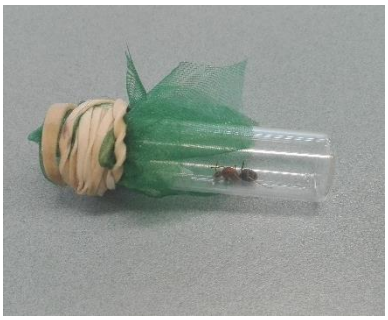
Biological processes influencing the success of invasive ants

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À mes parents, pour avoir toujours encouragé la curiosité de leurs enfants et leur soif d'apprendre

To Mory, for 10 years of continuing support and for sharing my Australian dream

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Intellectual support	Collaboration	My advisors Prof. Lori Lach, Dr Benjamin D. Hoffmann and Dr Wee Tek Tay collaborated throughout the duration of the PhD. I have mentioned other collaborators at the start of the individual chapters where appropriate
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Abstract

Despite invasive ants being a major threat to global conservation, most factors contributing to their success are unknown. The lack of information on the biology of most invasive ants is a major limitation to the study of ant invasions because it hinders our ability to distinguish between specific cases and general patterns. In my thesis, I used a combination of field experiments, laboratory experiments, and next generation sequencing techniques to investigate some of the processes that may hinder or facilitate ant invasions such as: (i) genetic bottleneck, (ii) disturbance, (iii) anthropogenic dispersal, and (iv) reproductive strategies. My first three chapters focused on the tropical fire ant (*Solenopsis geminata*) and my fourth chapter on the yellow crazy ant (*Anoplolepis gracilipes*), both highly invasive ants for which baseline information has been lacking.

In my first chapter, I determined the effects of diploid male production on colony founding in *S. geminata*. When an ant queen mates with a male carrying a complementary sex determining allele that is identical to the queen's, half of her worker-destined eggs will develop into diploid males, which do not contribute to colony labour, instead of workers. Invasive ant populations often go through a genetic bottleneck during their introduction, which causes the loss of sex determining alleles and triggers the production of diploid males. I found that in the Northern Territory (Australia), 8 out of 10 field *S. geminata* colonies produced diploid males presumably because of the small genetic diversity in this population. In a laboratory experiment, I assigned newly mated queens to nests consisting of 1, 2, 3, or 5 queens (n=95±9 replicates) and let them start a colony for 23 days. Diploid male producing colonies did not rear as many pupae and workers as non-diploid male producing colonies. I observed two mechanisms that could alleviate the cost of producing diploid males during colony founding: queens starting a nest together and cannibalism of diploid male larvae. Polyandry (queen mating with multiple males) could potentially reduce the occurrence of diploid male production, but I found that queens were always single mated.

In the Northern Territory of Australia, *S. geminata* is limited to disturbed habitats (e.g. road sides, park lawns) for reasons unknown. In my second chapter, I conducted two field experiments in which I introduced incipient *S. geminata* colonies or newly mated queens to disturbed and undisturbed sites in the Northern Territory to determine whether disturbance

facilitated the establishment of *S. geminata*. In one of the experiments, I introduced 447 *S. geminata* queens in individual cages that either allowed or prevented access to native ants to determine the relative roles of abiotic factors and biotic resistance on the establishment success of *S. geminata*. I found that the survival of newly mated queens was higher if they were introduced in disturbed sites and that unsuitable abiotic conditions, not biotic resistance, was the main driver of queen mortality.

In my third chapter, I used cutting-edge next generation sequencing techniques (double digest RADseq) on 177 *S. geminata* workers belonging to 28 colonies collected in 13 countries or islands to determine the ant's origin, invasion history, colony social structure, and geneflows between invasive populations. I found that *S. geminata* lost 38.5% of its genetic diversity during its introduction, that the ant was likely first dispersed from southwestern Mexico and that subsequent Indo-Pacific populations likely derive from Philippine populations, that all colonies in the invasive range were polygyne (i.e. with more than one queen), and that multiple geneflows have occurred between and among invasive populations.

In my fourth chapter, I used laboratory experiments and microsatellite analyses to investigate worker reproduction in *A. gracilipes*. I found that workers with a distended abdomen (i.e. physogastric) were more likely to contain yolky oocytes than normal workers and that the absence of queens triggered an increase in the percentage of physogastric workers. Physogastric workers were less likely to forage and less aggressive in interspecific aggression trials than normal workers. Despite being costly to colony productivity and defence, worker reproduction may increase the fitness of deceased *A. gracilipes* queens and orphaned workers as worker-produced males may be their last opportunity to contribute to the gene pool. I also found that the genetic pattern of the different castes (i.e. mostly homozygous queens and heterozygous workers) in this population suggest an unusual reproductive mode for this species.

The findings reported in my thesis improve our biological and ecological knowledge of two highly invasive ant species. In particular, I found that *S. geminata* queens can use strategies to overcome the cost of diploid male production following a 38.5% loss in genetic diversity. Anthropogenic dispersal has contributed to multiple geneflow events between and among *S. geminata* populations which may increase their genetic diversity and reduce inbreeding in the long term. Unsuitable abiotic conditions are probably the main factor preventing *S. geminata*

from establishing in undisturbed sites of the Northern Territory. Finally, worker reproduction in queenless *A. gracilipes* colonies can reduce their potential to become behaviourally dominant. Overall, my findings contribute to a better understanding of some of the foundational processes which influence the success of ant invasions.

Table of contents

Acknowledgements iv
Statement of contribution of others vi
Abstract viii
List of tables xvi
List of figures xvii
List of supplementary tables xx
List of supplementary figures xxii
General introduction 1
Genetic bottleneck 3
Disturbance 5
Anthropogenic dispersal 7
Reproductive strategies 9
Study species 11
The tropical fire ant, *Solenopsis geminata* 11
The yellow crazy ant, *Anoplolepis gracilipes* 12
Thesis overview 13
Chapter 1: Strategies of the invasive tropical fire ant (*Solenopsis geminata*) to minimize inbreeding costs 13
Chapter 2: Roles of biotic resistance and abiotic factors in the establishment of an invasive ant 14
Chapter 3: Invasion pathways and social structure of the invasive tropical fire ant (*Solenopsis geminata*) revealed by genome-wide SNP data analyses 15
Chapter 4: Origin, behaviour, and genetics of reproductive workers in the invasive yellow crazy ant *Anoplolepis gracilipes* 15
Chapter 5: Synthesis 16
Chapter 1 Strategies of the invasive tropical fire ant (*Solenopsis geminata*) to minimize inbreeding costs 17
Abstract 18

Introduction 18
Methods 22
Queen and male collection. 22
Spermatheca dissection 22
Colony founding experiment 23
DNA extraction and microsatellite genotyping 23
Statistical analysis 24
Results 25
Diploid males in field colonies 25
Queen mating frequency 26
Colony founding experiment 26
Discussion 33
Acknowledgements 38
Supplementary information 39
a. Collection sites and ploidy results of field-sampled males 39
b. PCR protocol for queen, larva and male DNA and microsatellite primer sequences 41
c. Figures on the colony founding experiment on the number of worker brood and adult worker per treatment, the number of worker brood and adult workers per queen, the number of DMP colonies executing diploid male larvae per treatment, and the weight distribution of newly mated queens. 43
d. Probability of double mating and queen and sperm genotypic distribution 47
Chapter 2 Roles of biotic resistance and abiotic factors in the establishment of an invasive ant 52
Abstract 53
Introduction 53
Methods 56
Site descriptions 56
Queen collection 57
Development of incipient colonies 57
Queen and colony introduction experiment 57
Caged queen experiment 58
Native ant sampling 59

Environmental variables 60
Data analysis 60
Results 62
Queen and colony introduction experiment 62
Caged queen experiment 63
Ant community results 70
Discussion 72
Acknowledgements 76
Supplementary information 77
Chapter 3 Invasion pathways and social structure of the invasive tropical fire ant (*Solenopsis geminata*) revealed by genome-wide SNP data analyses 83
Abstract 84
Introduction 84
Methods 87
Solenopsis geminata samples 87
Genomic DNA extraction 88
ddRAD library preparation 88
SNP calling and filtering 89
Population structure 90
Relatedness analysis 91
Results 92
Data summary 92
Genetic bottleneck 92
Origin and invasion history 92
Geneflow between and among invasive populations 95
Social structure 98
Discussion 99
Acknowledgements 103
Supplementary information 104
Chapter 4 Origin, behaviour, and genetics of reproductive workers in the invasive yellow crazy ant *Anoplolepis gracilipes* 115

Abstract 116
Introduction 116
Methods 119
Colony collection 119
Dissections of worker's ovaries of entire colonies 119
Colony observations 120
Morphometry and genotyping 120
Queen transfer experiment 122
Aggression tests 123
Data analysis 124
Results 127
Dissections of worker's ovaries of entire colonies 127
Colony observations 127
Male morphometry and genotyping 128
Queen transfer experiment 129
Aggression tests 131
Discussion 133
Acknowledgements 139
Supplementary information 140
Chapter 5 Synthesis 156
Thesis overview 156
Genetic bottleneck and strategies to minimize inbreeding costs 157
Disturbance facilitates the establishment of *S. geminata* mainly through abiotic conditions 158
Genome-wide sequencing, a powerful tool for the study of invasive ant populations 160
Costs and benefits of worker reproduction in an invasive ant 161
Limitations and recommendations for future work 162
Chapter 1: Strategies of the invasive tropical fire ant (*Solenopsis geminata*) to minimize inbreeding costs 162
Chapter 2: Roles of biotic resistance and abiotic factors in the establishment of an invasive ant 163

Chapter 3: Invasion pathways and social structure of the invasive tropical fire ant (*Solenopsis geminata*) revealed by genome-wide SNP data analyses 164

Chapter 4: Origin, behaviour, and genetics of reproductive workers in the invasive yellow crazy ant *Anoplolepis gracilipes* 165

Conclusion..... 165

References 167

List of tables

Table 1.1 Summary of generalized linear model results for each response variable in the colony founding experiment. ‘x’ represents the interaction terms. 32

Table 2.1 Summary of model results for the caged queen experiment. * p<0.05, ** p<0.01, *** p<0.001 64

Table 3.1 Estimates of average nestmate workers relatedness (Wang’s r), effective number of queens (Nqe) and social structure of colonies collected in the native and invasive range of *S. geminata*. We calculated Ne for two rs (average relatedness of worker offspring from the same matriline) values (see Table S3.3). 98

Table 4.1 Measurements of males from queenless and queenright colonies (mean ± SD) and results from type II Wald tests on LMM (df=1) * p<0.05, ** p<0.01 128

Table 4.2 Summary of generalized linear model results for each response variable in the queen transfer experiment. ‘x’ represents the interaction terms..... 130

List of figures

Figure 1.1 Mean queen loss of weight between the start and the end of the experiment for each queen treatment separated between DMP (diploid male producing) colonies and non-DMP colonies. The interaction between diploid male production and the number of queens had a significant effect on the mean queen weight loss (LM: ANOVA, $\chi 2=11.7166$, $P < 0.01$). **Indicates a significant difference between DMP colony and non-DMP colonies for the corresponding queen treatment (post hoc tests $P < 0.01$). One queen: non-DMP n=97, DMP n=5, two queens: non-DMP n=79, DMP n=16, three queens: non-DMP n=68, DMP n=29, five queens: non-DMP n=51, DMP n=35. 28

Figure 1.2 Number of worker pupae and adult workers produced for each queen treatment separated between DMP (diploid male producing) colonies and non-DMP colonies. DMP colonies had significantly fewer worker pupae and adult workers than non-DMP colonies (Table 1.1); * Indicates a significant difference between DMP colony and non-DMP colonies for the corresponding queen treatment (post hoc tests $P<0.05$). One queen: non-DMP n=97, DMP n=5, two queens: non-DMP n=79, DMP n=16, three queens: non-DMP n=68, DMP n=29, five queens: non-DMP n=51, DMP n=35. 30

Figure 2.1 Photos of two mesh cages from the caged queen experiment before being buried. a) Big mesh cage with a *S. geminata* queen in the white circle. The mesh size prevented the queen from escaping but allowed *S. geminata* workers and native ants (maximum width 1.23mm) to enter and leave the cage. b) Small mesh cage with its individual label. All ants were prevented from entering and leaving the cage. 59

Figure 2.2 Proportion of dead and live queens in the caged queen experiment at a) CSIRO and b) Girraween by site status (disturbed, undisturbed), cage mesh size (big, small) and retrieval time (7, 14, 25 days). See Table 2.1 for statistical results. 67

Figure 2.3 Proportion of queens dying due to abiotic factors and baseline mortality or biotic resistance (inferred) by exposure time and disturbance. 7 days: undisturbed n=37, disturbed n=40, 14 days: undisturbed n=51, disturbed n=40, 25 days: undisturbed n=60, disturbed n=61. 68

Figure 2.4 Principal component map of environmental variables (percentages of grass cover, litter cover, bare soil cover, shading at 10cm-3m above ground and shading at 3m above ground) for the caged queen experiment in all the sites. The first letter indicates the site: C for CSIRO, G for Girraween. The second letter indicates the disturbance: D for disturbed and U for undisturbed. The first axis represents 85% of the variation and both axes cumulatively represent 99.9% of the variation. 69

Figure 2.5 Non-metric multidimensional scaling analysis for ant community similarity in seven sites for the queen and colony introduction experiment. The sites with blue names were also used in the caged queen experiment (CSIRO and Girraween sites). Each cross represents an ant species. The open symbols show the seven sites sampled. The first letter indicates the location: C for CSIRO, G for Girraween, H for Humpty Doo and V for Vanderlin. The second letter

indicates the disturbance: D for disturbed and U for undisturbed. The polygons link the disturbed and undisturbed sites separately..... 70

Figure 2.6 Relative abundances and distributions of ant species from sites used in the caged queen experiment. CSIRO Disturbed: N=566 individuals and 38 species, CSIRO Undisturbed: N=487 individuals and 37 species, Girraween Disturbed: N=1318 individuals and 39 species, Girraween Undisturbed: N=561 individuals and 27 species. Species with a relative abundance lower than 5% for all sites were combined in "Others"..... 71

Figure 3.1 Locations of *S. geminata* specimens analysed in this study. Red: specimens collected in the invasive range. Blue: specimens collected in the native range. Circle: single specimen. Triangle: multiple specimens from a colony. Numbers show the number of colonies sampled if more than one originated from this location. 88

Figure 3.2 Demographic history of *S. geminata* populations for 155 specimens. Inferred Maximum Likelihood (ML) tree obtained with Treemix (Pickrell and Pritchard 2012). The arrows depict migration edges and show geneflow directions. The migration weight is shown by the colour of the arrows. The two migration edges both emerged from the Philippines. The branches indicate ancestry relationship and their length is proportional to the amount of genetic drift that has occurred on the branch. Populations without a branch indicate that genetic drift did not occur for these populations according to the ML tree. 93

Figure 3.3 ADMIXTURE analysis conducted on SNPs from 155 specimens showing the optimal number of cluster: K=12. Individual black horizontal bars show specimens belonging to the same colony if multiple specimens from more than one colony were collected from the same location. Abbreviations: M. Aus. Mainland Australia, Tiwi isl. Tiwi Islands, Chris. isl Christmas Island, Ash. r. Ashmore Reef, Mal. Malaysia, Micro. Micronesia, Camb. Cambodia, Phil. Philippines, Thai. Thailand. 94

Figure 3.4 Geneflow between and among populations according to the ADMIXTURE analysis (Figure 3.3). Panel a shows geneflows involving mainland Australia and Australian islands, panel b other populations in the Indo-Pacific region, and panel c Mexico and Hawai'i. Circle: single specimen. Triangle: multiple specimens from a colony. Abbreviations: Camb. Cambodia, Phil. Philippines, Thai. Thailand, Mal. Malaysia, Chris. Christmas Island, Ash. Ashmore Reef 96

Figure 3.5 Principal component map of the SNPs in 144 individual specimens from the invasive range of *S. geminata*. The first axis represents 20% of the variation and the second axis 9%..... 97

Figure 4.1 External morphology of the abdomen of (a) a normal worker and (b) a physogastric worker. Note the exposed intersegmental membranes of the physogastric worker..... 120

Figure 4.2 Average± SD percentage of physogastric and normal workers in the queen transfer experiment by colony status (QR=queenright, QL=queenless, N=10 for each) and number of days since the start of the experiment. *** indicates a significant difference between queenright and queenless colonies for the corresponding time (GLMM: binomial, Table 4.2, post-hoc tests ***$p<0.001$). 131

Figure 4.3 Number of fights initiated by *A. gracilipes* (YCA) and *O. smaragdina* (GTA) workers during the aggression tests between *O. smaragdina* workers and *A. gracilipes* normal or physogastric workers from queenright or queenless colonies. Not applicable indicates instances in which *O. smaragdina* and *A. gracilipes* workers did not fight. Normal workers initiated the fight more often than physogastric workers (GLMM: binomial, Type II Wald test: $\chi2=5.7882$, df=1, $p<0.05$) and workers from queenright colonies initiated the fight more often than workers from queenless colonies (GLMM: binomial, Type II Wald test: $\chi2=6.3087$, df=1, $p<0.05$). 133

List of supplementary tables

Table S1.1 Male collection sites…….39

Table S1.2 Number of heterozygous males scored as diploid for each field-collected colony. .. 40

Table S1.3 Microsatellite primer sequences, types of simple sequence repeats (SSR), annealing temperatures, allele size range for *S. geminata*, and references from which the primer sequences were adapted. 42

Table S1.4 Genotypic distribution of queens, -: amplification failure. 48

Table S1.5 Genotypic distribution of sperm samples, -: amplification failure. 50

Table S2.1 Number of individuals captured per site for each ant species. Sampling took place before the queen and colony introduction experiment. * indicates locations which were also used in the caged queen experiment. 80

Table S3.1 List of samples, sample type (single sample or multiple individuals from a colony), collection locality and coordinates. 104

Table S3.2 Oligo sequence of primer pairs for PCR reaction 109

Table S3.3 Number of loci for each colony in which multiple nestmates were collected, estimates of average relatedness of nestmate workers (Wang's r), effective number of queens (Nqe) for two rs (average relatedness of worker offspring from the same matriline) values. 112

Table S4.1 Colony collection sites, number of queens, workers, status and time in captivity for the dissections of worker's ovaries of entire colonies 140

Table S4.2 Site coordinates 141

Table S4.3 Colony collection sites, status, time in captivity and number of workers and queens during the colony observations. Status: QR queenright colonies, QL queenless colonies. Number of queens column: NA not applicable. Site column: Kearns K, Russett Park RP, Sandy Creek SC, Mount Peter MP, Gray's creek GC, Vindula V, Hussey H, Whites creek WC, Drapers creek DC, Franks Creek F, Harold H, Townsville T, Hervey Bay HB 142

Table S4.4 Range for the number of workers counted during the colony observations 147

Table S4.5 Colony collection date, sites and number of workers per replicate at the start of the queen transfer experiment. Nome: N, Sandy Creek: SC, GV: Gordonvale Hervey Bay: HB, B: Brisbane 147

Table S4.6 Colony collection date, sites and status of *A. gracilipes* colonies used in the aggression tests. Status: QR queenright colonies, QL queenless colonies. Gordonvale: GV, Sandy Creek: SC, Drapers Creek: DC 148

Table S4.7 Genotypes of males from queenright (N=38 males) and from queenless (N=82 males) colonies (N=8 queenright and N=14 queenless colonies). Allele sizes in red and bold: heterozygous at this locus, -: amplification failure 149

Table S4.8 Genotypes of workers from queenright (N=40 workers) and queenless colonies (N=54 workers) (N=8 queenright colonies and N=10 queenless colonies). -: amplification failure 152

Table S4.9 Genotypes of queens (N=10 queens from 6 colonies). Allele sizes in red and bold: heterozygous at this locus, -: amplification failure 154

List of supplementary figures

Figure S1.1 Map of the male collection sites in the Northern Territory, Australia. 40

Figure S1.2 Number of worker brood and adult worker produced for each queen treatment separated between DMP (diploid male producing) colonies and non-DMP colonies. There was no significant difference between DMP and non-DMP colonies across all queen treatments (Table 1.1). One queen: non-DMP n=97, DMP n=5, two queens: non-DMP n=79, DMP n=16, three queens: non-DMP n=68, DMP n=29, five queens: non-DMP n=51, DMP n=35..... 43

Figure S1.3 Number of brood and adult workers produced per queen (total number of brood and adult workers divided by the number of queens) for each queen treatment. The queen treatment had a significant effect on the number of worker brood and adult workers produced per queen but none of the pairwise comparisons between queen treatment were significant (LM: ANOVA, $\chi 2 = 36.038$, $P<0.01$; post hoc tests $P>0.05$ for all pairwise comparisons). One queen: n=102, two queens: n=95, three queens: n=97, five queens: n=86. 44

Figure S1.4 Proportion of DMP colonies executing diploid male larvae for each queen treatment. One queen: DMP n=5, two queens: DMP n=16, three queens: DMP n=29, five queens: DMP n=35. 45

Figure S1.5 Weight distribution of the newly mated queens used in the colony founding experiment before the start of the experiment (n=1,013). 46

Figure S1.6 Probability of not detecting double-mating calculated from 32 sperm genotype frequencies. 47

Figure S2.1 Map of the study locations in the Northern Territory, Australia. All four locations were used in the queen and colony introduction experiment and we only used the sites situated at CSIRO and Girraween in the caged queen experiment. 77

Figure S2.2 Principal component map of environmental variables (percentages of grass cover, litter cover, bare soil cover, shading at 10cm-3m above ground and shading at 3m above ground) for the queen and colony introduction experiment in all the sites. The first letter indicates the location: C for CSIRO, G for Girraween, H for Humpty Doo and V for Vanderlin. The second letter indicates the disturbance: D for disturbed and U for undisturbed. The first axis represents 79% of the variation and both axis cumulatively represent 99% of the variation. 78

Figure S2.3 Average temperature recorded for each probe at 7cm underground during the caged queen experiment. LM: ANOVA, $\chi 2=14.4759$, df=1, *** $P<0.001$. The bars show 50% of the data within the 25th and 75th percentiles, the line inside the bars is the median, and the whiskers show minimum and maximum data values..... 79

Figure S3.1 Principal component map of the SNPs in 147 individual specimens from the invasive range of *S. geminata*. We removed the circled specimens from the subsequent principal component map (Figure 3.5) to observe how the other invasive specimens clustered together. 110

Figure S3.2 Principal component map of the SNPs in 157 individual specimens. The specimens originating from Mexico are labelled with their individual code or their colony code. See Figure S3.3 for a map showing the origin of the Mexican specimens. 111

Figure S3.3 Map of the Mexican's sample origin with the sample codes. Circle: single specimen. Triangle: multiple specimens from a colony. 114

Figure S4.1 Average percentage of physogastric and normal workers in the queen transfer experiment separated between queenright (QR, until day 60, N=3) and queenless colonies (QL, until day 60, N=3) and number of days since the start of the experiment until 120 days after the start of the experiment. ⇩ indicates when queens have been moved from the queenright colonies to the queenless colonies. After this date, queenright colonies became queenless (QLm) and queenless colonies became queenright (QRm). * and ** indicates a significant difference between queenright and queenless colonies for the corresponding time (GLM: quasi-binomial, Table 4.2, post hoc tests *$p<0.05$ **$p<0.01$). Error bars indicate standard deviation. 155

General introduction

Biological invasions are a major issue globally in terms of their environmental, economic, health and social impacts (Simberloff et al. 2013). Invasive species are costly for the economy of many countries (Pimentel et al. 2001). For example, invasive species have been estimated to cost US$120 billion/year to the US economy in damages and control (Pimentel et al. 2005) and £1.7 billion/year to the British economy in damages (Williams et al. 2010). Invasive species have also contributed to 33% of animal extinctions and 25% of plant extinctions globally (Blackburn et al. 2019). For example, the introduction of the predatory brown tree snake (*Boiga irregularis*) in Guam has caused the local extinctions of several native vertebrates (Savidge 1987, Wiles et al. 2003) and the introduction of the Nile perch (*Lates niloticus*) in Lake Victoria resulted in the extinction of hundreds of native cichlids (Vitule et al. 2009). About one-sixth of the global land surface and 16% of the world's biodiversity hotspots are highly vulnerable to biological invasions (Early et al. 2016). Research into highly successful invasive species and the factors that contribute to their success is fundamental to limit the occurrence of biological invasions and their effects.

With over 150 species established outside their native range, ants are one of the most diverse and harmful group of invaders (McGlynn 1999, Suarez et al. 2010). Competition and predation from invasive ants can negatively affect native vertebrates (e.g. nesting disruption of birds and reptiles) and invertebrates (e.g. displacement of native ants) and lead to dramatic changes in ecosystems (reviewed in Holway et al. 2002a, Lach and Thomas 2008, Lach and Hooper-Bui 2010). The painful sting of some species, such as the red imported fire ant (*Solenopsis invicta*), can trigger anaphylactic shock and limit residents from undertaking outdoor activities, potentially reducing tourism revenue (Salin et al. 2000, Solley et al. 2002). The negative effects of ant invasions can generate severe economic costs (Pimentel et al. 2005). For example, *S. invicta* was estimated to cost the United States US$600 million in losses and damages annually (Pimentel et al. 2005). In Texas, the production costs of several crops have dramatically increased because of *S. invicta* (e.g. yield loss, equipment and infrastructure damages, medical expenses) (Wylie and Janssen-May 2016). Livestock health is also affected as the stings of *S. invicta* can trigger blindness and swelling in animals which can result in sale losses and animal

death (Hafi et al. 2014, Wylie and Janssen-May 2016). Eradication programs are also costly. For example, the eradication program for *S. invicta* in Queensland (Australia) will cost A$411.426 million over ten year and eradication failure could cost Australia A$1.65 billion per year (Wylie and Janssen-May 2016, Janssen 2017). Some of the costs included in the program are treatment and monitoring (about A$20 million per year), scientific support (about A$1 million per year), and communication and engagement (about A$1.5 million per year) (Janssen 2017).

Invasive ants are highly successful invaders, but we lack baseline information (e.g. region of origin, reproductive mode, colony founding strategies) for most species (Holway et al. 2002a). For example, the native ranges of the yellow crazy ant (*Anoplolepis gracilipes*) and the African big-headed ant (*Pheidole megacephala*) are unclear (Wetterer 2005, 2012, Drescher et al. 2007). The reproductive mode is unresolved for *A. gracilipes* (Drescher et al. 2007) and the social structure (i.e. number of queens per colony) of the tropical fire ant (*Solenopsis geminata*) remains to be determined for most of its invasive range. Such baseline information is paramount to the study of fundamental concepts of invasion biology. For example, knowing the native range of an invasive ant is crucial to determining whether key adaptation has occurred following its introduction (e.g. Rey et al. 2012). The reproductive mode of a species can contribute to its ecological dominance (e.g. clonal reproduction, Foucaud et al. 2009, Chifflet et al. 2018). Finally, the establishment potential of transported colony fragments depends on the colony social form as fragments from colonies with several queens are more likely to contain a queen (Tsutsui and Suarez 2003). The lack or absence of baseline information on most invasive ants is a major limitation that hinders our ability to distinguish between specific cases and general patterns for invasive ant species (Tsutsui and Suarez 2003, Krushelnycky et al. 2010).

To understand what makes some invasive ant species successful invaders and draw general patterns from this understanding, we need to look at some of the processes that may hinder or facilitate ant invasions such as: (i) genetic bottleneck, (ii) disturbance, (iii) anthropogenic dispersal, and (iv) reproductive strategies**.** First, invasive species often go through a genetic bottleneck because of their small founder size (Simberloff 2009). Inbreeding depression can occur because of genetic bottlenecks and may affect invasive ants' colony growth and survival (Ross and Fletcher 1985a, 1986, Ross et al. 1993). Second, disturbance may facilitate ant invasions through a combination of different factors by increasing resource availability and reducing the intensity of competition and predation from native species (i.e. biotic resistance)

(Elton 1958, Lozon and MacIsaac 1997, Walters and Mackay 2005, Krushelnycky et al. 2010, Moles et al. 2012). Third, anthropogenic dispersal facilitates the spread of invasive ants by providing opportunities for long distance dispersal and facilitating geneflow between geographically separated populations (e.g. Ascunce et al. 2011, Gotzek et al. 2015)**.** Finally, the reproductive strategies of invasive ants (e.g. clonal reproduction, colony budding) are often linked to their successful establishment and spread (Tsutsui and Suarez 2003, Chifflet et al. 2018). The overall objective of my thesis was to determine how these four processes may influence the success of invasive ants. Each process is the main subject of a data chapter. In the remainder of my introduction, I will give background information and identify research gaps for each of these processes. I will then give brief background information on the two invasive ant species I have studied (*S. geminata* and *A. gracilipes*). Finally, I will give an overview of my thesis chapters.

Genetic bottleneck

Many species invasions established from a small number of founder individuals (Allendorf and Lundquist 2003). As a result, the genetic diversity of non-native populations is often a fraction of native populations' diversity (i.e. genetic bottleneck) (Allendorf and Lundquist 2003, Dlugosch and Parker 2008a). For example, the allelic richness of invasive solitary sweat bee (*Lasioglossum leucozonium*) populations is reduced by 76% compared to the native populations' (Zayed et al. 2007). Theoretically, inbreeding would cause small populations to accumulate deleterious mutations and eventually go extinct (Lynch et al. 1995, Caballero et al. 2017). Reduced genetic diversity could also limit the ability of a population to adapt to new habitats (Allendorf and Lundquist 2003). However, paradoxically, inbreeding does not appear to affect the establishment and spread of successful invasive species (Allendorf and Lundquist 2003, Dlugosch and Parker 2008a). Although potential invaders may have failed to establish as a result of going through a bottleneck. How do invasive species overcome the potential effects of inbreeding depression?

Despite often losing genetic diversity during their introduction, invasive ants are successful invaders (Tsutsui and Suarez 2003). *Linepithema humile* and *S. invicta* both lost genetic variation during founding events (Ross et al. 1993, Tsutsui and Suarez 2003). The small genetic diversity of *L. humile* may have contributed to its success (Tsutsui and Suarez 2003). In their native range, *L. humile* workers can differentiate nestmates from outsiders because different colonies have

distinct cuticular hydrocarbon profiles (Brandt et al. 2009). In its invasive range, however, *L. humile* is unicolonial, presumably because the cuticular hydrocarbons of introduced *L. humile* populations became homogeneous as a consequence of genetic bottlenecks (Tsutsui et al. 2000, Brandt et al. 2009). In the absence of costs related to intraspecific territoriality, colonies in the invasive range are potentially able to direct more resources toward foraging, colony growth, and interspecific competition than colonies in the native range (Tsutsui and Suarez 2003).

Genetic bottlenecks may have contributed to the success of *L. humile*, but for *S. invicta*, they lead to adverse consequences by causing the loss of sex determining alleles, which disrupts the functioning of the sex determination system (Ross et al. 1993). The exact mechanism of sex determination in ants is unknown, but indirect evidence and genomic studies suggest that, as in most Hymenoptera that have been studied, the determination of sex is controlled by the genotype at the complementary sex determination (CSD) locus (or loci) (Ross and Fletcher 1985a, Ross et al. 1993, Cook and Crozier 1995, Heimpel and de Boer 2008, Wurm et al. 2011, Nipitwattanaphon et al. 2014). When sex determination is controlled by a CSD locus (or loci), haploid individuals from unfertilised eggs develop into males while diploid individuals from fertilised eggs develop into females (van Wilgenburg et al. 2006). However, when a queen mates with a male carrying a CSD allele that is identical to the queen's (i.e. match mating), half of her fertilized eggs will be homozygous at the CSD locus and develop into diploid males instead of workers if reared **successfully** (Crozier 1971, 1977, Ross et al. 1993). The only function of ant males is to mate and most diploid males are sterile (Ross and Fletcher 1986, Cook and Crozier 1995, Krieger et al. 1999). Therefore, diploid males are a waste of colony resource allocation and were found to reduce the colony growth rate of invasive *S. invicta* colonies established in the southern USA (Ross and Fletcher 1985a, 1986, Ross et al. 1993, Tschinkel 1998a). It is unlikely that diploid male production is common in the native range of *S. invicta*, as selection would favour diversity in CSD alleles (Ross & Fletcher, 1985a).

Diploid male production is especially detrimental for species with claustral founding queens (e.g. *S. invicta*, *S. geminata*). Claustral queens seal themselves in a chamber and rear their first brood on their fat reserves (Tschinkel and Howard 1983). To found a colony successfully, a queen must produce workers which will forage for her before her fat reserves are entirely depleted (Ross and Fletcher 1986). When a queen is match-mated, 50% of her worker-destined eggs will develop

into diploid males, a significant burden for founding queens (Ross and Fletcher 1985a, 1986, Ross et al. 1993, Tschinkel 1998a).

In theory, the high occurrence of match mating should decrease the colony founding success and hinder the establishment and further spread of invasive ants with small genetic diversity. Diploid male production is a significant mortality factor for *S. invicta* founding queens from the southern USA population (Ross and Fletcher 1986). However, the common occurrence of match-mated queens (up to 20%) has not prevented this population from rapidly expanding its range and reaching high population density (Ross and Fletcher 1986). In the laboratory, match mated *S. invicta* queens that found a colony on their own have lower growth and survival compared to queens that do not produce diploid males (Ross and Fletcher 1986). Presumably, match mated queens would only survive if they were adopted into a mature colony which could compensate for the loss in worker production (Ross and Fletcher 1985a, 1986, DeHeer and Tschinkel 1998). The effects of diploid male production on the colony founding success of invasive ants has mainly been studied for *S. invicta* populations established in the southern USA. We do not know how other invasive populations of *S. invicta* and other invasive ants successfully start colonies despite potentially producing diploid males. Studying strategies used by other invasive ants to overcome diploid male production during colony founding will provide further insight into how invasive species overcome inbreeding.

Disturbance

The idea that disturbance facilitates biological invasions is one of the foundational and most commonly accepted ideas in the field of invasion ecology (Moles et al. 2012). Disturbance is defined here as any discrete event that disrupts community structure and changes available resources, substrate availability, or the physical environment (Krebs 1994). Several studies have found a positive relationship between the presence of disturbance and the establishment of invasive terrestrial plants, animals, and marine organisms, but others find no or a negative relationship (Moles et al. 2012).

Disturbance may benefit biological invasions by altering abiotic conditions at the habitat and microhabitat scales and by reducing the intensity of biotic resistance (Elton 1958, Lozon and MacIsaac 1997, Walters and Mackay 2005, Krushelnycky et al. 2010, Moles et al. 2012). The biotic resistance hypothesis states that a native community may hinder the establishment of a

non-native species through interspecific competition or predation (Elton 1958, Lozon and MacIsaac 1997, Walters and Mackay 2005). The occurrence of anthropogenic disturbance and the modification of disturbance regimes may weaken biotic resistance by decreasing native species diversity and the competitive ability of native communities (Elton 1958, Lozon and MacIsaac 1997). Disturbance may also modify abiotic conditions and contribute to providing a suitable habitat to invasive taxa (Byers 2002, Krushelnycky et al. 2010). For example, disturbance can facilitate plant invasions by making more resources available and opening space for colonisation (Davis et al. 2000). To date, 70% (n=88/125) of studies on biotic resistance have focused on plants, and only about 25% of these studies found support for the biotic resistance hypothesis (Jeschke et al. 2012). In plants, biotic resistance can hinder or contain invasions but there is little evidence that plant invasions can be repelled by resistance from native communities (Levine et al. 2004). Invertebrates may be more susceptible to biotic resistance than plants. Only 16% (n=20/125) of studies on biotic resistance have focused on invertebrate invasions, but approximately 40% of these studies supported the biotic resistance hypothesis (Jeschke et al. 2012).

The successful establishment of invasive ants into new environments depends on a combination of abiotic and biotic factors which are sometimes linked to disturbance (Krushelnycky et al. 2010, Sanders and Suarez 2011). Unsuitable and suboptimal abiotic conditions can reduce the survival, limit the spread, and hinder the competitive ability of invasive ants (Holway et al. 2002b, Walters and Mackay 2003, Krushelnycky et al. 2005, Thomas and Holway 2005, Menke et al. 2007). The importance of abiotic conditions in the establishment success of invasive ants has mostly been studied for *L. humile* and depends on fine-scale variations in the abiotic environment. For example, *L. humile* workers stop foraging at temperatures below 5°C, which limits the northward expansion of this species in the United States (Brightwell et al. 2010). The range expansion of *L. humile* also depends on water availability. In a field experiment in California, artificial irrigation of dry environments facilitated the establishment of *L. humile* (Menke and Holway 2006, Menke et al. 2007). While it is clear that *L. humile*'s range is limited by water availability and extreme temperatures, the importance of abiotic factors in the successful establishment of other invasive ants remains to be tested.

Most research on the role of biotic resistance in the establishment success of invasive ants comes from laboratory and observational experiments. For example, a laboratory experiment showed

that workers from colonies of native ants (e.g. *Monomorium minimum, Solenopsis molesta*) could attack and eliminate colonies of *S. invicta* (Rao and Vinson 2002, 2004). Another lab experiment showed that native workers of *Tapinoma nigerrimum* were able to dominate food resources over *L. humile* and invade their nests (Blight et al. 2010). Field experiments in which invasive ant colonies are introduced and resident ant communities manipulated are ideal to investigate the role of biotic resistance on the successful establishment of invasive ants (Sanders and Suarez 2011), but only a few field studies have been conducted. In one such study, newly mated queens of *S. invicta* were introduced to combinations of ploughing, artificial shading, and resident ant community poisoning treatments (King and Tschinkel 2016). After 120 days, all the surviving colonies (5/980) were found in the tilled plots in which native ants had been eradicated. This experiment shows that soil disturbance and the absence of native ants play a role in the establishment success of *S. invicta* but does not permit conclusions on the importance of each factor for colony survival to be made. In another field experiment, different sizes (10, 100 or 1000 workers, 4-6 replicates) of *L. humile* colonies were introduced in containers in which workers, but not the queen, could leave (Sagata and Lester 2009). To determine the effect of abiotic conditions on colony survival, *L. humile* colonies were also introduced in control containers that prevented the workers from leaving (two replicates per colony size). Colonies in containers from which the workers could leave survived for an average of 25 days while colonies with 100 to 1000 workers introduced in control containers survived until the end of the experiment (3 months). These results suggest that biotic resistance from native ants may play an important role in the colony establishment success of *L. humile*. To improve our understanding of how disturbance influences the establishment success of invasive ants, conducting field experiments that allow us to distinguish between the role of abiotic conditions and biotic resistance is necessary. The biotic resistance hypothesis remains to be tested on most invasive ant species, and invasive ants other than *S. invicta* and *L. humile* may be especially vulnerable to biotic resistance as they may not be as behaviourally dominant as these two species (Tsutsui and Suarez 2003).

Anthropogenic dispersal

Anthropogenic dispersal has facilitated the spread of invasive species over long distances for hundreds of years, but the pace of this process has dramatically increased with the development

of global trade and human transport (Meyerson and Mooney 2007, Westphal et al. 2008, Hulme 2009). For example, over 60% of new introductions in the Great Lakes (U.S.) since 1960 has been attributed to commercial shipping (Horan and Lupi 2005). In fact, the greater the degree of international trade in a country, the higher the number of invasive species and the rate of new species introductions for that country (Levine and D'Antonio 2003, Westphal et al. 2008).

Retracing the origin and pathways of biological invasions is challenging but crucial to the development of biosecurity strategies and the study of fundamental concepts of invasion ecology (Dlugosch and Parker 2008a, Hulme 2009). For example, identifying main introduction pathways can assist the development of targeted monitoring and surveillance. Determining the native range of an invader can be used to determine whether an invasive species has lost genetic diversity during its introduction and/or undergone adaptive evolution following its introduction (Dlugosch and Parker 2008a). However, the development of international trade makes it increasingly difficult to determine main invasion pathways. Identifying the native range of ancient invaders can also be challenging. For example, ships have transported organisms unintentionally (e.g. fouling molluscs, algae) between continents since the 15th century, making the introduction history of these organisms difficult to retrace (Carlton 1999).

Genetic tools offer the best approach to retrace the origin of an invader as well as its main routes of introduction, and next generation sequencing methods represent a significant improvement to traditional genotyping methods (Baird et al. 2008, Peterson et al. 2012, Wagner et al. 2013, Nygaard and Wurm 2015, Andrews et al. 2016). As I previously detailed in the bottleneck section, the genetic diversity of invasive species is often low (Allendorf and Lundquist 2003, Dlugosch and Parker 2008a), which makes it difficult for researchers to find DNA markers with sufficient variability to draw conclusions about population dynamics. The development of new genotyping methods such as Restriction site-Associated DNA Sequencing (RADseq) has considerably improved our ability to identify the introduction routes and origin of invasive populations. For example, a RADseq study identified the native range and introduction history of an ambrosia beetle (*Xylosandrus crassiusculus*) (Storer et al. 2017) whereas a study using mitochondrial markers could only infer that the genetic distances among populations were large (Dole et al. 2010). Another RADseq study inferred the main routes of introduction and geneflows among invasive populations of the brown rat (*Rattus norvegicus*) (Puckett et al. 2016),

whereas a previous study using mitochondrial markers identified their centre of origin but was unable to resolve relationships among invasive populations (Song et al. 2014).

Although, RADseq methods are ideal to study the invasion pathways of invasive ants, such a study has never been attempted to the best of my knowledge. To date, studies on the origin of ant invasions and population movements have used microsatellite markers and/or sequenced portions of the mitochondrial genome to provide insights into the introduction routes and origin of invasive ants (e.g. Vogel et al. 2010, Ascunce et al. 2011, Wauters et al. 2018). By using a large number of individuals and microsatellite markers, Ascunce et al. (2011) successfully determined the global invasion history of *S. invicta* with high sensitivity. However, the conclusions of most studies are limited by the low number of individuals used and the low number and variability of the markers used due to invasive ant populations often having low genetic diversity (Ross et al. 1993, Tsutsui and Suarez 2003).RADseq methods are not limited by the low genetic variability of populations as they can generate hundreds to hundreds of thousands of genome-wide single nucleotide polymorphic (SNP) markers (Baird et al. 2008, Peterson et al. 2012, Wagner et al. 2013, Nygaard and Wurm 2015, Andrews et al. 2016). The higher resolution and sensitivity of genome-wide markers (Nygaard and Wurm 2015) lead to a greater ability to uncover global invasion patterns as well as colony-level structure. For example, one of the few ant RADseq studies conducted to-date determined worker relatedness and mating behaviour in ants living in symbiosis with *Acacia drepanolobium* (Boyle et al. 2018). Future studies using genome-wide sequencing to determine the origin, invasion history and gene flows of global ant invaders will certainly improve our understanding of invasion processes and assist the current efforts to limit the incidence of ant invasions by improving monitoring and surveillance strategies.

Reproductive strategies

The reproductive strategies of invasive species often contribute to their successful establishment and spread (Sakai et al. 2001). A meta-analysis revealed that invasive plants allocated more resources to reproduction than non-invasive plants (e.g. higher number of seeds and flowers per plants) (van Kleunen et al. 2010). Vegetative reproduction also contributes to the success of invasive plants because they can then successfully breed at low density and/or when pollinators are lacking (Barrett et al. 2008). For example, self-fertilisation has become the dominant reproductive mode in the highly invasive water hyacinth (*Eichhornia crassipes*) following its

introduction to regions from which suitable pollinators were lacking (Barrett et al. 1989). For invasive bivalves such as Asian clams (*Corbicula fluminea*) and zebra mussels (*Dreissena polymorpha*), high fecundity, early maturity, and rapid growth encourage rapid population recovery following reduction in population density (Mcmahon 2002). Reproductive strategies of the polyandrous Trinidadian guppy (*Poecilia reticulata*) also facilitate its range expansion (Deacon et al. 2011, Gasparini and Pilastro 2011). By storing sperm for up to six months and biasing paternity towards unrelated males, a single pregnant female can successfully establish a viable population (Deacon et al. 2011, Gasparini and Pilastro 2011).

Several aspects of invasive ant reproduction enhance invasive success, but the reproductive mode of most invasive ants remains to be described (Tsutsui and Suarez 2003, Drescher et al. 2007). Polygyny (i.e. colonies with multiple queens) contributes to the ability of invasive ants such as *S. invicta* to reach high densities and dominate ecosystems (Vargo and Fletcher 1989, Mackay et al. 1990, Holway et al. 2002a, Tsutsui and Suarez 2003). Furthermore, a displaced fragment of a polygyne colony is more likely to contain at least one queen and establish successfully than a fragment from a monogyne colony (i.e. with a single queen) (Vargo and Fletcher 1989, Tsutsui and Suarez 2003, Yang et al. 2012). The reproduction of the little fire ant or electric ant (*Wasmannia auropunctata*) is well-documented and has been found to contribute to its successful establishment in human-modified habitats (Foucaud et al. 2009, Chifflet et al. 2018). Some *W. auropunctata* populations reproduce clonally and produce queens by thelytokous parthenogenesis (i.e. from unfertilized eggs), males clonally (through queen eggs), while workers are still produced sexually. Therefore, clonality limits recombination and maintains a combination of male and female genotypes that results in the production of workers with high heterozygosity (Foucaud et al. 2009). These workers are suggested to be better-adapted to human-modified habitats than workers from sexual populations (Foucaud et al. 2009) and to contribute to the range expansion of *W. auropunctata* into disturbed habitats (Chifflet et al. 2018). The reproductive strategies of *W. auropunctata* clearly contribute to its invasive success but, we lack baseline information on the reproduction of most invasive ant species (e.g. occurrence of clonal reproduction, number of queens per colony, number of males queens mate with etc.) to make generalisations of how reproductive strategies contribute to invasiveness (Tsutsui and Suarez 2003, Krushelnycky et al. 2010).

Worker reproduction has an important influence on the social cohesion and efficiency of ant colonies (Bourke 1988), but this aspect of reproduction and its role in the success of invasive ants has been particularly overlooked. In most ant species, workers have retained functional ovaries (but not the ability to mate and store sperm) and are able to lay male eggs and/or trophic eggs (unviable eggs fed to the colony) (Bourke 1988, Hammond and Keller 2004). Worker reproduction can be costly for the colony because reproductive workers often contribute less time to colony labour and may engage in aggressive interactions with other workers for egg laying (Gobin et al. 2003, Heinze 2008). According to the kin selection theory, worker reproduction benefits reproducing workers because they are more related to their sons (average level of relatedness, r=0.5) than to their brothers (r=0.25) (Hamilton 1964). Queens however, are less related to their grandsons (r=0.25) than to their own sons (r=0.5) (Hamilton 1964). As a result, queen control (queen inhibition of worker fertility) can arise in queenright colonies, and the absence of a queen can trigger workers to produce males (Bourke 1988, Dietemann and Peeters 2000, Holman et al. 2010). Worker reproduction is particularly relevant to the study of invasive ants because of its link to queen death and colony decline (Bourke 1988), which can both be triggered by control treatments of invasive ant populations. Determining whether worker reproduction following queen death further hinders the survival of orphaned colonies and/or contributes to the production of viable sexuals that can mate with virgin queens will provide further insight into the role of reproductive strategies in the invasive success of ants.

