Exploring the regulatory role of behaviour and genome architecture in the socially polymorphic ant, *Leptothorax acervorum*

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Abstract

Eusocial species show incredible variation in all aspects of social living, which has facilitated their ecological success. Investigating the mechanisms which regulate variation in social traits is an important goal for evolutionary biology, since understanding fundamental mechanisms underpinning variation can inform social evolutionary theory. In this thesis, I investigate aggressive behaviour and genome architecture as essential mechanisms in regulating variation in the polymorphic social phenotype of the multiple queened ant species *L. acervorum*.

I investigated the role of enforcement behaviour in maintaining reproductive skew in functionally monogynous colonies. I show that in the absence of worker aggression (enforcement), functionally monogynous queens continue to engage in highly aggressive interactions and, crucially, high colony skew was not affected. Furthermore, I show that low skew is not affected by aggressive worker enforcement in polygynous colonies. Therefore, enforcement behaviour is likely to be important in regulating skew in functionally monogynous colonies but not in polygynous colonies, where potentially the loss of sensitivity to enforcement may be an evolved response to fitness benefits associated with the polygynous social phenotype.

Furthermore, I investigated the role of genome architecture in regulating variation between the two social phenotypes. I scanned the genomes of four populations (two polygynous and two functionally monogynous) for extreme population differentiation (F_{ST}) at SNP loci, which were associated with a difference in the social phenotype. I found a large (6.2Mb) contiguous region associated with different social phenotypes (the social region), which mapped to LG2 on the *S. invicta* linkage map. The social region displayed some similarities with the social chromosomes in *S. invicta* and *F. selysi*. Furthermore, the region contained potential gene candidates, such as odorant binding proteins, which have been associated with divergent social phenotypes in *S. invicta*.

The work presented in this thesis highlights the importance of different mechanisms, both behavioural and genomic, in regulating variation in fundamental social traits. Furthermore, it demonstrates the importance of understanding how mechanisms can bridge the gap between genotype and phenotype.

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Common Abbreviations

P:	Polygyny. A type of social organisation.
M:	Monogyny. A type of social organisation.
FM:	Functional monogyny. A type of social organisation.
MQ:	Multiple queen.
Q-Q:	Queen-queen interactions.
W-Q:	Worker-queen interactions.
OT:	Orihuela del Tremedal. A Spanish population sample site.
V:	Valdelinares. A Spanish population sample site.
PF:	Pla de la Font. A Spanish sample site.
SD:	Santon Downham. A UK population sample site.
NF:	New Forest. A UK population sample site.
vg:	Vitellogenin.
elf:	Elongation factor. A reference gene.
ubiq:	Ubiquitin. A reference gene.
rps18:	Ribosomal protein S18. A reference gene.
PHS:	Pollen hoarding syndrome
RGPH:	Reproductive ground plan hypothesis
RADseq:	Restriction site associated DNA sequencing.
SNP:	Single nucleotide polymorphism.
F_{ST}:	Population differentiation.
SoS:	Signature of selection.
DH:	Divergence hitchhiking

Chapter 1

Review: genetic mechanisms underpinning variation in complex social phenotypes

1.1 Introduction to social evolution

Understanding the genetic mechanisms underpinning variation in complex social phenotypes and explaining how genotypes and genomic architecture translate into phenotypes are important goals of evolutionary biology and behavioural ecology. The social insects represent some of the most successful organisms found in nature and amongst them it is the eusocial insects of the Hymenoptera (bees, wasps, sawflies and ants) that represent the best examples of complex social living (Smith et al., 2008, Fischman et al., 2011, Strassmann and Queller, 2007, Hölldobler and Wilson, 1990, Wilson, 1987). Eusocial societies are highly structured and feature a reproductive division of labour separating a small reproductive class from the majority of the functionally sterile worker class, which has facilitated the evolution of extraordinary levels of complexity (Smith et al., 2008, Bourke and Franks, 1995, Hölldobler and Wilson, 1990, Strassmann and Queller, 2007, Bourke, 2011a).

The evolution of eusociality has occurred independently in nature at least 24 times depending on the definition (Bourke, 2011a). Furthermore, the leap from solitary to group living is widely accepted as representing a major transition in evolutionary history (Szathmary and Smith, 1995b, Szathmary and Smith, 1995a). It has been argued that individuality is first required before a major transition can occur and that individuality is achieved, by all species, through three

common principles (Bourke, 2011a). First, the individual of the previous transition forms a social group. Second, the group evolves processes that are necessary to maintain a stable social group (Bourke and Franks, 1995). Finally, the evolutionary scaling of social integration through the development of complex social phenotypes eventually allows the group to be considered as an individual for the purposes of the next major transition. It follows, therefore, that by gaining greater understanding of the evolutionary processes driving variation in the complexity of social phenotypes, we can begin to fully explain the processes involved in major transitions (Bourke, 2011a).

