrood *et al*. 2008). However, direct comparison is different as sampling scales have been highly variable and the reduced genetic variation in my study may reflect the fact that my study was the only one that considered the differentiation of neighbouring stands of anthropogenically fragmented populations. The only studies that investigated genetic variation within estuaries besides my study was the study by Melville and Burchett (2002) who found low levels of genetic divergence within estuaries and the study by Melville *et al*. (2004) who found high levels of genetic divergence within estuaries.

6.3 *Standardization of research in pollination biology*

Some pollination studies are thoroughly performed (e.g. Rader *et al*. 2009; Micheneau *et al*. 2010) while others are not (e.g. Goodell *et al*. 2010; Griffin *et al*. 2009; Guzmán-Novoa *et al*. 2009), and the lack of standardization in research establishing pollinator plant associations' makes it difficult to draw any general conclusions based on comparisons between studies (Néeman *et al*. 2010). I emphasized (Chapter 2) that to be

able to compare pollination studies in general it is necessary to investigate both 1) body pollen loads down to body parts touching the stigma for individual species, 2) foraging behaviour estimated as what body parts of individual species that touches the stigma, and 3) how much pollen a pollinator removes from the anthers of a flower, and how many pollen grains it deposits on the stigma. In using this protocol (see chapter 2), it would be possible to compare pollination studies across studies and across species.

6.4 *Pollinators of temperate* A. marina

In general for terrestrial plants potential pollinator diversity is higher in tropical areas (Mishra et al. 2004) and lower in temperate areas (Smith-Ramirez *et al*. 2005), and I therefore predicted that this would also be the case for mangroves. However, if *Apis mellifera* is pollinator in tropical mangroves it is possible that it outcompetes a number of native pollinators in tropics as it did in the present study. In that case the difference in the diversity of pollinators in mangroves between tropical and temperate areas might be slight. In the present study I thoroughly investigated the pollination biology of temperate *A. marina* and found that of 38 species that acted as flower visitors, surprisingly, only the exotic *A. mellifera* was an effective pollinator (chapter 2), which is in contrast to the assumption that lots of generalized pollinators pollinate mangroves (Tomlinson 1986; Kathiresan & Bingham 2001). This is an aspect of pollination biology that *A. marina* has in common with lots of terrestrial plant species that are also dominated by *A. mellifera*, both in Australia (e.g. Paton 1993, 1996; Whelan *et al*. 2009; Lomov *et al*. 2010; Gross *et al*. 2010), and in other continents (e.g. Krend & Murphy 2003; Neves & Viana 2011; Dupont *et al*. 2004; Taha & Bayoumi 2009; Cayuela *et al*. 2011). Based on this the prediction that a diverse suite of generalized pollinators

typically pollinate mangroves (e.g. Tomlinson 1986; Kathiresan & Bingham 2001) needs further experimental support.

6.5 Apis mellifera *as dominating pollinator*

In Europe, US, New Zealand and other nations *A. mellifera* is becoming scarce because of a phenomenon called Colony Collapse Disorder (CCD) that makes honeybee workers desert from their hives (Glinski *et al*. 2012; Farooqui 2013). The main factors believed to be responsible for CCD is (1) the mite *Varroa destructor* that carries a virus that infects honeybees at all stages of development (Guzmán-Novoa *et al*. 2009) and suppresses honeybees hormonal and cellular immune responses (Glinski *et al*. 2012), and (2) biogenic amines-based pesticides resulting in olfactory dysfunction in honeybees (Farooqui 2013). If CCD is introduced to Australia consequences could be fatal for temperate *A. marina* that would loose its only effective pollinator, as it has happened for many terrestrial plant species in Europe and US (Guzmán-Novoa *et al*. 2009). If the native pollinators only are repressed, however, these may regain the position as dominant pollinators as it happened at the temperate Santa Cruz Island in California (Wenner & Thorp 1993). In such case the loss of *A. mellifera* would not have a harmful influence on *A. marina*, because this would lead to restoration of its historical mating system.

6.6 *Honeybees adapt their behaviour according to local conditions*

In terrestrial forests in Argentina Aizen & Feinsinger (1994*a*) showed that visits by honeybees were most consistent (‘varied the least among plants or over time’) in small stands. Aizen & Feinsinger (1994*a*) found that the total frequency of flower visits by

pollinators varied little with stand size, while the frequency of visits by native pollinators decreased and the frequency of visits by honeybees increased in small as compared to large stands, which is in contrast to the theory and many other observations (e.g. Aizen *et al*. 2002; Ghazoul 2005; Aguilar et al. 2006; Collinge 2009). Other experiments on the interactions between plants and bee-pollinators in fragmented plant populations have shown that reduced flower density may result in reduced flower visitation and allee effect (fitness), also in small as compared to large stands (reviewed by Ghazoul 2005), which may depend on the presence or absence of other species of flowering plants that could attract pollinators from or donate pollinators to a specific plant species (e.g. Feldman 2008; Ings *et al*. 2009). Finally, Menzel *et al*. (2005) concluded that honeybees can set course at any arbitrary location in their familiar area, and they can choose between at least two foraging sites. Pahl *et al*. (2011) found that honeybees use landmarks to recognize foraging sites in distances of up to 11 km from the hive and honeybees tend to choose the foraging site with the most distinct landmark. These results imply that honeybees most possible tend to return to the same foraging site (the one with the most distinct landmark), as I also concluded in the present study. Results like this indicate that honeybees in fragmented environments may adapt their behaviour according to local conditions to benefit maximally from their effort.