Study species

The tropical fire ant, *Solenopsis geminata*

My first three chapters focused on *S. geminata*. Originating from the Neotropics, this species is established in many tropical regions of the world but remains little studied in comparison with *L. humile* and *S. invicta* (Holway et al. 2002a, Wetterer 2011). Where it is established, *S. geminata* is a serious ecological and agricultural pest (Wetterer 2011). For example, workers have been observed attacking seabird and turtle hatchlings in Ashmore Reef (north western Australia), an important breeding site for seabirds and turtles (Hodgson and Clarke 2014). Recently, a genetic study used mitochondrial and microsatellite markers to investigate the invasion history of *S. geminata* for the first time (Gotzek et al. 2015). The markers were found to have low variability across the ant's invasive range which limited the study's conclusions, but did suggest that *S.*

geminata went through a genetic bottleneck during its invasion (Gotzek et al. 2015). The presence of diploid males has never been reported for this species, but the low genetic diversity of its invasive populations suggest that these populations would commonly produce diploid males. Interestingly, *S. geminata* is found in relatively undisturbed habitats in its putative native range (Tschinkel 1988, Plowes et al. 2007), but in the Northern Territory of Australia where this ant is invasive, it is limited to disturbed habitats (e.g. road sides, park lawns) for reasons unknown (Hoffmann and O'Connor 2004).

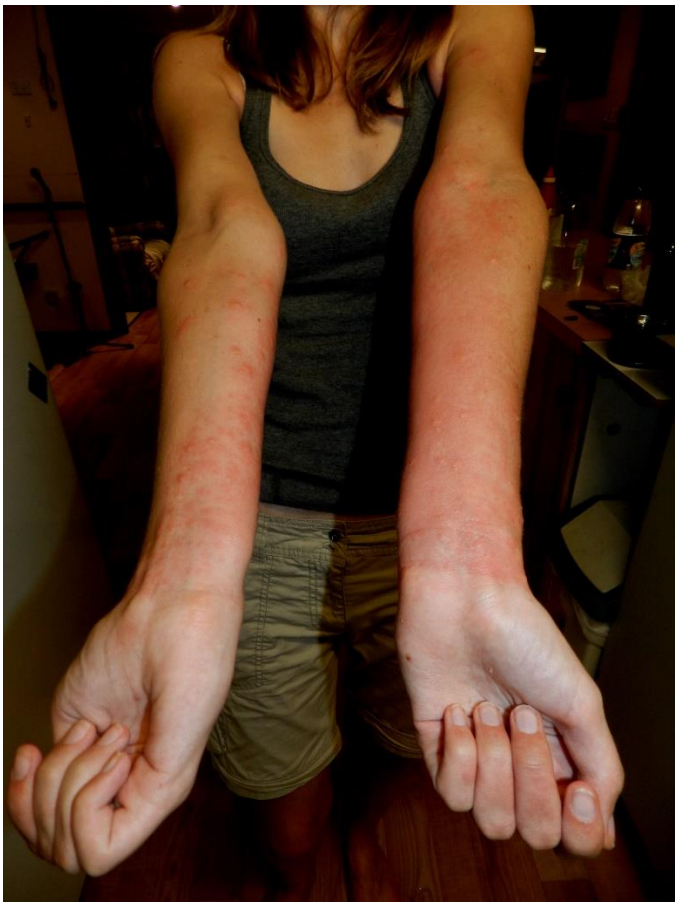

Figure 0.1 The author (PL) showing an allergic reaction to tropical fire ant stings.

The yellow crazy ant, *Anoplolepis gracilipes*

My fourth chapter focused on the yellow crazy ant, *A. gracilipes*. Listed among the world worst' invaders, *A. gracilipes* can have severe effects where it is introduced (Lowe et al. 2000). On Christmas island for example, the ant forms dense colonies (Abbott 2005) and has contributed to

the invasional meltdown of the ecosystem by depleting large areas of a keystone species, the red land crab (*Gecarcoidea natalis*) (Green et al. 1999, O'Dowd et al. 2003). Its native range is unknown but the relatively high mitochondrial DNA haplotype diversity of *A. gracilipes* in southeast Asia suggests that it originated from this region (Wetterer 2005, Drescher et al. 2007). The reproductive mode of *A. gracilipes* remains unresolved, and results from several genetic studies suggest that it is unusual (Drescher et al. 2007, Gruber et al. 2012, 2013). Unlike, *S. geminata* workers, *A. gracilipes* workers have retained ovaries (Bourke 1988, Hammond and Keller 2004), and worker reproduction was recently reported for the first time in queenless *A. gracilipes* colonies (Lee et al. 2017). It is unknown whether worker reproduction contributes to or hinders the invasive success of *A. gracilipes*.

Thesis overview

Each of my four data chapters is focused on a process that may influence the success of invasive ants, namely: genetic bottleneck, disturbance, anthropogenic dispersal, and reproductive strategies. Below, I identify research gaps for *S. invicta* for the first three processes and *A. gracilipes* for the last process. I have written each data chapter with the objective of submitting each to a peer-reviewed journal, which is why there is some repetition among the different chapters.

Chapter 1: Strategies of the invasive tropical fire ant (*Solenopsis geminata*) to minimize inbreeding costs

Inbreeding can disrupt the functioning of the sex determination system of ants and lead to the production of diploid males (Crozier 1971, 1977, Ross et al. 1993). The low genetic diversity of *S. geminata* across its invasive range (Gotzek et al. 2015) suggests that diploid males would be common. Diploid male production would be highly detrimental for the growth and survival of new *S. geminata* colonies as queens start colonies claustrally (Ross and Fletcher 1986). In this chapter, I used microsatellite DNA markers and conducted a lab experiment to determine the prevalence of diploid males in field colonies, determine whether diploid male production can hinder the growth and survival of founding colonies, and investigate whether strategies such as polyandry (queen mating with multiple males), pleometrosis (several queens starting a colony together), and diploid male execution can alleviate the potential burden of diploid male production. This work contributes to improving our understanding of how invasive ants

overcome the effects of inbreeding. This chapter has been published in Scientific Reports and is referenced in my thesis as Lenancker et al. 2019.

Figure 0.2 Benjamin D. Hoffmann and the author (PL) collecting tropical fire ants in the Northern Territory.

Chapter 2: Roles of biotic resistance and abiotic factors in the establishment of an invasive ant

The limited distribution of *S. geminata* to disturbed habitats in the Northern Territory could be due to strong biotic resistance, unsuitable abiotic factors or a combination of both. In this chapter, I conducted two field experiments in disturbed and undisturbed sites to determine (i) whether the survival of founding *S. geminata* queens is higher in disturbed habitats and (ii) the relative role of biotic resistance and abiotic factors in the survival of *S. geminata* queens during colony founding. By distinguishing the contribution of biotic and abiotic factors in the successful establishment of an invasive ant, this study improves our understanding of the mechanisms by which disturbance facilitates biological invasions.

Chapter 3: Invasion pathways and social structure of the invasive tropical fire ant (*Solenopsis geminata*) revealed by genome-wide SNP data analyses

The low genetic diversity of *S. geminata* across its invasive range (Gotzek et al. 2015) makes RADseq one of the best available methods to investigate its population genetics at the global and colony scale (Nygaard and Wurm 2015). A recent genetic study using microsatellite and mitochondrial markers investigated the invasion history of *S. geminata* for the first time (Gotzek et al. 2015) but was limited by the low variability of the markers for *S. geminata*. In this chapter, I used double digest RADseq (a variant method of RADseq) on *S. geminata* workers collected in the ant's native and invasive range to demonstrate the power of RADseq methods over traditional genotyping methods, determine the origin and invasion history of *S. geminata*, quantify how much genetic diversity this species has lost during its introduction, determine geneflow among invasive populations, and determine the social form (single or multiple queened colonies) of *S. geminata* across its invasive range. This work generated critical information regarding the invasion history and social form of *S. geminata*.

Chapter 4: Origin, behaviour, and genetics of reproductive workers in the invasive yellow crazy ant *Anoplolepis gracilipes*

Worker reproduction can greatly influence the social cohesion and efficiency of ant colonies (Bourke 1988). A recent study demonstrated that *A. gracilipes* workers were able to produce males by arrhenotokous parthenogenesis (Lee et al. 2017). In this chapter, I used microsatellite markers and ran a series of experiments and observations to investigate the characteristics, triggers, and costs of worker reproduction in *A. gracilipes* colonies. The potential role of worker reproduction in the success of invasive ants has been overlooked, and this work improves our understanding of the potential costs and benefits of worker reproduction for this highly invasive ant.

Figure 0.3 Yellow crazy ant queen and workers collected in Cairns, Queensland

Chapter 5: Synthesis

In this chapter, I summarize and synthesize the main results and conclusions of my research. I also address some of the limitations of my project and discuss opportunities for future research.

Chapter 1 Strategies of the invasive tropical fire ant (*Solenopsis geminata*) to minimize inbreeding costs

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Statement of contribution of others:

PL participated in the design of the study, carried out the lab work, analysed the data and drafted the manuscript. BH and LL coordinated the study and helped draft the manuscript. LL assisted with statistical analysis and WT with molecular lab work and analysis. See acknowledgments for other contributions.

Abstract

How invasive species overcome challenges associated with low genetic diversity is unclear. Invasive ant populations with low genetic diversity sometimes produce sterile diploid males, which do not contribute to colony labour or reproductive output. We investigated how inbreeding affects colony founding and potential strategies to overcome its effects in the invasive tropical fire ant, *Solenopsis geminata*. Our genetic analyses of field samples revealed that 13–100% of males per colony (n=8 males per 10 colonies) were diploid, and that all newly mated queens (n=40) were single-mated. Our laboratory experiment in which we assigned newly mated queens to nests consisting of 1, 2, 3, or 5 queens (n=95 ± 9 replicates) revealed that pleometrosis (queens founding their nest together) and diploid male larvae execution can compensate for diploid male load. The proportion of diploid male producing (DMP) colonies was 22.4%, and DMP colonies produced fewer pupae and adult workers than non-DMP colonies. Pleometrosis significantly increased colony size. Queens executed their diploid male larvae in 43.5% of the DMP colonies, and we hypothesize that cannibalism benefits incipient colonies because queens can redirect nutrients to worker brood. Pleometrosis and cannibalism of diploid male larvae represent strategies through which invasive ants can successfully establish despite high inbreeding.

Introduction

Populations arising from an introduction event often lose genetic variation because of their small founder population size (Simberloff 2009). Small populations are at risk of accumulating deleterious mutations via inbreeding and eventually going extinct (Lynch et al. 1995, Caballero et al. 2017). However, many populations of successful invaders began as small populations having also gone through a genetic bottleneck (Allendorf and Lundquist 2003) (e.g. cheatgrass: *Bromus tectorum*, Novak and Mack 1993; Argentine ant: *Linepithema humile*, Tsutsui et al. 2000; house finch: *Carpodacus mexicanus*, Hawley et al. 2006; solitary sweat bee: *Lasioglossum leucozonium*, Zayed et al. 2007; Asian honey bee: *Apis cerana*, Gloag et al. 2016). This genetic paradox is well-studied in *L. humile* and the red imported fire ant (*Solenopsis invicta*). Both species lost genetic variation during their introductions to various areas of the globe (Ross et al. 1993, Tsutsui and Suarez 2003) and yet they are listed among the world's most successful invaders (Lowe et al. 2000). The low genetic variability of *L. humile* in its invasive range may have resulted in selection for traits that encourage its spread and growth (Tsutsui and Suarez

2003). Within *L. humile*'s native range, workers from distinct colonies recognize and attack workers from other nests because ants from different nests have distinct cuticular hydrocarbon profiles (Brandt et al. 2009). The cuticular hydrocarbons of introduced *L. humile* populations became homogeneous, presumably as a consequence of genetic bottlenecks (Tsutsui et al. 2000, Brandt et al. 2009). Therefore, the ants are unable to distinguish nest mates from outsiders and form large, dense supercolonies of interacting nests (Tsutsui et al. 2000, Brandt et al. 2009). Supercolonies of *L. humile* are potentially able to direct more resources toward interspecific competition, foraging, and colony growth than colonies in their native range because of the absence of the cost of intraspecific territoriality (Tsutsui and Suarez 2003).

For other invasive Hymenoptera, such as *S. invicta*, genetic bottlenecks can lead to adverse consequences due to the reduction in sex-determining allele diversity in introduced populations (Ross et al. 1993, Gloag et al. 2016). The most common sex determination system of Hymenoptera is the haplodiploid sex determination system in which fertilized eggs develop into females that are diploid, and unfertilized eggs develop into males that are haploid. In some cases, the sex of the offspring is controlled by their genotype at the complementary sex determination (CSD) locus (or loci) (Heimpel and de Boer 2008). When diploid hymenopteran individuals are homozygous at the CSD locus, they develop into sterile diploid males (Crozier 1971, 1977). CSD and male diploidy have been found in over 60 species of Hymenoptera including *S. invicta* (van Wilgenburg et al. 2006).

When *S. invicta* queens mate only once and with a male with the same CSD genotype (i.e. matched-mating), half of their diploid offspring are homozygous at the CSD locus and develop into sterile diploid males if they are successfully reared to adulthood (Ross and Fletcher 1986, Cook and Crozier 1995). In some populations as much as 20% of *S. invicta* queens are match-mated and produce diploid males (Ross and Fletcher 1986, Cook and Crozier 1995). A low proportion of diploid males is fertile and their offspring are always triploid (Krieger et al. 1999). However, evidence of reproductive triploid queens has never been reported. This suggests that triploid queens are either sub-viable or executed by workers, and therefore fertile diploid males are likely a genetic dead-end (Krieger et al. 1999). Because the only function of males is reproduction, production of sterile diploid males represents an ineffective colony resource allocation and can reduce colony growth rate (Ross and Fletcher 1985a, 1986, Ross et al. 1993, Tschinkel 1998a).

Most of what we know about minimizing the cost of diploid male production comes from two species: *S. invicta* and *A. mellifera*. In *S. invicta*, polygyny (multiple-queened colonies) can help minimize the cost of sterile diploid male production induced by low genetic diversity (Ross and Fletcher 1986). Colonies of *S. invicta* can be either polygyne or monogyne (single-queened) (Adams et al. 1976, Mackay et al. 1990, Tschinkel 2006) but in the field diploid males are only ever found in polygyne populations (Ross and Fletcher 1985a). Between 11.9 and 19.6% of queens from monogyne populations produce diploid males, but these queens are unable to survive under field conditions (Ross and Fletcher 1985a, 1986). In the laboratory, single-queened *S. invicta* colonies founded by diploid male producing (DMP) queens were found to have lower growth and survival compared with non-DMP colonies because half of DMP queens' reproductive output were males that failed to develop beyond the larval stage, instead of workers that could contribute to colony labour (Ross and Fletcher 1986). In the field, DMP queens that attempt to start a colony on their own would invariably fail because they would invest half of their reserves in their sterile males instead of their workers (Ross and Fletcher 1985a, 1986). Presumably, only DMP *S. invicta* queens that are adopted into an existing polygyne colony or a queenless monogyne colony would survive because the worker force supplied by the non-DMP queens would compensate for the production of diploid males from DMP queens (Ross and Fletcher 1985a, 1986, DeHeer and Tschinkel 1998)

In addition to polygyny, there are three other strategies before or during colony founding that could mitigate the cost of diploid male production. Polyandry (queen mating with multiple males) could potentially reduce the occurrence of diploid male production by a match-mated queen because she could also have mated with males that do not share an allele at the CSD locus with her (Page 1980, Tarpy and Page 2002, Baer 2016, Gloag et al. 2016). Execution of diploid males early in their development is another strategy to reduce the cost of diploid male production and is employed by *A. mellifera* (Herrmann et al. 2005). However, workers of *S. invicta* seem unable to discriminate diploid males (Ross and Fletcher 1986). Finally, queens cooperatively founding a nest (pleometrosis) is another possible means of minimizing the diploid male load during colony founding because DMP queens would benefit from the worker force supplied by non-DMP queens.

The invasive tropical fire ant, *Solenopsis geminata*, is a serious agricultural and ecological pest closely related to *S. invicta* and has established in almost all tropical regions of the world

(Wetterer 2011), yet little is known of its social biology. *Solenopsis geminata* originates from the Neotropics (the exact extent of its native range is unclear) and has been spread through human commerce since the 16th century (Wetterer 2011, Gotzek et al. 2015). Even though diploid males have never been reported in *S. geminata*, its low genetic diversity across its invasive range (Gotzek et al. 2015) suggests that *S. geminata* potentially went through a genetic bottleneck and that diploid males are probably common. Both social forms, monogyne and polygyne, have been reported to occur in native populations of *S. geminata* (Mackay et al. 1994) and invasive populations in Florida (Adams et al. 1976, Ross et al. 2003) and the Galapagos islands (Wauters et al. 2018). Colonies have been found to be exclusively monogyne in Taiwan (Lai et al. 2015). Its social form remains unknown for the rest of its invasive range.

Initial observations of the Australian *S. geminata* population we used in our study indicated that newly mated queens dispersed via mating flights and were capable of independent founding when placed under laboratory or controlled field conditions. Independent founding is more frequent in monogyne ant populations but, in some species, queens from polygyne populations found colonies independently (Keller 1991). In some ant species, queens originating from polygynous colonies can found a colony under laboratory conditions in the absence of workers and other queens, demonstrating that the founding mode of polygynous ants may be more diverse than thought (e.g. *Solenopsis invicta*, DeHeer 2002; *Anoplolepis gracilipes*, Ito et al. 2016; *Pachycondyla villosa*, D'Ettorre et al. 2005; and *Lasius neglectus*, Espadaler and Rey 2001). We also observed in our *S. geminata* population that founding queens sometimes produced unusually large larvae, which in *S. invicta* typically indicates diploid male production (Ross and Fletcher 1985a). Queens of *S. geminata* found colonies claustrally, whereby the queen seals herself in a chamber and rears her first brood using her fat reserves (Tschinkel and Howard 1983). When the first workers emerge, they leave the nest to forage and feed the queen and her brood. Rapid growth is essential for the survival of claustrally founded colonies (Ross and Fletcher 1986). Therefore, diploid male production would likely be highly detrimental for these colonies' growth and survival. Whether *S. geminata* produces diploid males or has strategies that would minimize the costs of diploid male production and increase colony founding success has not previously been studied.

Our work aimed to establish whether *S. geminata* queens produced diploid males, how common diploid male production is, and whether colony founding strategies can compensate for the

genetic load associated with diploid male production. We used a combination of microsatellite analyses on field-collected *S. geminata* males and queens and a laboratory experiment in which we simulated a claustral founding scenario with haplometrosis and pleometrosis treatments to: (1) determine whether diploid males exist, and their prevalence, (2) estimate the number of males queens mate with, (3) determine whether diploid male production hinders colony growth and, (4) investigate whether strategies such as pleometrosis or brood cannibalism alleviate the potential burden of rearing sterile males. Our findings will improve our understanding of one of the most fundamental questions in invasion biology research: how invasive species overcome low genetic diversity and inbreeding.

Methods

Queen and male collection.

We collected newly mated queens of *S. geminata* after their nuptial flight, between March and April 2015 and 2016 at Humpty Doo (Figure S1.1), Northern Territory, Australia (−12.5722°, 131.0842°), as they flew to a veranda light and dropped to the ground to found a nest in the early evening. We used some of these queens for our colony founding experiment (see colony founding experiment section) and the remainder for spermathecal dissections (see spermatheca dissection section)

To determine the presence of diploid males in the field we collected eight adult males from each of ten nests at six sites in the Northern Territory (Table S1.1 and Figure S1.1) between January and March 2014. The males were placed in 99% ethanol at −18 °C until DNA extraction (see DNA extraction and microsatellite genotyping section).

Spermatheca dissection

We isolated sperm from 40 mated queens collected in 2015 following the method of Tay and Crozier (2001). Each sperm sample was stored in 10 µL isotonic buffer (1.76 mM NaCl) at -18°C until DNA extraction. The head and thorax of individual dissected queens were stored in 99% ethanol at -18°C until DNA extraction (see DNA extraction and microsatellite genotyping section).

Colony founding experiment

The 1187 queens collected in 2016 yielded 122±6 replicates per treatment. The queens were weighed and established in trials of 1, 2, 3 or 5 queens per colony within 15 to 40 hours after collection. Nests consisted of a 15-mL centrifuge tube, half filled with water retained by a cotton plug. Queens were retained by a second cotton plug inserted in the tube mouth. We inserted the nesting tubes into a polystyrene sheet to keep the nesting chambers in the dark. We allowed queens to initiate nests for 23 days, which was 1 day after the emergence of the first generation of workers in our pilot study. Because *S. geminata* queens are capable of founding their colonies claustrally (McInnes and Tschinkel 1995), they did not need to be fed during the experiment.

We recorded queen mortality and the cause of death three times per week. We considered dismembered queens to have been executed by other queens and dead intact queens to have died of natural causes. We also recorded the presence of distinctly large larvae and their position within the nest three times per week. On the 24th day we terminated the colonies by freezing, weighed each queen and all brood, and counted the number of eggs, larvae, worker pupae, and adult workers of the colonies in which all the queens survived (n=380, 95±9 replicate per treatment). Unusually large larvae distinguishable from the second instar larval stage were counted and weighed separately from the rest of the brood. As detailed in the Discussion, we considered large larvae to be males and not queens because investment in queen production is very unlikely during the colony founding stage (McInnes and Tschinkel 1995, Tschinkel 2006). We opportunistically selected 15 large larvae, out of the 109 we observed, for ploidy determination using microsatellite markers to determine whether they were haploid or diploid male larvae (see DNA extraction and microsatellite genotyping section). We left the other larvae in their nest to observe how the queens were treating them.

DNA extraction and microsatellite genotyping.

We extracted DNA from 80 adult males collected from ten field colonies (8 males per colony), 40 sperm samples from mated queens immediately following their nuptial flights, and 15 large larvae from the colony founding experiment. We also extracted DNA from the 40 queens that were dissected for sperm sampling, to enable distinction of multiple mating from potential maternal contamination. We washed larvae, males, and queens three times in distilled water to avoid contamination and used the Zymo Research Tissue and Insect DNA MiniPrepTM kit

following the supplier's instructions. For the sperm, we used the Qiagen DNeasy Blood and Tissue kit and the Zymo Research DNA Clean and ConcentratorTM following the supplier's instructions.

Based on the criterion of maximal allele detection (Gotzek et al. 2015), we chose six microsatellite markers (Table S1.3) for the adult males and three markers for the queen, sperm, and larvae. PCR conditions are described in Supplementary Information b. PCR for the queen, sperm and larvae were multiplexed and submitted to the Australian Cancer Research Foundation Biomolecular Resource Facility at the John Curtin School of Medical Research, Australian National University, for genotyping. We used the proprietary software 'Geneious®' (Biomatters Ltd., Auckland, New Zealand) to visualize trace files, fit the internal ladder, and identify microsatellite alleles. The highest peaks within the allele size range for *S. geminata* (Table S1.3) were determined. Heterozygous male larvae were scored as diploid. We inferred the queen's mating frequencies by scoring the alleles for the sperm and compared queen and sperm genotypes for contamination.

Statistical analysis

We analysed the colony founding experiment data using R version 3.3.2 (R Core Team 2018) and functions from the stats package (R Development Core Team 2009) unless otherwise mentioned. We used a generalized linear model (GLM, glm function) followed by ANOVA F-test (Anova function with test=F in the car package, Fox and Weisberg 2002) with the total number of brood and adult workers per colony as the response variable, the number of queens per colony (1, 2, 3 or 5), the presence of large larvae, and the mean initial queen weight as fixed factors. We used count rather than weight data as the response variable for our models because the two were highly correlated (Pearson's correlation, $r=0.82$, $t=27.516$, $df=378$, $P<0.0001$) and accurate measurement of brood weight can be difficult to achieve considering the size of the eggs and ease of damaging larvae. We used a quasi-Poisson error structure to account for overdispersion (Bolker et al. 2009, O'Hara and Kotze 2010).

To test the effect of large larvae production on egg, regular-sized larva, and the sum of worker pupae and adult worker production, we used three generalized linear models with a quasi-Poisson error structure followed by ANOVA F-test with the response variable being either the number of eggs, number of larvae, or sum of worker pupae and adult workers per colony and

with explanatory variables being the number of queens per colony (1, 2, 3 or 5), the presence of large larvae, and the mean initial queen weight. To test whether large larvae production and pleometrosis influenced the average weight loss of founding queens during the experiment, we used a linear model (lm function) with average queen weight loss as the response variable and the number of queens per colony and the presence of large larvae as explanatory variables. To test whether the number of brood and adult workers produced per queen (sum of brood and adult worker count divided by the number of queens) varied with queen treatment, we used a linear model with the number of brood and adult workers produced per queen as the response variable and the number of queens per colony and the presence of large larvae as explanatory variables. We compared the equivalent distribution of average queen weight in each treatment (i.e. number of queens per colony) with a Kruskal-Wallis test (kruskal.test function). We verified whether queen execution was higher in colonies with large larvae than in colonies without using a contingency chi-square test (chisq.test function).

We conducted additional analysis on the haplometrosis (single queen) treatment. We analysed the effect of the initial queen weight on the brood and adult worker count with a Wilcoxon signed-rank test (wilcox.test function). We tested whether weight loss differed between queens that did and did not have brood at the end of the experiment using Wilcoxon rank sum test (wilcox.test function). We tested whether queens that died of natural death were lighter than surviving queens using a Kruskal-Wallis test.

For all models, we plotted the residuals to check for their homoscedasticity, independence, and normality (plotresid function in the RVAideMemoire package Hervé 2016) and, where appropriate, we used post hoc Tukey tests to make pairwise comparisons (lsmeans function in the lsmeans package Lenth and Hervé 2015) which is the best available method when sample sizes are unequal (Dunnett 1980).

Results

Diploid males in field colonies.

Diploid males were found in 8 of the 10 field colonies (Table S1.2). According to microsatellite allele scoring, from one to all (13-100%) of the 8 sampled adult males per colony were diploid. Of the six microsatellite DNA markers that we used, two markers (i.e. Ms16C121 and

Ms33Sol11) were sufficiently polymorphic to detect heterozygosity. Out of the 80 male samples, we successfully amplified 69 male DNA samples at both these loci, and 8 at one locus while amplification failed at both loci for 3 samples. Heterozygosity at one locus indicates diploidy. In total, we found that 31 males (44.9%) were heterozygous, 26 at one locus and 5 at two loci. Five field colonies had more than two alleles at a given locus: H2 at 2 loci (Ms16C121 and Ms14C334), V1, E1, and S1 at one locus (Ms33Sol11 for V1 and E1 and, Ms14C334 for S1) while the remaining five had two alleles per locus at most.

Queen mating frequency.

We found all 40 queens to be monandrous (i.e. one allele per individual sperm sample), so there was no need to cross examine sperm DNA with maternal DNA to check for maternal contamination. We successfully amplified 31 of 40 sperm DNA samples for all three microsatellite loci, while the remaining 9 samples amplified successfully for two loci. We successfully amplified 15 queen DNA samples for all three microsatellite loci, 9 DNA samples for two loci, and 12 DNA samples for one locus. None of the queens were triploid. All sperm DNA samples had one allele at all loci or the locus for which they were successfully amplified. We used the sperm genotype frequency from 32 males (Table S1.5) which were successfully genotyped at all three loci to calculate the probability of n=1-32 queens having mated with two males sharing the same genotype (see Supplementary Information d for more details on the calculation). We found that the probability of missing one double-mated queen (i.e. double-mating frequency: 3.1%) was 0.121 (Figure S1.6). The probability of having missed double mating in 12.5% of the population and above was negligible (from 2.14×10^{-4} to 4.46×10^{-30}). Therefore, polyandry is unlikely in our population but could occur at low frequency.

Colony founding experiment

Of the 487 colonies reared (n=122±6 per treatment), 106 had queens that died before the end of the experiment. Of these, we deemed 67 colonies had queens die naturally, 28 had signs of queen execution, and 11 colonies had both. The frequency of queen mortality was too low to compare it across treatments. Queens were executed from day 2 to day 23 (mean ± SD: 13.2±6 days) of the experiment. In the single queen treatment, the initial weight of queens that died naturally was the same as surviving queens (Kruskal-Wallis test, Kruskal-Wallis $\chi2=29.601$, df=38, P=0.833).

We observed a total of 109 large larvae in 85 out of 380 colonies. None of these larvae developed into pupae before we terminated the colonies. Of the 15 large larvae we had selected for ploidy determination, 10 were successfully amplified at two to three markers. Three of these larvae were heterozygous at two markers and four at one marker and were scored as diploid. We detected one allele per marker for the remaining three larvae. We observed that 40% of the diploid male producing colonies had placed their large larvae in the refuse pile with the colony waste, away from the rest of the brood. We did not observe large larvae to be tended by queens, except on two occasions in which we observed queens in two different colonies tapping a larva placed in their respective refuse piles with their antennae. Following the results from our microsatellite DNA analyses, common behaviour displayed by queens towards large larvae and, additional evidence detailed in the Discussion, we considered all large larvae to be diploid males in the subsequent analyses. We hereafter refer to the queens and colonies that produced these large larvae as diploid male producing (DMP).

We found evidence of queens executing their diploid male larvae. We found that 73.4% (n=80/109) of diploid larvae disappeared between days 2 and 12 (mean ± SD: 3.8±1.8 days) after we had observed them. Large larvae disappeared in 43.5% of DMP colonies. There were too few occurrences to compare the number of executed diploid male larvae among treatments (Figure S1.4). Queens were the only adult ants in our colonies, which suggests that the queens cannibalized their diploid male larvae or fed them to other larvae. Queens from single and two-queened DMP colonies had lost less weight on average than non-DMP colonies but there was no difference in queen weight loss for three and five-queened colonies (Figure 1.1; LM: ANOVA, $\chi 2=11.7166$, $P<0.01$; post hoc tests $P<0.01$ for pairwise comparisons DMP vs non DMP in single queen and two-queened colonies, $P>0.05$ for all other pairwise comparisons). In 34.1% of DMP colonies (n=29/85), diploid male larvae were first found with the colony refuse before disappearing. We thought diploid male larvae could have died before being placed in the refuse pile by the queens. However, discarded larvae looked live and intact even after remaining in the refuse pile for several days, and we confirmed with a microscope that two of the discarded diploid male larvae were alive.

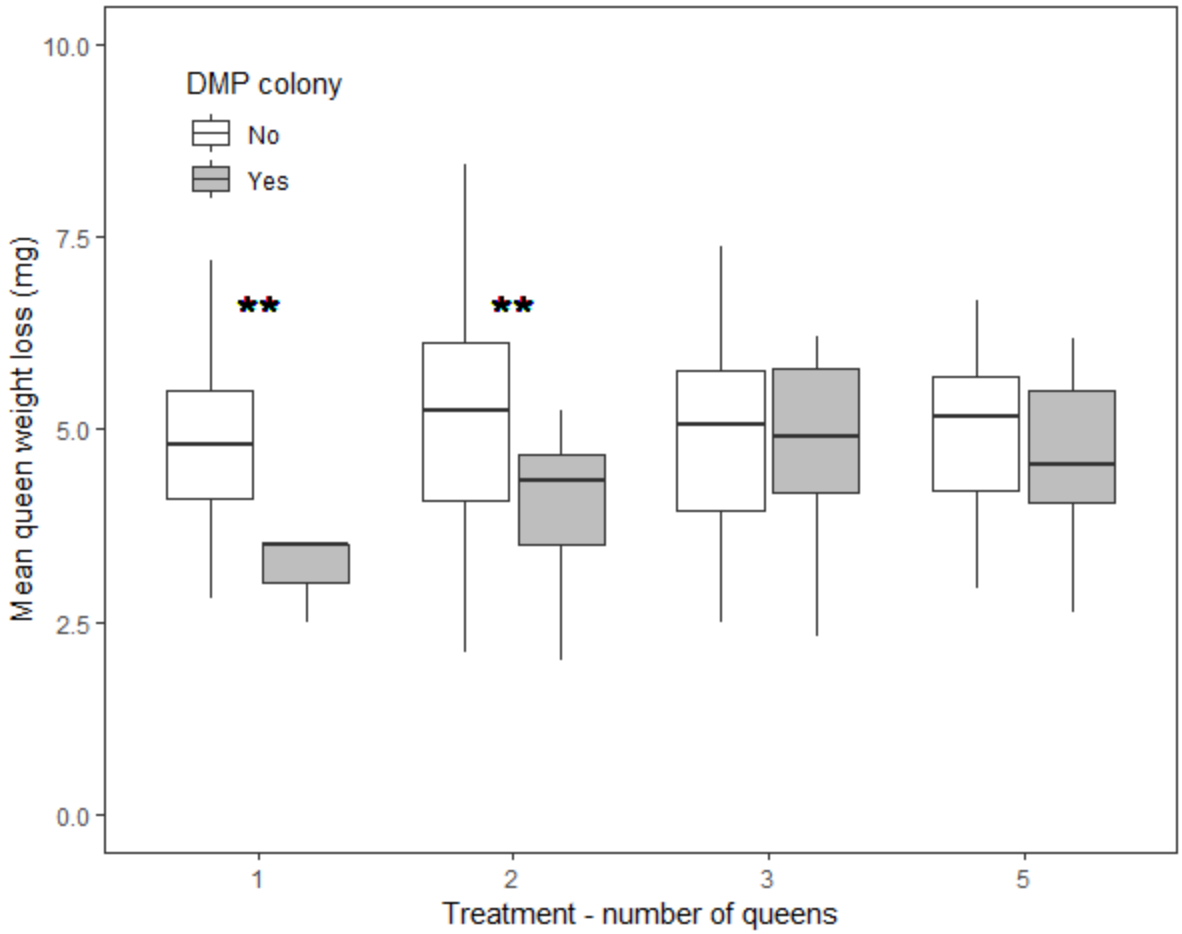

Figure 1.1 Mean queen loss of weight between the start and the end of the experiment for each queen treatment separated between DMP (diploid male producing) colonies and non-DMP colonies. The interaction between diploid male production and the number of queens had a significant effect on the mean queen weight loss (LM: ANOVA, $\chi 2=11.7166$, $P < 0.01$). **Indicates a significant difference between DMP colony and non-DMP colonies for the corresponding queen treatment (post hoc tests $P < 0.01$). One queen: non-DMP n=97, DMP n=5, two queens: non-DMP n=79, DMP n=16, three queens: non-DMP n=68, DMP n=29, five queens: non-DMP n=51, DMP n=35.

We estimated the proportion of DMP queens to range from 4.9% to 10.0%. We found that 4.9% of colonies with single queens produced diploid males, that would normally give us the proportion of DMP queens in the population. However, this number is likely an underestimate because 29.4% of queens in the single queen treatment did not produce any brood, and without any larvae it was not possible to differentiate non-DMP queens from DMP queens. If we take out queens that did not produce brood, 7.5% of single queens were DMP. We found that 16.8%,

29.9%, and 45.3% of 2-, 3- and 5-queen colonies, respectively, produced diploid males. To obtain another estimation of DMP queen frequency, we divided the frequency of DMP colonies by the number of queens for each multiple queen treatment. We obtained a DMP queen proportion of 8.4% for 2-queen colonies, 10% for 3-queen colonies and 9.1% for 5-queen colonies. We found that queen execution occurred in 10.9% of the pleometrotic colonies and that queen execution was not higher in DMP colonies than in non-DMP colonies (contingency chi-square test, $\chi 2=0.25721$, df=1, P=0.612).

The production of diploid males led to a reduction in the production of pupae and adult workers in DMP colonies which pleometrosis could alleviate. Our GLM with the number of worker pupae and adult workers as the response variable showed that DMP colonies had significantly fewer worker pupae and adult workers than non-DMP colonies (Figure 1.2; Table 1.1; post hoc tests $P<0.05$ for pairwise comparisons DMP vs non DMP in three-queened and five-queened colonies, $P>0.05$ for all other pairwise comparisons). Increasing the number of founding queens in DMP colonies increased the number of brood and adult workers at the end of the experiment (Figure S1.2; post hoc tests DMP colonies $P<0.001$ for pairwise comparisons two vs five queens, $P<0.01$ for one vs five and three vs five queens, $P<0.05$ for two vs three queens, $P>0.05$ for all other pairwise comparisons). There was no difference in the combination of brood and adult worker counts between DMP colonies and non-DMP colonies by treatment (Table 1.1 and Figure S1.2). We were able to distinguish worker from diploid male larvae from the second instar larval stage and excluded diploid male larvae we had identified from the brood count. We found similar results when analysing eggs and worker larvae separately (Table 1.1).

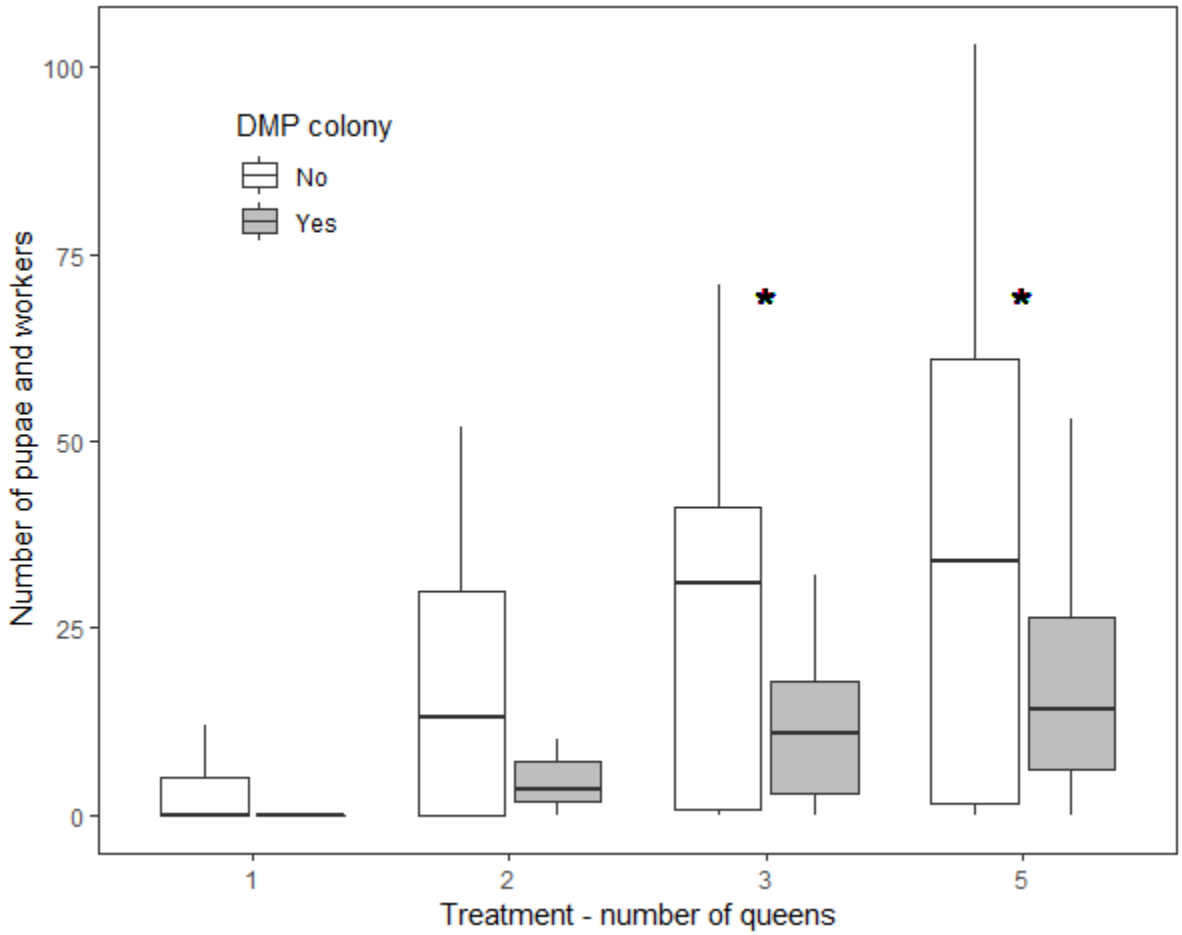

Figure 1.2 Number of worker pupae and adult workers produced for each queen treatment separated between DMP (diploid male producing) colonies and non-DMP colonies. DMP colonies had significantly fewer worker pupae and adult workers than non-DMP colonies (Table 1.1); * Indicates a significant difference between DMP colony and non-DMP colonies for the corresponding queen treatment (post hoc tests $P<0.05$). One queen: non-DMP n=97, DMP n=5, two queens: non-DMP n=79, DMP n=16, three queens: non-DMP n=68, DMP n=29, five queens: non-DMP n=51, DMP n=35.

The more queens founding a colony together, the more brood the colony had at the end of our experiment. Colonies with more queens had significantly more brood and adult workers (Table 1.1; Tukey post hoc tests $P<0.01$ for all pairwise comparisons) but the number of brood and adult workers per queen did not vary with the number of queens per colony (LM: ANOVA, $\chi 2=36.038$, $P<0.01$; post hoc tests $P>0.05$ for all pairwise comparisons, Figure S1.3). Colonies with more queens had more eggs (Table 1.1; Tukey post hoc tests $P<0.01$ for all pairwise

comparisons) and more larvae (Table 1.1; Tukey post hoc tests $P<0.01$ for all pairwise comparisons except for 2 queens vs 1 queen and 2 queens vs 3 queens for which $P>0.05$). We found no live brood in 29.4% of colonies in the single queen treatment and 1.8% of colonies in the multiple queen treatments. All of these colonies had eggs that were dead at the end of the experiment, and their nests were full of mould.

The initial weight of queens influenced some of the brood production. The weight distribution of newly mated queens before the start of the experiment (n=1,013) was unimodal (Figure S1.5). Most queens (99%) weighed between 11 and 16 mg while a few (1%) appeared to be outliers, weighing less than 11 mg. We did not notice lighter queens to be unusually smaller (microgynes) than heavier queens. The initial mean queen weight did not differ among treatments (Kruskal-Wallis test, Kruskal-Wallis $\chi2=2.7102$, df=3, $P=0.439$). The total number of brood and adult workers produced by each colony did not change with the average queen initial weight in pleometrotic treatments (Table 1.1). But in the single queen treatment, queens that were heavier at the start of the experiment had more brood and adult workers than lighter queens (Wilcoxon signed-rank test, $V=5253$, $P<0.0001$). Queens that produced brood lost more weight than brood-less queens. Haplometrotic queens that had brood at the end of the experiment lost 39±8.5% (mean ± SD) of their initial weight whereas queens that did not have brood lost 36±5.1% (Wilcoxon rank sum test, $W=789$, $P<0.05$). Regardless of treatments, colonies with heavier initial average queen weight had significantly more larvae than colonies with lighter average queen weight, whereas average initial queen weight had no significant effect on number of eggs and the sum of worker pupae and adult workers (Table 1.1). The number of queens per colony did not affect the average queen weight loss (LM: ANOVA, $\chi2=11.7166$, $P>0.05$).

Table 1.1 Summary of generalized linear model results for each response variable in the colony founding experiment. 'x' represents the interaction terms.

Variables	*df*	F	*p*
Number of worker brood and adult workers	quasi-poisson, n=380		
Number of queens per colony	3	141.52	<2.2e-16
Presence of diploid male larvae	1	3.60	0.059
Queens x diploid male larvae	3	0.57	0.63
Mean initial queen weight	1	2.55	0.11
Number of eggs	quasi-poisson, n=380		
Number of queens per colony	3	108.63	<2.2e-16
Presence of diploid male larvae	1	0.30	0.59
Queen x diploid male larvae	3	0.64	0.59
Mean initial queen weight	1	0.52	0.47
Number of larvae	quasi-poisson, n=380		
Number of queens per colony	3	46.68	<2.2e-16
Presence of diploid male larvae	1	0.29	0.59
Queen x diploid male larvae	3	0.39	0.76
Mean initial queen weight	1	4.02	0.04
Number of worker pupae and adult workers	quasi-poisson, n=380		
Number of queens per colony	3	54.89	<2.2e-16
Presence of diploid male larvae	1	34.76	<0.01
Queen x diploid male larvae	3	1.11	0.35
Mean initial queen weight	1	2.65	0.10

Discussion

Genetic bottlenecks can cause populations to accumulate deleterious mutations and be at risk of extinction (Lynch et al. 1995, Caballero et al. 2017). However, many invasive species successfully establish despite having experienced a genetic bottleneck (Tsutsui et al. 2000, Allendorf and Lundquist 2003, Gotzek et al. 2015). Understanding how invasive populations with low genetic diversity become successful invaders is one of the central questions of invasion biology. As expected given the low genetic diversity within our *S. geminata* population, we found that diploid males were common in the field (n=8/10 colonies) and that their frequency within colonies varied greatly (13-100% of the sampled adult males). Diploid males may be more common than we estimated. We found that 47.3% of queens (n=36) were homozygous. If the frequency of homozygosity is similar between queens and males, 47.3% of males scored as haploid may have been homozygous diploid males. Therefore, diploid male frequency could range from 54 to 100% instead of 13-100% of males sampled per colony. To our knowledge, this is the first evidence of diploid male production in *S. geminata*. We also found that *S. geminata* can use two strategies to minimize the cost of diploid male production: pleometrosis and execution of diploid male larvae. Polyandry could have potentially reduced the occurrence of DMP queens but, we found that the queens were always single-mated.

Our results confirmed that the genetic diversity of *S. geminata* in Australia was low, presumably because this population reflected the expected genetic bottleneck during the spread of *S. geminata* through the Indo-Pacific region (Gotzek et al. 2015). For example, we found that the three microsatellite DNA markers used on queen samples had 3 to 7 alleles per locus (Table S1.4) whereas the same markers used in a worldwide study of *S. invicta* had 13 to 22 alleles per locus in some sites situated within the native range of *S. invicta* (Ascunce et al. 2011). This genetic bottleneck most likely contributed to the production of diploid males. We estimated the frequency of DMP queens in our lab colonies to be between 4.9% and 10% which is lower than the newly mated DMP queen frequency of *S. invicta* in lab colonies collected in monogyne (11.9 to 19.6%, Ross and Fletcher 1985, 1986) and polygyne populations (13.9%, Ross and Fletcher 1985) originating from its invasive range. It is possible that triploid queens may be present in small numbers among the Australian population of *S. geminata*, but we failed to detect these queens among newly mated queens that were collected for this study. If triploid queens are absent in the field, it may indicate that diploid males are aspermic, contribute to the production

of sub-viable queens, or that reproductive triploid queens are executed by workers as suggested for *S. invicta* (Crozier 1977). The low genetic diversity in our population made diploidy difficult to detect in our large larvae. In *S. invicta*, the presence of large larvae with the worker brood of founding queens typically indicates diploid male production (Ross and Fletcher 1985a). Sexual larvae of *S. geminata* are distinctly larger than worker larvae as with *S. invicta* (Tschinkel 2006), and therefore, theoretically, a large larva could be either a male larva or a queen larva. However, it is highly unlikely that incipient colonies would invest in queen production (McInnes and Tschinkel 1995, Tschinkel 2006). Any large larvae would therefore be the result of either unmated queens that are only able to produce haploid males or match-mated queens producing diploid males. We dissected 101 newly mated queens' spermathecae as part of this study and found all to have mated. The lines of evidence taken as a whole suggest that these large larvae are diploid males which queens from our *S. geminata* population commonly produce because of the population's small genetic diversity.