Indeed, many complex phenotypes have evolved amongst the social insects, which have facilitated their success as a social group. Examples include; agricultural farming of aphids or fungi (Mueller et al., 1998, Phillips and Willis, 2005), warfare (Hölldobler and Wilson, 1977), heterospecific enslavement (Herbers and Foitzik, 2002), worker diversity in the division of labour (Smith et al., 2008, Huang et al., 2013, Fjerdingstad and Crozier, 2006), variation in colony size (Bourke, 2011a), social organisation (Keller, 1995, Bourke and Franks, 1995) and intricate inter-personal communication (Smith et al., 2008). Investigating the underlining genomic architecture of complex social phenotypes and exploring how the physical apparatus (gene structure, linkage and chromosomal rearrangements etc.) is influenced by selection is crucial to fully understanding how complex societies evolve, are maintained and finally transform into individuality (Bourke, 2011a). Furthermore, it is important to understand how different mechanisms, both genetic and behavioural, can bridge the gap between genotype and phenotype and how variation within these mechanisms can impact upon the social evolution of the species (Fischman et al., 2011, Toth and

Robinson, 2009, Smith et al., 2008, Robinson et al., 2005, Robinson et al., 2008, Donaldson and Young, 2008, Keller, 2009).

Complex phenotypes often require the coordination of multiple physiological, morphological and behavioural adaptations. For example, fungal farming in the leaf-cutting ant *Acromyrmex echinatior* involves complex interaction between behavioural, morphological and physiological characteristics across tens of thousands of individuals within a colony (Nygaard et al., 2011, Mueller et al., 1998). Although there is currently a large body of theoretical and mathematical work attempting to explain the major evolutionary transition from independent to eusocial living (e.g. Queller, 2011, Nonacs, 2011b, Gardner et al., 2011, Bourke, 2011b, Abbot et al., 2011, Nowak et al., 2010, Foster et al., 2006, Linksvayer and Wade, 2005, Queller and Strassmann, 1998, Hamilton, 1964, Smith et al., 2008, Bourke, 2011a, Bourke and Franks, 1995), it is not yet clear how variation in complex social phenotypes involving multiple biological traits is determined by genetic and molecular factors (Fischman et al., 2011).

In this chapter, I review the current knowledge of how genomic architecture plays an essential role in evolving complex social phenotypes. I also explore how variation within social traits can exist and operate within and between social species and how this variation can impact upon fundamental life history traits including colony organisation, division of labour and skew. Finally, I introduce the ant species *Leptothorax acervorum* as an excellent model for investigating the genome architecture underpinning variation in a fundamental social trait.

1.2 Pleiotropy and Regulatory Gene Networks

It is generally accepted that variation in complex social phenotypes is under the control of multiple genes, since social behaviours often involve the integration of behavioural, physiological and morphological characteristics. However, it is becoming increasingly apparent that single or just a few genes might be responsible for the diversification of some social traits (Bourke, 2011a). Some of these genes have been shown to be pleiotropic and so pleiotropy may play a key role in the evolution of complex social traits such as cooperation (Wagner and Zhang, 2011, Foster et al., 2004).

Pleiotropy describes a situation in which a single gene affects two or more phenotypes and can occur when a gene produces multiple functional products, a single product that is used in multiple processes or a transcription factor that regulates multiple genes (Stearns, 2010). Pleiotropy is an important and ubiquitous feature of genetics and has significant roles to play in evolution (Waxman and Peck, 1998, Barton, 1990, Otto, 2004), development (Hodgkin, 1998), ageing and disease (Crespi, 2010, Brunner and van Driel, 2004, Kirkwood, 2005, Solovieff et al., 2013). Pleiotropic genes are also considered to operate within many behavioural syndromes, which are correlated suites of traits that vary between individuals across context (Jandt et al., 2014).