6.7 *What is the native pollinator(s) of temperate* Avicennia marina?

In the present study one or more native pollinators may have been displaced. This might be caused by the high density and the 'aggressive behaviour' of honeybees, which is known to result in omission of indigenous pollinator species (Reviewed by Goulson 2003). In temperate *A. marina* possible indigenous pollinators could be insects of the

order Hymenoptera that includes bees and wasps. Native bee species like *Lipotriches excellens* of which I only captured two individuals, and the eleven species of wasps I captured, of which nine species were rare, or other species of Hymenoptera that might be hampered by *A. mellifera*, could be indigenous pollinators. Other possible native pollinators could be beetles, flies or species of Lepidoptera that includes butterflies and moths. The above mentioned insects are all recognised as common pollinators of terrestrial plant species (e.g. Willmer 2011). However, most of the rare species (species of which less than five individuals were captured – see Table 2.2) I observed and captured in the present study did not touch the stigma during observation, but because only few of each species were identified, intensive behavioural studies were impossible on these, and might reveal behaviour typical for pollinators. Finally, because honeybees are far the most common species, and remove the most pollen from a flower during a single visit (see chapter 2), the native pollinator(s) could still be there, but without carrying pollen (because honeybees already have removed it), or alternatively, native pollinators may be hampered by *A. mellifera*.

6.8 *Ecological and genetic effects are congruent*

I found the data of my genetic surveys of the current mating systems to support the data of my surveys testing the ecological consequences of the effects of stand size on pollination biology and reproductive output. In the present study the current mating systems revealed profound impacts of stand size on multilocus outcrossing rates of the small stands (as compared with the large stands), which from terrestrial systems are known to lead to a variety of impacts of stand size, such as reduced viability and reduced fruit set and quality in small stands (Ghazoul 2005; Aguilar *et al*. 2006;

Collinge 2009), exactly as I found in the present study. Likewise, the profound impacts of stand size on outcrossing rates are known consequences of reduced pollinator density, visitation and pollen deposition in terrestrial plant species (Ghazoul 2005; Aguilar *et al*. 2006; Collinge 2009), which I also found during my field surveys.

6.9 *Ecological consequences of the effects of stand size*

To test for significant differences in the ecological response of *A. marina* between small and large stands I investigated the effects of stand size on different ecological factors, such as pollinator services and behaviour and fruit set and quality, and the consequences for the production of seedlings. I found significantly lower pollinator abundance, flower visitation and pollen deposition, resulting in significantly lower fruit set with significantly smaller fruit in small stands, and the number of seedlings produced in small stands was also significantly lower. Such series of events leading to reduced production of fruit and seedlings are also known from terrestrial forest plants (Steffan-Dewenter & Tscharntke 1999; Aguilar *et al*. 2006; Barbeta *et al*. 2011). These ecological incidents, leading stepwise from one to the next, emphasize that significantly reduced pollinator abundance may have profound consequences for the production and fitness of the resultant fruit and seedlings (Burd 1994; Waser *et al*. 1996; Larson & Barrett 2000; Becker *et al*. 2011; Barbeta *et al*. 2011) and as predicted from the theory for ecological impacts on fragmented terrestrial flowering plants (Aizen *et al*. 2002; Ghazoul 2005; Aguilar *et al*. 2006; Collinge 2009).

In terrestrial plants reduced pollinator services in small stands cause higher levels of inbreeding and lead to reduced fruit production and inbreeding depression that result in reduced fruit quality (Aizen *et al*. 2002; Ghazoul 2005; Aguilar *et al*. 2006).

This again has profound influence on the production of seedlings in terrestrial plants (Ghazoul & McLeish 2001; Ghazoul 2005; Barbeta *et al*. 2011). In the present study the number of fruit (propagules) was significantly reduced in small stands, resulting in the fewer propagules, which may be the cause why the number of propagules per m^2 of the forest floor was significantly reduced in small stands and may to a great extent explain the significantly lower number of seedlings produced in small stands (chapter 4). However, another mechanism that may influence this is that the juvenile seedlings gain their nutrients for growth from two cotyledons that may vary in size. When released from the propagule, a seedling uses these nutrients for growth until a sufficient root net has been established (Tomlinson 1986). The smaller the cotyledons are the slower the seedlings grow (e.g. Saxton *et al*. 1997; Minchinton 2006), making seedlings from small stands (with significantly smaller cotyledons) less fit for survival (coursed by slower growth) than those from large stands, which may also be the case in the present study (chapter 4). Individual stands may be dependent on non-genetic, demographic factors as well, influencing negatively on the production of seeds and recruitment of seedlings of small stands of fragmented populations with a varying impact between different years. For example, Morgan (1999) found a significant effect of stand size on seed production (a potential impact on the seedling recruitment) of the endangered flowering plant *Rutidosis leptorrhynchoides* in two out of three years while no simple variation among stand sizes was found the third year. This emphasizes the importance of making long-term monitoring (over a number of years) of effects of fragmentation on fitness and reproductive capability of flowering plants. My observations of pollinators and pollinator services and my investigations of reproduction of temperate *A. marina*

were done over multiple seasons why the spatial and temporal replication of my study was great.