The production of diploid males imposes a cost to the colony during the founding phase according to the results from our colony founding experiment. The production of worker pupae and adult workers was lower in DMP colonies. This result is consistent with the idea that DMP colonies were unable to successfully rear as much worker brood to a late developmental stage as non-DMP colonies because the queen's body reserves were being depleted by rearing costly diploid male larvae. In the related *S. invicta*, the production of sterile diploid males also represents a burden to the colony (Ross and Fletcher 1985a, 1986, Ross et al. 1993, Tschinkel 1998a). However, we did not find deleterious effects of diploid male production on the total brood and adult workers produced. Extension of the colony founding experimental time frame, such as past the claustral period, may be needed to detect more pronounced effects of diploid male production on colony founding. We focused on the claustral phase so that we could draw conclusions about founding queen strategies without having to control for nascent worker foraging abilities.

We also found that pleometrosis was an efficient strategy to minimize the cost of diploid production and provided a distinct advantage to our colonies compared to haplometrosis. For example, three and five-queened colonies were 45.1-49.6% more likely to have worker pupae at the end of the claustral period and reared on average 5 to 7 times more pupae than haplometrotic colonies. The worker force supplied by DMP queens' nestmates increases their chances of

successful colony founding. Queens in the multiple queen treatments were also more likely to survive the claustral phase than queens in the single queen treatment. Most ant species are exclusively haplometrotic but, some species can found colonies using either mode of colony founding (Hölldobler and Wilson 1977). Queens of *S. invicta* in their invasive range are more likely to join each other during colony founding when local queen density is high (Tschinkel and Howard 1983). Queens founding a colony cooperatively are more successful during the claustral and incipient phase than single-founding queens, and pleometrosis increases brood production and queen survival for several ant species (e.g. *S. invicta*, Tschinkel 1993; *Iridomyrmex purpureus*, Hölldobler and Carlin 1985; *Myrmecocystus mimicus*, Bartz and Hölldobler 1982; and *Pogonomyrmex californicus,* Johnson 2004). Pleometrotic colonies can also begin foraging earlier than single-queen colonies (e.g. *Veromessor pergandei*, Rissing and Pollock 1987), and the benefit from the initial boost in brood production can remain long after the end of the claustral period (e.g. *M. mimicus*, Bartz and Hölldobler 1982). Because pleometrosis is more common in areas with high ant density, and cooperative founding increases colony establishment success (Bernasconi and Strassmann 2000), we would expect pleometrosis to be more common in successful invasive ant taxa. Pleometrosis would also especially benefit species with a high prevalence of DMP queens, but these hypotheses have not been investigated to the best of our knowledge.

Our findings about the benefits of pleometrosis are unlikely to be an artefact of a laboratory experiment. Combined results from our laboratory experiment and field obtained data indicate support for independent and pleometrotic colony founding in the field resulting in polygynous colonies. Four lines of evidence support independent pleometrotic founding in the field. First, field observations of our population indicate that queens disperse via mating flights. Second, most of our queens successfully founded a colony independently with or without other queens. For example, a large majority (68.1%) of our colonies successfully reared brood to the pupal stage. Third, queen execution was rare (10.9%) with most of the queens readily accepting each other and rearing their brood together. Finally, pleometrosis provided a clear advantage to the founding colonies and minimized the effect of diploid male production. We also have several lines of evidence in favour of polygyny in our population. First, five of our ten field colonies for which we genotyped males had more than two alleles per locus. Workers in the *Solenopsis* genus are unable to produce males because they do not have ovaries (Oster and Wilson 1978).

Therefore, these five colonies probably contained more than one queen. The remaining colonies had between one and two alleles per locus, probably due to the low diversity at our six loci (we detected between two and seven alleles at each locus). Second, the weight distribution of queens we used in the colony founding experiment was unimodal (Figure S1.5); in Florida, *S. geminata* queens originating from monogynous colonies are dimorphic, and the smaller queens are incapable of independent founding whereas the weight distribution of polygynous queens is unimodal (McInnes and Tschinkel 1995). Third, the presence of diploid males in the field may indicate polygyny as has been observed in the closely related *S. invicta*. In *S. invicta*, diploid males are only ever found in polygyne populations and are absent from monogyne populations in the field, although newly mated queens from monogyne populations produce diploid males under laboratory conditions (Ross and Fletcher 1985a, 1986). Our field and laboratory-based evidence suggest that the northern Australian *S. geminata* population is polygynous and its queens found new colonies independently. Independent founding is more frequent in monogyne ant populations but, in some species, queens from polygyne populations found colonies independently (Keller 1991). Several species with polygyne colonies are also capable of independent founding under laboratory conditions, which suggests that colony founding strategies might be more complex than previously suggested (Espadaler and Rey 2001, DeHeer 2002, D'Ettorre et al. 2005, Ito et al. 2016).

Selective execution of diploid male larvae appears to be a second strategy employed by *S. geminata* to lessen the cost of inbreeding. We found evidence in 43.5% of our DMP colonies that queens of *S. geminata* selectively cannibalize diploid male larvae or feed them to worker larvae. This behaviour may benefit DMP queens because it could prevent further investment in costly genetic dead-ends and allow queens to reclaim nutrients that they can redirect towards worker brood. The reclaiming of resources may explain why diploid male production did not increase queen mortality and why queens in single and two-queened DMP colonies lost less weight than non-DMP colonies. We acknowledge, however, that the low number of DMP colony replicates for these treatments (n=5 and n=16, for one and two-queened colonies, respectively) might have resulted in the significantly higher weight loss in non-DMP colonies. However, because we would expect that queens in DMP colonies would lose more weight than those in non-DMP colonies, even a finding of no significant difference, as we observed in the three and five-queened colonies, provides support for our hypothesis.

Larval execution is common in lab-reared *S. invicta* colonies with more than five queens (Tschinkel 1993), but *S. invicta* workers have not been found to discriminate diploid male larvae from worker brood for execution (Ross and Fletcher 1986). In fact, workers cannot differentiate diploid from haploid males at the adult stage either, even if diploid males are slightly larger than their haploid counterparts (Ross and Fletcher 1985a, 1986). That we found adult diploid males in our field survey indicates that workers rear diploid males and are probably unable to differentiate them from haploid males. Other Hymenoptera, such as *A. mellifera* workers, can recognize diploid drones hours after hatching and cannibalize them (Herrmann et al. 2005). *Monomorium pharaonis* workers can differentiate sexual from worker larvae and, when queens are present, workers selectively cannibalize sexual larvae (Edwards 1991). Workers of *Formica exsecta* eliminate male larvae before the pupal stage, probably as a response to resource limitation (Chapuisat et al. 1997). Some of our queens selectively eliminated their diploid male brood, and therefore *S. geminata* queens may be able to differentiate diploid male larvae from worker larvae based on size but, whether queens can differentiate diploid from haploid male larvae remains unknown.

Finally, although polyandry could reduce the occurrence of diploid male production and help overcome the cost of inbreeding, we found that this strategy was unlikely to occur in our population. Polyandry can potentially reduce the occurrence of match mating and increase the number of alleles at the CSD locus (or loci) carried by mated queens forming a founder group (Page 1980, Tarpy and Page 2002, Baer 2016, Gloag et al. 2016). All 40 sperm DNA samples we collected from queen spermatheca had one allele at all loci or the single locus for which they were successfully amplified. The probability of one queen having mated with two males sharing the same genotype, and therefore not being detected as having double-mated, was 0.121 for our samples. The probability that we failed to detect double mating drops rapidly. For example, the probability of having missed 12.5% of double mating is 2.14×10^{-4}. Therefore, most queens of *S. geminata* are likely to be single-mated in northern Australia. *Solenopsis geminata* queens were also found to be monandrous in Florida (Ross et al. 1988), which indicates that single mating might be the norm for this species. However, polyandry could potentially be more common in some populations. *Solenopsis invicta* queens were considered to be exclusively monandrous (Tschinkel 1998a) until Lawson et al. (2012) found that up to 20% of the queens are polyandrous in some populations.

Invasive species must overcome challenges linked to inbreeding to successfully establish and then maintain viable populations (Simberloff 2009). Pleometrosis and diploid male larvae execution are two strategies *S. geminata* queens can use to successfully establish new colonies despite high inbreeding that causes some queens to have half of their workers develop into sterile males. It would be useful to know whether queens can differentiate diploid from haploid male larvae, what type of recognition cue(s) they use, and whether this behaviour is restricted to the claustral period. It would also be illuminating to test whether selective larval execution only occurs in invasive populations of *S. geminata* as a response to the genetic load of sterile diploid males.

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Supplementary information

a. Collection sites and ploidy results of field-sampled males

Table S1.1 Male collection sites

Colony code	Site	Collection date	GPS coordinates	
			Latitude	**Longitude**
D1	**Douglas-Daly**	**29-Jan-14**	**-13.830017**	**131.226036**
D2	**Douglas-Daly**	**29-Jan-14**	**-13.834688**	**131.184547**
H1	**Humpty Doo**	**25-Jan-14**	**-12.548661**	**131.036591**
H2	**Humpty Doo**	**25-Jan-14**	**-12.548661**	**131.036591**
H3	**Humpty Doo**	**25-Jan-14**	**-12.548661**	**131.036591**
E1	**East Point**	**15-Feb-14**	**-12.412442**	**130.82912**
P1	**Pirlangimpi**	**01-Mar-14**	**-11.573308**	**130.581122**
S1	**Snake Bay**	**01-Mar-14**	**-11.42062**	**130.661118**
V1	**Virginia**	**25-Jan-14**	**-12.548661**	**131.036591**
V2	**Virginia**	**15-Feb-14**	**-12.547171**	**131.036639**

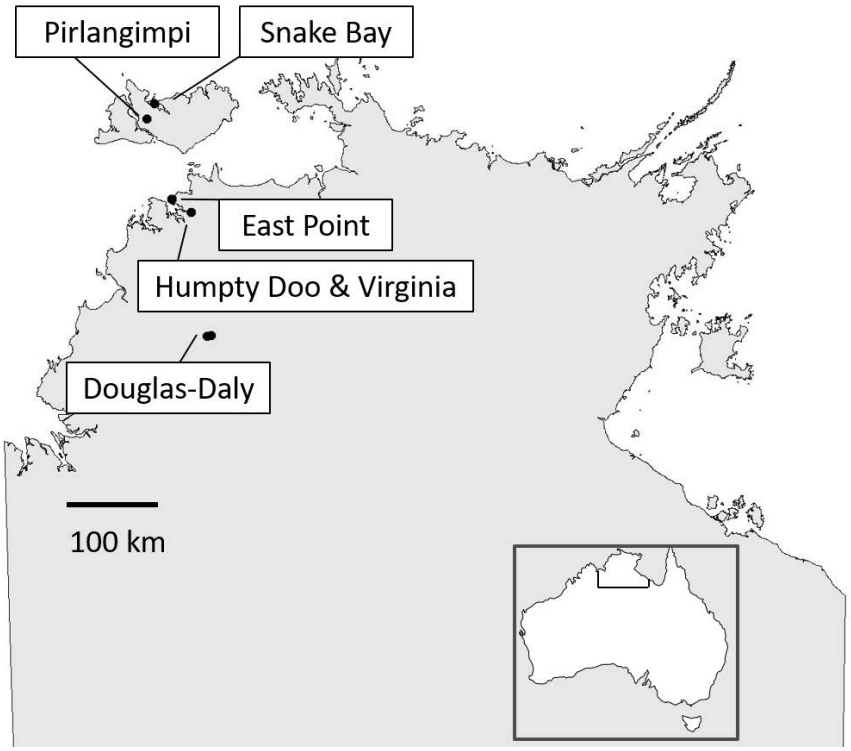

Figure S1.1 Map of the male collection sites in the Northern Territory, Australia.

Table S1.2 Number of heterozygous males scored as diploid for each field-collected colony.

Colony	D1	D2	H1	H2	H3	E1	P1	S1	V1	V2
1 marker	4	4	0	2	2	2	7	0	1	4
2 markers	1	4	0	0	0	0	0	0	0	0

Note: Total males sampled n=80, successful amplification for all six microsatellite loci n=48, five loci n=23, four loci n=1, three loci n=1, and one locus n=5 samples, failed amplification at all loci n=2.

b. PCR protocol for queen, larva and male DNA and microsatellite primer sequences

All DNA fragments were amplified using PCR based on the methods of Tay and Crozier (2000), modified and optimized for *S. geminata* microsatellite markers. Each amplification (in a final 10µL reaction volume) required 1.5 µL of DNA (undiluted for queens, larvae and males, 1:10 diluted for sperm), 0.2mM dNTPs, 0.2µM of each forward primer and reverse primer, 0.5 unit of Phusion® Taq DNA polymerase (5u/µL), 1mM $MgCl_2$, and 1x Phusion® Buffer. The PCR conditions were as follows: initial denaturation step at 95˚C for 2.5 minutes; 37 cycles comprising 30 seconds of DNA denaturing at 95˚C, specific primer annealing temperature (Table S1.1) for 30 seconds, 30 seconds DNA extension at 72˚C; and a final extension step of 5 minutes at 70˚C. Post PCR incubation was at 10˚C. For each individual sample, we multiplexed and submitted for genotyping 2.5 µL of each of the PCR amplicons for Ms19c367, Ms14c334 and Ms4Sol55 and, 2 µL for Ms16 C121 and 2.5 µL for Ms33 Sol11 and Ms41 i134.

Table S1.3 Microsatellite primer sequences, types of simple sequence repeats (SSR), annealing temperatures, allele size range for *S. geminata*, and references from which the primer sequences were adapted.

Locus	*Primer sequence*	*SSR units*	*Annealing temperature*	*Allele size range*	*Reference*
Ms16 C121[1]	F:NED-CGTGGAGGGACGATTTTCATACTC R[2]:GCTTCTCAAACTTCTCTTCGGTGACACTCT	$(GA)_{15}$	58˚C	175-197	Wang et al. 2007
Ms19 C367[1]	F:VIC-CCCGAACAATGAGACATCCT R:TTAAATCTTAGCGCCGACGA	$(GA)_7$	56˚C	244-253	Wang et al. 2007
Ms33 Sol11[1]	F:6FAM-ACTGGAGCCTCCGAGACC R:CACTCCGGAAGAGTAACTTTGC	$(TC)_{15}$	56˚C	132-180	Chen et al. 2003
Ms14 C334	F:PET-TTTCTTTCTTTCTGTCTCTTTCTCG R:ACGGAAGGCACGAATGAAGTC	$(TC)_{14}$	54˚C	197-220	Wang et al. 2007
Ms4 Sol55	F:6FAM-TGCGACAATGAATGAGAGTC R:TGCGAATATCCGGTCGAG	$(TC)_{15}$	48˚C	187-205	Chen et al. 2003
Ms41 i134	F:PET-CAGCTAAAAGTCAGGGACACG R:AGACGCTTTATTATCTGGGACAC	$(GT)_{10}$	56˚C	196-219	Ascunce et al. 2011

[1]Primers used for queens, larvae and sperm, [2]GCTTCT 'pigtail' sequence

c. Figures on the colony founding experiment on the number of worker brood and adult worker per treatment, the number of worker brood and adult workers per queen, the number of DMP colonies executing diploid male larvae per treatment, and the weight distribution of newly mated queens.

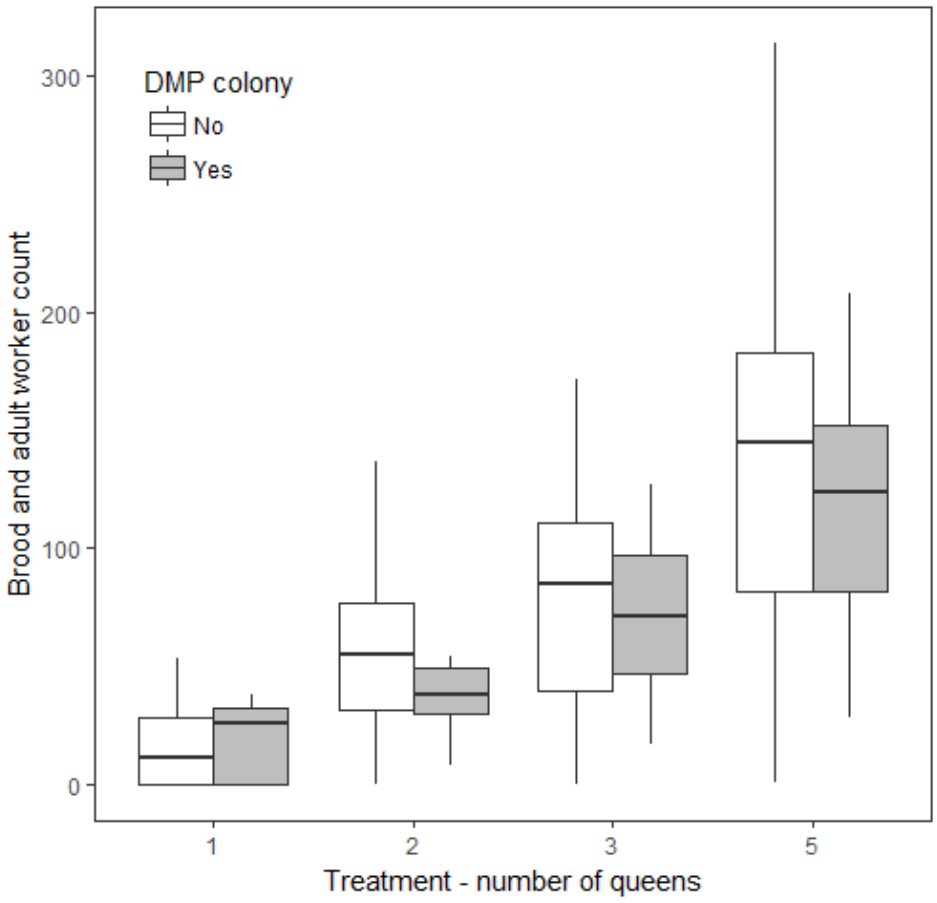

Figure S1.2 Number of worker brood and adult worker produced for each queen treatment separated between DMP (diploid male producing) colonies and non-DMP colonies. There was no significant difference between DMP and non-DMP colonies across all queen treatments (Table 1.1). One queen: non-DMP n=97, DMP n=5, two queens: non-DMP n=79, DMP n=16, three queens: non-DMP n=68, DMP n=29, five queens: non-DMP n=51, DMP n=35.

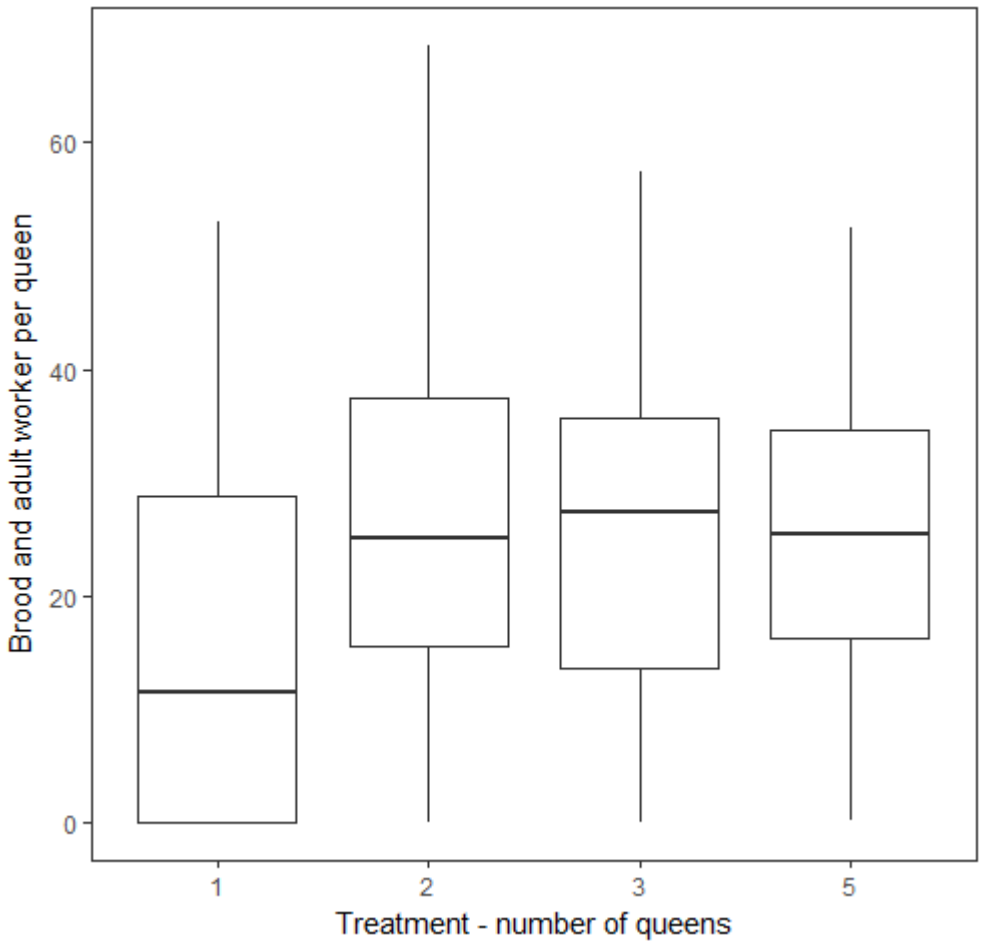

Figure S1.3 Number of brood and adult workers produced per queen (total number of brood and adult workers divided by the number of queens) for each queen treatment. The queen treatment had a significant effect on the number of worker brood and adult workers produced per queen but none of the pairwise comparisons between queen treatment were significant (LM: ANOVA, $\chi^2 = 36.038$, $P<0.01$; post hoc tests $P>0.05$ for all pairwise comparisons). One queen: n=102, two queens: n=95, three queens: n=97, five queens: n=86.

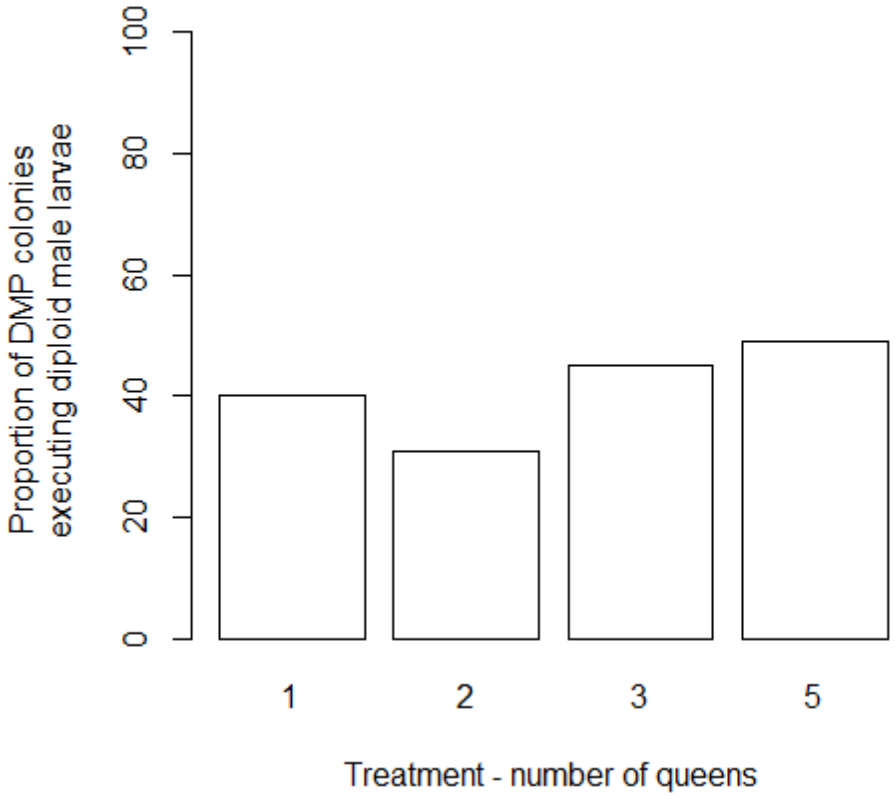

Figure S1.4 Proportion of DMP colonies executing diploid male larvae for each queen treatment. One queen: DMP n=5, two queens: DMP n=16, three queens: DMP n=29, five queens: DMP n=35.

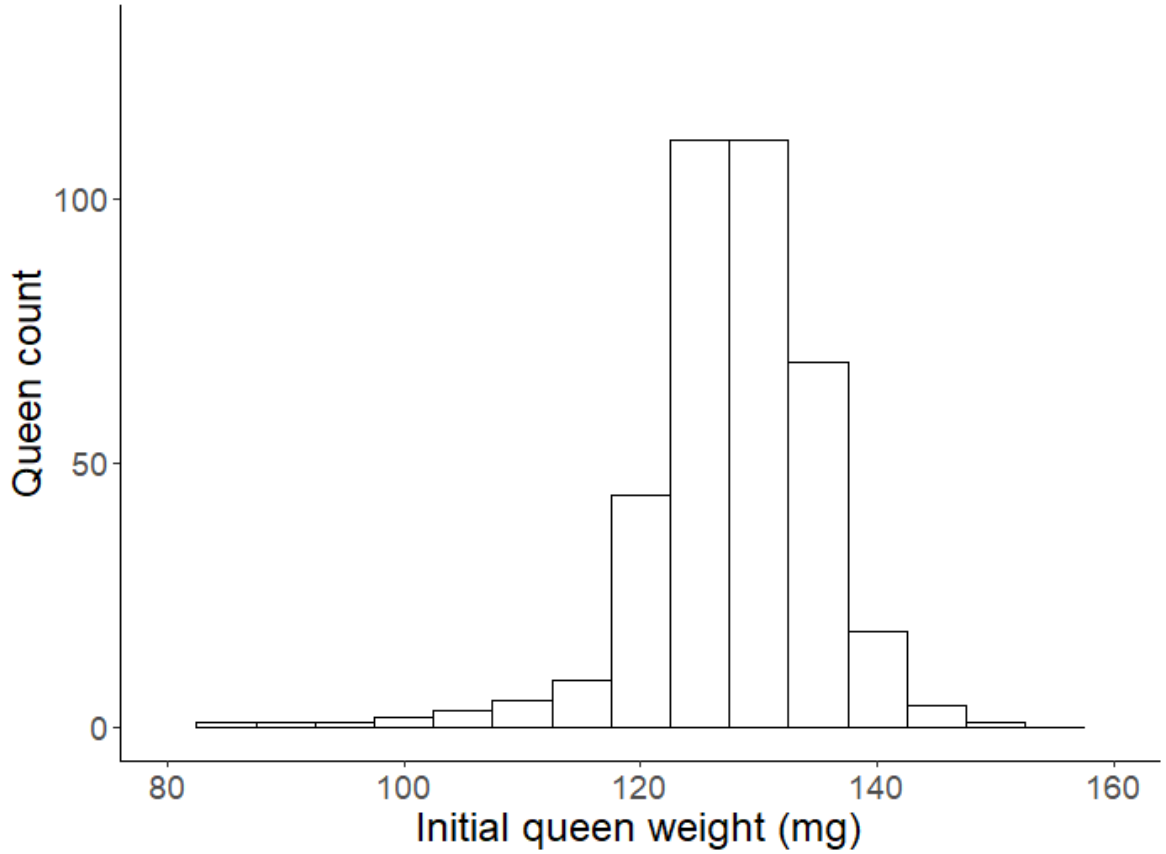

Figure S1.5 Weight distribution of the newly mated queens used in the colony founding experiment before the start of the experiment (n=1,013).

d. Probability of double mating and queen and sperm genotypic distribution

We calculated the probability of not having detected double-mating in our population using the sperm genotype frequency from 32 males (Table S1.5) which were successfully genotyped at all three loci. The probability of homogeneous mating for each genotype was calculated by squaring the genotypic frequency. To obtain the probability of not detecting a single double-mating event in our sample, we summed the probability of homogeneous mating for each genotype which was 0.121. To obtain the probability of not detecting more than one double mating event we raised 0.121 to the power of the number of double mating events (Figure S1.6). For example, the probability of not detecting that three queens were double mated (i.e. about 10% of double mating) is $0.121^3=1.8x10^{-3}$, ten double mated queens (i.e. about 30% double mating) $0.121^{10}=6.7x10^{-10}$ etc.

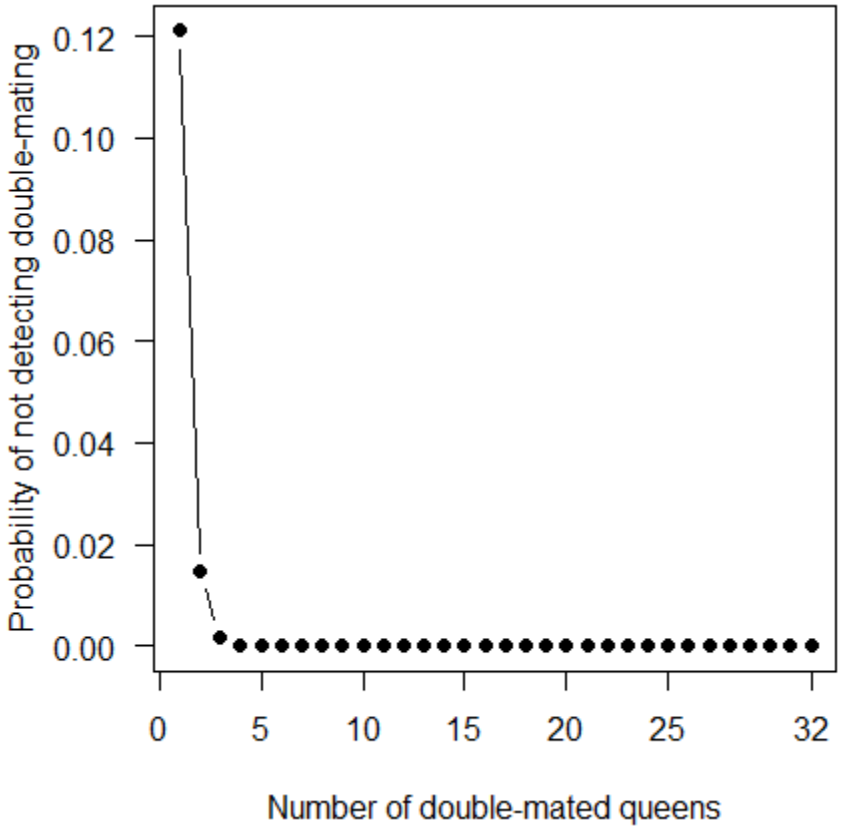

Figure S1.6 Probability of not detecting double-mating calculated from 32 sperm genotype frequencies.

Table S1.4 Genotypic distribution of queens, -: amplification failure.

ms33	Sol11	ms16	c121	ms41-i134		Sample size
132	132	190	190	202	202	1
134	134	190	190	202	220	1
134	134	190	190	202	221	3
134	134	190	190	221	221	1
134	134	192	192	220	220	1
134	163	188	188	220	220	1
134	179	190	190	202	202	1
163	163	190	190	221	221	1
163	179	190	190	202	220	1
177	177	190	190	202	221	1
179	179	192	192	202	220	1
134	177	190	190	221	221	2
129	129	190	190	-	-	1
179	179	190	190	-	-	1
163	179	190	190	-	-	1
134	179	192	192	-	-	1
134	177	190	190	-	-	2
135	135	-	-	202	202	1
177	177	-	-	202	202	1
-	-	190	190	202	202	1
179	179	-	-	-	-	1
134	134	-	-	-	-	5

134	179	-	-	-	-	2
-	-	190	190	-	-	1
-	-	-	-	202	220	2
-	-	-	-	202	221	1

Table S1.5 Genotypic distribution of sperm samples, -: amplification failure.

ms33 Sol11	ms16 c121	ms41-i134	Sample size
134	190	202	8
134	190	220	1
134	190	221	1
134	192	202	6
134	192	221	2
134	194	221	1
163	190	221	1
163	192	202	1
177	190	202	1
177	190	221	1
177	190	223	1
177	192	221	1
179	186	202	1
179	190	202	2
179	192	202	2
179	192	220	1
179	192	221	1
-	189	220	1
-	190	202	1
-	192	202	2
-	192	221	1
134	190	-	1

134	194	-	1
135	194	-	1
179	190	-	1

Chapter 2 Roles of biotic resistance and abiotic factors in the establishment of an invasive ant

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Statement of contribution of others:

PL conducted the field work and the data analysis and drafted the manuscript. BH and LL coordinated the study. All authors designed the study and edited the manuscript. See acknowledgments for other contributions.

Abstract

Several studies have found that disturbance facilitates invasions, but only a few have attempted to determine the relative importance of abiotic or biotic factors. We conducted two field experiments to determine whether disturbance facilitates the establishment of the invasive tropical fire ant (*Solenopsis geminata*) and the relative role of abiotic factors and biotic resistance on its establishment success. Our study sites were adjacent undisturbed (unmaintained savannah woodland) and disturbed (mowed field) sites situated at four locations near Darwin, Australia. The disturbed sites were either non-invaded or invaded by *S. geminata*. In our first experiment, we introduced 140 newly mated queens and 105 incipient colonies into four disturbed and three undisturbed sites. We lured workers to verify survival at 30 and 90 days. In our second experiment, we introduced 447 newly mated queens in individual cages that either excluded or allowed access to native ants into two undisturbed and two uninvaded disturbed sites. We retrieved the cages after 7, 14, or 25 days and determined queen survival. We had 19±1 replicates per treatment combination. For the first experiment, we found two live colonies at 30 and 90 days, all situated in invaded disturbed sites. In the second experiment, 157 queens had survived, and we found that abiotic factors and baseline mortality contributed the most to queen mortality (mean ± SD: 85.7±8%) followed by biotic resistance (14.2±8%). Isolation from native ants improved queen survival in undisturbed sites but not in disturbed ones (GLM:binomial $p<0.05$) suggesting that biotic resistance was stronger in undisturbed sites.

Introduction

One of the foundational and widely accepted ideas in invasion biology is that disturbance, here defined as any discrete event that disrupts community structure and changes available resources, substrate availability, or the physical environment (Krebs 1994), facilitates biological invasions (Moles et al. 2012). Some studies across taxonomic groups support this hypothesis (e.g. Polynesian rat, *Rattus exulans*, Rickart et al. 2011; Japanese stiltgrass, *Microstegium vimineum*, Eschtruth and Battles 2009; African daisy, *Senecio pterophorus*, Caño et al. 2008; Diplosoma tunicate, *Diplosoma listerianum*, Altman and Whitlatch 2007; and red imported fire ant, *Solenopsis invicta*, King and Tschinkel 2008) but, others find no or a negative relationship between disturbance and invasions (Moles et al. 2012).

Disturbance may benefit biological invasions by altering biotic resistance and abiotic conditions (Elton 1958, Lozon and MacIsaac 1997, Walters and Mackay 2005, Krushelnycky et al. 2010, Moles et al. 2012). Biotic resistance is the ability of a native community to hinder the establishment of a non-native species through interspecific competition or predation (Elton 1958, Lozon and MacIsaac 1997, Walters and Mackay 2005). Anthropogenic disturbance and the alteration of disturbance regimes may weaken biotic resistance by negatively affecting native species diversity and the competitive ability of native communities (Elton 1958, Lozon and MacIsaac 1997). For example, red crabs (*Gecarcoidea natalis*) limit the invasion of the giant African land snail (*Achatina fulica*) in undisturbed habitats of Christmas island through predation but, not in disturbed habitats where the red crabs are absent or present at low density (Lake and O'Dowd 1991). Disturbance may also modify abiotic conditions and contribute to providing a suitable habitat to invasive taxa (Byers 2002, Krushelnycky et al. 2010). For example, eutrophication benefits invasive plants such as *Hydrilla* and *Myriophyllum* which are adapted to low light level (Ruiz et al. 1999). A key challenge for invasion biology is to determine the relative importance of abiotic and biotic factors for the successful establishment of non-native species.

The successful establishment of invasive ants into new environments depends on a combination of biotic and abiotic factors which are sometimes linked to disturbance (Krushelnycky et al. 2010, Sanders and Suarez 2011). This introduction mainly focuses on the Argentine ant *(Linepithema humile)* and *S. invicta* because most research on topics relevant to our study has been directed at these two species of invasive ants. The most significant predators and competitors of invasive ants are often other ants (Thomas and Holway 2005, Krushelnycky et al. 2010). For example, in field experiments native ants slowed down the spread of *L. humile* (Menke et al. 2007) and *S. invicta* failed to establish where subterranean predators in the *Solenopsis* (*Diplorhoptrum*) group were present in high densities (Vinson and Rao 2004).

Unsuitable and suboptimal abiotic conditions can decrease the survival, hinder the range expansion, and limit the competitive ability of invasive ants (Holway et al. 2002b, Walters and Mackay 2003, Krushelnycky et al. 2005, Thomas and Holway 2005, Menke et al. 2007). For example, in the U.S. winter soil temperatures limit the spread of *L. humile* to north of North Carolina (Brightwell et al. 2010). In the laboratory, *L. humile* stopped foraging at temperature below 5°C, causing workers to die of starvation, and colonies to collapse after 8 days at 5°C

(Brightwell et al. 2010). In Western Australia, hot temperatures reduced the competitive performance of *L. humile* workers in the field (Thomas and Holway 2005). Workers were able to displace native ants from lures when ground temperatures were between 18-25°C but not when ground temperatures reached 30-40°C. Disturbance sometimes generate biotic and abiotic conditions which can facilitate ant invasions (Walters and Mackay 2003, King and Porter 2007, Krushelnycky et al. 2010, Sanders and Suarez 2011). For instance, irrigation and urban runoff facilitate the spread of *L. humile* into seasonally dry habitats in southern California by increasing water availability (Menke and Holway 2006, Menke et al. 2007). In Florida, disturbance treatments (mowing and ploughing) aided the establishment of transplanted *S. invicta* colonies (King and Tschinkel 2008).

In the Northern Territory of Australia, the invasive tropical fire ant, *Solenopsis geminata*, is limited to disturbed habitats (e.g. road sides, park lawns) and has not been observed spreading into natural habitats (Hoffmann and O'Connor 2004). For reasons unknown it is also absent from some disturbed parcels of land that are adjacent to invaded areas. However, within its putative native range in the southern U.S., *S. geminata* inhabits natural forested areas or, in urban areas, mature residential neighbourhoods with abundant plant cover (Tschinkel 1988, Plowes et al. 2007). Notably, the restriction of *S. geminata* to disturbed habitats in the Northern Territory is not caused by poor dispersal capacity or linked to this species being a recent invader in the process of expanding its range into natural environments. *Solenopsis geminata* queens may establish hundreds of meters away from their parent colony via nuptial flights (McInnes and Tschinkel 1995, personal observation) and this species has been present in the Northern Territory for about 150 years (Gotzek et al. 2015). Strong biotic resistance from native ants and unsuitable abiotic conditions could prevent the spread of *S. geminata* into undisturbed habitats in the Northern Territory. Australia hosts some of the world's most diverse and competitive ant communities (Andersen 2003) potentially making undisturbed habitats particularly resistant to ant invasions by less competitive invaders (Walters and Mackay 2005, Krushelnycky et al. 2010, Wittman 2014). *Solenopsis geminata* may be especially vulnerable to biotic resistance as it is not as dominant as other invasive ants such as *S. invicta* (Wetterer 2011). Unsuitable abiotic conditions could also limit the expansion of *S. geminata* to undisturbed habitats. For example, although *S. geminata* has been found in habitats with plant cover in the southern U.S. (Tschinkel 1988, Plowes et al. 2007), their establishment success could be reduced in shaded undisturbed

habitats as native colonies in central America have shown preference for open over shaded habitats (Torres 1984, Perfecto and Vandermeer 1996).

Our work aims to determine whether disturbance facilitates the establishment of *S. geminata*, why some disturbed areas seem more suitable to invasion than others, and the relative role of biotic resistance and abiotic factors in colony establishment. We conducted two complementary field experiments in which we introduced newly mated queens and/or incipient colonies to undisturbed (unmodified savannah woodland) or disturbed (mowed field, historically cleared savannah woodland) sites in the Northern Territory. The disturbed sites were either non-invaded or invaded by mature colonies of *S. geminata.* In our first experiment, we examined whether abiotic factors and/or the presence of a competitive native ant community influenced the establishment success of experimentally introduced queens and incipient colonies into undisturbed and disturbed sites (invaded or non-invaded). In our second experiment, we prevented or allowed access of resident ants to experimentally introduced queens to distinguish the contribution of biotic resistance to queen mortality from mortality due to the combination of abiotic factors and baseline mortality.

Methods

Site descriptions

We worked in four study locations near Darwin (Northern Territory, Australia). Darwin's climate is monsoonal with about two thirds of annual rainfall (1728mm) falling between January and March. We conducted our field experiments from April to May, at the end of the wet season (mean rainfall 132mm). Three locations (CSIRO, Girraween, and Humpty Doo) comprised an undisturbed and a disturbed site while the fourth location (Vanderlin) consisted only of a disturbed site (Figure S2.1). Disturbed and undisturbed sites at each location were adjacent to each other except at Humpty Doo where they were 500 metres apart because we could not find a suitable third location with adjacent disturbed and undisturbed sites. The undisturbed sites were all unmodified savannah woodland, dominated by Darwin stringybark (*Eucalyptus tetrodonta*). The trees and shrubs provided 40-60% shading. The ground was mostly covered in leaf litter (60-100%), followed by bare soil (5-30%), and grass (1-10%). The disturbed sites were historically cleared savannah woodland which have been maintained as open grassland. The herbaceous

layer provided virtually no shading apart from a low cover at 10cm-3m above ground (0-5%). The ground cover was mainly grass (80-100%) and bare soil (0-20%).

Queen collection

For each of the experiments, we collected newly mated queens of *S. geminata* after their nuptial flight in the early evenings between February and April at Humpty Doo, Northern Territory, Australia (-12.5722°, 131.0842°), as they flew to a veranda light and dropped to the ground to found a nest.

Development of incipient colonies

We placed 105 newly mated queens individually in an 8cm deep, 7.5cm diameter plastic container filled with 7cm moist soil and kept undercover in ambient conditions for 28 days (5 days after the emergence of the first workers; Lenancker et al. 2019). The colonies comprised 10 to 20 adult workers when introduced into the field.

Queen and colony introduction experiment

We conducted this field experiment to determine whether the establishment of newly mated queens and incipient colonies was facilitated by disturbance and whether abiotic factors and the native ant community differed between disturbed and undisturbed habitats and could potentially limit the establishment of *S. geminata*. We introduced 140 queens and 105 incipient colonies from April to May 2015 into four locations (CSIRO, Girraween, Humpty Doo and Vanderlin, Figure S2.1, see site descriptions section). We introduced 20 queens and 15 colonies per site. We introduced queens and incipient colonies over a one-month period, making sure we equitably introduced queens and colonies to each site during that time. We introduced the queens into the field within 24h of collection by placing each queen next to a 100x3mm (depth x diameter) wire-pierced hole in the ground which they quickly entered. We introduced the incipient colonies by burying their nesting container so that its base was 10cm underground. The base of the container had two 1.5mm holes to allow the ants to exit the container and dig their nest deeper. We positioned each queen and incipient colony replicate at least 10m apart. We assessed colony survival 30 and 90 days after introduction by placing a teaspoon of cat food as a lure at each introduction point (i.e. queen introduction hole or buried incipient colonies) between 8 and 10am in fine weather. We recorded the presence or absence of *S. geminata* workers after 30 minutes.

We chose to assess colony survival as early as 30 days because it corresponds to 7 days after the end of the claustral period. Therefore, we expected any workers produced from the introduced queens to be active and foraging. We lured each introduction point again after 90 days to avoid missing live colonies that were not active 30 days after introduction. In sites where *S. geminata* was already established, we distinguished our incipient colonies from resident colonies at the lures by looking at the abundance and size of foragers and by following their foraging trails back to their nest. Resident colonies had many large foragers at the lures and their nests were several meters away from the introduction points, whereas our experimental colonies had between 10 and 20 minim foragers at the lure stations and disappeared underground right beneath our introduction points. At the end of the experiment we drenched surviving colonies with Termidor® to kill them (Hoffmann et al. 2016), with a follow-up inspection 7 days later to confirm colony death.

Caged queen experiment

We conducted this field experiment to evaluate the relative contributions of biotic resistance and abiotic factors on the establishment success of newly mated queens of *S. geminata* into non-invaded disturbed and undisturbed habitats. We introduced 447 individually caged queens from April to May 2017 to Girraween and CSIRO (Figure S2.1, see site descriptions section). We weighed each queen within 40 hours of collection and placed her in a cage (45×35×10mm, length x width x height) of one of two mesh sizes. The smaller mesh size (0.32x0.19mm) excluded all ants from entering or leaving the cage, whereas the larger mesh (1.23x0.18mm) prevented the queen from leaving but allowed *S. geminata* workers and native ants (maximum width 1.23mm) to enter and leave the cage (Figure 2.1). *Solenopsis geminata* queens are claustral founders and did not need to leave the cage to feed during the experiment. We buried the cages at least 3 metres from each other and 7cm belowground which was the depth of the moist soil in the sealed containers in which queens successfully started a colony during the queen and colony introduction experiment. We minimized disturbance to the resident ant community by digging a hole just large enough to fit the cage. Queens were haphazardly assigned *a priori* to be collected after either 7, 14, or 25 days. There were 19±1 replicates per treatment combination (i.e. disturbed vs undisturbed site, big vs small mesh size and 7 vs 14 vs 25 days before retrieval) at each site (112±3 cages per site). We introduced the cages over a one-month period, making sure

queens collected during that period were equitably allocated to treatment combinations. Upon collection, we determined queen survival, weighed surviving queens, determined whether they had produced brood, and, if so, counted the number of eggs, larvae, pupae, and workers. We also recorded whether dead queens were still present in the big mesh cages and any evidence that they had been preyed on (e.g. missing limbs). We also collected and identified to species any other ants that were found inside the big mesh cages. Retrieved cages with queens that had survived were not placed back into the field because digging up the cages could disturb the queen, brood, and nearby resident ants. We inspected the sites one month after retrieving all cages to confirm they remained *S. geminata*-free.