The pollen hoarding syndrome (PHS) in honeybees (*Apis mellifera*) represents one of the best studied social pleiotropic gene networks (Rueppell, 2014, Amdam and Page, 2010, Hunt et al., 1995, Page et al., 2000, Ruppell et al., 2004, Rueppell et al., 2004, Rueppell et al., 2006, Wang et al., 2009). It is a highly complex phenotype involving the behavioural, physiological and neurological integration of thousands of different larvae, foragers and nurses. Two honeybee

strains have been successfully selected for high and low pollen hoarding behaviours (Page and Fondrk, 1995), which has facilitated invaluable insights into the pleiotropic architecture of the social phenotype (Page et al., 2012). High and low pollen hoarding bee colonies vary in a range of traits at the individual and colony level. High pollen hoarding foragers initiate foraging behaviour earlier, are more likely to return to the colony with a pollen load, and collect significantly more pollen per load (Page et al., 2012, Hunt et al., 1995, Page et al., 2000, Ruppell et al., 2004). High pollen hoarders are also highly sensitive to sucrose concentration and, as a result, will preferentially forage for nectar with lower sucrose concentrations (Hunt et al., 1995, Ruppell et al., 2004, Pankiw et al., 2002). Sucrose sensitivity is a trait of age dependent foraging initiation, therefore a high sensitivity to sucrose fits with the early initiation of foraging behaviours present in high pollen hoarders (Page et al., 2012). Finally, recruitment of foragers to pollen sources (Dreller, 1998) and the frequency of pollen waggle dances by scouts (Waddington et al., 1998) are significantly higher amongst colonies of high pollen hoarders. These traits all form a sensible syndrome of behaviours, which work towards producing the pollen hoarding phenotype.

However, a number of other phenotypic differences, when taken at face value, are difficult to reconcile with the PHS have been found to correlate with a difference in high and low pollen hoarding bees. These traits cover a wide range of biological characteristics. For example, differences in the morphology have included; worker size (Linksvayer et al., 2009), high locomotor activity after emergence (Humphries et al., 2005) and a higher sensitivity to stimuli (Tsuruda and Page, 2009). Variation in physiology includes; longer developmental times and late pupation (Amdam and Page, 2010), shorter longevity (Amdam et al., 2007), larger ovaries (Amdam et al., 2006c) and differences in brain biochemistry (Humphries et al., 2003, Amdam and Page, 2010,

Page and Amdam, 2007, Amdam et al., 2007). Finally, behavioural differences have been recorded, such as an increased learning performance in high pollen hoarders (Scheiner et al., 2001). These findings highlight the far reaching effects of pleiotropy upon systems outside of the social phenotype and how changes in a few genes can influence the diversification of not just highly complex social phenotypes but also fundamental life history traits. For example, candidate genes associated with the PHS include; the egg yolk precursor vitellogenin (Amdam et al., 2003a), a nuclear hormone receptor (Wang et al., 2009), a phosphoinositide-dependent kinase (Wang et al., 2009) and multiple genes involved in the insulin-like signalling pathway (Hunt et al., 2007, Amdam et al., 2007). All are likely to have pleiotropic effects within the PHS with particular emphasis on vitellogenin, which has been reported to affect multiple traits including; gustatory responsiveness, foraging specialisation and the age of transition from one foraging preference to another (Ihle et al., 2010, Amdam et al., 2007, Amdam et al., 2006b). Therefore, the presence of additional unwanted correlated traits associated with the principle role of the PHS highlights the potential evolutionary constraints associated with pleiotropy as adapting one trait may impact negatively on another.

Pleiotropy has clear advantages when diversifying complex social phenotypes as selection on a few pleiotropic genes has the potential to effectively link a complex suit of genes together. However, there have been many studies focused on the trade-offs that result from correlated traits and the evolutionary constraints due to genetic co-variance (Agrawal and Stinchcombe, 2009, Price and Langen, 1992, Wagner and Zhang, 2011). In essence, changes in any given trait are likely to impact upon all correlated traits within the pleotropic network and therefore influence the overall fitness of the phenotype (Rueppell, 2014, Jandt et al., 2014). For instance, colonies of

Pogonomyrmex barbatus vary in their foraging behaviour, which impacts upon the colony food storage potential and the amount of foraging conducted on a particular day, ultimately affecting colony fitness (Gordon et al., 2011). Within the context of behavioural phenotypes, constraints associated with pleiotropy should lead to a reduction in plasticity (Jandt et al., 2014, Sih et al., 2004), which should promote the coinheritance of correlated traits. However, this can cause potential problems in social insects because social insect colonies are affected by additional levels of constraint due to individual and colony levels of selection (Jandt et al., 2014). Behavioural plasticity is required when individuals are highly related and vary in caste, which suggests that plasticity in social insects might be an evolved response to limit the constraints of pleiotropy (Jandt et al., 2014, Gadagkar, 1997).