6.10 *Effect of stand size on outcrossing rates*

In the present study the adult mating systems revealed a strong connectedness among stands of all sizes while the effect of stand size on outcrossing rates of the current mating systems was significant (chapter 5). This is congruent to results from some fragmented terrestrial populations (e.g. Butcher *et al*. 2005), and can be explained by significantly reduced pollinator abundance and services (Aizen *et al*. 2002; Ghazoul 2005; Aguilar *et al*. 2006; chapter 3 and 4). In the present study the level of biparental inbreeding of the current mating system was equal high in all investigated stands (chapter 5), which may be explained by the foraging habits of *A. mellifera* (foraging within narrow surroundings for longer periods; Paton 1993; Whelan *et al*. 2009; chapters 2 and 3). In many terrestrial plants such impacts affect the fruit production and lead to inbreeding depression (Lennartsson 2002; Aizen *et al*. 2002), resulting in lower fruit quality and higher rates of fruit abortion (Ramsey & Vaughton 1996; Hauser & Siegismund 2000), which is equivalent to my findings (chapter 4). Influences like this may by time (over generations) alter the genetic constellation and lead to sub-division among stands (Reviewed by Loveless & Hamrick 1084) and reduce the viability of small stands (Burd 1994; Waser *et al*. 1996; Larson & Barrett 2000; Becker *et al*. 2011), making them more sensitive to environmental impacts from fragmentation. Such influences make temperate *A. marina* suitable for future management strategies.

6.11 *Conclusion*

The finding that *A. mellifera* is the only effective pollinator of temperate *A. marina* together with other field surveys of the life cycle of temperate *A. marina* from pollination to the production of seedlings of stands of varying size (chapter 3 & 4) and genetic surveys of the structure and mating systems also of stands of varying size (chapter 5), allow important comparisons of mangroves and terrestrial forests with respect to both the impact of *A. mellifera* and the consequence of existing small often anthropogenically generated stands of fragmented populations (e.g. see Table 2, 3 & 7). During these surveys I revealed, as also observed in many terrestrial plant species (e.g. Jennersten 1988; Aizen & Feinsinger 1994*ab*; Murcia 1996; Aizen 1998; Steffan-Dewenter & Tscharntke 1999; Ghazoul & McLeish 2001), that small stands experienced lower pollinator abundance, visitation and pollen deposition and altered pollinator behaviour (chapter 3 and 4), promoting higher levels of self-pollination or inbreeding (chapter 3 and 5), and in consequence displayed reduced fruit production and quality and reduced production of seedlings as compared to large stands (chapter 4). Moreover, the genetic survey revealed high levels of biparental inbreeding in all stands regardless of size (chapter 5) which can be explained by *A. mellifera's* foraging habits as it forage within small patches of few neighbouring trees (Paton 1993; Whelan 2009) transferring high amounts of near related pollen, which consequently may reduce the fitness of all stands.

6.12 *Future research*

My study suggests a number of topics for further research:

A greater effort should be made to investigate other effects of habitat fragmentation including degree of isolation, variability in plant density, influence from other plant species as for example *Aegiceras corniculatum*, influence of the matrix and influence of edge effects. This should be done both at the level of experimental field ecology and genetics and molecular ecology.

The current literature indicates that reduced genetic diversity and increased inbreeding, which have known effects on small stands of fragmented terrestrial populations, may reduce plant fitness and hence population viability (Ghazoul 2005). It is important to thoroughly determine what consequences reduced gene flow among populations may have on plant fitness and viability of mangroves. Such investigations however, would demand long-term studies, as plants may have to be followed from seedlings to late adulthood to be able to determine all aspects of this, because for self-compatible plants as temperate *A. marina* consequences for viability and fitness may occur throughout life.

Further research will be required to identify the most important environmental and habitat factors influencing the vigour of plants within small stands of temperate *A. marina* (and other mangroves) because low vigour may lead to disruption or decay of reproductive output and implement detrimental effects of genetic variability in small stands, which in most extreme cases of fragmented terrestrial forest populations has lead

to extinction (Aizen 2002). Knowledge from such research is vital for implementing a strategy of management for temperate *A. marina* and for mangroves in general.

Further research into the influence of pollinators and their effects on the mating system would be necessary, because the past research into this field has been too superficial in the past studies (see chapter 2), why this field of research needs further scrutiny.

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