Figure 2.1 Photos of two mesh cages from the caged queen experiment before being buried. a) Big mesh cage with a *S. geminata* queen in the white circle. The mesh size prevented the queen from escaping but allowed *S. geminata* workers and native ants (maximum width 1.23mm) to enter and leave the cage. b) Small mesh cage with its individual label. All ants were prevented from entering and leaving the cage.

Native ant sampling

We sampled the native ant community prior to the queen and colony introduction experiment. We positioned two pitfall traps and two subterranean traps at the corners of a 1m² quadrat centred on each queen or colony introduction point, yielding 70 traps of each type at each site. Pitfall traps were opened for 24 hours and consisted of 59x45mm (height x diameter) cylindrical

plastic containers filled with 70% ethylene glycol as a preservative, dug into the ground with their upper rims flushed with the ground surface. Subterranean traps were 40x10mm (height x diameter) capped plastic vials, with four 2mm diameter holes drilled below the cap to allow ant access (Andersen and Brault 2010). We smeared the inner walls above the access holes with a mix of honey, peanut butter and cat food to attract ants. The traps were operated for 48 hours and half filled with 70% ethylene glycol as a preservative. We identified the ants from each trap to species. We counted the individuals of each species for the pitfall traps but capped the abundance of each species at 100 to limit data distortions from a large number of ants from a single nearby colony falling into one trap. We only recorded presence absence data for the subterranean traps as these traps contained lures which typically attract many foragers of a single species at any one time. A full set of voucher specimens is held at the CSIRO laboratory in Darwin.

Environmental variables

We visually estimated the percentage of grass, bare soil, leaf litter, and rocks in a 1m² quadrat centred on each queen or colony introduction point. We also estimated the percentage of shading at 10cm-3m above ground and at 3m above ground for both experiments. These estimations were all done by the same person (PL) to ensure consistency. We recorded the underground temperature in each site every 30 minutes for the duration of the experiments using thermocrons (iButton temperature loggers). For the queen and colony introduction experiment, we buried three thermocrons across each site at 10cm (queen and colony introduction depth) and three at 50 cm. In the caged queen experiment, we buried 11 thermocrons across each site at 7cm (queen introduction depth).

Data analysis

Environmental data

We analysed our data in R version 3.3.2 (R Core Team 2018) and used functions from the stats package (R Development Core Team 2009) unless specified otherwise. We performed a Principal Component Analysis (PCA, prcomp function) for the queen and colony introduction experiment and another one for the caged queen experiment to visualise the variation in environmental variables (percentages of grass cover, litter cover, soil cover, shading at 10cm-3m above ground and shading at 3m above ground) between disturbed and undisturbed sites. We

generated a synthetic index from the first axis score (PC1) summarising the environmental variables for each experiment. We tested whether our resulting index differed between the disturbed and undisturbed sites for each experiment with a Kruskal-Wallis test (kruskal.test function). We used a linear model (lm function) for each experiment to test whether temperature varied with location and disturbed vs undisturbed sites. The response variable was the average site temperature during the queen and colony introduction experiment (4-6 probes per site) or during the caged queen experiment (6-8 probes per site). We were unable to retrieve data for some of the temperature loggers we had placed in the field which is why we do not have the same number of probes per site. For the queen and colony introduction experiment, the explanatory variables were depth, location, disturbance (disturbed or undisturbed sites), and the interaction between location and disturbance. For the caged queen experiment, they were location, disturbance, and the interaction between location and disturbance.

Ant community data

We used non-metric multidimensional scaling (NMDS, metamds function in the vegan package, Oksanen et al. 2015) to visually detect differences in ant species composition between locations and disturbance (disturbed or undisturbed sites). We also tested whether the community composition varied between disturbed and undisturbed sites using a permutational multivariate analysis of variance (PERMANOVA, adonis2 function in the vegan package). We tested whether ant species richness and Shannon index differed between all the disturbed and undisturbed sites with a Kruskal-Wallis test.

Survival data

For the queen and colony introduction experiment, we used two generalized linear models (GLM, glm function) with a binomial distribution followed by ANOVA F-test (Anova function with test=F in the car package, Fox and Weisberg 2002) with the outcome at 30 days (survival or death) as the response variable for the first model and outcome after 90 days for the second model. The fixed factors for both models were location, disturbance (disturbed or undisturbed), the interaction between location and site and, colony size (queen or incipient colony). We also tested which factors affected queen survival for the caged queen experiment using a GLM with a binomial distribution followed by ANOVA F-test. The outcome (death or survival) was the response variable, and fixed factors were the mesh size (big or small), exposure time (7, 14 or 25 days), queen weight before introduction, location, disturbance (disturbed or undisturbed), the

interaction between mesh size and site and the interaction between location and site, the fixed factors. We also tested the same explanatory variables for the survival of queens retrieved at 7, 14 or 25 days in three separate models. These three models were used to determine whether the factors affecting queen survival depended on exposure time. The final models did not include queen weight because its absence improved model fit for all four models based on the Akaike Information Criterion *(*AIC). The percentages of increased survival we report in the results for our models were obtained by adding the intercept coefficient to each model regression coefficient to obtain the predicted values for the corresponding condition. We used the following formula to transform the predicted values from log odds into percentage $\frac{e^{predicted\ value}}{1+e^{predicted\ value}} \times 100$ (Politzer-Ahles 2016). In order to test which factors affected the likelihood of having brood in the cages of surviving queens, we used a GLM with a binomial distribution followed by ANOVA F-test with the presence of brood as the response variable and mesh size, exposure time, disturbance (disturbed or undisturbed sites) and location as fixed factors. To test the effect of these variables on queen weight loss, we used a linear model with queen weight loss as the response variable and mesh size, exposure time, disturbance (disturbed or undisturbed sites) and location as fixed factors. We plotted the residuals to check for their homoscedasticity, independence, and normality when appropriate (plotresid function in the RVAideMemoire package, Hervé 2016). We used post-hoc Tukey tests to make pairwise comparisons (lsmeans function in the lsmeans package Lenth and Hervé 2015).

Results

Queen and colony introduction experiment

All surviving colonies and queens were found in disturbed sites where *S. geminata* was present before the experiment. We found two live colonies originating from our laboratory-reared incipient colonies in the Vanderlin drive site after 30 days. One of these colonies was still alive after 90 days but the other one had died. We also found a surviving colony 90 days post-introduction at the Humpty Doo site from one of our queen-only introductions. We did not find any significant effect of location, disturbance (disturbed or undisturbed sites), their interaction or colony size (queens vs incipient colonies) on the survival at 30 or 90 days (GLM binomials for survival at 30 and 90 days: ANOVA, all $p>0.05$) as we expected due to the very low number of surviving replicates.

The environmental variables we measured differed between disturbed and undisturbed sites. The average temperatures were 1.61±0.45°C and 1.7±0.46°C higher in disturbed compared to undisturbed sites at 10cm and 50cm belowground respectively (LM: ANOVA, $\chi 2=14.4759$, df=1, $P<0.001$), but the temperature did not differ significantly among the disturbed sites (post hoc test $p>0.05$ for pairwise comparisons of all the disturbed sites) or among the undisturbed sites (post hoc test $p>0.05$ for pairwise comparisons of all the undisturbed sites). The temperature did not differ with depth (10cm vs 50cm) (LM: ANOVA, $\chi 2=0.1007$, df=1, $p=0.75$). In the PCA (Figure S2.2) combining ground cover and shading, disturbed sites group on one side and the undisturbed sites group on the other side. The first PCA axis represents 79% of the variation among sites which made it a suitable index to summarize the environmental variation across our different sites. The PC1 score differed between disturbed and undisturbed sites (Kruskal-Wallis test, Kruskal-Wallis $\chi 2=4.5$, df=1, $p<0.05$).

Caged queen experiment

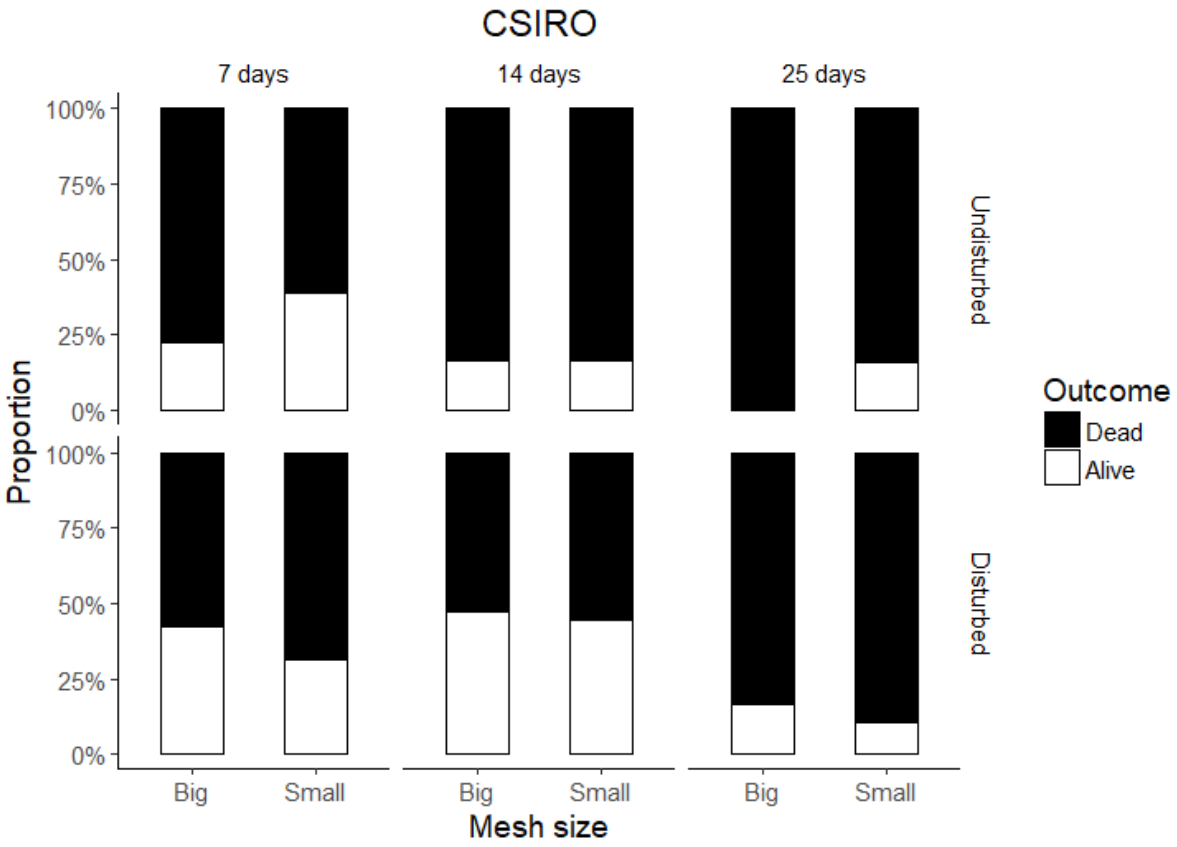
Of the 447 queens we introduced (n=19±1 per treatment), 157 were still alive when retrieved from the field. Mesh size, exposure time, disturbance (disturbed or undisturbed sites), and the interaction between disturbance and location all had a significant effect on queen survival (Table 2.1). Queens were 50.7% more likely to survive if they were in the small mesh and therefore isolated from other ants (91/223 queens introduced in the small mesh cages survived vs 66/223 for queens introduced in the big mesh cages, Table 2.1 and Figure 2.2). The overall interaction between mesh size and disturbance did not have a significant effect on queen survival (Table 2.1). However, post-hoc comparisons revealed that queens in small mesh cages introduced into undisturbed sites had higher survival than queens in big mesh, whereas mesh size did not affect queen survival in the disturbed sites (post hoc test undisturbed site: $p=0.03$ small vs big mesh, disturbed site $p=0.13$ small vs big mesh). Queens that were introduced in the Girraween location were 49.6% more likely to survive than queens introduced in the CSIRO location (Girraween 101/224 queens survived vs 56/222 for CSIRO, Table 2.1 and Figure 2.2). We found that queens in the CSIRO disturbed site were 21.9% more likely to survive than queens introduced in the undisturbed site (CSIRO disturbed site 36/112 queens survived vs 20/112 for the undisturbed site, Table 2.1 and Figure 2.2). In Girraween, however, we did not find any difference in survival between disturbed and undisturbed sites.

Table 2.1 Summary of model results for the caged queen experiment. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Overall survival **GLM: binomial distribution, n=447**	***df***	**F**	***p***
Mesh size	1	6.93	0.009**
Location	1	21.17	5.5e-6***
Exposure time	2	15.68	2.7e-7***
Disturbance	1	0.31	0.57
Mesh size: Disturbance	1	0.33	0.56
Disturbance: Location	1	7.73	0.006**
Survival at 7 days **GLM: binomial distribution, n=149**	***df***	**F**	***p***
Mesh size	1	0.05	0.83
Location	1	12.00	7.0e-4***
Disturbance	1	0.22	0.64
Mesh size: Disturbance	1	0.05	0.82
Disturbance: Location	1	1.53	0.22
Survival at 14 days **GLM: binomial distribution, n=147**	***df***	**F**	***p***
Mesh size	1	2.09	0.15
Location	1	2.72	0.10
Disturbance	1	3.20	0.08
Mesh size: Disturbance	1	0.19	0.66
Disturbance: Location	1	4.09	0.04*
Survival at 25 days **GLM: binomial distribution, n=150**	***df***	**F**	***p***
Mesh size	1	10.43	0.002**
Location	1	7.91	0.006**

Disturbance	1	0.30	0.58
Mesh size: Disturbance	1	0.004	0.95
Disturbance: Location	1	1.78	0.18
Weight loss **LM, n=157**	*df*	$\chi 2$	*p*
Mesh size	1	0.778	0.38
Exposure time	1	137.072	<2.2e-16***
Location	1	0.779	0.37
Disturbance	1	0.016	0.9
Presence of brood **GLM: binomial distribution, n=157**	*df*	F	*p*
Mesh size	1	6.5698	0.011*
Exposure time	1	0.0578	0.81
Location	1	1.4512	0.23
Disturbance	1	0.5528	0.46

Queen survival declined with retrieval time (Table 2.1 and Figure 2.2). Queens retrieved after 25 days in the field were 13.3% more likely to be dead than queens retrieved after 7 and 14 days (72/149 queens survived at 7 days, 56/147 at 14 days and 29/150 at 25 days, post hoc tests $p<0.001$ for pairwise comparisons 7 vs 25 days and 14 vs 25 days, $p>0.05$ for 7 vs 14 days). Factors affecting queen survival depended how long the queens had been in the field (Table 2.1). At 25 days, queens were 20.9% more likely to be alive if they had been kept in small mesh (22/75 queens survived in the small mesh vs 7/75 in the big mesh), but mesh size did not affect survival at 7 and 14 days. At 14 days, queens were 13.1% more likely to survive if they had been introduced to the CSIRO disturbed site rather than the undisturbed one (17/37 queen survived at CSIRO's disturbed site vs 6/36 at the undisturbed one, post hoc tests $p<0.01$ for pairwise comparisons CSIRO disturbed vs undisturbed, $p>0.05$ for Girraween disturbed vs undisturbed), but not at 7 and 25 days.


CSIRO
7 days
14 days
25 days
100%
75%
50%
25%
0%
Proportion
Undisturbed
Disturbed
Big
Small
Mesh size
Outcome
Dead
Alive


b
Girraween
7 days
14 days
25 days
100%
75%
50%
25%
0%
Proportion
Undisturbed
Disturbed
Big
Small
Mesh size
Outcome
Dead
Alive

Figure 2.2 Proportion of dead and live queens in the caged queen experiment at a) CSIRO and b) Girraween by site status (disturbed, undisturbed), cage mesh size (big, small) and retrieval time (7, 14, 25 days). See Table 2.1 for statistical results.

We examined each dead queen in the big mesh cages (n=157) to determine her cause of death. We found signs of predation (e.g. missing body parts or small pieces of cuticle remaining in the cage) in 89.8% of these cages while 1.9% of the dead queens seemed intact (i.e. no body parts missing, abdomen and thorax looking undamaged). The remaining 8.3% had no signs of queens. We checked these cages for potential gaps in the mesh that would have allowed the queen to escape but did not find any. Therefore, we assumed that all body parts of queens from such replicates were carried away by other ants and counted such replicates as dead queens. On three occasions, we found native ants inside big mesh cages as we were retrieving them. In the undisturbed sites, we observed *Nylanderia sp3 obscura group* workers inside a cage at CSIRO and a dozen *Carebara affinis* workers inside a cage at Girraween. In the Girraween disturbed site, we observed *C. affinis* workers moving pieces of a dead queen.

Because the small mesh cages successfully excluded native ants, we were able to deduce the queens' cause of death at different exposure times and between disturbed and undisturbed sites (Figure 2.3). Queen mortality in the small mesh cages can be attributed to unsuitable abiotic factors and baseline mortality, whereas big mesh queen mortality is a combination of abiotic factors, baseline mortality, and biotic resistance. Therefore, the proportion of deaths due to biotic resistance can be estimated by subtracting the relative number of dead queens in small mesh from the number of dead queens in big mesh. For example, 26/38 or 68.4% of queens in small mesh introduced at undisturbed sites and retrieved at 25 days died. 34/38 (89.5%) of queens in big mesh cages introduced in undisturbed sites and retrieved at 25 days died, so if we assume the same percentage of queens in big and small mesh cages died from abiotic factors and natural causes, then 8 queens or 21% died due to biotic resistance. Overall, abiotic factors and baseline mortality drove queen mortality (mean ± SD: 85.7±8% mortality assigned to abiotic factors and baseline mortality) while biotic resistance played a minor role in queen mortality (mean ± SD: 14.2±8% mortality assigned to biotic resistance) (Figure 2.3). Death due to biotic resistance was lower in the first days than at the later stage of colony founding. The difference between death

due to biotic resistance at undisturbed and disturbed sites was low but always higher in undisturbed sites (Figure 2.3).

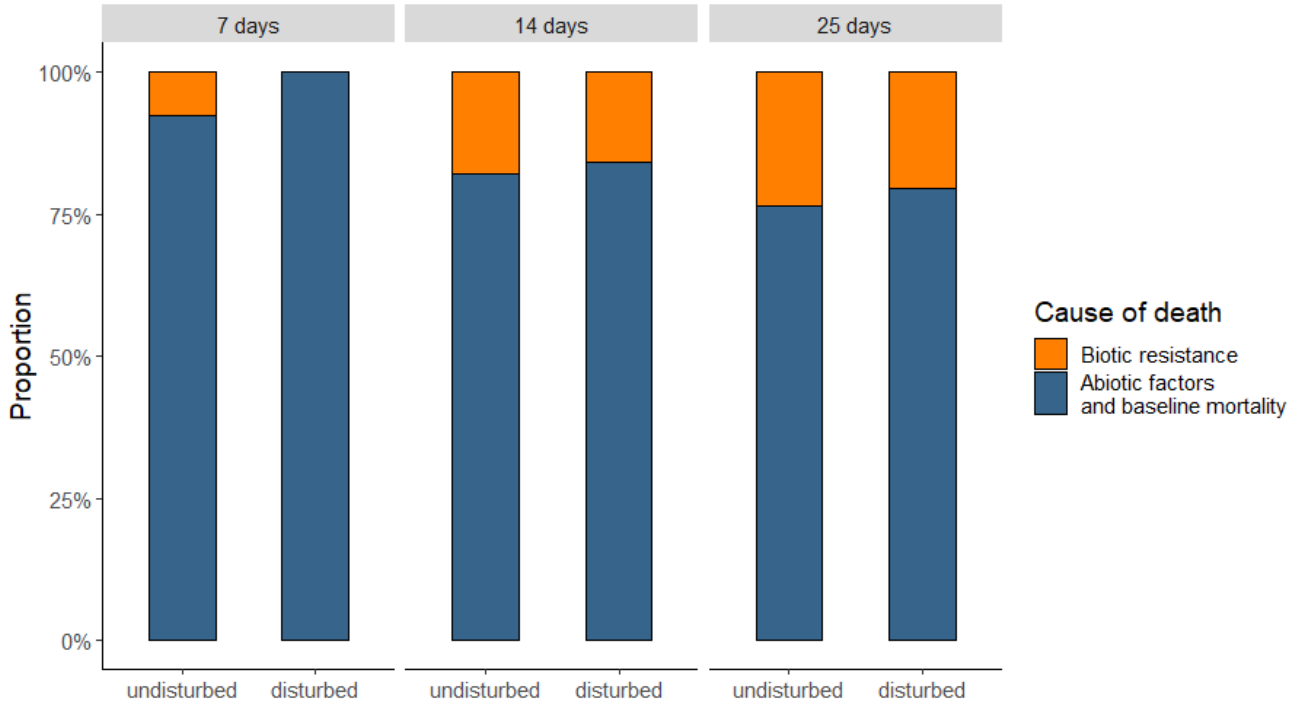


Figure 2.3 Proportion of queens dying due to abiotic factors and baseline mortality or biotic resistance (inferred) by exposure time and disturbance. 7 days: undisturbed n=37, disturbed n=40, 14 days: undisturbed n=51, disturbed n=40, 25 days: undisturbed n=60, disturbed n=61.

We found brood in 26 of the 157 cages that had surviving queens. Surviving queens in the small mesh cages had brood more often (23.1%) than queens in big mesh cages (7.6%, Table 2.1), and pupae were only present in the small mesh cages. Other factors such as exposure time, disturbance (disturbed or undisturbed sites), and location did not affect brood presence in the cages (Table 2.1). Eggs, but no other brood, were present in 16.7% of cages retrieved after 7 days. Cages collected after 14 days contained eggs and/or larvae (12.5%). We found worker pupae only in the cages that spent 25 days in the field. The adult worker stage was only present in one small mesh cage in the CSIRO disturbed site which contained a single worker as well as 22 eggs, 12 larvae and 11 pupae. Most surviving queens (91.1%) had lost weight (mean ± SD: 20.1±13% loss from their initial weight) while in the field, but 8.9% were heavier (mean ± SD: 5.9±14.6% heavier than their initial weight) when retrieved from the field than when we

introduced them. The longer a queen remained in the field, the more weight she lost, but mesh size, location, and disturbance (disturbed or undisturbed sites) did not affect queen weight loss (Table 2.1).

Abiotic conditions differed between disturbed and undisturbed sites. It was 1.5±0.4°C (mean ± SD) hotter in disturbed sites compared to undisturbed sites (Figure S2.3; LM: ANOVA, χ^2=37.013, df=1, p<0.0001) but the temperature did not differ significantly between the two disturbed sites (post hoc test p>0.05 for pairwise comparisons of both disturbed sites) or between the two undisturbed sites (post hoc test p>0.05 for pairwise comparisons of both undisturbed sites). The combined first two axis from the PCA of the environmental variables accounted for 99.9% of the total variation (Figure 2.4). The PCA combining ground cover and shading shows that one group consisted of the disturbed sites and the second one of the undisturbed sites, however the PC1 score did not differ significantly between disturbed and undisturbed sites (Kruskal-Wallis test, Kruskal-Wallis χ2=2.4, df=1, p=0.12).

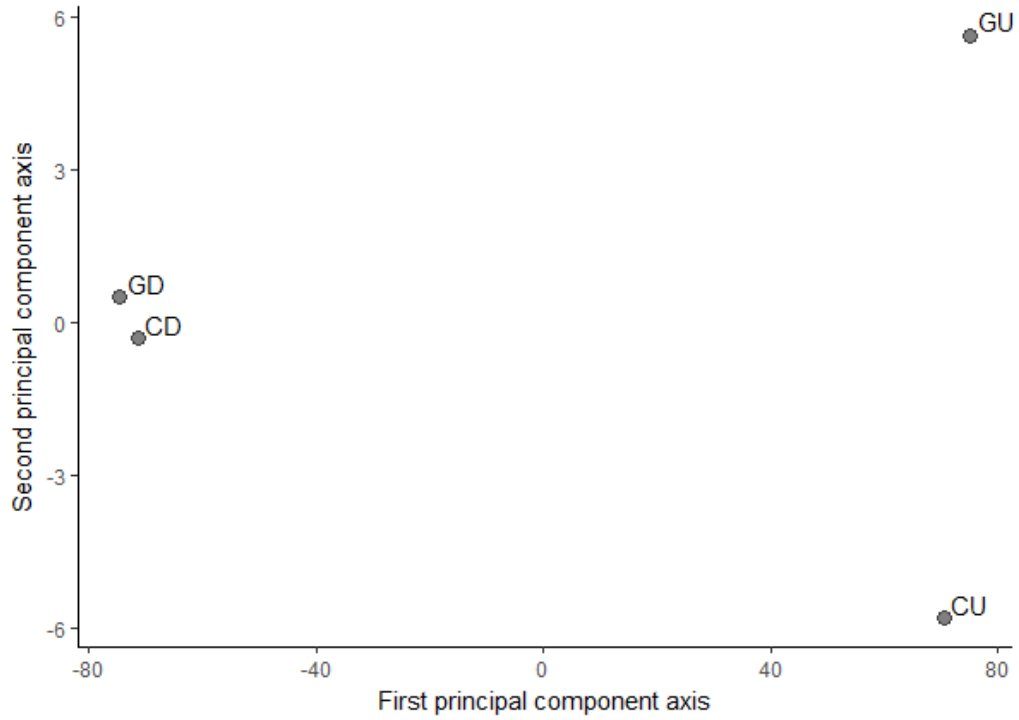


Figure 2.4 Principal component map of environmental variables (percentages of grass cover, litter cover, bare soil cover, shading at 10cm-3m above ground and shading at 3m above ground) for the caged queen experiment in all the sites. The first letter indicates the site: C for CSIRO, G for Girraween. The second letter indicates the disturbance: D for disturbed and U for

undisturbed. The first axis represents 85% of the variation and both axes cumulatively represent 99.9% of the variation.

Ant community results

Queen and colony introduction experiment sites

Ant species richness did not differ between disturbed and undisturbed sites, but species composition did (Figure 2.5, Table S2.1). We found between 25 and 39 species (mean ± SD: 33±5.8) in the four disturbed sites and between 27 and 38 species (mean ± SD: 33.3±4.5) in the three undisturbed sites (Kruskal-Wallis test, $\chi 2=0.125$, df=1, p=0.724). The Shannon index did not differ between undisturbed (Table S2.1, mean ± SD: 2.2±0.18) and disturbed sites (mean ± SD: 2.6±0.43, Kruskal-Wallis test, $\chi 2=0.5$, df=1, p=0.480). The NMDS ordination of the community composition (Figure 2.5) resulted in disturbed and undisturbed sites grouping separately and therefore indicating that their community compositions differ. However, the ant community composition did not differ significantly between disturbed and undisturbed sites according to our PERMANOVA (p=0.19).

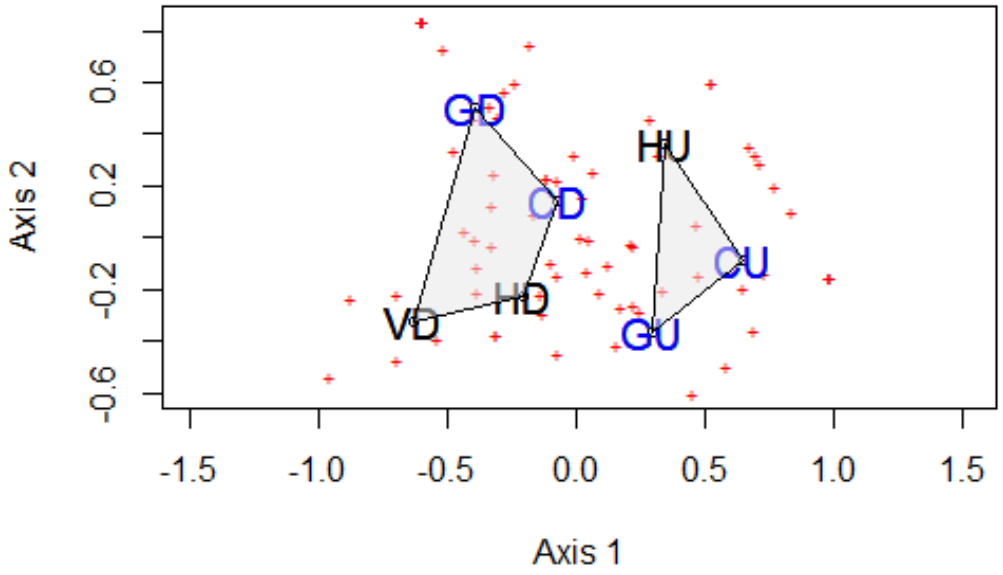


Figure 2.5 Non-metric multidimensional scaling analysis for ant community similarity in seven sites for the queen and colony introduction experiment. The sites with blue names were also used in the caged queen experiment (CSIRO and Girraween sites). Each cross represents an ant

species. The open symbols show the seven sites sampled. The first letter indicates the location: C for CSIRO, G for Girraween, H for Humpty Doo and V for Vanderlin. The second letter indicates the disturbance: D for disturbed and U for undisturbed. The polygons link the disturbed and undisturbed sites separately.

Caged queen experiment sites

The species richness was similar between disturbed and undisturbed sites, but the community composition differed (Figure 2.5, Figure 2.6, Table S2.1). We found 37 and 27 ant species in the undisturbed sites and 38 and 39 ant species in the disturbed sites. The disturbed sites clustered separately from the undisturbed sites on the NMDS ordination of the community composition (Figure 2.5). The two ant species that we observed attacking *S. geminata* queens in big mesh cages (*C. affinis* and *Nylanderia sp3 obscura group*) were more common in undisturbed sites than in disturbed ones (Figure 2.6). *Carebara affinis* was the most abundant species at undisturbed sites and was absent from disturbed sites while the relative abundance of *N. sp3 obscura group* was 10 times higher on average in undisturbed (mean ± SD: 0.15±0.08) than in disturbed sites (0.014±0.006).

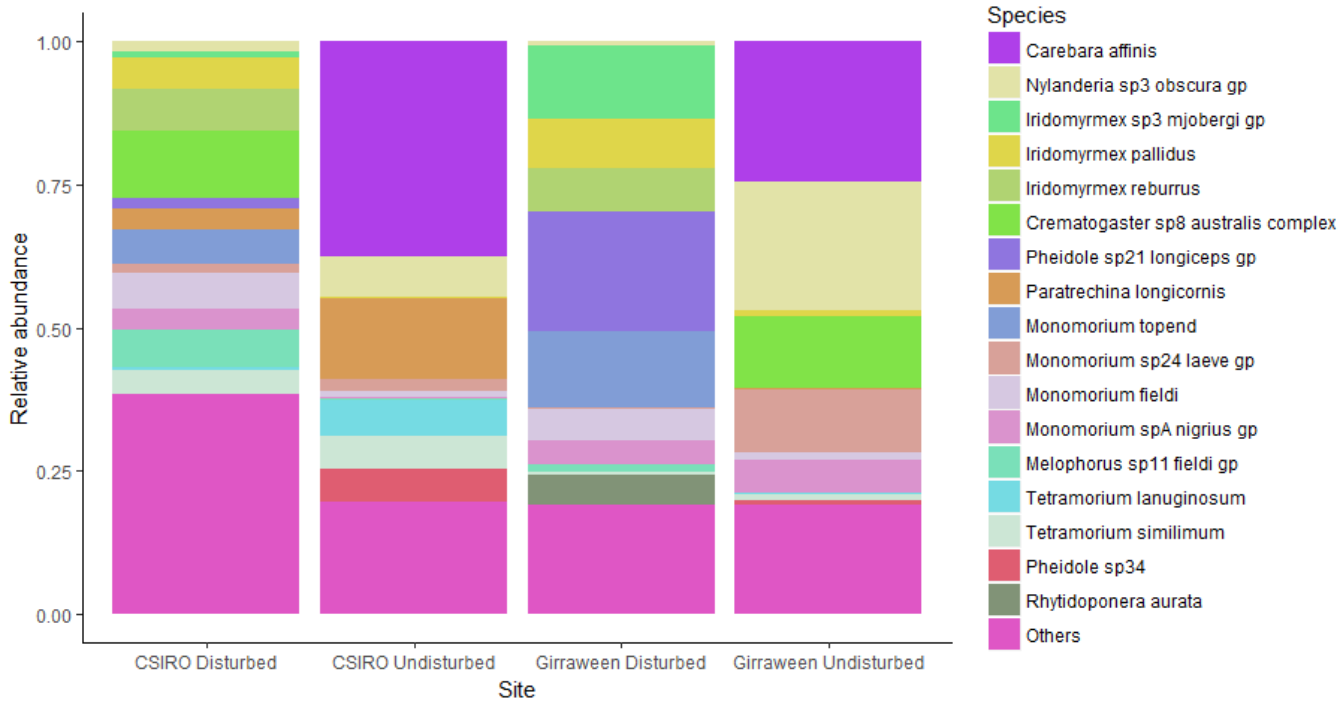


Figure 2.6 Relative abundances and distributions of ant species from sites used in the caged queen experiment. CSIRO Disturbed: N=566 individuals and 38 species, CSIRO Undisturbed:

N=487 individuals and 37 species, Girraween Disturbed: N=1318 individuals and 39 species, Girraween Undisturbed: N=561 individuals and 27 species. Species with a relative abundance lower than 5% for all sites were combined in "Others".

Discussion

Determining which factors contribute to making some areas more susceptible to biological invasions than others is one of the foundational goals of invasion biology. Disturbance can facilitate biological invasions through a combination of biotic and abiotic factors but, only a few experimental studies have focused on quantifying the relative importance of such factors in determining the suitability of an area for invasion (Menke et al. 2007). The low survival rate in the queen and colony introduction experiment (0.82% at 30 and 90 days in disturbed sites) did not allow us to make strong conclusions, so we focus our discussion on results from the caged queen experiment. With our caged queen experiment we investigated the relative importance of abiotic factors and biotic resistance on *S. geminata* queen survival and colony establishment at the founding stage. We found that the combination of abiotic factors and baseline mortality were responsible for 85.7±8% (mean ± SD) of queen mortality. Biotic resistance was not the main driver of queens' failure to establish but was still important for queen survival with queens being 50.7% more likely to survive if they were introduced in the small mesh cages. We found evidence for stronger biotic resistance in undisturbed sites; being protected from native ants improved queen survival in undisturbed sites but not in disturbed sites and aggressive subterranean ant species were more common in undisturbed sites. Therefore, biotic resistance from the native community may contribute to preventing the establishment of *S. geminata* in undisturbed habitats.

Unsuitable abiotic conditions were the main driver of queen mortality. Although we were unable to distinguish between death caused by abiotic factors and baseline mortality in our field experiment, in a lab experiment, 20.3% of single-founding newly mated queens of *S. geminata* collected at the same location as the queens we used here, died before the end of the claustral period (Lenancker et al. 2019). Assuming that the baseline mortality in our field experiment was the same or similar to that of the laboratory experiment, abiotic factors were the main cause of queen mortality, followed by baseline mortality, and biotic resistance. Suitable abiotic conditions, not lack of biotic resistance, are also the main determinant of Argentine ants'

successful establishment in their invasive range (Menke and Holway 2006, Menke et al. 2007). In manipulative experiments in California, *L. humile* failed to invade dry plots even in the absence of native ants while, in irrigated plots, the native ant community slowed, but did not prevent, the spread of *L. humile* (Menke et al. 2007). Water availability does not seem to be a strong determinant of *S. geminata* success according to results from a small-scale pilot experiment we conducted prior to the queen and colony introduction experiment. In this pilot, we found that artificial irrigation did not improve the survival of introduced *S. geminata* queens and colonies. Differences in temperature and shading between disturbed and undisturbed habitats could have affected the establishment success of queens.

Significant differences in the underground temperatures between disturbed and undisturbed sites could have influenced the success of *S. geminata*. Temperature strongly influences the establishment success of ants (Bertelsmeier et al. 2013), particularly by affecting colony development (Hartley and Lester 2003) and brood survival (Abril et al. 2010). The average temperatures we recorded at queens depth (7cm) during the caged queen experiment for disturbed (mean ± SD: 28.3±0.8°C) and undisturbed sites (mean ± SD: 26.8±0.47°C) are well within the range preferred by *S. geminata* in a laboratory experiment conducted in Texas. At 100% relative humidity, *S. geminata* workers with brood preferred temperatures ranging from 25 to 33°C and 22 to 25°C at 0% relative humidity (Cokendolpher and Francke 1985). It was 1.5±0.4°C hotter on average in disturbed compared to undisturbed sites. Even a few degrees Celsius increase in temperature can significantly accelerate brood development and increase the egg laying rate of queens. For example, it took 25 days for eggs laid by founding *S. invicta* queens to develop into adults at 28°C but only 20 days at 30°C (Porter 1988). Producing workers rapidly is crucial for founding queens as the longer the claustral period, the longer the queen is exposed and depleting reserves. Rate of oviposition can also increase with temperature as shown for *L. humile* (Abril et al. 2008). *Linepithema humile* queens reared under monogynous conditions laid 10 eggs a day on average at 26°C but 20 eggs a day at 28°C.

Unsuitable shading conditions could also be responsible for the absence of *S. geminata* in undisturbed habitats. Undisturbed sites were shaded savannah woodland (mean ± SD: 33.1±5.9% of shading at 10cm-3m above ground and 39.4±0.3 above 3m) whereas disturbed sites were mowed fields with virtually no shading (mean ± SD: 1.1±1.3% of shading at 10cm-3m above ground and 0% above 3m). Colonies of *S. geminata* show a strong preference for open habitats.

In central America, its putative native range, *S. geminata* was observed to avoid shaded habitats. In Costa Rica, field colonies avoided placing their nest in the shade and moved their brood chambers to open areas within 24 hours of becoming artificially shaded (Perfecto and Vandermeer 1996). In Puerto Rico, *S. geminata* was only ever found in agricultural areas or grassland and ten colonies introduced in a forest were gone after one month while seven of the ten colonies introduced in grassland were still present (Torres 1984). In the southern US, its putative native range, *S. geminata* is established in habitats with dense plant cover presumably because it has been displaced from open habitats by the invasive *S. invicta* (Tschinkel 1988, Plowes et al. 2007). Queens of *S. invicta* prefer to establish in open habitats, presumably because their survival declines when shaded (King and Tschinkel 2016).

Unsuitable abiotic conditions in undisturbed habitats were not the only determinant of queen survival, biotic resistance in undisturbed sites may have also affected queen survival. We found that isolation from native ants improved queens' survival in undisturbed sites but not in disturbed ones. This suggests that biotic resistance was stronger in undisturbed sites than in disturbed ones. We found that the proportion of queen death assigned to biotic resistance increased with the time queens spent in the field. Therefore, suitable abiotic conditions seem critical in the first days of colony founding whereas, even though the effect of biotic resistance on queen mortality remains low, it appears to be taking a greater role towards the end of the claustral period, when the first workers start emerging. Native ants not only attacked the queens, they also likely preyed on the brood. Brood was more common in small mesh cages than in big mesh ones and we only found pupae in small mesh cages. The lack of brood in big mesh cages could ultimately have led to colony failure past the claustral phase.

Dominant subterranean ant species may have contributed to an increase in biotic resistance in undisturbed sites. Only small subterranean ants (maximum width 1.23mm) could have entered our big mesh cages to attack the queens and their brood. *Carebara affinis* is a small ant that forages above and below ground and can recruit large numbers of nest mates to overcome competitors (Berghoff et al. 2003*a*, 2003*b*). Interestingly, our ant survey showed that *C. affinis* was the most abundant species at our undisturbed sites and was absent from disturbed sites, which suggest that this species could be responsible for lower survival of queens in big mesh in undisturbed sites. Our field observations of *C. affinis* workers in disturbed sites inside a big mesh cage and transporting dead queen parts out of a cage support our hypothesis. *Nylanderia sp3*

obscura group is also of interest because its relative abundance was higher in undisturbed sites than disturbed ones and because we found workers in a big mesh cage situated at CSIRO's undisturbed site. Species of *Nylanderia* often arrive at baits before other competitors but can seldom defend resources against other competitors (Lapolla et al. 2011) which suggest that *N. sp3 obscura group* opportunistically scavenged on *S. geminata* queens rather than attacked and killed them. Native species in the *Solenopsis (Diplorhoptrum)* group are small and inconspicuous subterranean predators (Pacheco and Mackay 2014) that have been reported to attack *S. invicta* newly mated queens and incipient nests (Buren 1983, Nichols and Sites 1991). Even though we found that the cumulative abundance of subterranean *Solenopsis* was low in disturbed and undisturbed sites, it is likely that native *Solenopsis* occasionally preyed on *S. geminata* brood. Species in the *Iridomyrmex* genus are often identified as limiting invasive ant species establishment in Australia (Thomas and Holway 2005, Wittman 2014) but these ants were not able to predate on our queens as they are mostly epigeic and too large to penetrate our cages. However ants belonging to this genus most likely play a role at a later stage in the establishment of colonies, for example by competing for food resources as with *L. humile* (Thomas and Holway 2005). It would be interesting to test whether various ant species affect the establishment success of *S. geminata* at different colony life stages.

We found that disturbance increase *S. geminata* queen survival. In the caged queen experiment, survival was 21.9% greater at CSIRO's disturbed site compared to the undisturbed site. The only queen that successfully reared an adult worker was also found at this site in a small mesh cage. In a manipulative experiment testing the effect of various disturbance treatments on the establishment success of newly mated *S. invicta* queens, survival also increased with disturbance (King and Tschinkel 2016). During our queen and colony introduction experiment, all surviving replicates at 30 and 90 days were found in disturbed sites in which resident *S. geminata* had already established. However, we did not find live colonies in disturbed uninvaded sites. This suggests that the absence of *S. geminata* from some disturbed sites is not due to a lack of introduction opportunities but that abiotic and/or biotic factors may prevent its establishment in some disturbed sites. Further experimental work (e.g. introduction of caged queens in invaded vs non-invaded sites) is required to determine why some disturbed areas may be unsuitable to *S. geminata*.

Disturbance can facilitate biological invasions through a combination of biotic and abiotic factors but only a few studies have attempted to quantify their relative roles in the establishment success of an invasive species (Menke et al. 2007). We found that unsuitable abiotic conditions were the main contributor to tropical fire ant queen mortality. Strong interspecific competition from Australian native ants in undisturbed habitats has been suggested to hinder the establishment of invasive ants (Walters and Mackay 2005, Sanders and Suarez 2011, Wittman 2014). We found that biotic resistance may have contributed more to queen mortality in undisturbed than in disturbed habitats, but that the contribution of biotic resistance to queen mortality was relatively low. It would be interesting to test whether the relative contribution of biotic and abiotic factors on colony survival differs between biogeographical regions and at different colony life stages.

Acknowledgements

We are grateful to Moryfing Sylla, Léo Blondet, Mirzha Mendez and Russell Graham for their help in the field. We thank Luke and Daniel Hoffmann who helped to collect queens. We are also grateful to Greg Payne for allowing access to the Girraween field site. We thank Gabriela B. Arcoverde and Peter Yeeles for their advice regarding data analysis. This work was supported by a Holsworth Wildlife Research Endowment from the Ecological Society of Australia (ESA) and an ESA student research award to PL.

Supplementary information

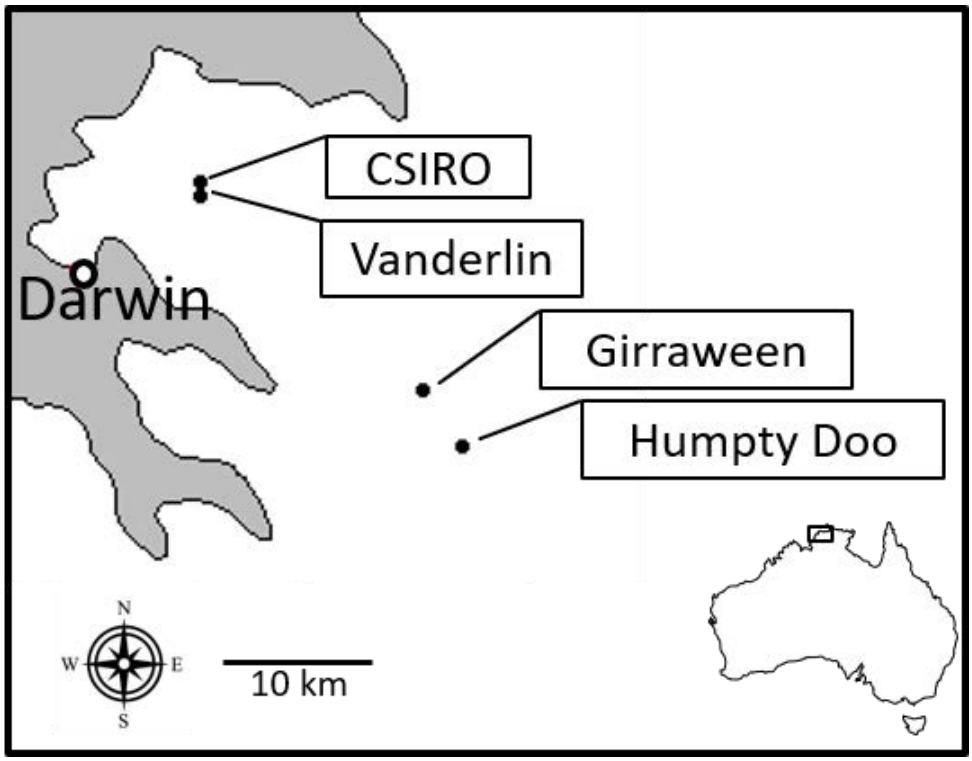


Figure S2.1 Map of the study locations in the Northern Territory, Australia. All four locations were used in the queen and colony introduction experiment and we only used the sites situated at CSIRO and Girraween in the caged queen experiment.