1.2.1 Gene regulatory networks

Social insects provide powerful models for comparing the activation of gene regulatory networks (GRNs) and elucidating the developmental mechanisms and genomic architecture of complex social phenotypes (Fischman et al., 2011). A GRN describes the connections and regulatory logic between a sequence of regulatory elements and target genes (Linksvayer et al., 2012). Many of these genes are pleiotropic and therefore influence many downstream targets of the network (Molodtsova et al., 2014). Furthermore, the strength and type of selection has been shown to vary between elements depending on their position within the network, with the more pleiotropic genes experiencing the strongest negative selection (Molodtsova et al., 2014).

Many powerful genomic techniques are now available, which allow cross comparisons between a wide range of divergent lineages (Fischman et al., 2011). They include; whole-genome comparisons, candidate gene approaches and comparative analysis of protein coding sequence

(Fischman et al., 2011). Whole genome comparisons are especially useful in revealing new molecular targets associated with social phenotypes. Indeed, at present, there are eight known available genomes for eusocial species including the honeybee (Weinstock et al., 2006), three *Nasonia* parasitoid wasps (Werren et al., 2010) and seven ant species (Bonasio et al., 2010, Wurm et al., 2011, Smith et al., 2011b, Smith et al., 2011a, Nygaard et al., 2011, Suen et al., 2011), which hold vast potential in revealing insights into the genomic architecture underpinning complex social phenotypes (Gadau et al., 2012).

Important candidate genes involved in GRNs have been associated with variation in social phenotypes in social insects. In the following sections, I describe in detail how two of the best studied genes, *vitellogenin* and *foraging*, operate within their networks to facilitate phenotypic variation.

1.2.2 Vitellogenin: roles in foraging

Amongst the worker caste of some social insect species, *vitellogenin* (*vg*) has been linked to age-related foraging behaviour, a behavioural phenotype completely decoupled from its established roles in reproduction (Page et al., 2012, Ihle et al., 2010, Amdam and Page, 2010, Amdam et al., 2006a, Amdam et al., 2004, Amdam and Omholt, 2003, Amdam et al., 2003a, Nelson et al., 2007, Amdam et al., 2003b). Importantly, the association with *vg* expression and foraging behaviour has evolved independently using different mechanisms. For example, the differential expression of a single *vg* gene is linked to variation in foraging behaviour in honeybees (Ihle et al., 2010, Amdam and Page, 2010, Nelson et al., 2007) and in some ants, variation in foraging behaviour is underpinned by the differential expression of genotypically diverged *vg* duplicates (Morandin et al., 2014, Corona et al., 2013, Wurm et al., 2011).

Vitellogenin codes for an egg yolk storage precursor, which is important for the maturation of eggs in the ovary and its expression is often associated with the activation of the ovaries in queens before reproduction (Tufail and Takeda, 2008). More specifically in insects, vitellogenin is synthesised by the fat body and released into the haemolymph where it travels to the ovaries (Tufail and Takeda, 2008). The importation of *Vg* into the ovaries is achieved via receptor-mediated endocytosis and to date a number of different vertebrate and invertebrate *Vg* receptors have been characterised (Chen et al., 2004, Tufail and Takeda, 2008). *Vitellogenin* has been found to be highly conserved amongst many different taxa (Spieth et al., 1991, James et al., 1982), although multiple copies of the Vg gene are known to exist in some ant species (Wurm et al., 2011, Morandin et al., 2014, Corona et al., 2013).

Early studies on vitellogenin (*vg*) expression in social insects were conducted on honeybees (*Apis mellifera*) and focused on variation in foraging behaviour (Amdam and Omholt, 2003, Amdam et al., 2003a). Initially, *vg* was shown to play a key role in determining the transition between nest based nursing and foraging behaviour in worker bees (Nelson et al., 2007). RNAi knock down of *vg* expression in 5-7 day old worker bees caused early initiation of foraging behaviour compared to the controls, which demonstrated that *vg* was acting to inhibit the transition from nest tasks to foraging (Amdam et al., 2003b, Nelson et al., 2007). Further observations revealed that *vg* knock-down bees placed a significant bias on nectar collection in comparison to control bees of the same age (Nelson et al., 2007). Mechanistically, variation in food bias was associated with an antagonistic relationship between Vg and JH titre, the balance of which biased workers towards either pollen or nectar gathering (Ihle et al., 2010, Nelson et al.,

2007). Furthermore, the pleiotropic nature of Vg was demonstrated when observations of high *vg* expression in foragers also correlated with a higher sensitivity to sucrose (Tsuruda et al., 2008).