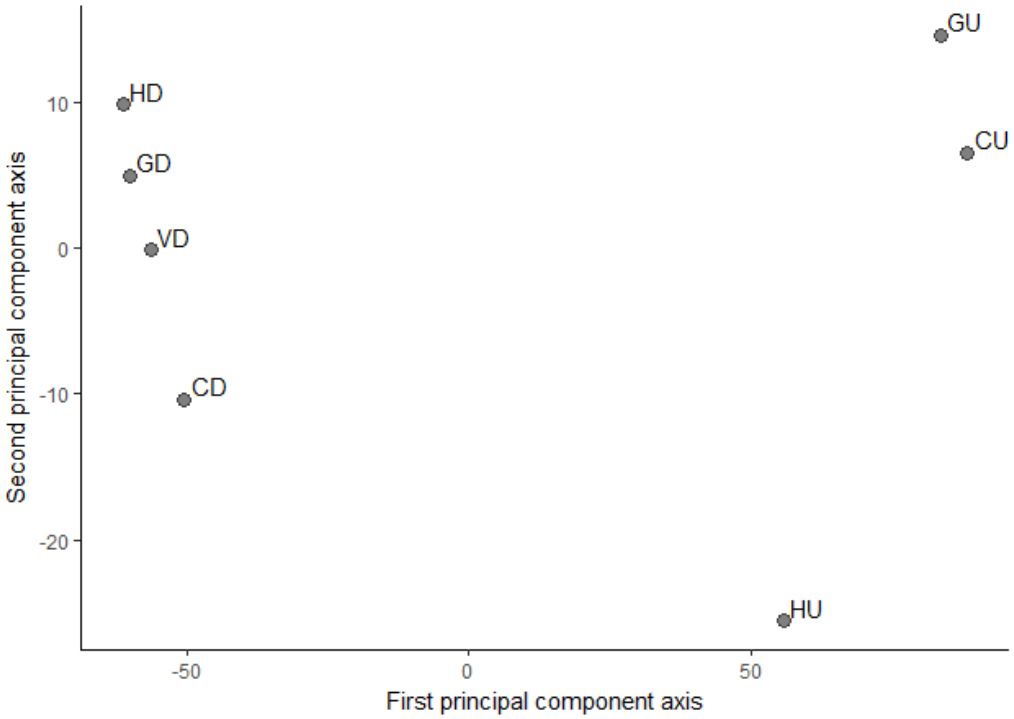


Figure S2.2 Principal component map of environmental variables (percentages of grass cover, litter cover, bare soil cover, shading at 10cm-3m above ground and shading at 3m above ground) for the queen and colony introduction experiment in all the sites. The first letter indicates the location: C for CSIRO, G for Girraween, H for Humpty Doo and V for Vanderlin. The second letter indicates the disturbance: D for disturbed and U for undisturbed. The first axis represents 79% of the variation and both axes cumulatively represent 99% of the variation.

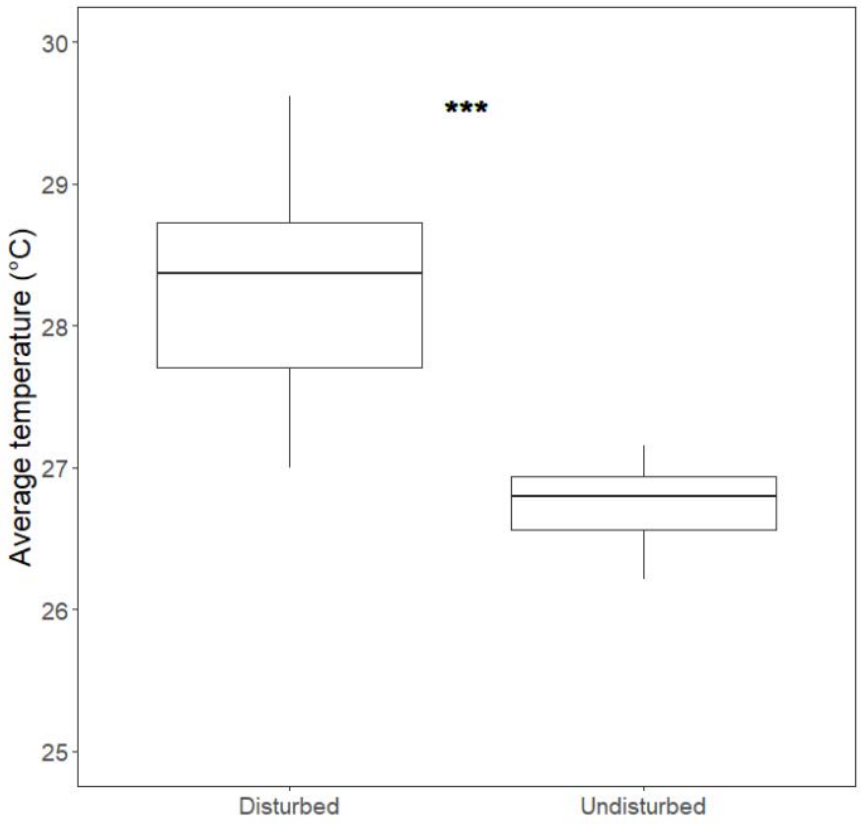


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Figure S2.3 Average temperature recorded for each probe at 7cm underground during the caged queen experiment. LM: ANOVA, $\chi 2=14.4759$, df=1, *** P<0.001. The bars show 50% of the data within the 25th and 75th percentiles, the line inside the bars is the median, and the whiskers show minimum and maximum data values.

Table S2.1 Number of individuals captured per site for each ant species. Sampling took place before the queen and colony introduction experiment. * indicates locations which were also used in the caged queen experiment.

	CSIRO*		Girraween*		Humpty Doo		Vanderlin
Ant species	Disturbed	Undisturbed	Disturbed	Undisturbed	Disturbed	Undisturbed	Disturbed
Anochetus rectangularis	1	0	0	0	0	0	0
Bothroponera sp1 porata gp	0	0	0	1	0	0	0
Camponotus sp9 novaehollandiae gp	0	0	0	2	1	5	1
Camponotus spA	0	1	0	0	0	2	0
Cardiocondyla atalanta	1	0	0	0	25	0	8
Carebara affinis	0	183	0	137	0	1	0
Carebara sp1	0	3	0	1	0	0	1
Crematogaster sp2 australis complex	0	2	0	0	0	0	0
Crematogaster sp8 australis complex	67	0	0	70	0	0	0
Ectatomminae rhytidoponera spB	0	0	0	0	0	1	0
Ectomomyrmex ruficornis	0	2	0	0	0	1	0
Iridomyrmex angusticeps	15	0	10	0	3	3	13
Iridomyrmex pallidus	31	1	114	6	15	240	27
Iridomyrmex reburrus	41	0	98	0	88	155	238
Iridomyrmex sp1 anceps gp	19	0	47	3	57	163	190
Iridomyrmex sp2 mjobergi gp	10	0	1	0	0	0	21
Iridomyrmex sp3 mjobergi gp	5	1	169	0	3	1	1
Leptogenys adlerzi	2	0	1	0	0	0	0
Melophorus sp1 aeneovirens gp	21	0	65	0	0	5	0
Melophorus sp11 fieldi gp	37	1	18	0	0	0	2
Melophorus spI	11	0	10	0	0	0	0
Meranoplus sp4	0	6	0	0	0	1	0
Meranoplus sp6 insolescens gp	0	0	1	0	0	0	0
Meranoplus sp8 F gp	0	1	0	0	1	0	0
Monomorium bifidum	0	0	16	0	0	0	0

Monomorium donisthorpei	26	0	0	0	0	0	0
Monomorium fieldi	36	5	73	7	70	41	213
Monomorium floricola	0	1	0	0	0	0	0
Monomorium sp14	14	3	6	5	8	1	0
Monomorium sp18 nigrius gp	26	1	7	23	22	3	4
Monomorium sp23 laeve gp	9	2	4	0	0	0	0
Monomorium sp24 laeve gp	9	11	4	62	1	10	13
Monomorium sp7 insolescens gp	0	1	0	0	0	3	0
Monomorium spA insolescens gp	0	0	1	0	0	0	0
Monomorium spA nigrius gp	20	1	54	32	28	0	76
Monomorium topend	34	0	176	0	10	0	3
Nylanderia sp3 obscura gp	11	34	11	126	4	4	0
Nylanderia sp4 vaga gp	3	17	1	19	0	2	0
Nylanderia sp5 vaga gp	4	7	2	0	7	0	1
Odontomachus sp nr turneri	7	10	3	7	7	5	0
Oecophylla smaragdina	0	12	0	10	0	3	0
Ooceraea sp19	1	0	0	0	0	2	0
Opisthopsis haddoni	1	1	0	1	0	2	0
Paraparatrechina sp1 minutula gp	0	5	0	0	0	0	0
Paraparatrechina tapinoma	0	0	0	0	0	0	0
Paratrechina longicornis	21	68	0	2	0	0	58
Pheidole sp13 ampla gp	16	0	5	5	0	12	1
Pheidole sp2 onifera gp	0	0	5	0	0	1	0
Pheidole sp21 longiceps gp	10	0	274	0	0	0	0
Pheidole sp26 F gp	0	3	0	0	0	4	0
Pheidole sp3 variabilis gp	0	1	0	5	0	0	0
Pheidole sp34	0	28	0	5	0	3	0

Pheidole sp5 C gp	5	4	6	0	1	1	0
Pheidole sp9 B gp	0	0	11	0	0	0	0
Polyrhachis senilis	0	0	0	0	0	2	0
Rhytidoponera aurata	0	0	70	0	3	7	0
Rhytidoponera borealis	2	3	1	0	0	2	0
Rhytidoponera reticulata	0	0	14	0	0	0	0
Solenopsis geminata	0	0	0	0	224	0	324
Solenopsis sp1	15	4	2	13	4	7	0
Solenopsis sp10	0	0	0	0	2	1	0
Solenopsis sp2	5	2	3	9	6	1	0
Solenopsis sp3	1	0	21	0	16	7	3
Solenopsis spF	0	0	0	0	1	0	0
Tapinoma melanocephalum	0	0	0	0	1	0	0
Tapinoma sp2	0	0	1	0	0	0	0
Tetramorium lanuginosum	3	30	0	1	14	0	1
Tetramorium similimum	23	29	5	6	189	3	16
Tetramorium sp1	0	2	0	2	2	0	2
Tetramorium sp2 striolatum gp	3	0	4	1	1	0	0
Tetramorium sp3 striolatum gp	0	0	4	0	0	0	15
Tetramorium spF	0	0	0	0	0	0	1
Tetraponera nitida	0	1	0	0	0	0	0
Total number of species	**38**	**37**	**39**	**27**	**30**	**36**	**25**
Shannon index	**3.22**	**2.38**	**2.65**	**2.34**	**2.29**	**1.97**	**2.08**

Chapter 3 Invasion pathways and social structure of the invasive tropical fire ant (*Solenopsis geminata*) revealed by genome-wide SNP data analyses

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Statement of contribution of others:

PL, DG and SE conceived the study. All authors assisted with the study design. SM optimised and conducted the laboratory work. PL and SE conducted the data analysis with suggestions from WT and TW. SM drafted the protocol for the laboratory work and PL drafted the rest of the manuscript. All authors edited the manuscript and SE coordinated the study. See acknowledgments for other contributions.

Abstract

Restriction site-Associated DNA Sequencing (RADseq) can generate thousands of genome-wide single nucleotide polymorphic (SNP) markers. RADseq constitutes a significant improvement to genotyping methods traditionally used to study the invasion pathways of invasive species such as microsatellite and mitochondrial markers, which often have low variability for invasive species with low genetic diversity. The tropical fire ant, *Solenopsis geminata*, is a global invader with low genetic diversity, making RADseq one of the best available methods to investigate its population genetics. We used double digest RADseq (a variant method of RADseq) to generate 3,834 SNPs and determine the origin, invasion history, geneflows, loss of genetic diversity, and social structure from 177 workers belonging to 28 colonies collected in 13 countries or islands. We found that invasive *S. geminata* populations lost 38.5% of their genetic diversity across their genome compared to native populations and that a single native colony contained more genetic diversity than all invasive specimens combined. Our analysis also revealed that the populations established in Australian islands (i.e. Christmas island, Ashmore Reef and Tiwi islands) probably originated from the Australian mainland. We identified multiple geneflow events between and among virtually all our sampled invasive populations and found that all colonies in the invasive range were polygyne (i.e. with more than one queen) suggesting that polygyny increases the invasion success of *S. geminata*. Future studies using genome wide sequencing to determine the origin, invasion history and geneflows of global ant invaders will shed light on their main invasion pathways and improve our understanding of invasion processes.

Introduction

Determining the origin of invasive populations, their main introduction pathways, and traits promoting invasion success is crucial to enable targeted biosecurity strategies (Hulme 2009). Establishing the origin and invasion pathways of invasive populations is also critical to the study of some of the fundamental questions in the field of invasion ecology, such as whether invasive populations display evidence of adaptive evolution following their introduction and whether they experience reductions in genetic diversity (Dlugosch and Parker 2008a). For example, comparisons of growth and flowering traits among native and invasive populations of the ornamental shrub *Hypericum canariense* showed that increased growth and a latitudinal cline in flowering phenology have evolved in the invasive range (Dlugosch and Parker 2008a, 2008b).

Comparisons between the genetic diversity of native and invasive populations of the house finch (*Carpodacus mexicanus*) revealed that invasive populations went through a genetic bottleneck and lost 17.5% of their allelic richness because they established from relatively low numbers of individuals (Hawley et al. 2006).

As commonly observed in invasive species, populations of invasive ants often go through a genetic bottleneck during their introduction which results in a low level of genetic diversity in invasive populations (Tsutsui and Suarez 2003) and makes the study of their population genetics challenging. Despite often losing genetic variation, over 150 species of ants have successfully established outside their native range, making ants one of the most diverse and harmful group of invaders (McGlynn 1999, Suarez et al. 2010). The low genetic diversity of invasive ant populations makes it difficult for researchers to find DNA markers with sufficient variability to determine their origin and invasion pathways. For example, the mitochondrial DNA region often shows low variability because invasive ant populations typically originate from a few maternal lineages: the tropical fire ant (*Solenopsis geminata*) was found to have four *mtCOI* haplotypes throughout its invasive range vs 57 in its native range while only one mtDNA *cyt b* haplotype was found in yellow crazy ant (*Anoplolepis gracilipes*) specimens from New Zealand (Corin et al. 2007, Gotzek et al. 2015). To date, studies on the origin of ant invasions and population movements have relied predominantly on limited numbers of microsatellite markers and/or sequenced portions of the mitochondrial genome. Such studies have provided initial insights into the introduction routes and origin of invasive populations (e.g. global studies: *S. geminata,* Gotzek et al. 2015; *Solenopsis invicta,* Ascunce et al. 2011; *Wasmannia auropunctata,* Foucaud et al. 2010; *Linepithema humile,* Vogel et al. 2010; local studies: *S. geminata* on the Galápagos Islands, Wauters et al. 2018, *L. humile* in New Zealand, Corin et al. 2007). However, the conclusions of these studies may be limited due to the small number of loci used (i.e. 12-66 microsatellite loci for the studies cited above) and their low variability. For example, the mean allelic richness (± SD) of *S. geminata* at 12 microsatellite loci was 1.3±0.1 in the Galápagos islands (invasive range) and 2.9±1.2 in its native range (Wauters et al. 2018).

Genome-wide approaches are needed to overcome challenges linked to the low variability of mtDNA and microsatellite markers in invasive ants.

Restriction site-Associated DNA Sequencing (RADseq) is a significant improvement to traditional genotyping methods: it is cost-effective, does not require a genome for data analysis, requires small amounts of DNA (50 to 100ng) and can generate hundreds to hundreds of thousands of genome-wide single nucleotide polymorphic (SNP) markers (Baird et al. 2008, Peterson et al. 2012, Wagner et al. 2013, Nygaard and Wurm 2015, Andrews et al. 2016). RADseq has been used in a wide range of studies in ecology, conservation and evolutionary biology (e.g. Wagner et al. 2013: phylogeny of cichlid fish, Hohenlohe et al. 2013: patterns of introgression between a native and invasive trout species, Dierickx et al. 2015: geneflows between populations of endangered albatross). Only a few studies have used RADseq on ants. For example it was used to determine worker relatedness and mating behaviour in ants living in symbiosis with *Acacia drepanolobium* (Boyle et al. 2018) and to inform the taxonomy of two phragmotic ant species (Fischer et al. 2015). RADseq is ideal for the study of invasive ants with low genetic diversity because it can generate thousands of SNP markers compared to a few dozen for microsatellite markers (Foucaud et al. 2010, Ascunce et al. 2011, Gotzek et al. 2015).

The low genetic diversity in invasive tropical fire ant, *S. geminata,* populations (Gotzek et al. 2015, Lenancker et al. 2019) makes RADseq one of the best available method to investigate its population genetics at the global and colony scale (Nygaard and Wurm 2015). *Solenopsis geminata* is one of the world's six most widespread, abundant, and damaging invasive ants, being established globally throughout tropical regions (Holway et al. 2002a, Wetterer 2011, Hodgson and Clarke 2014). A recent study using microsatellite and mitochondrial markers concluded that *S. geminata* was likely transported from Acapulco in southwestern Mexico to Manila in the Philippines in the 16th century and, from there to the rest of the Old World (Gotzek et al. 2015). However, the study is limited by the lack of samples collected around Acapulco (450 km away for the closest sample) and the low variability of the microsatellite and mitochondrial markers across the invasive range of *S. geminata*: four *mtCOI* haplotypes and 4±2.7 alleles per microsatellite markers on average (±SD) (Gotzek et al. 2015).

Solenopsis geminata is a highly successful invasive species with a wide global range and its success has been attributed to its ability to form polygyne colonies (i.e. with multiple queens) (Mackay et al. 1990, Holway et al. 2002a, Tsutsui and Suarez 2003). Colonies of *S. geminata* can be either monogyne (single queened) or polygyne (Holway et al. 2002a). Polygyny has been suggested to enable populations of *S. geminata* to reach high densities and dominate ecosystems

(Mackay et al. 1990, Holway et al. 2002a). Furthermore, transported fragments of polygyne colonies are more likely to contain at least one queen and establish successfully (Vargo and Fletcher 1989, Tsutsui and Suarez 2003). Colonies of *S. invicta* can also be either monogyne or polygyne (Holway et al. 2002a) and polygyne *S. invicta* colonies have been suggested to be associated with the successful establishment and range expansion of this ant in southeast China (Yang et al. 2012). The social structure of *S. geminata* has been determined in a few populations. Both monogyne and polygyne colonies appear to be present in the native and invasive range of *S. geminata* (Adams et al. 1976, Mackay et al. 1990, Williams and Whelan 1991, Ross et al. 2003, Trible et al. 2018, Wauters et al. 2018, Lenancker et al. 2019) but, the invasive population established in Taiwan appears to be exclusively monogynous (Lai et al. 2015).

We used ddRADseq (double digest RADseq, Peterson et al. 2012), a variant method of RADseq, to generate genome-wide SNPs and investigate the geneflows, invasion history, potential bottleneck and social structure of *S. geminata* at a global scale. Some of our samples were collected in invasive populations for which the population genetics and social structure had not previously been studied (e.g. Cambodia, Malaysia, Ashmore Reef). Our samples from the native range were collected in four locations in southwestern Mexico. Our objectives were to (i) quantify how much genetic diversity *S. geminata* has lost, (ii) determine the social structure of *S. geminata* across its invasive range and establish whether polygyne colonies are more common in its invasive range, and (iii) demonstrate the power of RADseq methods over traditional genotyping methods to determine the origin, invasion history, and geneflow among populations for an invasive ant with low genetic diversity.

Methods

Solenopsis geminata samples

We genetically analysed 177 *S. geminata* workers from 13 countries or islands. We used a single worker per colony or 3 to 8 workers per colony (mean ± SD: 7±1) depending on the availability of the material (Figure 3.1, Table S3.1). Most samples were from the Indo-Pacific region where *S. geminata* is considered invasive. We analysed seven workers belonging to a single colony and three individuals from three other colonies in southwestern Mexico, which was previously identified as the most-likely source of the invasive populations (Gotzek et al. 2015).

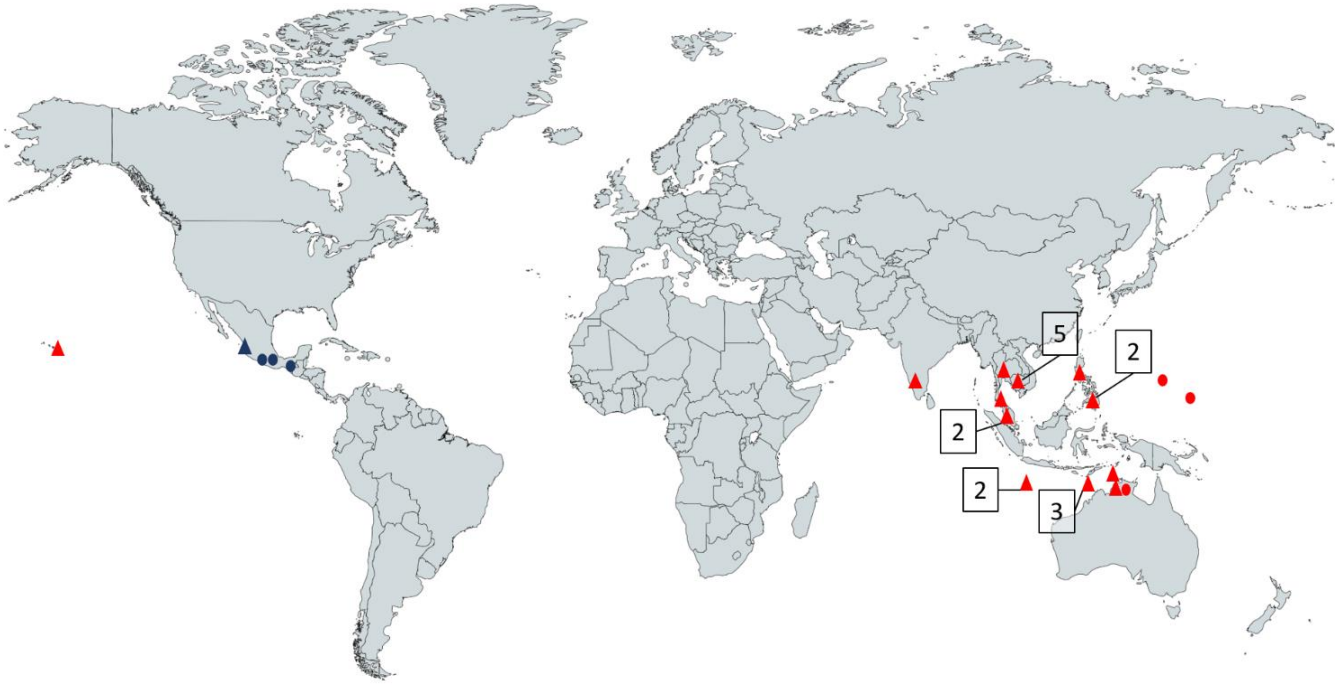


Figure 3.1 Locations of *S. geminata* specimens analysed in this study. Red: specimens collected in the invasive range. Blue: specimens collected in the native range. Circle: single specimen. Triangle: multiple specimens from a colony. Numbers show the number of colonies sampled if more than one originated from this location.

Genomic DNA extraction

We placed workers individually in a 1.5 ml Eppendorf tube, snap froze them in liquid nitrogen and homogenized them. We extracted total genomic DNA (gDNA) using the Qiagen DNeasy® Blood and Tissue kit modifying the manufacturer's instructions slightly (half the lysis buffer volumes and eluted the gDNA in 20µl Elution Buffer containing 10mM Tris-HCL). We quantified gDNA with a Qubit® 3.0 fluorometer (Life Technologies, Eugene, Oregon, USA) and selected gDNA samples with the highest concentrations.

ddRAD library preparation

The ddRADseq protocol was adapted from Peterson et al. (2012) and DaCosta and Sorenson (2014). Briefly, we prepared double-digest of 50-100ng of gDNA with 20 units each of high-fidelity restriction enzymes, *PstI* and *EcoRI* (New England Biolabs, Ipswich MA, USA), in

CutSmart buffer in a final volume of 50µL. Samples were digested in a thermocycler for 30 min at 37°C, then heat inactivated for 20 min at 65°C and slowly cooled to room temperature and held at 4°C. We assessed and quantified the fragment size distribution on the 2100 BioAnalyzer (Agilent, CA, USA) using the High Sensitivity DNA chips. We ligated 50µL of each digested gDNA sample to uniquely barcoded P1 and P2 adapters in a 70µL reaction containing 4µL of 50 nM P1 adapter, 12µL of 50 nM P2 adapter, 1µL of 2000 units of T4 DNA ligase (New England Biolabs, Ipswich MA, USA), 0.6µL of 0.15 mM rATP (Promega, Madison, WI, USA), 2µL of 10x NEBuffer 2 and 0.4µL of water. We ligated the samples on a thermal cycler for 30 min at 22°C, then heat inactivated for 20 mins at 65°C, and slowly cooled to room temperature and held at 4°C. Five µL of ligated gDNA from each sample were combined to create a pool of individuals. The samples were size-selected with 0.65x volume of Agencourt® AMPure® XP magnetic bead solution (Beckman Coulter, Brea, CA, USA), and suspended in 20µL elution buffer (Qiagen, Hilden, Germany). We prepared the final library amplification in a 60µL PCR reaction with 15µL of size selected DNA, 30µL of Phusion High Fidelity 2x Master Mix (New England Biolabs, Ipswich MA, USA), 3µL of 10 µM each of P1 and P2 primers (Table S3.2) and 9µL water. We ran the samples at 98°C for 30 s, followed by 20 cycles of 98°C for 10 s, 60°C for 30 s and 72°C for 40 s, with the final elongation at 72°C for 5 min. We cleaned the PCR product with 1.8x volume of Agencourt® AMPure® XP magnetic bead solution (Beckman Coulter, Brea, CA, USA), and suspended in 40µL elution buffer (Qiagen, Hilden, Germany). We quantified the ddRAD libraries on the Qubit 3.0 fluorometer (Life Technologies, Eugene, Oregon, USA) and assessed the size fragments on a Tape Station 2200 (Agilent, CA, USA) using the High-Sensitivity DNA screen tapes. We normalized the final library to 4nM for sequencing on the HiSeq platform (Illumina, CA, USA).

SNP calling and filtering

We used FastQC version 0.11.8 (Andrew 2010) to assess the quality of the raw fastq sequences. We dropped 10 low quality samples based on the FastQC reports, and trimmed the remaining sequences by quality (Phred quality score higher than 20) to a length of 50bp using Trimmomatic version 0.38 (Bolger et al. 2014).

We conducted SNP calling using a *de novo* approach on the 164 samples that passed the data filtering step. We used the software pipeline stacks version 2.2 (Catchen and Amores 2011,

Catchen et al. 2013) for SNP calling. We first used ustacks to align our short-read sequences into stacks with a minimum depth of coverage of 3. We used the final coverage information given at the end of the ustacks step to eliminate samples (n=7) with less than 50% reads. We then used ctsacks on the 157 remaining samples to build a catalogue of consensus loci with 2 mismatches allowed between sample loci. We used sstacks to search the putative loci built by ustacks against the catalog produced by ctsacks. We then used tsv2bam to orientate the data by locus and gstacks to align reads to the locus and call SNPs. We used the last stacks pipeline step, populations, to generate population genetic statistics and test three loci filtering scenarios. The minimum percentage of individual required to process a locus (r) for our population was conducted for three threshold levels 20, 50 and 70%. We then compared the summary statistics between the three variant call format (VCF) SNP files. The VCF file with r=70% resulted in the least amount of missing data in the VCF and was kept for the subsequent analysis. We excluded sites with more than 50% missing data (option max-missing) from the resulting SNP VCF file by feeding it to VCFtools version 0.1.16 (Danecek et al. 2011). The amount of gDNA of the final set of samples ranged from 50 to 100ng. Samples that contained more DNA were more likely to pass the filtering steps (Fisher's Exact Test for Count Data, $p<0.05$), but most samples passed these steps successfully regardless of the amount of DNA (50ng: 85%, 70ng: 89.4%, 100ng: 91.6%).

Population structure

We used several approaches to determine the population structure of *S. geminata*. All analyses requiring R were conducted in R version 3.5.0 (R Core Team 2018). We conducted a Principal Component Analysis (PCA, snpgdsPCA function in the SNPRelate package, Zheng et al. 2012) based on our SNP data for 157 *S. geminata* samples to visualize how the samples from different locations clustered. To visualize clusters within the invasive populations, we also conducted a PCA excluding the native samples (i.e. Mexican) and another one without three additional samples (two from Ashmore Reef and one from India, Figure S3.1) because they clustered away from the remaining 144 specimens and prevented us from looking at the structure of the invasive specimens. We used Treemix version 1.13 (Pickrell and Pritchard 2012) on 155 individuals to infer the history of *S. geminata*'s invasion. For this analysis, we removed the two most distant Mexican samples based on the PCA results (Figure S3.2). We used 100 bootstrap replicates and allowed up to five migration events to build a population tree which allows for geneflow and

population splits. We identified episodes of geneflow from the residual covariance matrix. We used ADMIXTURE version 1.3.0 (Alexander et al. 2009) to estimate genetic ancestry on the same 155 individuals used for the Treemix analysis. ADMIXTURE uses a maximum likelihood approach to estimate the number of genetic clusters and the proportion of derived alleles in each sample from each of the K populations. We ran ADMIXTURE multiple times and varied the K value from 2 to 20. We used a cross-validation test to determine the optimal K value.

Relatedness analysis

We used the populations program implemented in stacks (Catchen and Amores 2011, Catchen et al. 2013) to obtain the SNP data from 151 workers belonging to 22 colonies. We used r=1 to avoid biased estimation of relatedness from missing data (Kraemer and Gerlach 2017). This step resulted in 84 and 922 loci per colony and 42 to 461 SNPs per colony. We estimated relatedness using Wang's moment estimator of relatedness (Wang 2002) implemented in Coancestry 1.0.1.9 (Wang 2011). Wang's relatedness estimator is more reliable than other indices for biallelic loci with allele frequencies of 0.5 (Wang 2002). We estimated the effective mean number of queens (N_{qe}) per colony as

$$N_{qe} = (4r_s - r_q - 2r_{ml})/(4r_{xy} - r_q - 2r_{ml})$$

where r_s is the average relatedness of worker offspring from the same matriline, r_q the average relatedness of nestmate queens, r_{xy} the average relatedness of nestmate workers, and r_{ml} the average relatedness of the mates of nestmate queens (Ross 1993, Seppä 1994, Tay et al. 2011). We estimated r_{xy} from our data. We cannot determine r_s, r_q, and r_{ml} with our data and therefore relied on published data on polygyne *S. invicta* populations established in the USA to estimate their values. The invasive *S. invicta* is related to *S. geminata* and both species mate during nuptial flights (Hung et al. 1977, Hoffmann and O'Connor 2004). The social structure of *S. invicta* has been well-studied, whereas such information is lacking for *S. geminata.*

We used $r_s = 0.75$ which is the average value for full sisters under male haploidy and outbreeding conditions (Crozier 1970) as expected for *S. invicta*. We used $r_q = 0$ because queens in polygyne colonies of *S. invicta* have a null average relatedness (Goodisman and Ross 1997). Previous studies on the mating behaviour of *S. invicta* have found no evidence of local inbreeding (Ross and Fletcher 1985b, Ross et al. 1987, Ross 1993). The mating flights of *S.*

invicta and *S. geminata* involve alates from many colonies distributed over large areas which suggests panmixia (Lenancker et al. 2019). However, given the low genetic diversity of *S. geminata* (Gotzek et al. 2015, Lenancker et al. 2019), we expect some individuals from different colonies to be related. Therefore, we used two values of r_{ml}, $r_{ml} = 0$ (mates of nestmate queens are unrelated) and $r_{ml} = 0.5$ (mates are brothers).

Results

Data summary

From the 177 samples used to prepare the ddRAD libraries. we used a final sample set of 157 for the analysis after filtering the data (see Data filtering and SNP calling filtering sections in the methods) and obtained a total of 3,834 SNPs.

Genetic bottleneck

Our results show that invasive *S. geminata* populations went through a strong genetic bottleneck, losing 38.5% of their genetic diversity across their genome. We obtained a total of 2,358 SNPs in the specimens collected from the invasive populations vs 3,834 SNPs for the specimens collected from the invasive and native populations. The scores of specimens collected in the ant's native range are interspersed among all the scores on the PCA (Figure S3.2) whereas the scores of invasive samples form a compact group. The PCA shows that there was more genetic diversity within the FC1 colony from Mexico than from all specimens combined from the entire invasive range of *S. geminata.* Additionally, the number of SNPs in FC1 (461) exceeds that of any other colonies in the invasive range (average ± SD: 170.8±77.1, Table S3.3).

Origin and invasion history

Our results designate Acapulco as the primary origin and the Philippines as the secondary origin of this species' invasive populations. The Mexican samples that were collected closest to Acapulco were the closest to the invasive cluster (Figure S3.2 and Figure S3.3). In the Treemix analysis, Mexico and the Philippines are linked by a branch that denotes an ancestry relationship between the two populations and points to Mexico as the direct origin of the Philippines' population (Figure 3.2). The two migration edges show strong geneflows from the Philippines to the other invasive populations (Figure 3.2). ADMIXTURE analysis reveals that all the invasive

populations with evidence of geneflow (except Thailand) received geneflow from the Philippines (Figure 3.3, Figure 3.4). The Hawaiian population may not share this common history as it shows signatures of geneflow from the JDA264 cluster (the closest Mexican sample to Acapulco) as well as the Philippines (Figure 3.3, Figure 3.4). These results indicate a possible mixed Mexican and Philippine origin of the Hawaiian population.

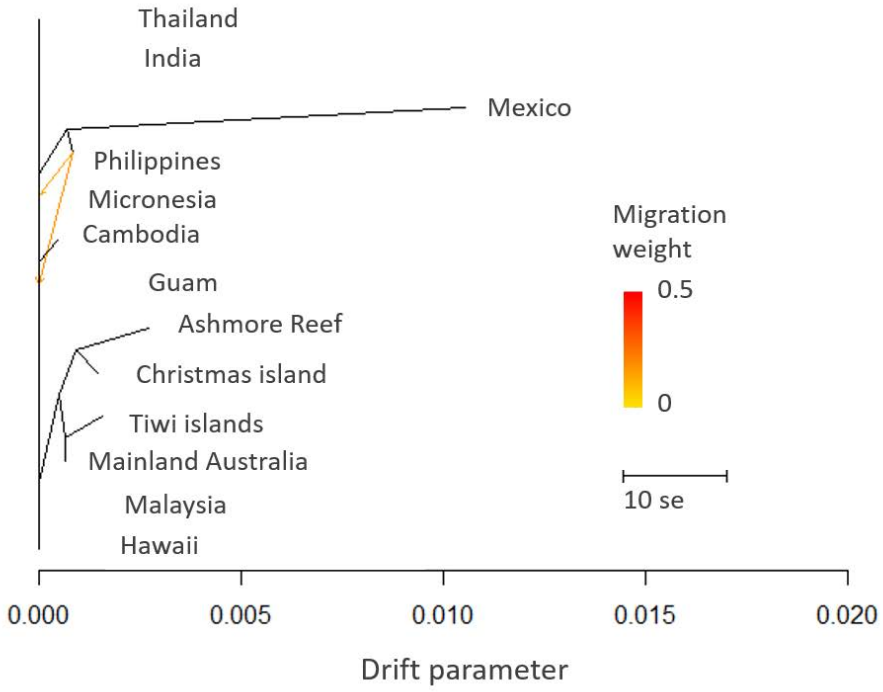


Figure 3.2 Demographic history of *S. geminata* populations for 155 specimens. Inferred Maximum Likelihood (ML) tree obtained with Treemix (Pickrell and Pritchard 2012). The arrows depict migration edges and show geneflow directions. The migration weight is shown by the colour of the arrows. The two migration edges both emerged from the Philippines. The branches indicate ancestry relationship and their length is proportional to the amount of genetic drift that has occurred on the branch. Populations without a branch indicate that genetic drift did not occur for these populations according to the ML tree.

Our analysis also revealed that populations from the Australian mainland and its islands (Tiwi Islands, Christmas Island and Ashmore Reef) share a common introduction history. In the Treemix analysis, mainland Australia, Tiwi Islands, Ashmore Reef and Christmas Island are all linked by branches denoting ancestry relationship among the four populations (Figure 3.2).

Mainland Australia and Tiwi Islands belong to the same ADMIXTURE cluster (Figure 3.3) and are linked by a branch on the Treemix figure (Figure 3.2) suggesting that the invasion of Tiwi Islands originated from the Australian mainland. Data from specimens from the three main islands of Ashmore Reef revealed that the ants on West and East islands both represent a single cluster whereas the ants of the Middle Island belong to a mix of the West and East Island clusters, which indicates potential geneflow from the West and East Island to the Middle Island.

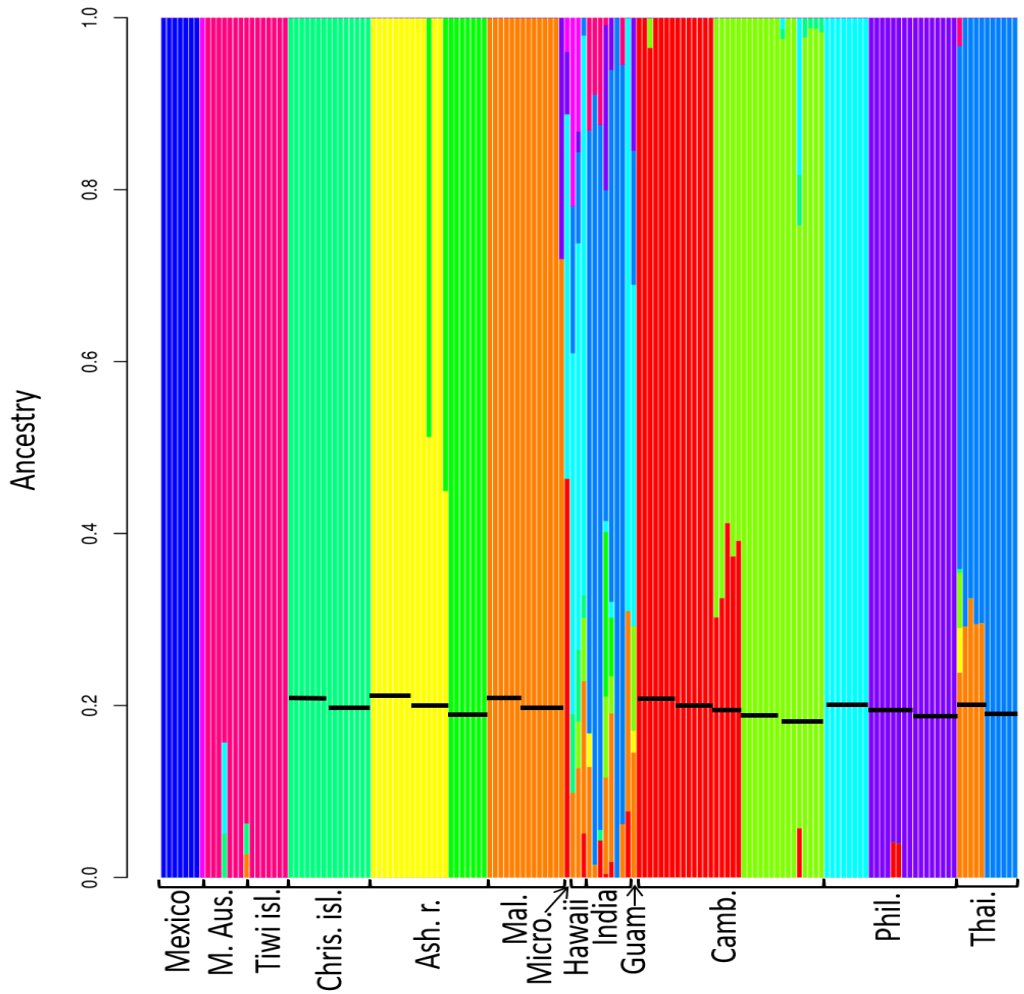
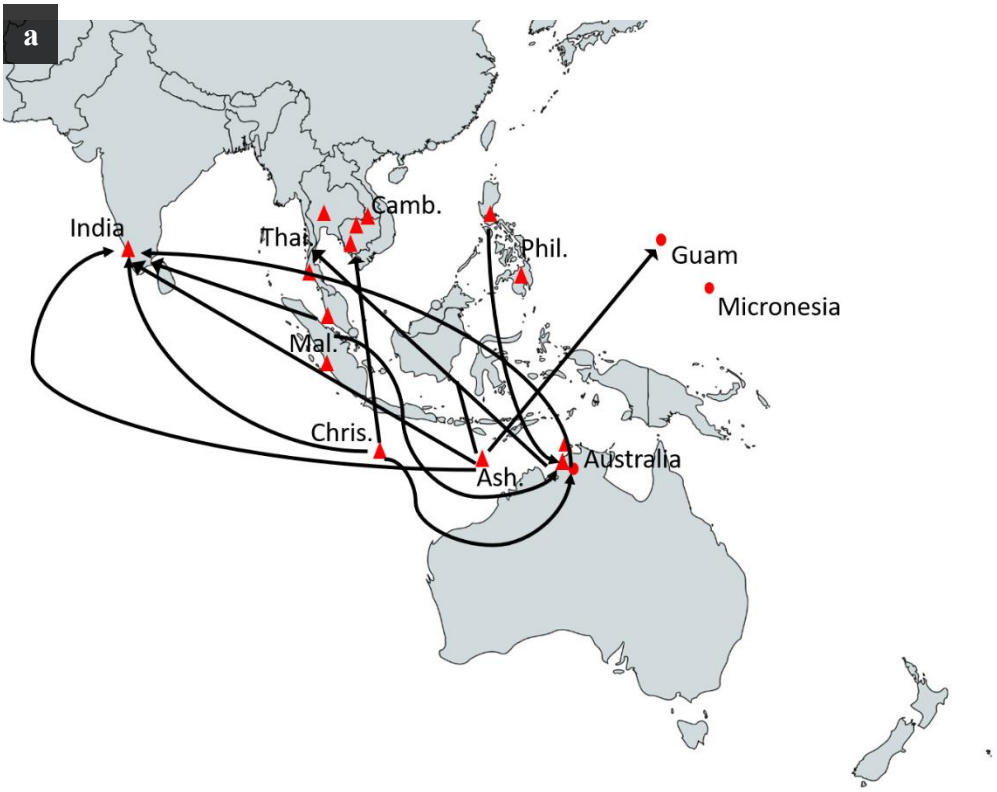


Figure 3.3 ADMIXTURE analysis conducted on SNPs from 155 specimens showing the optimal number of cluster: K=12. Individual black horizontal bars show specimens belonging to the same colony if multiple specimens from more than one colony were collected from the same location. Abbreviations: M. Aus. Mainland Australia, Tiwi isl. Tiwi Islands, Chris. isl Christmas Island, Ash. r. Ashmore Reef, Mal. Malaysia, Micro. Micronesia, Camb. Cambodia, Phil. Philippines, Thai. Thailand.

Geneflow between and among invasive populations

We found evidence of multiple geneflow among the invasive populations. The ADMIXTURE analysis shows that some populations (Micronesia, Hawai'i, India and Guam) did not belong to any cluster and received geneflow from several populations (Figure 3.3, Figure 3.4). Other populations either belong to single clusters (Mexico, mainland Australia, Tiwi Islands, Christmas Island, Malaysia and Thailand) or two clusters (Ashmore Reef, Cambodia and the Philippines) and most of them showed evidence of geneflow.



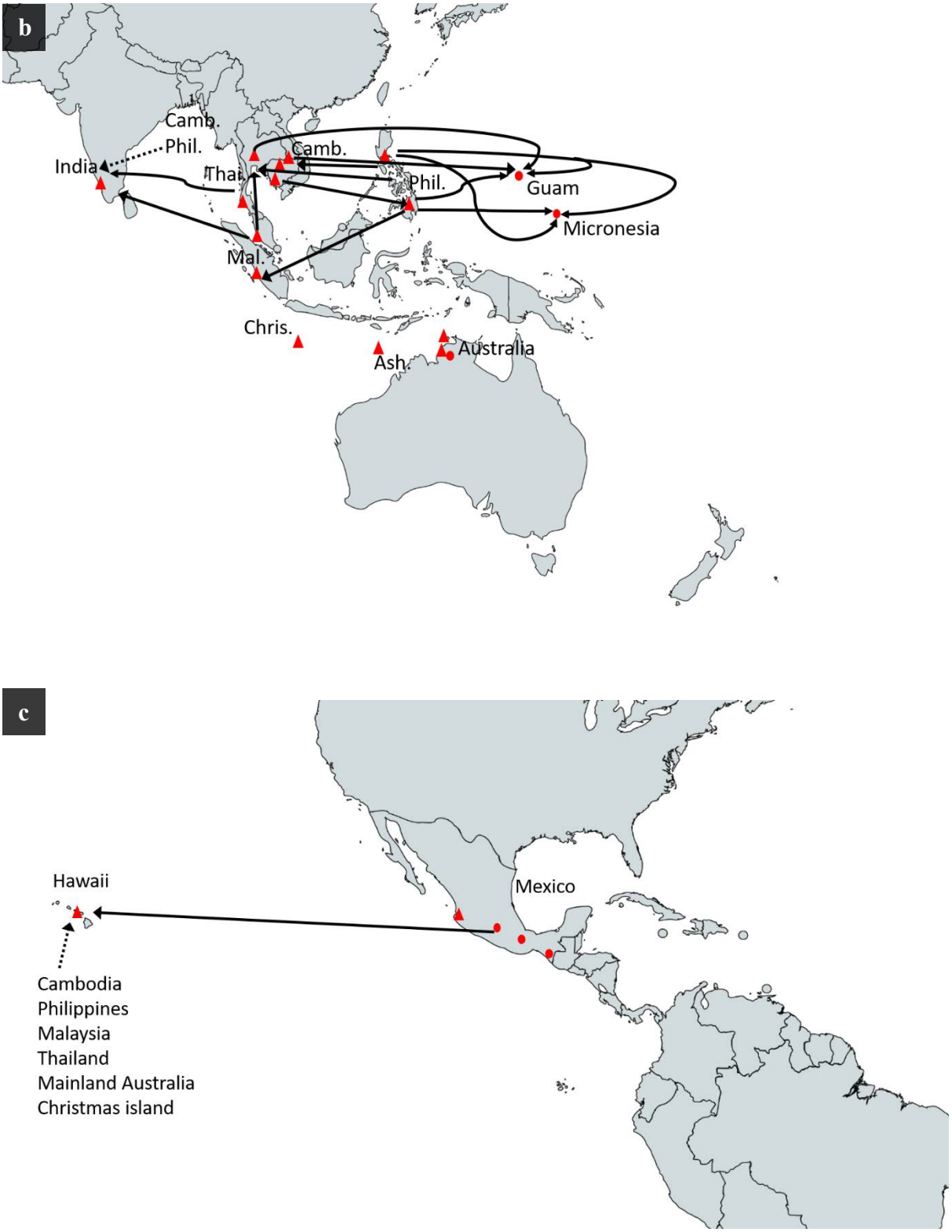


Figure 3.4 Geneflow between and among populations according to the ADMIXTURE analysis (Figure 3.3). Panel a shows geneflows involving mainland Australia and Australian islands, panel b other populations in the Indo-Pacific region, and panel c Mexico and Hawai'i. Circle:

single specimen. Triangle: multiple specimens from a colony. Abbreviations: Camb. Cambodia, Phil. Philippines, Thai. Thailand, Mal. Malaysia, Chris. Christmas Island, Ash. Ashmore Reef

The PCA analysis also indicates potential geneflow among invasive populations (Figure 3.5). The PCA with 144 specimens belonging to invasive populations shows that specimens from Ashmore Reef, Cambodia, and the Philippines each form distinct groups. Specimens from the other invasive populations are all mixed in one large group, albeit with individuals with the same geographic origin tending to group closer together.