Vitellogenin expression in conjunction with genotypic differentiation plays a similar regulatory role in the division of labour in the fire ant, *Solenopsis invicta* (Gadau et al., 2012, Wurm et al., 2011) and the harvester ant, *Pogonomyrmex barbatus* (Corona et al., 2013). Both *S. invicta* and *P. barbatus* have been shown to possess multiple copies of *vg*, which are differentially expressed between workers and queens (Wurm et al., 2011, Corona et al., 2013). Differential expression between Vg copies was also found to occur between nurses and foragers in *P. barbatus* (Corona et al., 2013). Furthermore, multiple *vg* copies were also found in seven *Formica* species (Morandin et al., 2014). However, differential expression for each *vg* copy was highly variable between castes, with the exception of Vg-like-C which was consistently worker biased (Morandin et al., 2014). Interestingly, the study showed that newly duplicated Vg copies experienced positive selection, leading to the acquisition or loss of protein coding domains (Morandin et al., 2014). This demonstrates that genotypic changes within a pleiotropic gene and not just gene expression differences, can be important in driving adaptation in new social phenotypes (Morandin et al., 2014).

1.2.2.1 Evolutionary co-option of the reproductive gene network to diversify social behaviour

Explaining the evolutionary mechanism behind the pleotropic effects of *vg* in promoting variation in foraging behaviour has been difficult since conserved reproductive physiology is not traditionally associated with behaviour (Amdam and Omholt, 2003, Amdam and Page, 2010, Amdam et al., 2004). The main evolutionary problem postulates that selection should repress

reproductive traits within the worker caste as organisms adopt greater degrees of sociality since complex societies cannot arise if there is significant conflict over reproduction (Khila and Abouheif, 2008). However, the reproductive ground plan hypothesis (RGPH) in tandem with double repressor hypothesis (DRH) (Amdam and Page, 2010, Amdam and Omholt, 2003, Amdam et al., 2004) has emerged as the leading theory explaining how reproductive traits within the worker caste can be co-opted through selection to perform a behavioural function rather than be repressed.

The RGPH argues that female reproductive biology can act as a potent source of behavioural adaptation by acting as a target for natural selection, which then has the flexibility to achieve the advanced levels of labour specialisations seen amongst sterile workers (Amdam et al., 2004). In essence, the existing reproductive regulatory scaffold that in solitary ancestors and queens regulated reproductive biology is co-opted by selection to regulate division of labour in workers. This is achieved mechanistically through an antagonistic interaction with Vitellogenin (Vg) and Juvenile Hormone (JH), which regulates the temporal expression of foraging behaviour as described by the DRH (Amdam and Omholt, 2003). Initially, high Vg expression in young nurse bees represses the expression of JH. However, Vg expression naturally decreases over time as workers get older (similarly to queen bee reproduction), which disinhibits the expression of JH. Therefore, the increase in JH expression further suppresses Vg expression and leads to high titres of JH in older bees, which correlates with the transition from nurse tasks to foraging tasks (Ihle et al., 2010, Amdam and Page, 2010, Amdam and Omholt, 2003).

Tests of the RGPH and DRH have successfully used RNAi knockdown on strains of high and low pollen-hoarding bees to test the validity of the DRH and the role of Vg titre (Ihle et al., 2010,

Amdam et al., 2003b, Amdam and Omholt, 2003, Nelson et al., 2007, Guidugli et al., 2005). As expected high Vg titre was repeatedly associated with the inhibition of foraging behaviour and that inhibition is released and foraging behaviour initiates early in Vg knockdown bees (Ihle et al., 2010, Nelson et al., 2007, Amdam et al., 2007).