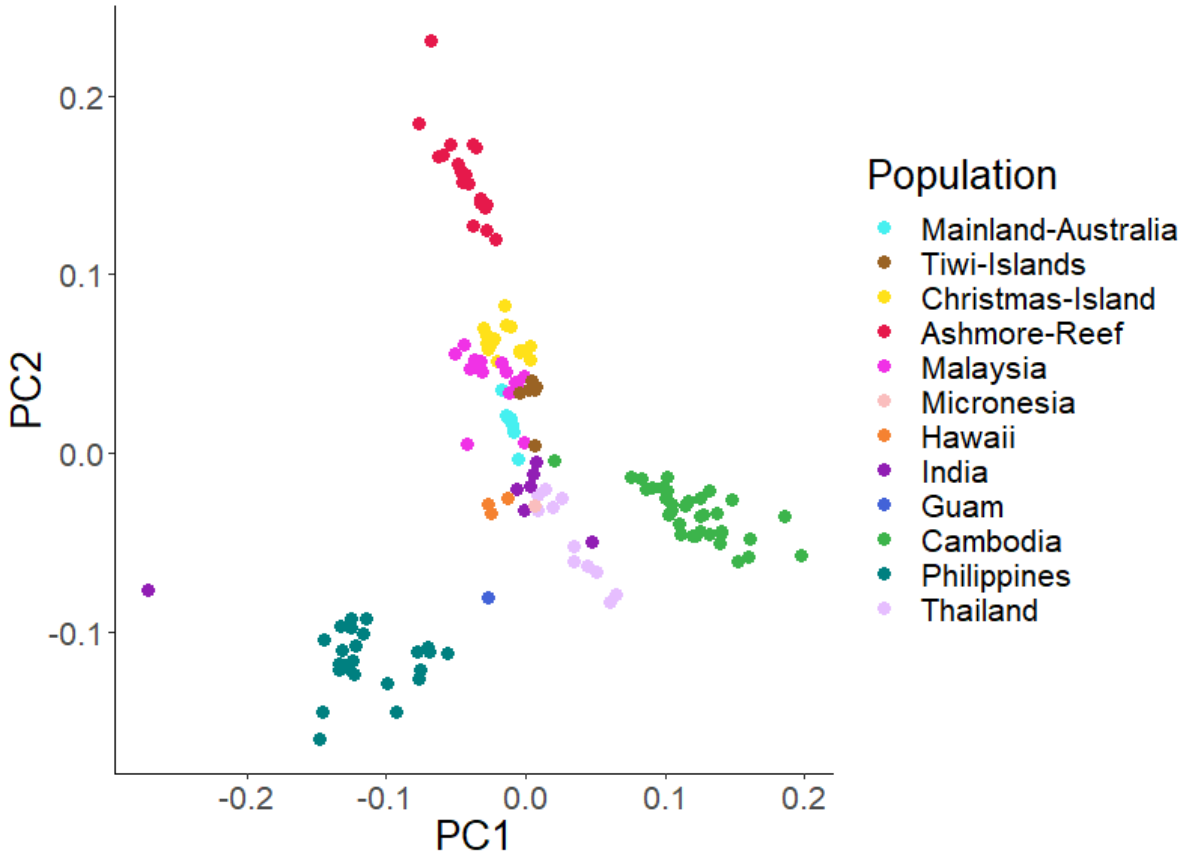


Figure 3.5 Principal component map of the SNPs in 144 individual specimens from the invasive range of *S. geminata*. The first axis represents 20% of the variation and the second axis 9%.

Social structure

We found that all 22 colonies were polygyne (Table 3.1). The relatedness value r_{xy} (± SD) ranged from 0.26 ±0.13 to 0.68±0.11 (average ± SD: 0.12±0.05). We used an average (± SD) of 361.9±186.8 markers per colony for relatedness estimates. The effective number of queens (N_{qe}) of polygyne colonies ranged from 1.1 to 40 queens per colony. N_{qe} varied depending on the r_{ml} values (Table 3.1).

Table 3.1 Estimates of average nestmate workers relatedness (Wang's r), effective number of queens ($\boldsymbol{N_{qe}}$) and social structure of colonies collected in the native and invasive range of *S. geminata*. We calculated $\boldsymbol{N_e}$ for two $\boldsymbol{r_{ml}}$ (average relatedness of the mates of nestmate queens) values (see Table S3.3).

Origin	Colony code	Number of workers	Wang's r ±SD	N_{qe}	Social structure
Mexico	FC1	7	0.29±0.25	2.6-14.1	polygyne
mainland Australia	B1	7	0.68±0.12	1.1-1.2	polygyne
Tiwi Islands	Ti3	7	0.57±0.18	1.3-1.6	polygyne
Christmas Island	Pink_House	7	0.68±0.08	1.1-1.2	polygyne
Christmas Island	Temple_Road	8	0.62±0.08	1.2-1.3	polygyne
Ashmore Reef	E5A	7	0.54±0.15	1.4-1.7	polygyne
Ashmore Reef	M1A	7	0.46±0.18	1.6-2.4	polygyne
Ashmore Reef	W6A	7	0.53±0.08	1.4-2.8	polygyne
Malaysia	DAG582	6	0.61±0.08	1.2-1.4	polygyne
Malaysia	SCCY14-07	8	0.68±0.11	1.1-1.2	polygyne
Hawai'i	HDA	3	0.26±0.13	2.9-40	polygyne
India	HB-gem	8	0.41±0.11	1.8-3.2	polygyne
Cambodia	DAG623	7	0.58±0.1	1.3-1.5	polygyne

Cambodia	DAG618	7	0.62±0.08	1.2-1.4	polygyne
Cambodia	DAG621	7	0.65±0.1	1.2-1.3	polygyne
Cambodia	DAG622	5	0.55±0.08	1.4-1.6	polygyne
Cambodia	DAG637	8	0.51±0.11	1.5-1.9	polygyne
Philippines	IRRI-700	8	0.52±0.11	1.4-1.8	polygyne
Philippines	Phil1	8	0.53±0.18	1.4-1.8	polygyne
Philippines	Phil2	8	0.61±0.04	1.2-1.4	polygyne
Thailand	DAG561	5	0.54±0.07	1.4-1.7	polygyne
Thailand	SH1	6	0.53±0.16	1.4-1.8	polygyne

Discussion

The use of ddRADseq rather than traditional genotyping methods allowed us to determine how much diversity *S geminata* has lost during its introduction, geneflows among and between invasive populations and the social structure of colonies collected across the ant's invasive range. Our study also provides strong support for the invasion scenario first described in Gotzek et al. (2015) and new knowledge about *S. geminata* invasion to Australia and its islands.

Use of genome-wide SNP analysis enabled us to estimate that *S. geminata* has lost 38.5% of its genetic diversity across its genome during its introduction, which likely has repercussions for colony fitness. We also found that the genetic diversity in one native colony was higher than in all invasive specimens combined. Previous research using mitochondrial and microsatellite markers could not estimate the loss of genetic diversity for *S. geminata* as the results differed between marker types (mitochondrial: 93% *mtCOI* haplotype loss in the invasive range, microsatellite: 63% allele loss on average in invasive colonies) (Gotzek et al. 2015). The loss of genetic diversity can lead to severe consequences on the colony growth of invasive ants as shown for *S. invicta* and *S. geminata* (Ross and Fletcher 1986, Lenancker et al. 2019). Genetic bottleneck can lead to the loss of CSD (complementary sex determination) alleles which disrupts the functioning of the sex determination system (Ross et al. 1993). Generally in the

Hymenoptera, when a queen and the male she mated with share the same CSD genotype, half of the queen's offspring resulting from this mating will develop into diploid males instead of workers (Crozier 1971, 1977, Heimpel and de Boer 2008). Adult diploid males do not contribute to the reproductive output of the colony or its workload (Ross and Fletcher 1986, Cook and Crozier 1995). In *S. geminata* and *S. invicta* diploid male production can reduce colony growth and survival (Ross and Fletcher 1986, Cook and Crozier 1995, Lenancker et al. 2019). Diploid male production is common in Australian *S. geminata* populations but strategies during colony founding (pleometrosis, execution of diploid male larvae) can minimize its effects during colony founding (Lenancker et al. 2019). Another invasive ant, *Brachyponera chinensis*, was found to be pre-adapted to potential inbreeding depression following invasion (Eyer et al. 2018). Both native and invasive populations have low genetic diversity because *B. chinensis* queens preferably mate with their brothers. Sibmating in the native range may reduce the potential cost of inbreeding for *B. chinensis* by purging deleterious alleles prior to invasion (Eyer et al. 2018).

Despite the small genetic diversity of *S. geminata* in its invasive range, we were able to analyse a total of 3,834 SNPs giving us unprecedented power to identify geneflow events in invasive *S. geminata* populations. We found evidence of multiple geneflow events between and among virtually all our sampled invasive populations. These populations were geographically separated, suggesting that human-mediated transport has been the main mode of dispersal of *S. geminata* across Indo-Pacific countries and islands. In Gotzek et al. (2015), all the colonies collected in the Indo-Pacific and Australia were assigned to a single cluster. Therefore, it was not possible to determine whether geneflow events took place among these populations. In the long term, connection among invasive populations and multiple introductions can increase the genetic diversity in invasive populations and reduce the effects of inbreeding (Dlugosch and Parker 2008a). Continuous geneflow among invasive *S. geminata* populations may lead to an increase in the diversity of CSD alleles and decrease the occurrence of diploid male production in some populations over a long period of time, potentially increasing their colony growth and spread.

The use of ddRADseq also enabled us to determine the origin and invasion history of this ant and provide strong support to conclusions from Gotzek et al. (2015) as well as reveal the common history of Australian populations. The analysis of such a high number of SNPs constitutes a significant improvement to the 45 microsatellite markers and one *mtCOI* 646bp portion used to investigate the global invasion of *S. geminata* previously (Gotzek et al. 2015). Our study

provides strong evidence that Acapulco in southwestern Mexico was the primary origin and the Philippines the secondary origin of the Indo-Pacific populations of *S. geminata.* These results support the hypothesis from Gotzek et al. (2015) that *S. geminata* was introduced to tropical Asia by Spanish galleons departing every year from Acapulco to Manila (Philippines) and Canton (China) from the 16th to the 18th century (Flynn and Giráldez 1995, Bjork 1998, Fradera 2004). This transoceanic route and trade connection between Manila and the rest of tropical Asia provided the opportunity for *S. geminata* to colonize the Old World, probably transported in the rock, sand and soil from the ballast (Carlton 1999, Gotzek et al. 2015). Ship trade is probably responsible for dispersing many global invaders prior to the 1800s as ships provided many opportunities for different organisms to be transported among continents (e.g., gastropods in rock ballast, bivalves on the ship's hull; Carlton 1999). Early records show that some ant species were established globally by the 19th century (e.g., *A. gracilipes*, Wetterer 2005; *Paratrechina longicornis*, Wetterer 2008; *Monomorium pharaonis*, Wetterer 2010; *P. megacephala*, Wetterer 2012). It is highly likely that other species originating from the New World could have been dispersed along the same route as *S. geminata* considering that filling ballast with soil was common until the early 20th century and that ants could also have been transported in goods and inside the ship's framework (Carlton 1999, Gotzek et al. 2015). Our results also indicate that the invasion of Australian islands (i.e. Christmas island, Tiwi islands and Ashmore Reef) probably originated from mainland Australia. Previous analyses were unable to reveal invasion history at such a fine scale and could only determine that Australia and Christmas Island belonged to the same cluster (Gotzek et al. 2015). Interestingly, specimens collected from Ashmore Reef represent two clusters in the ADMIXTURE analysis suggesting that *S. geminata* was introduced to Ashmore Reef from mainland Australia on more than one occasion.

We found that all colonies in the invasive range were polygyne suggesting that polygyny plays an important role in the invasion success and dispersal of *S. geminata*. Polygyny can increase colony growth rate and the establishment potential of transported colony fragments which are more likely to contain a queen (Vargo and Fletcher 1989, Tsutsui and Suarez 2003). Polygyne populations of invasive ants can also reach higher densities than monogyne ones, which contributes to their ability to dominate ecosystems (Macom and Porter 1996, Holway et al. 2002a). For example, in Mexico, polygyne *S. geminata* nest density was estimated to be more than 40 times higher than nest density of an adjacent monogyne population (Mackay et al. 1990).

In southeast China, most *S. invicta* colonies situated at isolated infestation sites were polygyne, which suggests that this social form plays an important role in the successful establishment and range expansion of this ant to new areas (Yang et al. 2012). The effective mean number of queens from our polygyne colonies was low (1.1 to 40 queens depending on the r_{ml} values). Likewise, colonies from the Galapagos Islands and Costa Rica were estimated to have an average of 3.7 queens per colony based on worker relatedness values (Wauters et al. 2018). The average (± SD) number of queens (7±2.5) excavated from nests situated in Mexico was higher (Mackay et al. 1990). In Costa Rica, colonies were estimated to contain 11 to 293 queens based on the caste composition of three 500g colony samples extrapolated to the total mass of the colony (Trible et al. 2018). Such variations in queen number between excavations and estimations could be due to the presence of non-egg laying queens in the colonies, which represented 8% of the excavated queens in Mexico (Mackay et al. 1990). A recent study has revealed that in a *S. geminata* population in Florida, most queens in polygyne colonies are produced asexually and workers sexually, whereas both castes are produced sexually in monogyne colonies (Lacy et al. 2019). Additional investigations into the reproductive systems of *S. geminata* will reveal whether this mixed reproductive system associated with colony social structure is present in other populations and its role in the invasive success of *S. geminata*.

The use of ddRADseq enabled us to use more than 3,500 SNPs to look at the invasion history, introduction pathway and social structure of *S. geminata*. This technique is ideal for small taxa as samples with as little as 50ng of DNA passed the filtering steps with a high success rate (85%). High-throughput sequencing methods are a considerable improvement from microsatellite and mtDNA markers which to-date have been the preferred methods to study the population genetics of global ant invaders (Ascunce et al. 2011, Peterson et al. 2012, Nygaard and Wurm 2015). Future studies using high throughput sequencing to determine the origin, invasion history and geneflow between populations of global ant invaders will certainly improve our understanding of invasion processes and shed light on their main invasion pathways.

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Supplementary information

Table S3.1 List of samples, sample type (single sample or multiple individuals from a colony), collection locality and coordinates.

Specimen Code	Sample type	Colony code	Country	State/County	Population	Latitude	Longitude
AVS2433	Individual	na	Mexico	Oaxaca	Mexico	17.070753	-96.726595
B1.1	Colony	B1	Australia	Northern Territory	Mainland Australia	-12.572264	131.084244
B1.2	Colony	B1	Australia	Northern Territory	Mainland Australia	-12.572264	131.084244
B1.3	Colony	B1	Australia	Northern Territory	Mainland Australia	-12.572264	131.084244
B1.4	Colony	B1	Australia	Northern Territory	Mainland Australia	-12.572264	131.084244
B1.6	Colony	B1	Australia	Northern Territory	Mainland Australia	-12.572264	131.084244
B1.7	Colony	B1	Australia	Northern Territory	Mainland Australia	-12.572264	131.084244
B1.8	Colony	B1	Australia	Northern Territory	Mainland Australia	-12.572264	131.084244
Col.1	Individual	na	Mexico	Chiapas	Mexico	14.983333	-92.166667
DAG561.10	Colony	DAG561	Thailand	Krabi	Thailand	8.012769	98.842447
DAG561.2	Colony	DAG561	Thailand	Krabi	Thailand	8.012769	98.842447
DAG561.3	Colony	DAG561	Thailand	Krabi	Thailand	8.012769	98.842447
DAG561.4	Colony	DAG561	Thailand	Krabi	Thailand	8.012769	98.842447
DAG561.8	Colony	DAG561	Thailand	Krabi	Thailand	8.012769	98.842447
DAG582.1	Colony	DAG582	Malaysia	Negeri Sembilan	Malaysia	2.491341	101.841248
DAG582.10	Colony	DAG582	Malaysia	Negeri Sembilan	Malaysia	2.491341	101.841248
DAG582.2	Colony	DAG582	Malaysia	Negeri Sembilan	Malaysia	2.491341	101.841248
DAG582.4	Colony	DAG582	Malaysia	Negeri Sembilan	Malaysia	2.491341	101.841248
DAG582.5	Colony	DAG582	Malaysia	Negeri Sembilan	Malaysia	2.491341	101.841248
DAG582.6	Colony	DAG582	Malaysia	Negeri Sembilan	Malaysia	2.491341	101.841248
DAG618.1	Colony	DAG618	Cambodia	Ratanakiri	Cambodia	13.97623	106.81185
DAG618.2	Colony	DAG618	Cambodia	Ratanakiri	Cambodia	13.97623	106.81185
DAG618.3	Colony	DAG618	Cambodia	Ratanakiri	Cambodia	13.97623	106.81185
DAG618.5	Colony	DAG618	Cambodia	Ratanakiri	Cambodia	13.97623	106.81185

DAG618.6	Colony	DAG618	Cambodia	Ratanakiri	Cambodia	13.97623	106.81185
DAG618.7	Colony	DAG618	Cambodia	Ratanakiri	Cambodia	13.97623	106.81185
DAG618.8	Colony	DAG618	Cambodia	Ratanakiri	Cambodia	13.97623	106.81185
DAG621.10	Colony	DAG621	Cambodia	Stung Treng	Cambodia	13.52879	105.97082
DAG621.12	Colony	DAG621	Cambodia	Stung Treng	Cambodia	13.52879	105.97082
DAG621.2	Colony	DAG621	Cambodia	Stung Treng	Cambodia	13.52879	105.97082
DAG621.3	Colony	DAG621	Cambodia	Stung Treng	Cambodia	13.52879	105.97082
DAG621.4	Colony	DAG621	Cambodia	Stung Treng	Cambodia	13.52879	105.97082
DAG621.7	Colony	DAG621	Cambodia	Stung Treng	Cambodia	13.52879	105.97082
DAG621.8	Colony	DAG621	Cambodia	Stung Treng	Cambodia	13.52879	105.97082
DAG622.12	Colony	DAG622	Cambodia	Kampot	Cambodia	10.63199	104.17026
DAG622.3	Colony	DAG622	Cambodia	Kampot	Cambodia	10.63199	104.17026
DAG622.4	Colony	DAG622	Cambodia	Kampot	Cambodia	10.63199	104.17026
DAG622.6	Colony	DAG622	Cambodia	Kampot	Cambodia	10.63199	104.17026
DAG622.7	Colony	DAG622	Cambodia	Kampot	Cambodia	10.63199	104.17026
DAG623.1	Colony	DAG623	Cambodia	Stung Treng	Cambodia	13.28907	105.92434
DAG623.10	Colony	DAG623	Cambodia	Stung Treng	Cambodia	13.28907	105.92434
DAG623.11	Colony	DAG623	Cambodia	Stung Treng	Cambodia	13.28907	105.92434
DAG623.12	Colony	DAG623	Cambodia	Stung Treng	Cambodia	13.28907	105.92434
DAG623.3	Colony	DAG623	Cambodia	Stung Treng	Cambodia	13.28907	105.92434
DAG623.7	Colony	DAG623	Cambodia	Stung Treng	Cambodia	13.28907	105.92434
DAG623.9	Colony	DAG623	Cambodia	Stung Treng	Cambodia	13.28907	105.92434
DAG637.1	Colony	DAG637	Cambodia	Kampong Speu	Cambodia	11.724286	104.055783
DAG637.3	Colony	DAG637	Cambodia	Kampong Speu	Cambodia	11.724286	104.055783
DAG637.4	Colony	DAG637	Cambodia	Kampong Speu	Cambodia	11.724286	104.055783
DAG637.5	Colony	DAG637	Cambodia	Kampong Speu	Cambodia	11.724286	104.055783
DAG637.6	Colony	DAG637	Cambodia	Kampong Speu	Cambodia	11.724286	104.055783
DAG637.7	Colony	DAG637	Cambodia	Kampong Speu	Cambodia	11.724286	104.055783
DAG637.8	Colony	DAG637	Cambodia	Kampong Speu	Cambodia	11.724286	104.055783
DAG637.9	Colony	DAG637	Cambodia	Kampong Speu	Cambodia	11.724286	104.055783
E5A.10	Colony	E5A	Australia	Ashmore Reef	Ashmore Reef	-12.261432	123.096493
E5A.12	Colony	E5A	Australia	Ashmore Reef	Ashmore Reef	-12.261432	123.096493
E5A.13	Colony	E5A	Australia	Ashmore Reef	Ashmore Reef	-12.261432	123.096493

E5A.4	Colony	E5A	Australia	Ashmore Reef	Ashmore Reef	-12.261432	123.096493
E5A.5	Colony	E5A	Australia	Ashmore Reef	Ashmore Reef	-12.261432	123.096493
E5A.6	Colony	E5A	Australia	Ashmore Reef	Ashmore Reef	-12.261432	123.096493
E5A.8	Colony	E5A	Australia	Ashmore Reef	Ashmore Reef	-12.261432	123.096493
EF1.16PL	Individual	na	Australia	Northern Territory	Mainland Australia	-13.800463	131.343937
FC1.1	Colony	FC1	Mexico	Jalisco	Mexico	20.596667	-105.231111
FC1.2	Colony	FC1	Mexico	Jalisco	Mexico	20.596667	-105.231111
FC1.3	Colony	FC1	Mexico	Jalisco	Mexico	20.596667	-105.231111
FC1.4	Colony	FC1	Mexico	Jalisco	Mexico	20.596667	-105.231111
FC1.5	Colony	FC1	Mexico	Jalisco	Mexico	20.596667	-105.231111
FC1.7	Colony	FC1	Mexico	Jalisco	Mexico	20.596667	-105.231111
FC1.8	Colony	FC1	Mexico	Jalisco	Mexico	20.596667	-105.231111
HB-gem.10	Colony	HB-gem	India	Karnataka	India	12.41525	76.596059
HB-gem.2	Colony	HB-gem	India	Karnataka	India	12.41525	76.596059
HB-gem.3	Colony	HB-gem	India	Karnataka	India	12.41525	76.596059
HB-gem.4	Colony	HB-gem	India	Karnataka	India	12.41525	76.596059
HB-gem.5	Colony	HB-gem	India	Karnataka	India	12.41525	76.596059
HB-gem.6	Colony	HB-gem	India	Karnataka	India	12.41525	76.596059
HB-gem.7	Colony	HB-gem	India	Karnataka	India	12.41525	76.596059
HB-gem.9	Colony	HB-gem	India	Karnataka	India	12.41525	76.596059
HDA7.5	Colony	HDA	USA	Hawaii	Hawaii	20.883898	-156.476422
HDA7.6	Colony	HDA	USA	Hawaii	Hawaii	20.883898	-156.476422
HDA7.8	Colony	HDA	USA	Hawaii	Hawaii	20.883898	-156.476422
IRRI-700.10	Colony	IRRI-700	Philippines	Laguna	Philippines	14.170267	121.258393
IRRI-700.11	Colony	IRRI-700	Philippines	Laguna	Philippines	14.170267	121.258393
IRRI-700.2	Colony	IRRI-700	Philippines	Laguna	Philippines	14.170267	121.258393
IRRI-700.3	Colony	IRRI-700	Philippines	Laguna	Philippines	14.170267	121.258393
IRRI-700.4	Colony	IRRI-700	Philippines	Laguna	Philippines	14.170267	121.258393
IRRI-700.5	Colony	IRRI-700	Philippines	Laguna	Philippines	14.170267	121.258393
IRRI-700.7	Colony	IRRI-700	Philippines	Laguna	Philippines	14.170267	121.258393
IRRI-700.8	Colony	IRRI-700	Philippines	Laguna	Philippines	14.170267	121.258393
JDA264	Individual	na	Mexico	Guerrero	Mexico	17.953889	-99.439444
M1A.2	Colony	M1A	Australia	Ashmore Reef	Ashmore Reef	-12.264966	123.033136
M1A.3	Colony	M1A	Australia	Ashmore Reef	Ashmore Reef	-12.264966	123.033136
M1A.4	Colony	M1A	Australia	Ashmore Reef	Ashmore Reef	-12.264966	123.033136
M1A.5	Colony	M1A	Australia	Ashmore Reef	Ashmore Reef	-12.264966	123.033136

M1A.6	Colony	M1A	Australia	Ashmore Reef	Ashmore Reef	-12.264966	123.033136
M1A.8	Colony	M1A	Australia	Ashmore Reef	Ashmore Reef	-12.264966	123.033136
M1A.9	Colony	M1A	Australia	Ashmore Reef	Ashmore Reef	-12.264966	123.033136
Phil1.1	Colony	Phil1	Philippines	Mindanao	Philippines	6.6905556	125.355
Phil1.2	Colony	Phil1	Philippines	Mindanao	Philippines	6.6905556	125.355
Phil1.3	Colony	Phil1	Philippines	Mindanao	Philippines	6.6905556	125.355
Phil1.4	Colony	Phil1	Philippines	Mindanao	Philippines	6.6905556	125.355
Phil1.5	Colony	Phil1	Philippines	Mindanao	Philippines	6.6905556	125.355
Phil1.6	Colony	Phil1	Philippines	Mindanao	Philippines	6.6905556	125.355
Phil1.8	Colony	Phil1	Philippines	Mindanao	Philippines	6.6905556	125.355
Phil1.9	Colony	Phil1	Philippines	Mindanao	Philippines	6.6905556	125.355
Phil2.1	Colony	Phil2	Philippines	Mindanao	Philippines	6.2555556	125.0908333
Phil2.10	Colony	Phil2	Philippines	Mindanao	Philippines	6.2555556	125.0908333
Phil2.12	Colony	Phil2	Philippines	Mindanao	Philippines	6.2555556	125.0908333
Phil2.3	Colony	Phil2	Philippines	Mindanao	Philippines	6.2555556	125.0908333
Phil2.5	Colony	Phil2	Philippines	Mindanao	Philippines	6.2555556	125.0908333
Phil2.6	Colony	Phil2	Philippines	Mindanao	Philippines	6.2555556	125.0908333
Phil2.8	Colony	Phil2	Philippines	Mindanao	Philippines	6.2555556	125.0908333
Phil2.9	Colony	Phil2	Philippines	Mindanao	Philippines	6.2555556	125.0908333
Pink_House.1	Colony	Pink_House	Australia	Christmas Island	Christmas Island	-10.502	105.652
Pink_House.2	Colony	Pink_House	Australia	Christmas Island	Christmas Island	-10.502	105.652
Pink_House.3	Colony	Pink_House	Australia	Christmas Island	Christmas Island	-10.502	105.652
Pink_House.4	Colony	Pink_House	Australia	Christmas Island	Christmas Island	-10.502	105.652
Pink_House.6	Colony	Pink_House	Australia	Christmas Island	Christmas Island	-10.502	105.652
Pink_House.7	Colony	Pink_House	Australia	Christmas Island	Christmas Island	-10.502	105.652
Pink_House.8	Colony	Pink_House	Australia	Christmas Island	Christmas Island	-10.502	105.652
RC800.SOL.1	Individual	na	USA	Guam	Guam	13.504801	144.878596
RC803.SOL.1	Individual	na	Micronesia	Chuuk	Micronesia	7.447917	151.883833
SCCY14-07.1	Colony	SCCY14-07	Malaysia	Penang or Kuala Lumpur	Malaysia	4.238372	101.059778
SCCY14-07.2	Colony	SCCY14-07	Malaysia	Penang or Kuala Lumpur	Malaysia	4.238372	101.059778

SCCY14-07.3	Colony	SCCY14-07	Malaysia	Penang or Kuala Lumpur	Malaysia	4.238372	101.059778
SCCY14-07.4	Colony	SCCY14-07	Malaysia	Penang or Kuala Lumpur	Malaysia	4.238372	101.059778
SCCY14-07.6	Colony	SCCY14-07	Malaysia	Penang or Kuala Lumpur	Malaysia	4.238372	101.059778
SCCY14-07.7	Colony	SCCY14-07	Malaysia	Penang or Kuala Lumpur	Malaysia	4.238372	101.059778
SCCY14-07.8	Colony	SCCY14-07	Malaysia	Penang or Kuala Lumpur	Malaysia	4.238372	101.059778
SCCY14-07.9	Colony	SCCY14-07	Malaysia	Penang or Kuala Lumpur	Malaysia	4.238372	101.059778
SH1.10	Colony	SH1	Thailand	Pathum Thani	Thailand	14.036219	100.734414
SH1.11	Colony	SH1	Thailand	Pathum Thani	Thailand	14.036219	100.734414
SH1.5	Colony	SH1	Thailand	Pathum Thani	Thailand	14.036219	100.734414
SH1.7	Colony	SH1	Thailand	Pathum Thani	Thailand	14.036219	100.734414
SH1.8	Colony	SH1	Thailand	Pathum Thani	Thailand	14.036219	100.734414
SH1.9	Colony	SH1	Thailand	Pathum Thani	Thailand	14.036219	100.734414
Temple_Road.10	Colony	Temple_Road	Australia	Christmas Island	Christmas Island	-10.492	105.647
Temple_Road.2	Colony	Temple_Road	Australia	Christmas Island	Christmas Island	-10.492	105.647
Temple_Road.4	Colony	Temple_Road	Australia	Christmas Island	Christmas Island	-10.492	105.647
Temple_Road.5	Colony	Temple_Road	Australia	Christmas Island	Christmas Island	-10.492	105.647
Temple_Road.6	Colony	Temple_Road	Australia	Christmas Island	Christmas Island	-10.492	105.647
Temple_Road.7	Colony	Temple_Road	Australia	Christmas Island	Christmas Island	-10.492	105.647
Temple_Road.8	Colony	Temple_Road	Australia	Christmas Island	Christmas Island	-10.492	105.647
Temple_Road.9	Colony	Temple_Road	Australia	Christmas Island	Christmas Island	-10.492	105.647
Ti3.11	Colony	Ti3	Australia	Tiwi Islands	Tiwi Islands	-11.417832	130.672318

Ti3.12	Colony	Ti3	Australia	Tiwi Islands	Tiwi Islands	-11.417832	130.672318
Ti3.13	Colony	Ti3	Australia	Tiwi Islands	Tiwi Islands	-11.417832	130.672318
Ti3.15	Colony	Ti3	Australia	Tiwi Islands	Tiwi Islands	-11.417832	130.672318
Ti3.16	Colony	Ti3	Australia	Tiwi Islands	Tiwi Islands	-11.417832	130.672318
Ti3.17	Colony	Ti3	Australia	Tiwi Islands	Tiwi Islands	-11.417832	130.672318
Ti3a	Colony	Ti3	Australia	Tiwi Islands	Tiwi Islands	-11.417832	130.672318
W6A.1	Colony	W6A	Australia	Ashmore Reef	Ashmore Reef	-12.241836	122.966004
W6A.3	Colony	W6A	Australia	Ashmore Reef	Ashmore Reef	-12.241836	122.966004
W6A.4	Colony	W6A	Australia	Ashmore Reef	Ashmore Reef	-12.241836	122.966004
W6A.6	Colony	W6A	Australia	Ashmore Reef	Ashmore Reef	-12.241836	122.966004
W6A.7	Colony	W6A	Australia	Ashmore Reef	Ashmore Reef	-12.241836	122.966004
W6A.8	Colony	W6A	Australia	Ashmore Reef	Ashmore Reef	-12.241836	122.966004
W6A.9	Colony	W6A	Australia	Ashmore Reef	Ashmore Reef	-12.241836	122.966004

Table S3.2 Oligo sequence of primer pairs for PCR reaction

P1	AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC G
P2	CAA GCA GAA GAC GGC ATA CGA GAT CGT GAT GTG ACT GGA GTT CAG ACG TGT GC

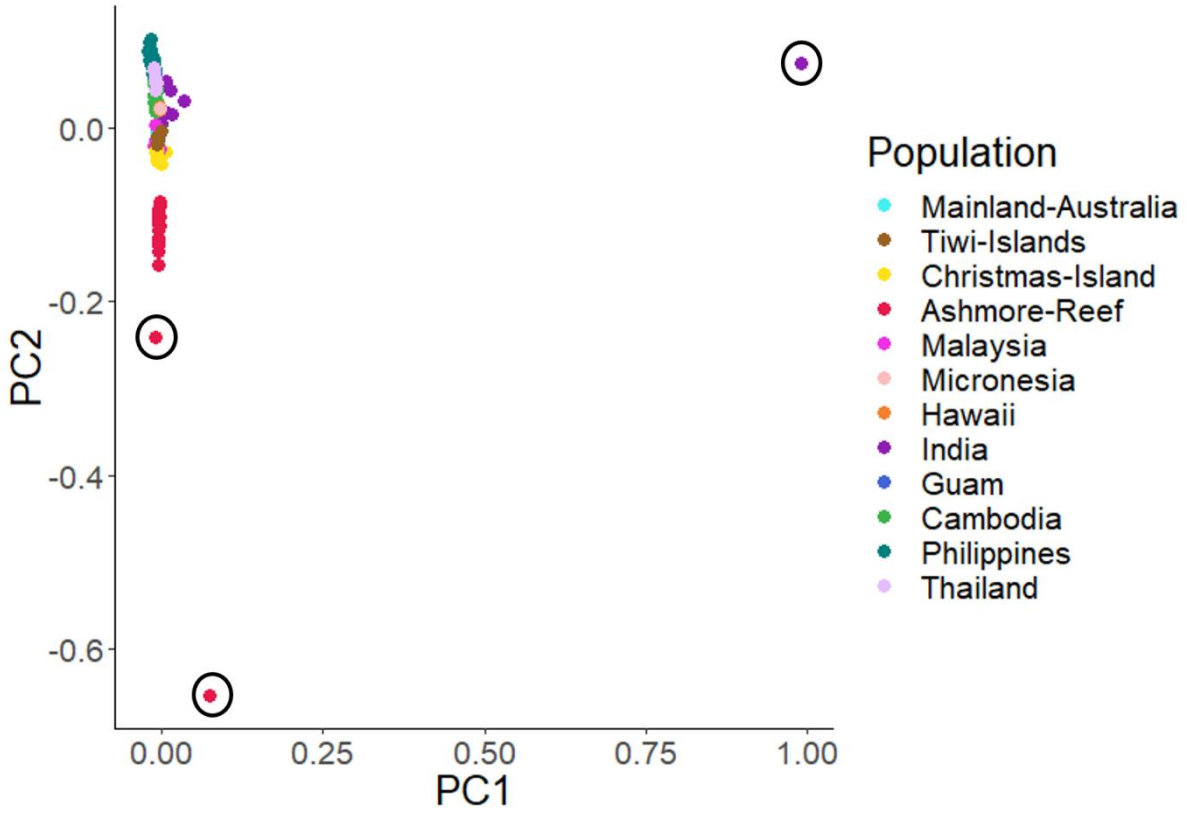

Figure S3.1 Principal component map of the SNPs in 147 individual specimens from the invasive range of *S. geminata*. We removed the circled specimens from the subsequent principal component map (Figure 3.5) to observe how the other invasive specimens clustered together.

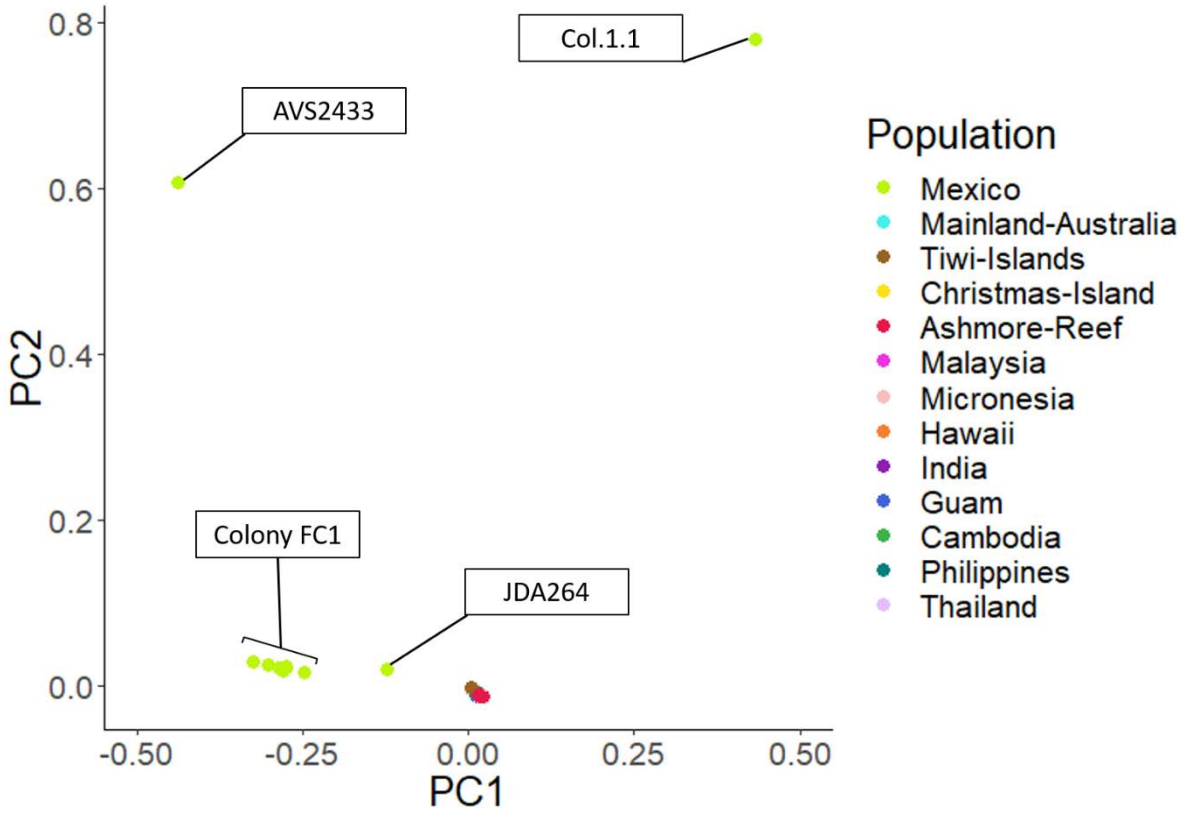

Figure S3.2 Principal component map of the SNPs in 157 individual specimens. The specimens originating from Mexico are labelled with their individual code or their colony code. See Figure S3.3 for a map showing the origin of the Mexican specimens.

Table S3.3 Number of loci for each colony in which multiple nestmates were collected, estimates of average relatedness of nestmate workers (Wang's r), effective number of queens ($\boldsymbol{N_{qe}}$) for two $\boldsymbol{r_{ml}}$ (average relatedness of the mates of nestmate queens) values.

Origin	Colony code	SNPs/loci	Wang's r ±SD	N_{qe}	
				r_{ml}=0	r_{ml}=0.5
Mexico	FC1	461/922	0.29±0.25	2.6	14.1
Mainland Australia	B1	54/108	0.68±0.12	1.1	1.2
Tiwi Islands	Ti3	79/158	0.57±0.18	1.3	1.6
Christmas Island	Pink_House	202/404	0.68±0.08	1.1	1.2
Christmas Island	Temple_Road	169/338	0.62±0.08	1.2	1.3
Ashmore Reef	E5A	164/396	0.54±0.15	1.4	1.7
Ashmore Reef	M1A	298/396	0.46±0.18	1.6	2.4
Ashmore Reef	W6A	241/482	0.53±0.08	1.4	2.8
Malaysia	DAG582	178/356	0.61±0.08	1.2	1.4
Malaysia	SCCY14-07	65/130	0.68±0.11	1.1	1.2
Hawai'i	HDA	198/396	0.26±0.13	2.9	40
India	HB-gem	60/120	0.41±0.11	1.8	3.2
Cambodia	DAG623	126/251	0.58±0.1	1.3	1.5
Cambodia	DAG618	263/526	0.62±0.08	1.2	1.4

Cambodia	DAG621	151/302	0.65±0.1	1.2	1.3
Cambodia	DAG622	207/414	0.55±0.08	1.4	1.6
Cambodia	DAG637	42/84	0.51±0.11	1.5	1.9
Philippines	IRRI-700	187/374	0.52±0.11	1.4	1.8
Philippines	Phil1	194/388	0.53±0.18	1.4	1.8
Philippines	Phil2	213/426	0.61±0.04	1.2	1.4
Thailand	DAG561	329/657	0.54±0.07	1.4	1.7
Thailand	SH1	167/334	0.53±0.16	1.4	1.8

Figure S3.3 Map of the Mexican's sample origin with the sample codes. Circle: single specimen. Triangle: multiple specimens from a colony.

Chapter 4 Origin, behaviour, and genetics of reproductive workers in the invasive yellow crazy ant *Anoplolepis gracilipes*

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Statement of contribution of others:

PL, LL and HF conceived the study. PY, BH, and WT assisted with the study design. PL, AS, MG and AH carried out the field and laboratory work. PL conducted the data analysis and drafted the manuscript. LL, HF, PY, BH, and WT assisted with the data analysis. All authors edited the manuscript and LL coordinated the study. See acknowledgments for other contributions.

Abstract

Worker reproduction has an important influence on the social cohesion and efficiency of social insect colonies, but its role in the success of invasive ants has been overlooked. The production of males by workers of the invasive yellow crazy ant (*Anoplolepis gracilipes*) was recently reported for the first time. We used lab experiments and genetic analyses to investigate the conditions for worker reproduction and its potential cost on colony productivity and defence. Our laboratory experiment and ovary dissections showed that queen removal triggered an increase in the proportion of physogastric workers which have a higher reproductive potential than normal workers. We observed 35.8% of queenless colonies (N=24/67) producing males about five months after becoming queenless. Worker reproduction may generate a cost on colony defence and productivity as physogastric workers were less aggressive during interspecific aggression tests and foraged less than normal workers. The head width, Weber's length and wing length of worker-produced males were 2.6 to 4.8% greater on average than those of males from queenright colonies. Our microsatellite DNA analyses indicate that heterozygous males were present in queenless and queenright colonies which suggests the presence of diploid males and/or sex mosaics in *A. gracilipes*. Further research is necessary to resolve uncertainties in the reproductive mode of *A. gracilipes* and determine whether it contributes to its success as an invasive species.

Introduction

In social Hymenoptera, a caste of less reproductive individuals (i.e. workers) contributes to colony labour while fecund individuals (i.e. queens) produce offspring, but workers sometimes challenge the reproductive primacy of the queen by producing male-destined eggs (Wilson 1971, Bourke 1988). In most species, including *Apis* honeybees, Meliponinae stingless bees, Vespinae wasps, *Bombus* bumblebees, and most ants, workers possess ovaries but cannot mate (Bourke 1988). Through the haplodiploid sex determination system of Hymenoptera, in which females (i.e. workers and queens) are diploid and originate from fertilized eggs while males are haploid and originate from unfertilised eggs (arrhenotoky), these workers can produce male-destined eggs (Crozier 1977, Heimpel and de Boer 2008).

Despite worker reproduction not being beneficial to social Hymenoptera queens, a review found that worker reproduction accounted for 0-100% of the males produced in queenright colonies in

90 species for which workers had functional ovaries (Hamilton 1964, Wenseleers and Ratnieks 2006). According to kin selection theory, worker reproduction is beneficial at the worker level because workers are more related to their own sons (degree of relatedness, r=0.5) than to their brothers (i.e. queen's sons, r=0.25) (Hamilton 1964). However, in the case of queens mating multiple times, workers are more related to their brothers (r=0.25) than to other workers' sons (r<0.25) which favours workers to police eggs laid by other workers (Wenseleers et al. 2004, Ratnieks et al. 2006). The queen should always prefer to invest in her own sons which are more related to her (r=0.5) than her grandsons (r=0.25). As a result, workers are most reproductive in the absence of a queen because queen control (i.e. queen inhibition of worker fertility) can arise in queenright colonies (Bourke 1988). If the queen dies, the production of males by workers advantages both workers and queens because it is the last opportunity for the deceased queen to contribute to the gene pool. Workers from most ant species have retained functional ovaries and are able to lay male-destined eggs (Bourke 1988, Hammond and Keller 2004). Queens from several species can regulate the ovarian activity of workers with pheromones and their absence can trigger worker reproduction (Bourke 1988 e.g. *Neoponera apicalis* Dietemann and Peeters 2000, *Camponotus floridanus* Endler et al. 2004, *Lasius niger* Holman et al. 2010).

Ant workers with functional ovaries can also produce trophic eggs (unviable eggs fed to the colony) (Bourke 1988). Trophic eggs are used to transfer proteins and nutrients to members of the colony (especially queens and larvae) and can be an important source of nutrition for colony members (Hölldobler and Carlin 1989, Wheeler 1994, Dietemann and Peeters 2000, Khila and Abouheif 2008). In some species, workers switch from trophic egg to male-destined egg production in the absence of queens (Bourke 1988). For example, *Oecophylla longinoda* workers produce trophic eggs in queenright colonies and begin laying male-destined eggs one to two months after being separated from the queen (Hölldobler and Wilson 1983).

Worker reproduction has an important influence on the social cohesion and efficiency of colonies (Bourke 1988), but its potential role in the success of invasive ants has been overlooked. The production of males by workers can be costly and disrupt the social organisation of the colony (Heinze 2008). Colony productivity may decrease due to workers laying male-destined eggs instead of contributing to colony labour (Cole 1986, Gobin et al. 2003). For example, worker reproduction led to a 15% reduction in time spent on brood care for queenless colonies of *Temnothorax allardycei* (Cole 1986). For orphaned ant colonies, the fitness benefit of

contributing to the gene pool through worker-produced males could outweigh the costs of worker reproduction to colony labour and brood care. In the case of non-invasive ants, the study of worker reproduction has long been underrated, because it is often associated with queen death or colony decline (Bourke 1988). Studying what happens when the queen dies and/or the colony declines is particularly relevant to the study of invasive ants to understand the potential effects of control treatments on worker reproduction and colony efficiency.

Worker reproduction was recently reported for the first time in the yellow crazy ant (*Anoplolepis gracilipes*) (Lee et al. 2017), one of the world worst' invaders for which baseline information about reproduction is lacking (Holway et al. 2002a, Drescher et al. 2007, Lee et al. 2017). Workers with an unusually distended abdomen (i.e. physogastric) from queenless *A. gracilipes* colonies collected in Taiwan had ovaries that were more developed than those of other workers and laid male and trophic eggs (Lee et al. 2017). The reproductive mode of *A. gracilipes* remains unresolved, and results from several genetic studies suggest that it is unusual (Drescher et al. 2007, Gruber et al. 2012, 2013). Where the genetics of this species has been studied, workers are typically heterozygous, queens homozygous, and heterozygous males common (Borneo: Drescher et al. 2007, Christmas Island: Thomas et al. 2010, Arnhem land in Australia: Gruber et al. 2012, 2013, Taiwan: Lee et al. 2017). Heterozygous males have been suggested to be either diploid or sex mosaics (i.e. gynandromorphs) (Drescher et al. 2007, Gruber et al. 2012, 2013). We do not know whether worker reproduction contributes or represents a hindrance to the invasive success of *A. gracilipes*. *Anoplolepis gracilipes* being a highly successful invader (Holway et al. 2002a, Wetterer 2005), worker reproduction may be too rare to impose a cost on colony success or the benefits of worker reproduction (e.g. production of fertile males) may outweigh its costs (e.g. reduction in colony labour).

In Queensland (Australia), where this ant is invasive, we observed that our queenless laboratory colonies contained workers with unusually large abdomens (physogastric workers) and produced males. Following these observations, we ran a series of experiments and observations to investigate the attributes, potential triggers and costs of worker reproduction in *A. gracilipes*. We used observations and ovary dissections to determine how to objectively visually differentiate potentially reproductive from non-reproductive workers without the need for dissection. We compared the prevalence of male production in colonies with or without queens. We conducted experiments to determine whether the absence of queens triggers workers to become

reproductive and whether the contribution to colony labour and the ability to defend the colony differ between potentially reproductive and non-reproductive workers. We used microscopy and genetic analyses to determine whether the size and observed heterozygosity of males from queenless and males from queenright colonies differ and to determine whether more than one worker produces males in queenless colonies. We also genotyped queens and workers to determine whether the level of heterozygosity differs between castes.

Methods

Colony collection

Colonies of *A. gracilipes* were collected in Queensland, Australia. Nests were visually located and partially excavated to collect queens, workers and brood.

Dissections of worker's ovaries of entire colonies

In this preliminary experiment, we dissected the ovaries of all the workers from two queenright (26-29 workers per colony containing 6-8 queens) and two queenless colonies (22-64 workers per colony, queenless for 100-143 days) to objectively define physogastry and determine whether there is a link between workers' physogastry and their reproductive status (Table S4.1, Table S4.2). Colonies were reared in a 150x220x320mm (height x width x depth) box with two 50ml nesting tubes (length x diameter: 93x60mm) containing moist cotton and a 35x100x150mm (height x width x depth) piece of cardboard egg carton. The colonies were kept in a constant temperature room at 26±0.2°C (mean ± SD) and 59.5±4.4% humidity and at a 12:12h day:night regime.

The colonies were alternately fed either a mealworm or a cricket twice a week and 50% sugar water ad-libitum. We counted the number of ovarioles and yolky (i.e. opaque) oocytes and determined the presence of yellow bodies for each worker. Prior to dissecting each worker, we visually evaluated its physogastry by counting the number of exposed intersegmental membranes on its gaster (Figure 4.1). We avoided bias by keeping the dissector (PL in all cases) blind to colony status (queenless or queenright). Following the results from this preliminary experiment (see Results section for more details), we define physogastric workers as having a conspicuously enlarged gaster with two to four exposed intersegmental membranes. We used this definition of physogastric workers for the queen transfer experiment and the aggression tests.

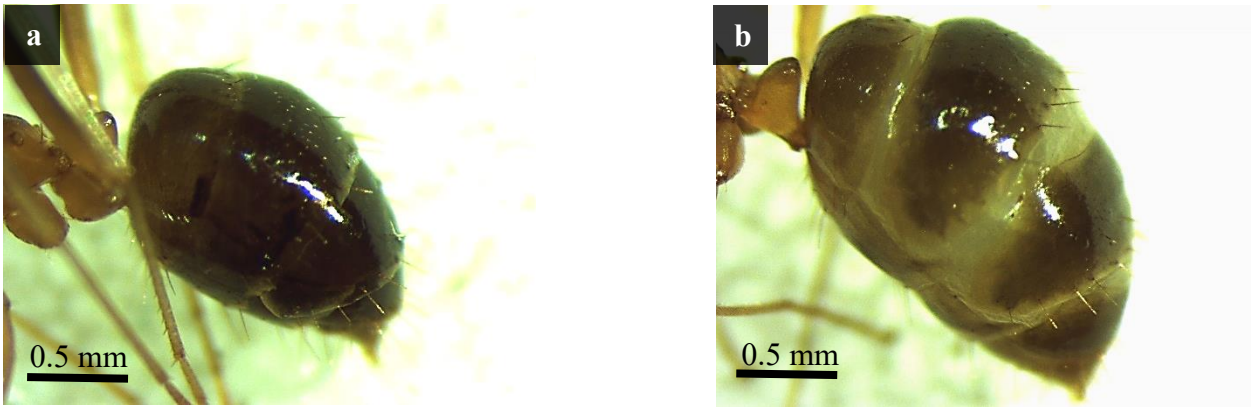

Figure 4.1 External morphology of the abdomen of (a) a normal worker and (b) a physogastric worker. Note the exposed intersegmental membranes of the physogastric worker.

Colony observations

We conducted observations from May 2016 to December 2017 on 127 colonies collected from May 2016 to July 2017 at 13 sites in Queensland (11 near Cairns, 1 near Townsville, 1 near Hervey Bay, Table S4.2) to compare male production between queenright (n=60) and queenless (n=67) colonies (Table S4.3). Queen number ranged from 1 to 35 queens per colony at the time of observations (Table S4.3). The number of workers ranged from 10 to more than 650 workers in queenright colonies and 10 to more than 550 workers in queenless colonies at the time of observations (Table S4.3). Colonies were kept in the same conditions as described in the Dissections of worker's ovaries of entire colonies section. We counted the number of males and queens and categorized the number of workers weekly (Table S4.4). To check for the presence of males, we scrutinized the nest and checked dead ant piles. Workers of *A. gracilipes* take approximately 60 days to develop at 17.5-26.2°C (Rao and Veeresh 1991). We do not know how long *A. gracilipes* males take to develop. To prevent including field-laid males in our analysis, we only included observations of males if they were observed at least 100 days from colony collection for queenright colonies and 100 days since queen's absence for queenless colonies.

Morphometry and genotyping

Measurements

The live and dead adult males that were found during the colony observations (protocol described above) were collected, placed in ethanol, and measured under a stereomicroscope. We

measured the head width, Weber's length, wing width and wing length of 37 males from 8 queenright colonies and 84 males from 14 queenless colonies. Not all the measurements could be obtained from some males which were collected dead because of missing parts (e.g. missing head). All the colonies from which the samples originated were fed the same diet and were kept in the same rearing conditions (see Dissections of worker's ovaries of entire colonies section for more details on the diet and rearing conditions).

Microsatellite analysis

We genotyped 38 males from 8 queenright colonies and 82 males from 14 queenless colonies. We also genotyped 3-7 workers from 8 of the queenright colonies, 2-17 workers from 10 of the queenless colonies, 9 queens from 5 of the queenright colonies, and 1 queen from another queenright colony. We washed each individual samples three times in distilled water to avoid contamination and extracted the DNA using Puregene DNA isolation kits (Gentra Systems) with minor modifications to the supplier's protocol (addition of 20µl of 200µg/ml Proteinase K and 2-hours water bath incubation after addition of the cell lysis solution). We resuspended the DNA pellets in 30µl DNA hydration solution and stored the DNA samples at 4°C. We used seven microsatellite markers which have been found to be polymorphic for *A. gracilipes*: Ano1, Ano3, Ano4, Ano5, Ano6, Ano8 (Feldhaar et al. 2006), and Ano9 (5′−3′ forward: TCATGCTACCCTGAAGC, reverse: CGTCTTATGGCAGTCG). We conducted the polymerase chain reaction (PCR) amplification as in Drescher et al. (2007). We used a total reaction volume of 12.5µl with approximately 10ng of template DNA (2µl), 1 × PCR-buffer (1.25µl), 160µM dNTPs (1µl), 2.5µM of each primer (0.25µl) and 0.5U of Taq DNA polymerase (0.1µl, PEQLAB GmbH). The forward primers were labelled with fluorescent IR-700 or IR-800 dye (LI-COR). The cycle parameters were as follow: initial denaturation step at 3 min at 94°C, 30 cycles comprising 40 seconds of DNA denaturing at 94˚C, specific primer annealing temperature (annealing temperature of Ano9 was 52°C, see Feldhaar et al. 2006 for Ano1-8) for 40 seconds, 40 seconds DNA extension at 72˚C, and a final extension step of 3 minutes at 72˚C. We conducted a 1:10 dilution on the PCR products and analysed them on a LI-COR 4300 DNA Analyzer.

Queen transfer experiment

Experimental design

We conducted a laboratory experiment to determine whether the absence of queens triggers workers to become physogastric and lay eggs. We evenly split workers and brood from 14 colonies between two treatments. The first treatment (n=14) contained a single queen and 121 to 200 workers depending on the size of the original colony (Table S4.5). The second treatment (n=14) also contained 121 to 200 workers but no queen. The colonies were collected from September to November 2017 at five sites in Queensland (2 sites near Cairns, one near Nome, one near Hervey Bay, and one near Brisbane, Table S4.2).

Each colony setup comprised a 150x220x320mm (height x width x depth) box with two 50ml nesting tubes (length x diameter: 93x60mm) containing moist cotton and a 35x100x150mm (height x width x depth) piece of cardboard egg carton. Colonies were kept at 23.7±0.78°C (mean ± SD) and at ambient photoperiod. They were fed one mealworm biweekly and were provided with 50% sugar water ad libitum.

Ten queens (out of 14) survived until day 60 and were moved to their corresponding queenless colony to determine whether moving the original queen back to the queenless treatment reduced the percentage of physogastric workers and stopped workers from laying eggs. When moving the queen between colonies, we recorded workers behaviour towards the queen every 10 minutes for 1 hour and recorded the queen's position within the colony box after 24h. We scored behaviour toward the queen according to Lai et al. (2015) as: 0 ignoring, 1 touching, 2 avoiding, 3 holding, 4 aggression, and 5 fighting. At day 120 for all but the six queenless colonies with the most workers, we dissected the ovaries of 15 workers selected haphazardly among all the remaining workers, or all the workers if the colony was smaller than 15 workers. We continued monitoring six queenless colonies with the most remaining workers for another 60 days to determine whether they would produce males. We checked for the presence of yellow bodies and counted the number of ovarioles and yolky oocytes of each dissected worker. To avoid bias, we kept the dissector (PL in all cases) blind to colony status (queenless or queenright).

Colony observations

We counted the number of dead workers, brood (eggs, larvae, pupae) and trophic eggs (sub-spherically shaped eggs, Lee et al. 2017) weekly, and recorded the position (outside, under the

egg carton or inside the nesting tubes) of physogastric and normal workers every two weeks to determine whether the behaviour of physogastric and normal workers differ.

At the start of the experiment and at days 30, 60, 90, and 120 we determined queen egg production by isolating each queen for 24h in a 10x50mm (diameter x length) glass vial that we left in the colony container. We closed off the vial with tulle fabric to allow communication between the queen and her workers. After 24h we counted the number of eggs laid by the queen and removed the tulle to allow her to join the workers.

Aggression tests

We conducted aggression trials between *A. gracilipes* and native green tree ant (*Oecophylla smaragdina*) workers to determine whether *A. gracilipes* worker aggression toward a competitor differed depending on physogastric state (physogastric or normal) and colony state (queenless or queenright) in a fully factorial design. *Oecophylla smaragdina* is a native dominant species that matched the competitive ability of *A. gracilipes* in aggression tests in Borneo (Drescher et al. 2011), although *A. gracilipes* displaces *O. smaragdina* in northern Australia (Lach and Hoffmann 2011). Workers from queenless *A. gracilipes* colonies originated from the six colonies we had kept monitoring for 60 days after the end of the queen transfer experiment (Table S4.3, Table S4.6). These colonies were collected around Cairns and had been queenless for three to seven months. We did not have remaining queenright colonies from the queen transfer experiment, so we used six laboratory queenright colonies also collected around Cairns at the same dates as the queenless colonies (Table S4.6) to avoid variation caused by time spent in the laboratory and colony origin. We collected *O. smaragdina* workers before each trial from a single tree on the James Cook University campus in Cairns to eliminate variation in *O. smaragdina* worker aggression due to workers originating from a different colony. We only selected minor workers that exhibited defensive behaviour (i.e. lifting their gaster to spray acid) when approached by our forceps. We let the *O. smaragdina* workers acclimatize to the lab for 10 minutes after collection. We used each *A. gracilipes* and *O. smaragdina* worker only once.

We measured aggressive interactions between three *A. gracilipes* workers that were either all physogastric or normal and a single O. *smaragdina* worker. We decided on this ratio based on a pilot experiment in which *O. smaragdina* workers always overcame *A. gracilipes* in 1:1 or 1:2 interactions. We decided on a 1:3 ratio to enable us to detect differences between the aggression

level and survival of physogastric and normal *A. gracilipes* workers. We replicated the aggression tests three times for both physogastric states (normal or physogastric) for each of the six queenless colonies and six queenright colonies except for one queenless colony in which we only found three normal workers and therefore ran only one aggression test between normal workers and one *O. smaragdina* worker for this colony. Thus, we ran 18 tests each of normal workers and physogastric workers from queenright colonies and physogastric workers from queenless colonies, and 16 tests for normal workers from queenless colonies (n=70 tests in total).

We conducted the 60-minute aggression tests in fluon-coated 60x93mm (diameter x height) PVC cylinders separated into two halves with a laminated paper card. The arenas were placed on a plastic tray that was washed with non-scented soap after each trial. We placed one *O. smaragdina* worker on one side and three *A. gracilipes* workers on the other side and let the ants acclimatize for five minutes before removing the dividing wall. For the first five minutes, we noted the highest aggression score between the two species at 30s intervals according to the method used in Lai et al. 2015 and described above. We then checked the arena every 5 minutes for the remaining 55 minutes and recorded whether *O. smaragdina* or *A. gracilipes* workers had died. At the end of the trial, we collected the three *A. gracilipes* workers (dead or alive), placed them in ethanol, and dissected their ovaries to record the presence of yellow bodies and the number of ovarioles and yolky oocytes. We avoided bias by keeping the dissector (PL in all cases) blind to colony status (queenless or queenright) and aggression test outcome.

Data analysis

We analysed our data in R version 3.5.0 (R Core Team 2018) and used functions from the stats package (R Development Core Team 2009) unless specified otherwise.

Dissections of worker's ovaries of entire colonies

We tested whether physogastric workers were more likely to have oocytes than normal workers with a binomial GLM (generalized linear model, glm function) followed by likelihood ratio tests (Anova function in the car package, Fox and Weisberg 2002) with presence and absence of mature oocytes as the response variable and colony status (queenless or queenright) and worker type (physogastric or normal) as explanatory variables. We tested whether physogastric workers were more common in queenless colonies with a binomial GLM with worker type (physogastric or normal) as the response variable and colony status as the explanatory variable.

Colony observations

We tested whether males were more common in queenless than in queenright colonies using a GLM followed by ANOVA F-test (Anova function with test=F) with presence of males as the explanatory variable and colony status as the response variable. We used a quasi-binomial error structure to account for overdispersion (Venables and Dichmont 2004).

Morphometry and genotyping

We compared the head width, Weber's length, wing width and wing length between males from queenless and queenright colonies with a linear mixed-effects model (LMM, lmer function in the lme4 package, Bates et al. 2015) for each measurement followed by Type II Wald $\chi 2$ tests (Anova function). For each of the four models we used each measurement as the response variable, the colony status of the males (queenless or queenright) as the fixed explanatory variable and the colony of origin as random variable to account for possible covariance within colonies.

Queen transfer experiment

We analysed data from replicates in which the queens survived until day 60 (10 queenright and 10 queenless colonies) separately from data obtained from replicates in which the queens survived until day 120 (3 queenright and 3 queenless colonies) because of the low queen survival rate until day 120. For colonies with queens that survived until day 60, we used a binomial GLMM (generalized linear mixed model) followed by a Type II Wald $\chi 2$ test to test whether physogastric workers became more common following queen removal (glmer function in the lme4 package, Bates et al. 2015). The response variable was the proportion of normal workers and the fixed explanatory variables were the number of days since the beginning of the experiment, the colony status (queenright or queenless) and the interaction between the number of days since the beginning of the experiment and colony status. We included colony of origin (i.e. colony which was originally split in two treatments, 10 levels) as a random variable to account for variation due to colony of origin. We added a random factor with each level corresponding to one observation to account for overdispersion.

For colony replicates with queens that survived until day 120, we used a GLM followed by an ANOVA F-test to determine whether moving the queen back to the queenless treatment at day 60 reduced the proportion of workers that were physogastric. The response and explanatory

variables were the same as the model in which queens survived until day 60. We also included colony of origin as a fixed factor. We did not include it as a random factor because it only had three levels (Bolker 2019). We used post-hoc Tukey tests to make pairwise comparisons for both models (emmeans function).

We used a GLMM followed by a Type II Wald $\chi 2$ test to test which worker type (physogastric or normal) was observed more often in the foraging area. We used presence or absence of workers in the foraging area as the response variable, worker type and colony status as fixed explanatory variables and colony of origin as a random variable to account for variation in worker behaviour between different colonies. We used a binomial GLMM followed by a Type II Wald $\chi 2$ test to test whether physogastric workers dissected at 120 days were more likely to have mature oocytes. The response variable was presence and absence of mature oocytes, the explanatory variables were colony status (queenless or queenright) and worker type (physogastric or normal) and the random variable was colony of origin.

We used a GLMM followed by a Type II Wald $\chi 2$ test to test whether the average egg production of queens changed with time since the start of the experiment. We used queen egg production at 0, 30, and 60 days as response variable, number of days since the start of the experiment as fixed explanatory variable and colony of origin as random factor. We did not include queen egg production at 90 and 120 days because only five and two queens had survived until day 90 and 120, respectively.

Aggression tests

We used a proportional odds mixed model to analyse our aggression score data (Hervé 2014, Christensen 2018). We tested whether the maximal aggression score varied between physogastric and normal workers and workers from queenless and queenright colonies, and their interaction using the clmm function (package ordinal, Christensen 2018) followed by likelihood ratio tests. The response variable was maximal aggression score, the fixed explanatory variables colony status, worker type and their interaction, and the random variable was colony of origin to account for possible variation in aggression of workers from different colonies. We used a binomial GLMM followed by a Type II Wald $\chi 2$ test to test whether *O. smaragdina* and *A. gracilipes* were less likely to fight if *A. gracilipes* workers were physogastric or from queenless colony fragments than if they were normal or from queenright colonies. The response variable was

presence or absence of a fight (presence of fight aggression score: 3-5, absence: 0-2, Lai et al. 2015), the fixed explanatory variables were colony status, worker type, and the random variable colony of origin. We also used two binomial GLMMs to test whether the survival of *O. smaragdina* and *A. gracilipes* changed with *A. gracilipes* colony status and worker type. We used a binomial GLMM followed by a Type II Wald $\chi 2$ test with survival of the *O. smaragdina* worker or survival of all three *A. gracilipes* workers as response variables. The fixed explanatory variables were colony status, worker type, and their interaction, and the random variable was colony of origin. We tested whether physogastric workers were more likely to have mature oocytes with a binomial GLMM followed by a Type II Wald $\chi 2$ test with presence and absence of mature oocytes as the response variable, colony status (queenless or queenright), and worker type (physogastric or normal) as explanatory variables, and colony of origin as a random variable.

Results

Dissections of worker's ovaries of entire colonies

Workers with two to four exposed intersegmental membranes (hereafter physogastric worker) had a conspicuously enlarged gaster that was more likely to contain yolky oocytes than workers that had zero to one exposed intersegmental membrane (hereafter normal worker). The proportion of physogastric workers was higher in queenless (mean ± SD: 78.6±10.4%) than in queenright colonies (mean ± SD: 53.9±11.6%, GLM: binomial, ANOVA: $\chi 2=14.093$, df=1, $p<0.001$). Physogastric workers were more likely to have yolky oocytes (54.9%, N=56/102) than normal workers (25.6%, N=10/39, GLM: binomial, ANOVA: $\chi 2=4.7578$, df=1, $p<0.05$). Yellow bodies were only observed in physogastric workers (10.7% in queenright colonies and 12.9% in queenless colonies). Considering that the differences between normal and physogastric workers were visually conspicuous in live workers and that these results show that these two worker categories have different reproductive potential, we categorized workers as physogastric or normal in the subsequent experiments by counting their exposed intersegmental membranes.

Colony observations

Queenless colonies were more likely to produce males than queenright colonies (male producing queenless colonies 24/67, queenright colonies 8/60, GLM quasibinomial, $\chi 2=8.12$, df=1,

p<0.01). Male-producing queenless colonies (N=24) started producing males 146±46.2 days (mean ± SD) after becoming queenless. There was no significant difference in the number of males produced by queenless (mean ± SD: 15.2±15.6) and queenright colonies (7.8±7.3, GLM quasi-Poisson F test, F=2.063, df=1, p=0.16). Queenless colonies had 10 to more than 380 workers when we started observing adult males and queenright colonies 10 to more than 330 workers.

Male morphometry and genotyping

Measurements of males from queenless colonies were 2.6 to 4.8% greater on average than those of males from queenright colonies except for wing width (Table 4.1).

Table 4.1 Measurements of males from queenless and queenright colonies (mean ± SD) and results from type II Wald tests on LMM (df=1) * p<0.05, ** p<0.01

	Head width (mm)	Weber's length (mm)	Wing width (mm)	Wing length (mm)
Queenless colonies	0.77±0.04 n=56	1.79±0.12 n=84	1.37±0.1 n=50	3.11±0.19 n=55
Queenright colonies	0.75 ±0.03 n=28	1.71±0.11 n=37	1.30±0.09 n=28	2.96±0.2 n=28
$\chi 2$	6.7248	4.7851	3.5321	7.8537
P	0.0095**	0.029*	0.06	0.005**

We found that most males from queenright and queenless colonies were homozygous, that more than one worker produced males in one of the queenless colonies, and that some males were conspicuously deformed. We found that 28.9% of queenright males (n=11/38) and 4.9% of queenless males (n=4/82) were heterozygous for at least one locus (Table S4.7). These heterozygous males were from 5 out of 8 queenright colonies and 3 out of 14 queenless colonies. We genotyped more than one male in 11 queenless colonies. We found three different alleles at Ano5 in one of these colonies (colony 0407Kearns13, Table S4.3) which indicates that more than one worker produced males in this colony. Six males from queenless colonies had conspicuous deformities: 2 out of 56 queenless males for which we measured the head width had one eye

which was oversized compared to the other eye and 4 out of 55 queenless males with wings had abnormal wings (black and stubby wings or underdeveloped wing tips). All six of these deformed workers were homozygous (or haploid). Two out of 28 queenright males for which we measured the head had one oversized eye and both were heterozygous for at least one locus.

Most workers were heterozygous for at least one locus while most queens were homozygous. We found that 95% (n=38/40) and 98.1% (n=53/54) of workers from queenright and queenless colonies were heterozygous, respectively (Table S4.8). Nine queens out of ten were homozygous at all 4-6 loci and one queen was heterozygous at Ano4 (Table S4.9).

Queen transfer experiment

The number of physogastric workers increased following queen removal (Table 4.2, Figure 4.2, Figure S4.1). We found similar proportions of physogastric workers in queenright and queenless colonies with queens that survived until 60 days from day 0 until 15 days after the start of the experiment (Figure 4.2, Table 4.2, post hoc tests $p>0.05$ between queenright and queenless colonies for 0 and 15 days since the experiment started). At day 30 and until day 60, there were more physogastric workers in queenless than in queenright colonies (Figure 4.2, Table 4.2, post hoc tests $p<0.001$ between queenright and queenless colonies for day 30, day 45 and day 60).

Workers readily accepted the six queens that were moved back to each of their corresponding queenless treatment at day 60. The queens were either ignored or touched by workers for the first hour after translocation (aggression score 0-1, Lai et al. 2015), and all six queens were tended by workers (aggression score 1) inside a nest or under the egg carton after 24 hours. Three queens survived until day 120, and there were more normal workers in the now queenright colonies than in the corresponding colonies from which the queen was removed (Figure S4.1, Table 4.2, post hoc tests $p<0.05$ between queenright and queenless colonies for day 120). At day 120, colonies that became queenless had 53 to 467 workers per colony and queenright colonies 42 to 113. Yolky oocytes were not significantly more common in physogastric workers (N=51/119) than in normal workers (N=13/52) dissected at 120 days (GLMM: binomial, Type II Wald test: $\chi 2=3.5061$, df=1, $p=0.061$). Only the ovaries of physogastric workers had yellow bodies (7.8% had yellow bodies in queenright and 8.6% in queenless colonies).

Table 4.2 Summary of generalized linear model results for each response variable in the queen transfer experiment. 'x' represents the interaction terms.

Proportion of normal workers until day 60	**GLMM binomial, n=100 observations**		
Variables	***df***	**$\chi 2$**	***p***
Colony status (queenright or queenless)	1	45.475	<0.001
Time (since the beginning of the experiment)	4	13.446	<0.01
Colony status x Time	4	14.488	<0.01
Proportion of normal workers until day 120	**GLM quasibinomial, n=54 observations**		
Variables	***df***	**F**	***p***
Colony status (queenright or queenless)	1	0.7622	0.39
Time (since the beginning of the experiment)	8	8.3808	<0.001
Colony status x Time	4.6001	8	<0.001
Colony of origin	2.7679	2	0.077

We observed a difference in behaviour between normal and physogastric workers. Normal workers were observed in the foraging area outside of the nesting tubes more often than physogastric workers regardless of whether the colony was queenright or queenless (GLMM: binomial, Type II Wald test, worker type: $\chi 2$=55.5766, df=1, p<0.001, colony status: $\chi 2$=0.6344, df=1, p=0.43). We observed normal workers outside the nests during colony monitoring 81 times (n=100) and physogastric workers only 5 times (n=100).

We isolated queens for 24h every 30 days from day 0 to determine their egg production. The average number of eggs produced over 24 hours by queens was highest at day 0 (13.6±15.7 eggs per queen, 0.14±0.52 at day 30, 5.8±10.6 at day 60, GLMM: poisson, Type II Wald test, $\chi 2$=81.458, df=2, p<0.001). Surviving queens did not produce any eggs from day 90. We did not

observe male production in any of the colonies, including the six queenless colonies we monitored for the additional 60 days (i.e. until day 180).

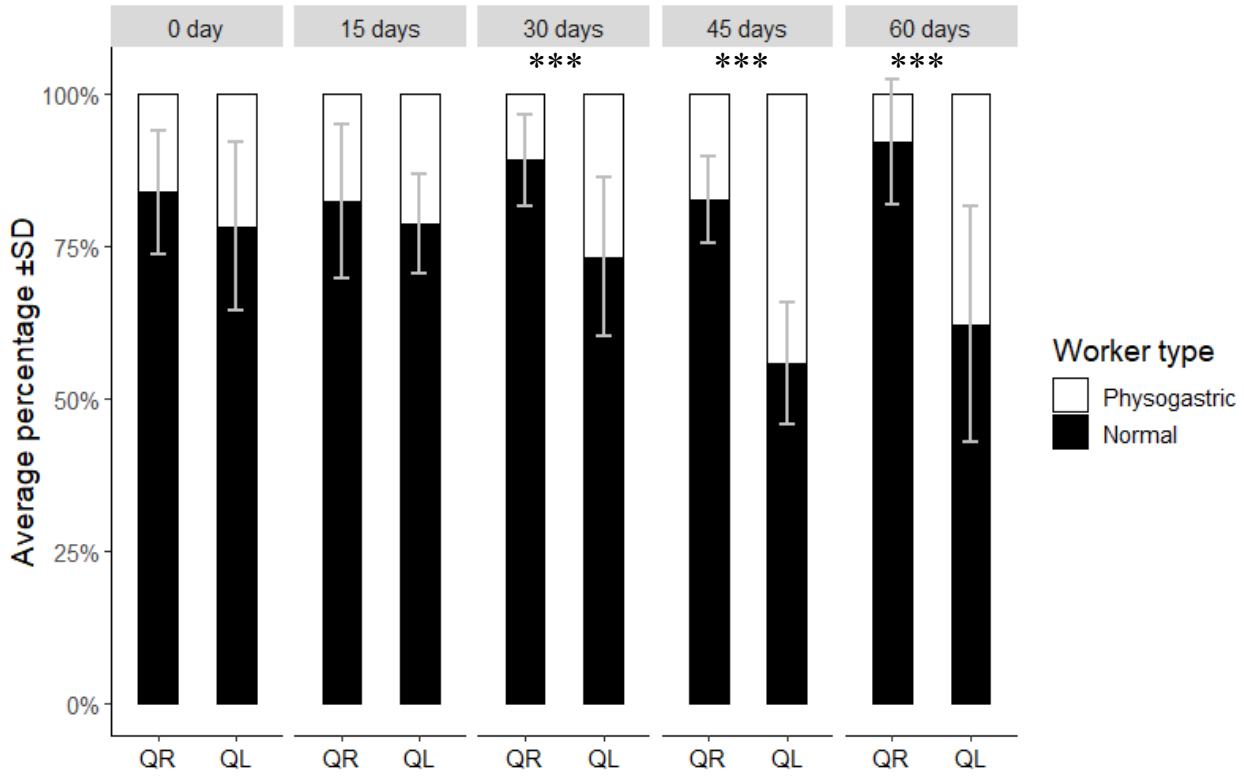

Figure 4.2 Average± SD percentage of physogastric and normal workers in the queen transfer experiment by colony status (QR=queenright, QL=queenless, N=10 for each) and number of days since the start of the experiment. *** indicates a significant difference between queenright and queenless colonies for the corresponding time (GLMM: binomial, Table 4.2, post-hoc tests ***p<0.001).

Aggression tests

Physogastric workers and workers from queenless colonies were less aggressive than normal workers and workers from queenright colonies. The highest aggression scores of trials with normal workers (median: 5, range: 0-5, N=34) was higher than the aggression score of trials with

physogastric workers (median: 4, range: 0-5, N=36) (POMM, likelihood ratio test: $\chi2=6.6341$, df=1, $p<0.05$). The highest aggression score of trials with workers from queenright colonies was also higher (median: 5, range: 0-5, N=36) than when the workers were from queenless colonies (median: 4, range: 0-5, N=34) (POMM, likelihood ratio test: $\chi2=5.8243$, df=1, $p<0.05$). *Anoplolepis gracilipes* workers were more likely to initiate the fight if they were from queenright colonies and normal (Figure 4.3, GLMM: binomial, Type II Wald test: $\chi2=6.3087$, df=1, $p<0.05$ for queenright vs queenless and $\chi2=5.7882$, df=1, $p<0.05$ for normal vs physogastric workers). *Oecophylla smaragdina* workers were more likely to die if they were fighting against workers from queenright colonies (GLMM: binomial, Type II Wald test: $\chi2=12.2473$, df=1, $p<0.001$) but physogastry did not influence the survival of *O. smaragdina* (GLMM: binomial, Type II Wald test: $\chi2=0.6466$, df=1, $p=0.42$). The survival of *A. gracilipes* was not influenced by physogastry and whether they originated from a queenless or queenright colony (GLMM: binomial, Type II Wald test: $\chi2=1.0203$, df=1, $p=0.31$ for queenright vs queenless and $\chi2=1.0203$, df=1, $p=0.31$ for normal vs physogastric workers). Physogastric workers dissected after the aggression trials were more likely to have mature oocytes (N=40/108) than normal workers (N=2/102, GLMM: binomial, Type II Wald test: $\chi2=22.7$, df=1, $p<0.001$). We only observed yellow bodies in physogastric workers (9.3% had yellow bodies in queenright colonies and 12.3% in queenless colonies).

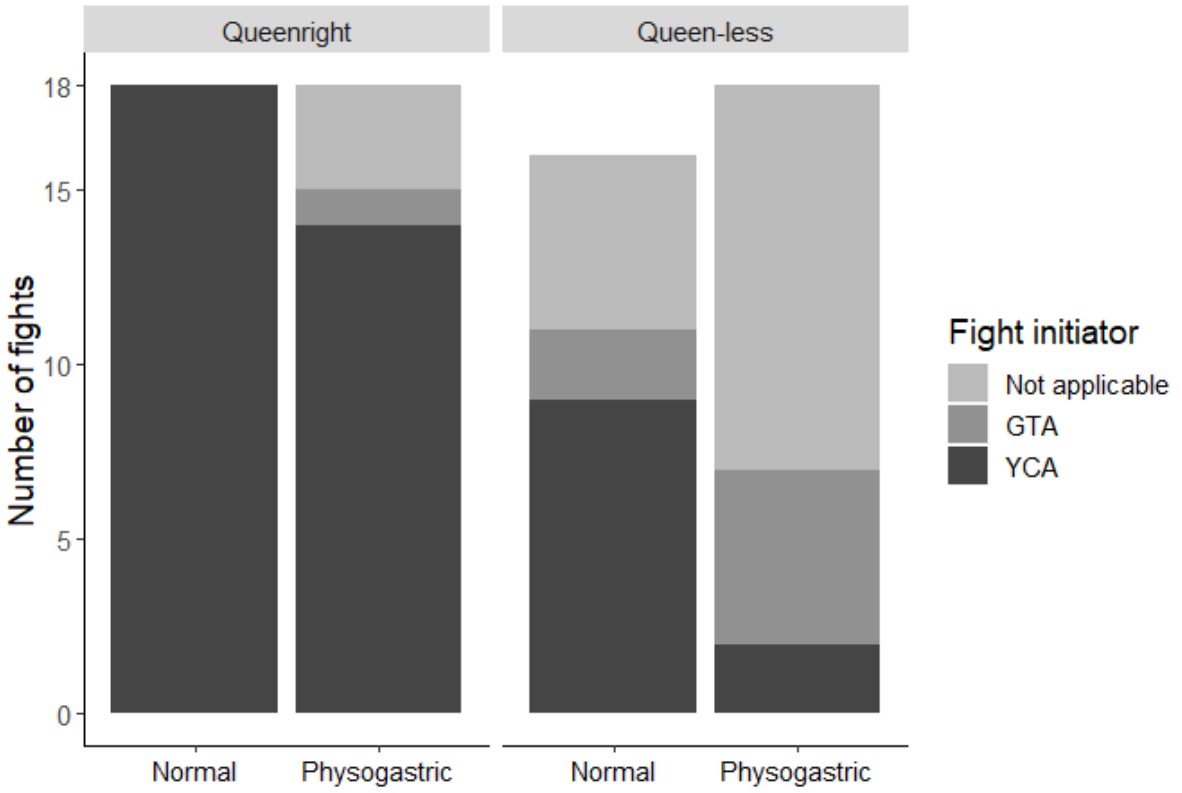

Figure 4.3. Number of fights initiated by *A. gracilipes* (YCA) and *O. smaragdina* (GTA) workers during the aggression tests between *O. smaragdina* workers and *A. gracilipes* normal or physogastric workers from queenright or queenless colonies. Not applicable indicates instances in which *O. smaragdina* and *A. gracilipes* workers did not fight. Normal workers initiated the fight more often than physogastric workers (GLMM: binomial, Type II Wald test: $\chi2=5.7882$, df=1, $p<0.05$) and workers from queenright colonies initiated the fight more often than workers from queenless colonies (GLMM: binomial, Type II Wald test: $\chi2=6.3087$, df=1, $p<0.05$).

Discussion

Our experimental work provides crucial information on worker reproduction in the invasive *A. gracilipes*. We found that the absence of queens triggered an increase in physogastric workers which have the potential to produce males. We also found that physogastric workers were less aggressive and less likely to forage than normal workers, which indicates that their presence may be costly to colony productivity and defence. Our genetic results indicate that the reproduction of *A. gracilipes* is unusual and may involve diploid males and/or sex mosaics as previously suggested (Drescher et al. 2007, Gruber et al. 2013).

Physogastric workers had more reproductive potential than normal workers and queen removal triggered an increase in the number of physogastric workers. Physogastric workers dissected as part of the dissections of worker's ovaries of entire colonies and following the aggression tests were more likely to have yolky oocytes than normal workers. However, there was no difference in yolky oocytes presence at the end of the queen transfer experiment, but this is probably because colonies in the queen transfer experiment had been queenless for a relatively short period (60-120 days vs 100-143 days for dissections of worker's ovaries of entire colonies and 102-212 days for aggression tests). We only observed yellow bodies in physogastric workers (7.8-12.9%). Yellow bodies can indicate oviposition of viable eggs, although they are sometimes observed in trophic egg-layers (Billen 1985, Gobin et al. 1999, Dietemann et al. 2002). Physogastric *A. gracilipes* workers originating from Taiwan also had a higher reproductive potential than normal workers (Lee et al. 2017). They had more well-developed ovaries and more yolky oocytes than normal workers (Lee et al. 2017). Yellow bodies were also only observed in physogastric workers (13%) (Lee et al. 2017). The number of physogastric workers increased after we removed the queen in our queen transfer experiment suggesting that the presence of queens limits workers from becoming physogastric.

Additional results from our colony observations also suggest that queens limit worker reproduction, although worker reproduction could also be controlled by worker policing. Queenless colonies started producing males about five months after becoming queenless. This finding is consistent with Lee et al. (2017) who found that adult males were produced in one *A. gracilipes* queenless colony six months after being queenless. We did not observe trophic eggs during the queen transfer experiment, but any trophic eggs produced by physogastric workers would likely have been fed to the queen and brood immediately after being laid as observed in queenright *A. gracilipes* colonies by Lee et al. (2017). Observations from Lee et al. (2017) and our results suggest that physogastric workers switch from producing trophic eggs in queenright conditions to producing viable male eggs in queenless conditions. Worker reproduction could also be controlled through the policing of reproductive workers (Wenseleers et al. 2004, Wenseleers and Ratnieks 2006). For example, queens and workers could aggressively behave towards egg layers or destruct worker-laid eggs (Ratnieks et al. 2006). Considering our evidence for the existence of queen control over worker reproduction, we will hereafter consider males in queenright colonies to be queen-produced and males in queenless colonies to be worker-

produced. Workers belonging to other ant species also switch from trophic egg production to male egg production when the queen dies or disappears (e.g. *Aphaenogaster senilis*, Ichinose and Lenoir 2009; *Aphaenogaster cockerelli*, Smith et al. 2008; *Prolasius advena*, Grangier et al. 2013; *N. apicalis*, Dietemann and Peeters 2000; *O. longinoda* and *O. smaragdina*, Hölldobler and Wilson 1983). Social insect queens can inhibit worker reproduction with queen pheromones, i.e. chemical signals indicating the reproductive status of the queen (Bourke 1988, Holman 2018). Several experiments with ants, wasps, and some bees have shown that applying synthetic queen pheromones to queenless colonies inhibits worker reproduction by preventing workers from activating their ovaries and by causing secondary oocyte resorption (Holman et al. 2010, 2013, Van Oystaeyen et al. 2014, Holman 2018). It would be interesting to generate a synthetic queen pheromone for *A. gracilipes* and test whether this pheromone would prevent workers from becoming physogastric in queenless colonies.

We found that behavioural differences between physogastric and normal workers could be costly for the colony. Physogastric workers in queenright and queenless colonies were infrequently observed in the foraging area and were mostly observed inside the nesting tubes. These observations suggest that physogastric workers do not contribute to colony labour as much as normal workers. Physogastry may also affect the ability of workers to defend the colony during interspecific conflicts. We found that physogastric workers were less aggressive towards *O. smaragdina* workers and were less likely to engage in a fight than normal workers, which would reduce the potential of *A. gracilipes* to become behaviourally dominant. The production of males by workers also generates costs for other ant species (Heinze 2008). For example, in *Neoponera obscuricornis* colonies, two costs are associated with worker reproduction following queen removal: an increase in energetic cost associated with aggressive interactions between workers for egg-laying and a decrease in productivity due to reproductive workers contributing less time to colony labour (Gobin et al. 2003). Costly worker conflicts about which workers become reproductive and which workers continue to contribute to colony labour also take place in *Aphaenogaster senilis* (Ichinose and Lenoir 2009). It remains unclear whether conflicts take place among *A. gracilipes* workers for becoming physogastric and among physogastric workers for male production. Interestingly, adult males may not originate from a single dominant physogastric worker in queenless *A. gracilipes* colonies as our genetic results indicate that males originated from more than one worker in at least one of our queenless colonies. This result is

supported by male genetic data for *A. gracilipes* in Taiwan which showed four different alleles at one locus (Ano10) in one queenless colony (Lee et al. 2017).

We found that queen control probably regulated the percentage of physogastric workers in queenright colonies. Despite the costs associated with their lack of contribution to colony labour and defence, the role of physogastric workers as trophic egg layers in queenright colonies may be significant (Lee et al. 2017). Colony observations have shown that trophic eggs may represent a major part of the larval diet (Lee et al. 2017). Without a queen, *A. gracilipes* colonies are doomed because reproductive workers are unable to lay worker eggs due to their lack of spermatheca (Lee et al. 2017). The only chance of survival for a queenless colony would be to merge with a queenright colony and/or adopt a queen. Our workers in queenless colonies readily accepted their original queen back in the nest after being separated for 60 days. In the Northern Territory (Australia), laboratory-kept *A. gracilipes* queenless colonies were successfully merged with queenright colonies from a different source colony (Hoffmann 2014). Orphaned colonies may therefore merge with other colonies and/or adopt a queen from a different colony in the field. However, the increase in proportion of physogastric workers following queen death could precipitate the demise of orphaned colonies before such opportunity arises.

Despite being costly to colony productivity and defence, worker reproduction may increase the fitness of deceased *A. gracilipes* queens and orphaned workers as it is their last opportunity to contribute to the gene pool. In Taiwan, the seminal vesicles of *A. gracilipes* worker-produced males contained viable sperm suggesting that they are able to mate (Lee et al. 2017). Although the reproductive mode of *A. gracilipes* is unresolved, genetic data and lab observations suggest that intranidal mating is the main mode of reproduction for this species (Drescher et al. 2010, Thomas et al. 2010). Therefore, if queen brood or virgin queens were present in the colony at the time of the queen's death and did not inhibit the production of males by workers, intranidal mating between worker-produced males and virgin queens could occur. Such a strategy could prolong the life of a colony after the queen's death.

We found that worker-produced males were slightly larger than queen-produced males which may provide worker-produced males with a competitive advantage (Davidson 1982). Our size difference estimation between queen-produced and worker-produced males (2.6-4.8%) is lower than the size difference previously reported (12.7-14.5%, Lee et al. 2017). All the males we

measured (queenright N=28-37, queenless N=50-84 depending on the measurements) were reared in the same standard conditions, whereas the males measured in Lee et al. (2017) were not. Their worker-produced males (N=14) originated from laboratory colonies fed *ad libitum* and their queen-produced males (N=20) from field colonies. Therefore, the larger difference between worker- and queen-produced males in Lee et al. (2017) may be explained by an excess in food supply in queenless colonies. We do not know whether *A. gracilipes* queens select the males they mate with and whether this selection involves male sizes. It would be interesting to test whether *A. gracilipes* queens choose larger males, potentially selecting worker-produced over queen-produced males.

We found that most males from queenright and queenless colonies were homozygous and that heterozygous males were present at a significantly higher proportion in queenright colonies (28.9% of genotyped males) than in queenless colonies (4.9%). Our findings are different to those of Lee et al. (2017) who found all 14 *A. gracilipes* worker-produced males from a single queenless colony to be homozygous at all loci and most of the 20 males from one queenright colony to be heterozygous. Elsewhere they have been genotyped, field-collected heterozygous *A. gracilipes* males were found to be common (Borneo: Drescher et al. 2007, Christmas Island**:** Thomas et al. 2010, Arnhem land Australia: Gruber et al. 2013). For example, about 50% of males collected in Borneo were heterozygous (Drescher et al. 2007). A heterozygous genotype would typically indicate that these males are diploid and suggest a high level of inbreeding in *A. gracilipes* populations, but heterozygous males could also be sex mosaics.

An increase in diploid male load can occur in ant populations that have gone through a genetic bottleneck and consequently lost alleles at the complementary sex determination (CSD) locus (or loci) (Crozier 1971, 1977, Heimpel and de Boer 2008). Matings between queens and males which share the same CSD genotype results in the production of mostly sterile diploid males instead of workers (Crozier 1971, 1977, Heimpel and de Boer 2008) and can lead to a reduction in colony growth and survival (Ross and Fletcher 1986, Cook and Crozier 1995, Lenancker et al. 2019). In some ant species, a low proportion of diploid males produces sperm and can father triploid progeny (Krieger et al. 1999, Cournault and Aron 2009). In *A. gracilipes*, dissections of the seminal vesicles of 16 putative diploid males revealed that all of them possessed functional sperm (Lee et al. 2017). Given that heterozygous males (putatively diploid) are apparently common for this species, we would expect a high prevalence of triploid workers resulting from

successful mating between a queen and a diploid male. However, evidence of triploid *A. gracilipes* individuals has never been reported (Drescher et al. 2007, Thomas et al. 2010, Gruber et al. 2012, 2013, Lee et al. 2017). This suggests that heterozygous males may not be diploid.

Another explanation for the common occurrence of heterozygous males in our and other studies (Drescher et al. 2007, Thomas et al. 2010, Gruber et al. 2013) could be linked to sex mosaics. Sex mosaics, or gynandromorphs, can occur in Hymenoptera and combine the morphological features of males and females (Jones and Phillips Jr. 1985, Wcislo et al. 2004, Skvarla and Dowling 2014). In sex mosaics, the male and female tissue are spread in patches across the body (Yoshizawa et al. 2009, de Campos et al. 2011). Matings with sex mosaics can result in viable non-mosaic offspring as shown for *Cardiocondyla kagutsuchi*, but the reproductive potential of sex mosaics in other ant species remains unclear (Yoshizawa et al. 2009). Heterozygous *A. gracilipes* males could therefore be haploid with cells inherited from paternal and maternal sources (Gruber et al. 2013). Our four males that had an oversized eye may be sex mosaics with a conspicuous phenotype. In ants, sex mosaics sometimes present an enlarged eye (female) on one side of the head and a smaller eye (male) on the other side (Yoshizawa et al. 2009, Skvarla and Dowling 2014). Future research exploring sex mosaics in *A. gracilipes* could help determine whether heterozygous males are not diploid but are instead sex mosaics, and whether sex mosaics hinder or contribute to the success of invasive ants.

We found that most workers were heterozygous for at least one locus and that most queens were homozygous. This genetic pattern is typical of *A. gracilipes* populations and suggests that female castes are determined by a genetic component for this species (Drescher et al. 2007, Thomas et al. 2010, Gruber et al. 2012, 2013, Lee et al. 2017). A potential caste determination system could be linked to gynandromorphy in males. Gynandromorph males could produce sperm from their inherited paternal or maternal cells and the determination of female castes could be determined by a combination of male and female alleles (Gruber et al. 2013). The reproductive mode of *A. gracilipes* may contribute to the ecological dominance of this ant by maintaining high level of heterozygous workers which may be better adapted to human-modified environments as suggested for the little fire ant or electric ant (*Wasmannia auropunctata*) (Foucaud et al. 2009, Chifflet et al. 2018). Additional investigations into the reproductive mode of *A. gracilipes* are necessary to resolve current uncertainties on worker and queen castes determination as well as

the occurrence of sex mosaics and to determine whether this potentially unusual reproductive mode contributes to the invasive success of *A. gracilipes*.

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Supplementary information

Table S4.1 Colony collection sites, number of queens, workers, status and time in captivity for the dissections of worker's ovaries of entire colonies

Colony code	Collection Site	Number of queens	Number of workers	Number of days in captivity	Status
20171104Nome-TSV4	Townsville	8	29	349	Queenright
B420171020	Brisbane	6	26	175	Queenright
GC200417	Gray's creek	na	22	368	Queenless (143 days)
20171127GV1	Gordonvale	na	64	125	Queenless (108 days)

Table S4.2 Site coordinates

Site	Latitude	Longitude
Kearns	-17.0044	145.7173
Russett Park	-16.8030	145.5968
Sandy Creek	-17.0561	145.734
Mount Peter	-17.0498	145.7282
Gray's creek	-17.0616	145.7410
Vindula	-17.0524	145.7292
Gordonvale	-17.0831	145.7961
Hussey	-17.0636	145.7395
Whites creek	-19.3676	146.9549
Drapers Creek	-17.0833	145.7563
Franks Creek	-17.0452	145.7275
Harold	-17.0554	145.7293
Townsville	-19.2589	146.8169
Hervey Bay	-25.2882	152.7677
Brisbane	-27.3914	153.1183
Nome	-19.3736	146.9065

Table S4.3 Colony collection sites, status, time in captivity and number of workers and queens during the colony observations. Status: QR queenright colonies, QL queenless colonies. Number of queens column: NA not applicable. Site column: Kearns K, Russett Park RP, Sandy Creek SC, Mount Peter MP, Gray's creek GC, Vindula V, Hussey H, Whites creek WC, Drapers creek DC, Franks Creek F, Harold H, Townsville T, Hervey Bay HB

Colony code	Days in captivity	Site	Status	Male producing	Number of workers	Number of queens
0606RPT2-S3	266	RP	QR	Yes	10-190	1
0606RPT3-S4	266	RP	QR	Yes	10->450	1-3
0711WC3	134	WC	QR	Yes	20->330	5-7
1905MPRF6	361	MP	QR	Yes	20->410	2-3
20170112RPT3-S10	285	RP	QR	Yes	10-250	1-4
20170215RPT3-S6	209	RP	QR	Yes	10-120	1
20170316GCT4-9	249	GC	QR	Yes	10->460	1
20170717DCT2-10	163	DC	QR	Yes	220->560	1
0308Franks3	335	F	QR	No	10-140	1-4
0308Franks5	278	F	QR	No	10-140	1-8
0308Harold8	391	H	QR	No	50->290	1-5
0407Franks	222	F	QR	No	10->420	1-7
0407RPT2-S8	265	RP	QR	No	10-500	1
0407RPT3-S6	385	RP	QR	No	10-420	1-2
0407SC2	322	SC	QR	No	10-280	1-3
0606Kearns1	252	K	QR	No	10->280	1
0606Kearns3	324	K	QR	No	10->380	1
0711WC1	274	WC	QR	No	10-150	3-5
0711WC2	416	WC	QR	No	20-240	5-8
0809Kearns14	333	K	QR	No	20-260	1-5
1009Townsville	263	T	QR	No	10-100	3-4
1011RPT1-S2	356	RP	QR	No	20->290	1
1011RPT2-S3	404	RP	QR	No	10->390	1

1905MPS05	270	MP	QR	No	10-230	1
20170112SC13	249	SC	QR	No	10->570	1-9
20170112SC3	242	SC	QR	No	10->470	1-7
20170112SC8	165	SC	QR	No	10->650	5-7
20170316FrankT12-1	74	F	QR	No	>220->470	1-2
20170316FrankT1-6	287	F	QR	No	20->280	1-2
20170316FrankT1-7	286	F	QR	No	50-160	1-2
20170316FrankT2-1	265	F	QR	No	10->300	1-2
20170410Kearns13	261	K	QR	No	10->290	1-2
20170412FR12	260	F	QR	No	10-380	2-6
20170412SC13	260	SC	QR	No	130-280	1
20170412SC14	259	SC	QR	No	10-300	2-7
20170418RPT2-S8	253	RP	QR	No	140->300	1
20170418RPT5-S8	209	RP	QR	No	20->260	1-3
20170421Hussey2	255	H	QR	No	10->360	1-3
20170803Harold8	461	H	QR	No	10-150	1-2
20170815FR13	135	F	QR	No	20->220	1-3
20170815SC14	134	SC	QR	No	50-310	2
20170815SC5	98	SC	QR	No	230->460	2
20170815SC9	134	SC	QR	No	220-320	1
20170816RPT3-1	133	RP	QR	No	120->290	1
20170921SC3	98	SC	QR	No	100-340	7-9
20170921SC5	68	SC	QR	No	>200->260	3-4
20170921SC8	98	SC	QR	No	20-120	2-3
20170921SC9	98	SC	QR	No	100->231	1
2109RPT1-S9	342	RP	QR	No	10->290	1-2
2109SC8	363	SC	QR	No	10->290	1-8
2110RPT1-S8	283	RP	QR	No	10-260	1-2

2110RPT2-S1	342	RP	QR	No	10-290	1-4
2110RPT2-S4	424	RP	QR	No	10-290	1-6
2110RPT2-S5	432	RP	QR	No	110-310	1
2110RPT2-S7	298	RP	QR	No	10-300	1
2110SC12	283	SC	QR	No	10-290	1-8
2110SC15	432	SC	QR	No	20->280	1-35
2110SC16	304	SC	QR	No	10->350	1-17
2110SC20	290	SC	QR	No	10->310	2-18
0308Kearns12	314	K	QL	Yes	10-260	NA
0308Kearns2	293	K	QL	Yes	10-520	NA
0308RPT4-S6	328	RP	QL	Yes	10-340	NA
0407Kearns13	303	K	QL	Yes	10->450	NA
0407Kearns15	296	K	QL	Yes	10-310	NA
0407Kearns19	238	K	QL	Yes	10-550	NA
0407RPT3-S4	303	RP	QL	Yes	10-310	NA
0606Kearns17	273	K	QL	Yes	10->320	NA
0606RPT1-S2	301	RP	QL	Yes	10->310	NA
0606RPT3-S3	357	RP	QL	Yes	20->550	NA
0606RPT3-S5	259	RP	QL	Yes	10-260	NA
0606SC10	280	SC	QL	Yes	10->520	NA
0712RPT2-S6	386	RP	QL	Yes	20->460	NA
0905MPRF1	259	MP	QL	Yes	20-180	NA
1011RPT4-S9	229	RP	QL	Yes	20-330	NA
1108Kearns6	319	K	QL	Yes	10->260	NA
20170112Kearns20	221	K	QL	Yes	20->320	NA
20170112RPT3-S6	243	RP	QL	Yes	10->360	NA
20170316GCT4-1	193	GC	QL	Yes	10-350	NA
20170410GCT2-3	211	GC	QL	Yes	10-470	NA

20170410VIT2-5	242	V	QL	Yes	10-280	NA
20170503Hussey1	239	H	QL	Yes	30->360	NA
20170503RPT2-S8	223	RP	QL	Yes	10-90	NA
20170508SC1	203	SC	QL	Yes	10-300	NA
0308Franks20	244	F	QL	No	10-40	NA
0308Harold3	278	HB	QL	No	10-70	NA
0308Kearns13	209	K	QL	No	10-150	NA
0308Kearns7	321	K	QL	No	10-270	NA
0308RPT1-S6	286	RP	QL	No	10-170	NA
0308RPT2-S6	328	RP	QL	No	10-180	NA
0308RPT3-S2	278	RP	QL	No	10-80	NA
0308RPT4-S4	285	RP	QL	No	10-230	NA
0407Kearns5	245	K	QL	No	10-80	NA
0407RPT1-S2	253	RP	QL	No	10-240	NA
0407RPT2-S5	260	RP	QL	No	10-320	NA
0407RPT4-S1	176	RP	QL	No	10-100	NA
0407SC7	296	SC	QL	No	10-240	NA
0606RPT1-S6	266	RP	QL	No	10->280	NA
0606RPT4-S9	252	RP	QL	No	10->260	NA
0606SC1	244	SC	QL	No	10-280	NA
0606SC18	204	SC	QL	No	10-270	NA
0712RPT4-S1	202	RP	QL	No	10-190	NA
0809HerveyBay	193	HB	QL	No	10-50	NA
1108Kearns14	305	K	QL	No	10-170	NA
1108Kearns16	242	K	QL	No	10-130	NA
1108Kearns18	298	K	QL	No	10-140	NA
1108Kearns20	277	K	QL	No	10-170	NA
1905MPRF11	229	MP	QL	No	10->230	NA

1905MPRF12	229	MP	QL	No	10->280	NA
1905MPRF15	229	MP	QL	No	10-140	NA
20170112RPT4-S6	165	RP	QL	No	20-250	NA
20170215Kearns18	223	K	QL	No	20-140	NA
20170215RPT1-S1	236	RP	QL	No	10-80	NA
20170215RPT2-S5	131	RP	QL	No	20-130	NA
20170316FrankT2-3	102	F	QL	No	>225->440	NA
20170316GCT4-5	201	GC	QL	No	10->430	NA
20170316SC1	201	SC	QL	No	20->460	NA
20170410GC1	177	GC	QL	No	80-350	NA
20170410Kearns9	231	K	QL	No	10-150	NA
20170418RPT1-S8	202	RP	QL	No	10-150	NA
20170505VIT1-10	222	V	QL	No	10-230	NA
20170508SC7	219	SC	QL	No	10-240	NA
20170621Kearns17	190	K	QL	No	70-230	NA
20170717DCT1-6	163	DC	QL	No	20-250	NA
20170717DCT2-7	147	DC	QL	No	10-190	NA
20170816RPT4-1	134	RP	QL	No	20-230	NA
2109Kearns4	278	K	QL	No	10-240	NA
2110RPT4-S10	333	RP	QL	No	10-340	NA

Table S4.4 Range for the number of workers counted during the colony observations

Number of workers	Category
0	a
1-10	b
11-20	c
21-50	d
51-100	e
101-200	f
>201	g

Table S4.5 Colony collection date, sites and number of workers per replicate at the start of the queen transfer experiment. Nome: N, Sandy Creek: SC, GV: Gordonvale Hervey Bay: HB, B: Brisbane

Collection date	Colony code	Site	Workers per replicate at day 0
November 2017	20171104NomeTSV2	N	135
November 2017	20171104NomeTSV4	N	200
November 2017	20171109SC1	SC	121
November 2017	20171104NomeTSV31	N	160
November 2017	20171113GV1	GV	150
November 2017	20171109GV1	GV	200
November 2017	20171127GV1	GV	200
November 2017	20171104NomeTSV4	N	200
October 2017	20171023HB2	HB	200
October 2017	20171014NomeTSV2	N	160
October 2017	20171020B4	B	170
September 2017	20170910GV1	GV	200
September 2017	20170910SC5	SC	150
September 2017	20170910GV2	GV	200

Table S4.6 Colony collection date, sites and status of *A. gracilipes* colonies used in the aggression tests. Status: QR queenright colonies, QL queenless colonies. Gordonvale: GV, Sandy Creek: SC, Drapers Creek: DC

Collection date	Colony code	Site	Status
November 2017	20171109GV1	GV	QL
November 2017	20171109GV2	GV	QL
November 2017	20171127GV1	GV	QL
September 2017	20170910SC5	SC	QL
September 2017	20170910SC5	SC	QL
September 2017	20170910GV2	GV	QL
November 2017	20171101DC2	DC	QR
November 2017	20171101D3	DC	QR
November 2017	20171113DC1	DC	QR
November 2017	20171109DC1	DC	QR
September 2017	20170921SC3	SC	QR
September 2017	20170921SC9	SC	QR

Table S4.7 Genotypes of males from queenright (N=38 males) and from queenless (N=82 males) colonies (N=8 queenright and N=14 queenless colonies). Allele sizes in red and bold: heterozygous at this locus, -: amplification failure

Colony status	Sample size	Ano1		Ano3		Ano4		Ano5		Ano6		Ano9	
Queenright	2	**101**	**105**	**160**	**166**	156		118		**116**	**130**	**153**	**155**
Queenright	1	**101**	**105**	160		156		120		-		153	
Queenright	1	**101**	**105**	**160**	**168**	**156**	**174**	120		-		155	
Queenright	2	**101**	**105**	**160**	**166**	156		**118**	**120**	-		**153**	**155**
Queenright	1	103		**160**	**140**	**156**	**164**	122		-		149	
Queenright	1	101		168		-		120		-		**149**	**155**
Queenright	1	-		140		164		**118**	**120**	-		-	
Queenright	1	-		**160**	**140**	**156**	**164**	118		-		-	
Queenright	1	-		-		**156**	**174**	-		-		155	
Queenright	1	-		-		**156**	**174**	-		-		-	
Queenright	1	105		160		156		120		116		155	
Queenright	1	105		160		156		120		116		153	
Queenright	1	105		166		174		118		114		-	
Queenright	1	105		160		156		120		-		155	
Queenright	1	101		166		174		118		-		155	
Queenright	2	99		166		156		-		-		155	
Queenright	1	105		160		-		118		-		153	
Queenright	1	105		160		156		122		-		-	
Queenright	2	103		-		156		122		-		155	
Queenright	1	105		160		156		-		-		149	
Queenright	1	99		-		174		118		-		-	
Queenright	1	99		160		-		-		114		153	
Queenright	1	99		140		-		-		-		155	
Queenright	1	-		160		156		120		-		153	
Queenright	1	-		160		156		-		-		153	
Queenright	1	-		-		156		-		114		149	
Queenright	1	105		160		-		-		-		149	
Queenright	1	103		-		156		122		-		-	
Queenright	1	-		166		174		118		-		-	

Queenright	1	99	-	-		-	114	-
Queenright	1	-	-	-		-	114	155
Queenright	1	103	-	-		122	-	-
Queenright	1	-	-	156		-	-	-
Queenright	1	103	-	-		-	-	-
Queenright	1	99	-	-		-	-	-
Queenless	1	99	160	**156**	**174**	118	-	153
Queenless	1	99	166	**156**	**174**	-	114	153
Queenless	1	-	166	**156**	**174**	118	128	155
Queenless	1	-	-	**156**	**174**	-	128	-
Queenless	1	105	166	174		116	114	155
Queenless	1	105	166	174		118	114	153
Queenless	2	105	166	174		116	-	155
Queenless	1	99	166	174		118	-	153
Queenless	2	105	160	174		118	-	153
Queenless	1	-	166	156		118	128	153
Queenless	1	99	166	174		118	114	-
Queenless	1	105	160	-		122	-	155
Queenless	1	99	-	156		118	114	-
Queenless	1	105	166	-		118	114	-
Queenless	1	105	166	156		-	114	-
Queenless	1	99	166	174		118	-	-
Queenless	1	105	160	174		118	-	-
Queenless	1	99	166	156		116	-	-
Queenless	1	99	166	-		118	-	155
Queenless	1	99	160	-		118	-	155
Queenless	1	99	166	-		116	-	155
Queenless	2	105	166	174		-	-	155
Queenless	1	99	-	174		-	128	153
Queenless	1	105	160	-		-	114	153
Queenless	1	105	-	-		-	114	153
Queenless	1	105	166	-		122	-	153
Queenless	1	99	166	-		116	-	153
Queenless	2	99	160	-		116	-	153

Queenless	1	99	-	174	118	128	-
Queenless	1	99	-	174	116	128	-
Queenless	1	-	166	156	-	-	155
Queenless	1	-	-	-	116	114	153
Queenless	2	-	-	156	-	114	153
Queenless	1	-	166	-	-	128	155
Queenless	3	-	-	174	-	114	155
Queenless	1	-	-	156	118	-	155
Queenless	1	105	-	156	-	-	155
Queenless	1	105	-	174	-	128	-
Queenless	1	105	-	-	118	128	-
Queenless	1	-	166	156	-	-	153
Queenless	2	105	-	-	118	114	-
Queenless	1	99	-	-	116	114	-
Queenless	1	105	-	156	-	114	-
Queenless	1	105	-	174	116	-	-
Queenless	1	105	166	-	118	-	-
Queenless	1	105	160	-	118	-	-
Queenless	1	99	160	-	118	-	-
Queenless	1	-	166	174	116	-	-
Queenless	1	105	166	156	-	-	-
Queenless	1	105	160	156	-	-	-
Queenless	1	99	160	156	-	-	-
Queenless	1	-	-	-	116	128	-
Queenless	1	105	-	-	-	128	-
Queenless	1	-	-	-	116	114	-
Queenless	1	-	-	174	-	114	-
Queenless	1	105	-	-	-	114	-
Queenless	1	-	166	156	-	-	-
Queenless	1	-	-	174	118	-	-
Queenless	1	-	-	156	118	-	-
Queenless	1	-	-	156	116	-	-
Queenless	2	99	-	174	-	-	-
Queenless	1	105	166	-	-	-	-

Queenless	1	105	160	-	-	-	-
Queenless	3	99	160	-	-	-	-
Queenless	1	-	-	156	-	-	-
Queenless	1	-	166	-	-	-	-
Queenless	1	-	-	174	-	-	-
Queenless	1	105	-	-	-	-	-
Queenless	1	99	-	-	-	-	-
Queenless	1	-	-	-	118	-	-

Table S4.8 Genotypes of workers from queenright (N=40 workers) and queenless colonies (N=54 workers) (N=8 queenright colonies and N=10 queenless colonies). -: amplification failure

Colony	Sample												
status	size	Ano1		Ano3		Ano4		Ano5		Ano6		Ano9	
Queenright	1	101	103	160	140	156	164	122	106	116	130	149	155
Queenright	4	101	105	160	140	156	174	118	120	116	130	153	155
Queenright	5	101	105	160	166	156	174	118	120	116	130	153	155
Queenright	3	101	105	160	168	156	174	118	120	116	130	153	155
Queenright	1	101	105	166	140	156	164	118	120	116	130	155	155
Queenright	1	101	105	160	140	-	-	118	120	116	130	153	155
Queenright	1	101	105	160	140	156	164	118	120	-	-	153	155
Queenright	1	101	105	160	166	-	-	118	120	116	130	153	155
Queenright	1	101	105	160	168	-	-	118	120	116	130	153	155
Queenright	4	99	105	160	166	156	174	-	-	114	128	153	153
Queenright	1	101	105	160	140	-	-	118	120	-	-	153	155
Queenright	1	101	105	166	140	156	164	118	120	-	-	-	-
Queenright	2	99	105	-	-	156	174	-	-	114	128	153	153
Queenright	2	99	105	160	166	156	174	-	-	-	-	153	153
Queenright	1	-	-	160	166	156	174	-	-	114	128	153	153

Queenright	1	-	-	160	168	156	174	118	120	116	130	-	-
Queenright	1	99	103	-	-	156	174	-	-	-	-	149	155
Queenright	1	99	105	-	-	156	174	-	-	-	-	153	153
Queenright	1	-	-	160	140	-	-	-	-	116	130	153	155
Queenright	1	-	-	160	140	156	164	-	-	116	130	-	-
Queenright	1	99	99	-	-	-	-	-	-	128	128	-	-
Queenright	1	-	-	160	140	156	164	-	-	-	-	-	-
Queenright	1	99	99	-	-	-	-	-	-	-	-	-	-
Queenright	1	-	-	-	-	-	-	-	-	-	-	149	155
Queenright	1	-	-	-	-	-	-	-	-	114	128	-	-
Queenright	1	-	-	160	140	-	-	-	-	-	-	-	-
Queenless	1	99	99	160	166	156	174	-	-	114	128	155	155
Queenless	1	99	105	160	166	156	174	-	-	114	128	153	153
Queenless	11	99	105	160	166	156	174	-	-	114	128	153	155
Queenless	2	99	105	160	166	156	174	-	-	114	128	155	155
Queenless	2	99	105	166	166	156	174	-	-	114	128	153	153
Queenless	10	99	105	166	166	156	174	-	-	114	128	153	155
Queenless	1	-	-	160	166	156	174	-	-	114	128	153	155
Queenless	1	-	-	160	166	156	174	-	-	114	128	155	155
Queenless	1	-	-	166	166	156	174	-	-	114	128	153	155
Queenless	2	99	105	-	-	156	174	-	-	114	128	153	153
Queenless	5	99	105	-	-	156	174	-	-	114	128	153	155
Queenless	2	99	105	160	166	156	174	-	-	-	-	153	153
Queenless	1	99	105	166	166	156	174	-	-	114	128	-	-
Queenless	1	99	107	-	-	156	174	-	-	114	128	153	155
Queenless	1	99	107	166	166	156	174	-	-	-	-	153	155
Queenless	1	-	-	-	-	156	174	-	-	114	128	153	155
Queenless	1	-	-	166	166	-	-	-	-	114	128	153	153
Queenless	1	-	-	166	166	156	174	-	-	114	128	-	-

Queenless	1	99	99	-	-	156	174	-	-	114	128	-	-
Queenless	1	99	105	-	-	156	174	-	-	-	-	153	153
Queenless	1	99	105	-	-	156	174	-	-	-	-	153	155
Queenless	1	99	105	-	-	156	156	-	-	-	-	-	-
Queenless	1	-	-	-	-	-	-	-	-	114	128	-	-
Queenless	1	-	-	-	-	-	-	-	-	116	122	-	-
Queenless	2	-	-	-	-	156	174	-	-	-	-	-	-
Queenless	1	99	99	-	-	-	-	-	-	-	-	-	-

Table S4.9 Genotypes of queens (N=10 queens from 6 colonies). Allele sizes in red and bold: heterozygous at this locus, -: amplification failure

Sample size	Ano1		Ano3		Ano4		Ano5		Ano6		Ano9	
1	-	-	160	160	**156**	**174**	118	118	-	-	153	153
1	105	105	160	160	156	156	118	118	116	116	153	153
1	105	105	160	160	156	156	120	120	116	116	153	153
1	105	105	160	160	156	156	120	120	116	116	155	155
1	105	105	166	166	156	156	122	122	116	116	155	155
1	103	103	160	160	156	156	122	122	116	116	149	149
1	103	103	160	160	156	156	122	122	116	116	153	153
1	103	103	160	160	156	156	122	122	-	-	149	149
1	105	105	160	160	-	-	120	120	116	116	153	153
1	105	105	-	-	-	-	120	120	116	116	153	153

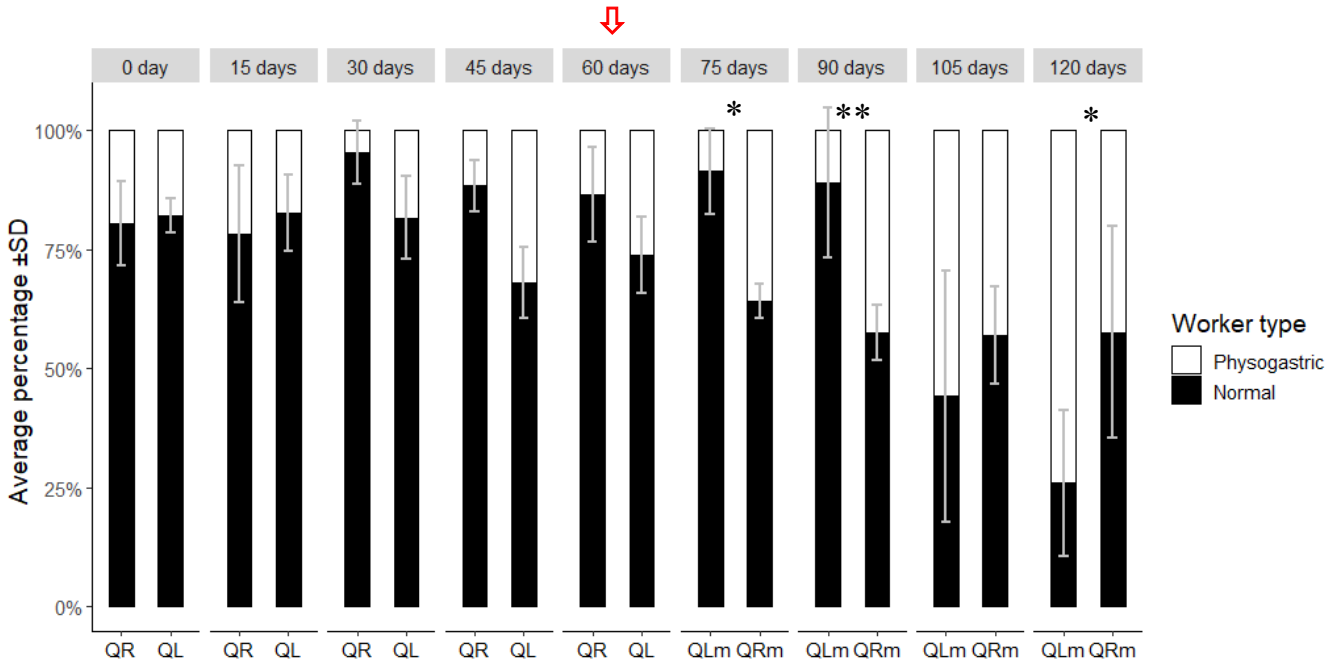

Figure S4.1 Average percentage of physogastric and normal workers in the queen transfer experiment separated between queenright (QR, until day 60, N=3) and queenless colonies (QL, until day 60, N=3) and number of days since the start of the experiment until 120 days after the start of the experiment. ⇩ indicates when queens have been moved from the queenright colonies to the queenless colonies. After this date, queenright colonies became queenless (QLm) and queenless colonies became queenright (QRm). * and ** indicates a significant difference between queenright and queenless colonies for the corresponding time (GLM: quasi-binomial, Table 4.2, post hoc tests *p<0.05 **p<0.01). Error bars indicate standard deviation.

Chapter 5 Synthesis

Thesis overview

Understanding the processes that hinder or contribute to the success of ant invasions is vital to limit their occurrence and effects. In my thesis I investigated how four processes may facilitate or hinder the successful establishment and dispersal of two highly successful ant species: (i) genetic bottleneck, (ii) disturbance, (iii) anthropogenic dispersal, and (iv) reproductive strategies. Each of this process is linked to some of the most foundational concepts, paradoxes and/or hypotheses of invasion biology:

(i) One of the paradoxes of invasion biology is that many invasive populations are successful despite having gone through a genetic bottleneck (Allendorf and Lundquist 2003, Dlugosch and Parker 2008a). Invasive species often lose genetic variation when they establish new populations due to the small number of founding individuals (Allendorf and Lundquist 2003, Dlugosch and Parker 2008a). In theory, inbreeding would cause small populations to accumulate deleterious mutations and eventually going extinct (Lynch et al. 1995, Caballero et al. 2017).

(ii) One of the most foundational and widely accepted ideas of invasion ecology is that disturbance facilitates biological invasions (Moles et al. 2012). Disturbance may benefit invaders by modifying abiotic conditions and weakening biotic resistance (Elton 1958, Lozon and MacIsaac 1997, Walters and Mackay 2005, Krushelnycky et al. 2010, Moles et al. 2012). One of the key challenges in studying the role of disturbance in the successful establishment of non-native species is to determine the relative importance of abiotic and biotic factors. Distinguishing the role of abiotic and biotic factors can potentially provide key insights into the factors facilitating invasions.

(iii) The long distance spread of invasive species has been facilitated by anthropogenic dispersal for hundreds of years (Hulme 2009). Retracing the origin and pathways of biological invasions is crucial to investigate some of the major concepts of invasion ecology such as whether genetic diversity was lost following invasion and whether geneflow occurs between and among invasive populations and contributes to their success (Dlugosch and Parker 2008a, Hulme 2009).

(iv) Several reproductive strategies are associated with the success of invasive species (e.g. vegetative reproduction, high fecundity) (Sakai et al. 2001). Investigating how some aspects of reproduction may facilitate or hinder the establishment and spread of invasive species is crucial to better understand what makes some species such successful invaders.

I conducted laboratory experiments, field experiments and genetic analyses on *S. geminata* and *A. gracilipes* to address current research gaps linked to each of these processes. In the rest of this chapter, I synthesise some of my main findings, address some of the limitations of my project that were not discussed in individual chapters, and give recommendations for future research.

Genetic bottleneck and strategies to minimize inbreeding costs

Solenopsis geminata went through a severe genetic bottleneck during its introduction (Chapter 3). This genetic bottleneck contributed to the production of diploid males in its invasive range, a significant genetic burden for founding queens (Lenancker et al. 2019). Using a genome-wide sequencing technique (double digest RADseq), I found that *S. geminata* lost 38.5% of its genetic diversity across its genome during its introduction (Chapter 3). As a result of this genetic bottleneck, invasive *S. geminata* populations most likely lost sex determining alleles, disrupting the functioning of their sex determination system and triggering the production of diploid males (Crozier 1971, 1977, Ross et al. 1993). Queens of *S. geminata* found colonies claustrally, thus the rapid production of workers before the queen's fat reserves are depleted is essential for the colony to survive (Ross and Fletcher 1986). The production of diploid males was common in field *S. geminata* colonies (n=8/10) and was a significant burden to founding queens as DMP colonies produced fewer pupae and workers during the claustral phase than non-DMP colonies (Lenancker et al. 2019).

Several mechanisms may limit the costs and occurrence of diploid male production. I found that pleometrosis (queens founding their nest together) increased colony size and could help compensate for the cost of diploid male production in *S. geminata* (Lenancker et al. 2019). I also found that 43.5% of DMP colonies likely executed diploid male larvae during the claustral phase (Lenancker et al. 2019). Diploid male execution may benefit the colony by redirecting nutrients to the worker brood. This cannibalistic behaviour may be restricted to the claustral phase as I found adult diploid males in the field. Colonies in the native range of *S. geminata* are less likely to produce diploid males because they have not experienced a genetic bottleneck (Ross and

Fletcher 1985a). Indeed, I found that one native colony contained more genetic diversity than all invasive specimens combined (Chapter 3). It would be interesting to test whether selective larval execution only occurs in invasive populations of *S. geminata* as a response to the cost of diploid male production to incipient colonies. In the long term, continuous geneflow among invasive populations could contribute to increasing the number of sex determining alleles in some populations and thus decrease the occurrence of diploid male production (Dlugosch and Parker 2008a). I found evidence of geneflow between and among invasive *S. geminata* populations throughout the Indo-Pacific region (Chapter 3). It would be interesting to determine whether diploid male production is less common in populations with a high rate of geneflow (e.g. India, Thailand) than in populations with low geneflow rates (e.g. Malaysia, Christmas Island).

Diploid male production is a clear negative consequence of inbreeding depression in invasive ants, but a genetic bottleneck may not always limit the success of invasive species. Loss in genetic variation following introduction may not result in a decline in adaptation opportunities or trait variations for invasive populations (Dlugosch and Parker 2008a). For example, despite going through a strong genetic bottleneck, invasive populations of the ornamental shrub *Hypericum canariense* have shown adaptation in flowering phenology and growth rate (Dlugosch and Parker 2008a, 2008b). Bottlenecked populations of graylings (*Thymallus thymallus*) also display evidence of adaptive evolution, although they have experienced severe genetic bottlenecks (Koskinen et al. 2002). For the Argentine ant (*Linepithema humile*), losing genetic variability following its introduction may have contributed to reduced intraspecific aggression among colonies and facilitated the formation of large and dense interacting nests (Tsutsui et al. 2000, Brandt et al. 2009).

Disturbance facilitates the establishment of *S. geminata* mainly through abiotic conditions

According to results from Chapter 2, disturbance increased the survival of *S. geminata* queens. In my caged queen experiment, queens were more likely to survive if they were introduced in cleared savannah woodland maintained as open grassland than in savanna woodland. Disturbance may modify abiotic conditions and contribute to providing a suitable habitat to invasive taxa (Byers 2002, Krushelnycky et al. 2010). It may also weaken biotic resistance by

decreasing native species diversity and the competitive ability of native communities (Elton 1958, Lozon and MacIsaac 1997).

Abiotic conditions were the primary drivers of *S. geminata* queen mortality in my caged queen field experiment which suggest that the spread of *S. geminata* to undisturbed sites in the Northern Territory is mainly prevented by unsuitable abiotic conditions. (Chapter 2). The combination of abiotic factors and background mortality accounted for 85.7±8% (mean ± SD) of queen mortality (Chapter 2). Based on results from my lab rearing experiment, background mortality accounts for about 20% of mortality in single-founding *S. geminata* queens during the claustral period (Lenancker et al. 2019). Temperature and shade have been suggested to be a strong determinant of *S. geminata* success (Cokendolpher and Francke 1985, Perfecto and Vandermeer 1996) while temperature and water availability are the main limiting factors to the spread and establishment of *L. humile* (Menke and Holway 2006, Menke et al. 2007, Brightwell et al. 2010). Experiments manipulating various abiotic conditions and testing their effects on the survival of *S. geminata* would be useful to establish which abiotic factors limit the success of *S. geminata* in undisturbed habitats.

Biotic resistance from the native ant community also played a role in limiting the establishment success of *S. geminata* in undisturbed habitats. Isolation from native ants improved the survival of queens in undisturbed sites but not in disturbed ones (Chapter 2). The ant species composition was different between disturbed and undisturbed sites, but species richness was not (Chapter 2). Aggressive subterranean taxa that are sensitive to disturbance (e.g. *Carebara affinis*) may limit the establishment of colonies in undisturbed habitats during the claustral phase by attacking newly mated queens and their brood (Berghoff et al. 2003*a*, 2003*b*, Chapter 2). Determining whether resident species most likely to limit the success of incipient vs mature invasive ant colonies differs could further our understanding of the mechanisms of biotic resistance. For example, species in the *Iridomyrmex* genus are not likely to predate on *S. geminata* queens during the claustral period because these ants are mostly epigeic, but they may compete for food resources with foraging *S. geminata* workers.

Genome-wide sequencing, a powerful tool for the study of invasive ant populations

The use of double digest RADseq (ddRADseq) rather than traditional genotyping methods (i.e. mitochondrial and microsatellite markers) gave me unprecedented power to study the population genetics of *S. geminata* at global and colony scales (Chapter 3). Comparing my results with results obtained by Gotzek et al. (2015), who investigated the invasion history of *S. geminata* using mitochondrial and microsatellite markers, demonstrates the power of genome-wide sequencing approaches over traditional methods. However, it is possible to reach high sensitivity by using a large number of microsatellite markers to genotype many individuals. For example, Ascunce et al. (2011) successfully reconstructed the global invasion history of *S. invicta* by genotyping 2144 samples with 66 microsatellite markers.

I identified multiple geneflow events between and among virtually all my sampled invasive populations (Chapter 3). Gotzek et al. (2015) were unable to find evidence of geneflow because their power to detect clusters was limited. Thus, all the samples from the Indo-Pacific and Australia were assigned to a single cluster (Gotzek et al. 2015). Regarding the introduction history of *S. geminata*, I provided strong support to the invasion scenario described by Gotzek et al. (2015) and confirmed that the ant was first dispersed from Acapulco in southwest Mexico and that subsequent Indo-Pacific populations likely derive from Philippine populations (Gotzek et al. 2015, Chapter 3). I was also able to provide new information regarding the invasion history of *S. geminata* to Australian islands. I found that populations established in Australian islands probably originated from the Australian mainland, and that Ashmore Reef may have been invaded on two occasions (Chapter 3). I was also able to estimate the number of queens per colony (Chapter 3). Gotzek et al. (2015) did not attempt to determine the social structure of *S. geminata* colonies as they used a single worker per colony in their study. Using mitochondrial markers to estimate the number of queens per colony in invasive ants may not always be appropriate due to invasive ant populations often originating from a few maternal lineages (Corin et al. 2007, Gotzek et al. 2015). The number of queens per colony can however be estimated with highly variable microsatellite markers.

RADseq methods such as ddRADseq are ideal to study the population genetics of small taxa with low genetic diversity such as invasive ants: they are cost effective, require as little as 50ng

of DNA per sample, and can generate thousands of markers (Baird et al. 2008, Peterson et al. 2012, Chapter 3). Comparing my results (Chapter 3) with results from Gotzek et al. (2015) demonstrates the use of genome-wide sequencing approaches to determine the geneflow, invasion history, and social structure of global ant invaders. Studies using such approaches could resolve current uncertainties regarding the native range of invaders such as *A. gracilipes* and *Pheidole megacephala* (Wetterer 2005, 2012, Drescher et al. 2007).

Costs and benefits of worker reproduction in an invasive ant

The reproduction of *A. gracilipes* workers can be costly for the productivity and defence of the colony (Chapter 4), but the production of males by workers can also benefit orphaned colonies. I found that physogastric workers were more likely to have yolky oocytes than normal workers (e.g. 37% of physogastric workers in the aggression trials had yolky oocytes vs 2% of normal workers) and therefore had more reproductive potential (Chapter 4). Physogastric workers were rarely observed in the foraging area (5/100 observations vs 81/100 for normal workers) suggesting that they do not contribute to colony labour as much as normal workers. They were also less aggressive than normal workers during interspecific conflicts (median aggression score physogastric workers: 4, normal workers: 5, range both worker type: 0-5). Therefore, a high percentage of physogastric workers in a colony can potentially reduce the ability of a colony to defend itself and become behaviourally dominant. Sixty days after the start of my queen transfer experiment, physogastric workers represented 37.8±0.2% (mean ± SD) of workers in queenless colonies whereas they accounted for 7.8±0.1% of workers in queenright colonies (Chapter 4). These results suggest that queen control limits the percentage of physogastric workers in queenright colonies. According to kin selection theory, worker reproduction should not benefit queens because they are more related to their own sons than their grandsons (Hamilton 1964). When the queen dies, however, worker reproduction should benefit both workers and queens because worker-produced males are the last opportunity for workers and the deceased queen to contribute to the gene pool (Bourke 1988). Furthermore, if queen brood or virgin queens were present in the nest at the time of the queen's death, and did not inhibit worker reproduction, intranidal mating between worker-produced males and virgin queens could prolong the life of the colony. The ability of workers to switch to male production when the queen dies may therefore contribute to the invasive success of *A. gracilipes*.

The reproductive mode of *A. gracilipes* has long been a mystery but genetic results suggest that it is unusual and may involve gynandromorphy and/or clonal reproduction (Drescher et al. 2007, Gruber et al. 2012, 2013, Chapter 4). Gynandromorphs or sex mosaics combine the morphological features of males and females (Jones and Phillips Jr. 1985, Wcislo et al. 2004, Skvarla and Dowling 2014). Heterozygous males present in queenright (28.9%) and queenless colonies (4.9%) could be diploid or sex mosaics (Jones and Phillips Jr. 1985, Wcislo et al. 2004, Skvarla and Dowling 2014). It is unlikely that heterozygous males were all diploid as hymenopteran diploid males are often sterile (Cook and Crozier 1995) and heterozygous *A. gracilipes* males were found to produce viable sperm (Lee et al. 2017). Additionally, four of my male specimens had a potential gynandromorph phenotype (Chapter 4). As found in other *A. gracilipes* populations, most workers were heterozygous and queens homozygous which suggest that castes are determined by a genetic component for this species (Drescher et al. 2007, Thomas et al. 2010, Gruber et al. 2012, 2013, Lee et al. 2017, Chapter 4). This reproductive system could result in the production of workers with high levels of heterozygosity and contribute to the success of *A. gracilipes*. In the little fire ant or electric ant (*Wasmannia auropunctata*), some populations produce males and queens clonally and workers sexually (Foucaud et al. 2009, Chifflet et al. 2018). This clonal reproductive system results in a high proportion of heterozygous workers, which have been suggested to be better adapted to human-modified habitats and contribute to the expansion of *W. auropunctata* into disturbed habitats (Foucaud et al. 2009, Chifflet et al. 2018). The increase of genetic studies on the reproductive systems of ants has revealed that ant reproductive systems are far more variable than previously thought (Heinze 2008). For example, a recent study has revealed the existence of a mixed reproductive system in a *S. geminata* population in Florida (Lacy et al. 2019). In this population, most queens in polygyne colonies are produced asexually and workers sexually, whereas both castes are produced sexually in monogyne colonies (Lacy et al. 2019). Further research into the complex reproductive systems of ants will certainly reveal never-before described systems and will contribute to a better understanding of their role in the success of invasive ants.

Limitations and recommendations for future work

As with any research projects, my project had limitations which I will discuss here, along with recommendations for future research.

Chapter 1: Strategies of the invasive tropical fire ant (*Solenopsis geminata*) to minimize inbreeding costs

In my colony founding experiment, I chose not to let DMP colonies continue past the claustral phase (i.e. 23 days) because losing DMP replicates for my colony founding experiment would have increased the risk of being unable to detect differences between DMP and non-DMP colonies. Letting some of the DMP colonies continue past the claustral phase instead of stopping them all at 23 days could have brought further insights into the colony founding strategies of *S. geminata*. I may have been able to determine additional effects of diploid male production and identify whether there is a specific stage at which *S. geminata* colonies rear diploid males to adulthood instead of executing them.

An interesting follow-up experiment to my work would be to determine whether queens choose to found a colony on their own or associate with other queens in the field. The protocol of such an experiment could be modelled after Tschinkel (1998) in which newly mated *S. invicta* queens were offered to nest in pre-formed holes with or without another queen.

Chapter 2: Roles of biotic resistance and abiotic factors in the establishment of an invasive ant

One of the objectives of my queen and colony introduction experiment was to determine which factors affect the survival of *S. geminata* colonies past the claustral phase, but I was unable to reach a strong conclusion due to the low survival of colonies in the field (2% at one and 1% at three months). If I were to design a similar experiment, I would introduce colonies in containers from which the workers, but not the queen, could leave. I would model such containers after the big cages from my caged queen experiment and the field cage design of Sagata and Lester (2009). To distinguish between the effect of biotic resistance and abiotic factors on colony survival, I would experimentally remove the native ant community from half of the sites in which I would introduce colonies. I could also test the effect of propagule pressure on colony establishment by introducing different colony sizes as Sagata and Lester (2009) did in their *L. humile* introduction experiment. Considering the low survival rate of colonies after one and three months, I would monitor colony survival every seven days for three months instead of twice in three months to have sufficient power to determine which factors affected the survival of colonies over that period.

Chapter 3: Invasion pathways and social structure of the invasive tropical fire ant (*Solenopsis geminata*) revealed by genome-wide SNP data analyses

In this chapter, I prioritised using samples from the invasive range of *S. geminata* and samples collected in southwestern Mexico because this region had been identified by Gotzek et al. (2015) as the origin of the invasive populations. The cost of ddRADseq is still relatively high and if I had additional financial resources, I would have also used samples collected across the native range of *S. geminata* to determine geneflows between and among native populations and the social structure of colonies across the native range. Besides the cost of ddRADseq, one of the difficulties of using this technique is data analysis, which requires good skills in bioinformatics and access to high performance computing for SNP calling.

I successfully determined the origin, invasion history, geneflow, and social structure of *S. geminata* by analysing my ddRADseq SNP data *de novo* (i.e. without a reference genome). Using a reference genome for data analysis can increase the number of sequence data kept, although ddRADseq is more flexible and robust in region recovery compared to other RADseq approaches (Peterson et al. 2012, Andrews et al. 2016). The genome of *S. geminata* is currently being assembled (J. Wang, personal communication) and could be used to re-analyse my data and provide further information on the biology of *S. geminata*. For instance, a genome would provide information on the relative positions of the markers used, their relationships to genes and the potential gene functions (Nygaard and Wurm 2015). Sites with low individual and population variability could be identified and support the development of novel *S. geminata* control methods such as RNA interference (Taning et al. 2017). Additionally, I could determine whether a single amino-acid is responsible for the emergence of polygyne populations in *S. geminata* as previously suggested (Ross et al. 2003, Krieger and Ross 2005).

Another potential avenue of research would be to conduct a similar study to mine for multiple other invasive ant species to determine their origin and invasion pathways. The management of introduction pathways is a key challenge in the prevention of biological invasions (Hulme 2009). A comparative analysis of invasion pathways across invasive ants could serve as the foundation of pathway risk assessments for ant invasions.

Chapter 4: Origin, behaviour, and genetics of reproductive workers in the invasive yellow crazy ant *Anoplolepis gracilipes*

In this chapter, the low variability of microsatellite markers was limiting. For example, it is likely that adult males in queenless colonies originated from more than one worker given that each colony contained more than one physogastric worker with active ovaries (Lee et al. 2017, Chapter 4). However, I could only confirm that males were produced by more than one worker in a single queenless colony due to the low variability of male genotypes. Genome-wide sequencing methods were not available to me at the time of the laboratory work for this chapter, but they would certainly resolve the issues of low marker variability.

Further investigations into worker reproduction in *A. gracilipes* offer many opportunities for experimental work. For example, it would be interesting to conduct observations of queenless colonies to determine whether intranidal aggression occurs between physogastric workers for the right to reproduce and whether the attacker and attacked ant differ in sizes and cuticular hydrocarbon profiles as shown for *Aphaenogaster senilis* (Ichinose and Lenoir 2009).

Conclusion

Together, my findings improve our understanding of some of the foundational processes that influence the success of ant invasions such as genetic bottleneck, disturbance, anthropogenic dispersal, and reproductive strategies. In particular, I found that *S. geminata* queens can overcome diploid male production following a genetic bottleneck through two mechanisms: pleometrosis and execution of diploid male larvae. I have also shown that this ant is limited to disturbed habitats in the Northern Territory mainly by abiotic factors while biotic resistance may play a minor role in preventing its successful establishment. Using state-of-the-art genome-wide sequencing techniques, I found that anthropogenic-assisted dispersal has contributed to multiple geneflow events between and among virtually all my sampled invasive populations. Finally, my investigations of worker reproduction in *A. gracilipes* suggest that colony productivity and defence decrease when workers reproduce, and that queen control limits worker reproduction. Overall, my findings contribute to improving our knowledge of the biology and ecology of two highly invasive ant species for which baseline information has been lacking. Further investigations into the fascinating biology of invasive ants and the processes which influence

their successful establishment will provide crucial information towards preventing ant invasions and their effects.

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