However, variation in the sensitivity to Vg titre and its effects on foraging behaviour does exist between different strains of bees. Bees artificially selected for low pollen-hoarding behaviour showed no significant difference in foraging onset in the presence of Vg knockdown when compared to controls (Ihle et al., 2010). Although it is not known precisely which factors are responsible for sensitivity to Vg titre, it is likely that there is some overlap between them and the endocrine apparatus of the DRH (Ihle et al., 2010). Furthermore, anarchistic bees which show elevated levels of worker reproduction and are likely to possess a high Vg titre, showed no bias towards pollen foraging and initiated foraging behaviour significantly later in comparison to wild type bees (Oldroyd and Beekman, 2008). However, the reproductive biology of anarchistic bees has been selected to overcome queen released pheromones, which suppress oviposition (Amdam and Page, 2010, Tsuruda et al., 2008), and is therefore different to the selection regime of wild type honeybees. Consequently, different selection regimes can significantly affect the phenotypic outcome of gene regulatory networks like the DRH. Importantly, this highlights the possibility for variation within these ancestral frameworks to exist, allowing the diversification of traits within complex social phenotypes.

1.2.3 *Foraging*: roles in foraging behaviour and behavioural plasticity

Foraging (*for*), is a cGMP-activated protein kinase gene (PKG) and has become widely associated with behavioural plasticity in foraging behaviour across many diverse taxa (Tobback et

al., 2011, Lucas and Sokolowski, 2009, Tobback et al., 2008, Fujiwara et al., 2002, Ben-Shahar et al., 2002, Osborne et al., 1997, Hofmann et al., 2006, Hong et al., 2008, Ingram et al., 2005, Ben-Shahar et al., 2003, Lucas et al., 2010). Behavioural plasticity is not only important as a response to environmental pressures (West-Eberhard, 1989), but it is also important in the division of labour in social insects (Seid and Traniello, 2006). Furthermore, the pleiotropic effects of *foraging* have also been found to be highly diverse, differing in function, mechanism, genotype and expression across varied taxa (Ingram et al., 2011).

1.2.3.1 Genotypic differences can underpin variation in foraging behaviour and regulate behavioural plasticity

The *foraging* gene (*for*)was initially shown to be involved in the foraging behaviour of larvae in *D. melanogaster* (Osborne et al., 1997). Differences in the rates of foraging behaviour were associated with a polymorphic allele, with possessors of the "rover" allele expressing higher rates of foraging behaviour than possessors of the "sitter" allele (Pereira and Sokolowski, 1993, Debelle and Sokolowski, 1987, Sokolowski et al., 1997, Sokolowski, 1980, Debelle et al., 1993, Debelle et al., 1989, Osborne et al., 1997). Furthermore, alternate alleles had associated expression differences, with upregulated *for* in "rover" flies in comparison to "sitter" flies, suggesting that genotypic differences in the structure of the *for* gene caused differential expression, which ultimately translated into the observed variation in foraging behaviour.

In social insects, the *foraging* gene ortholog *Amfor* has been heavily implicated in the behavioural division of labour (Tobback et al., 2011, Lucas and Sokolowski, 2009, Tobback et al., 2008, Ingram et al., 2005, Ben-Shahar, 2005), which is a fundamental aspect of eusociality (Smith et al., 2008). Crucially, it is differential expression in an evolutionary conserved gene that

determines variation in foraging behaviour and not genotypic differences as seen in *D. melanogaster* (Ingram et al., 2011). Honeybee workers initiate foraging behaviour at different life stages, which has been shown to be correlated with differential levels of *Amfor* expression (Ben-Shahar, 2005, Ben-Shahar et al., 2002). Upregulated expression of *Amfor* is associated with the transition from nursing to foraging and experimental treatment of one day old workers with cGMP protein (the gene product of *Amfor*) caused them to switch to a behavioural phenotype typified by older workers (Ben-Shahar et al., 2002). Similar results have been found in populations of bumblebees (*Bombus terrestris*), which show an age and size related mode of division of labour. In these populations, older and larger foragers were found to have elevated levels of *foraging* expression than the much smaller and younger nurse bees (Tobback et al., 2011).

Interestingly, the relationship between foraging expression and worker task has been shown to be reversed in a harvester ant species, *Pogonomyrmex barbatus* (Ingram et al., 2011, Ingram et al., 2005). The harvester ant *foraging* ortholog, *Pbfor*, was found to be expressed more highly in workers not performing foraging tasks such as brood care (Ingram et al., 2005), although these results may be explained by differences in temporal expression (Ingram et al., 2011). Similar results have also been found in *Vespula vulgaris* wasps and *Pheidole pallidula* ants (Tobback et al., 2008, Lucas and Sokolowski, 2009). In both cases, workers involved in non-foraging related tasks were found to up-regulate *foraging* orthologs when compared to the dedicated foragers. These are interesting conclusions as they suggest that natural selection has operated on the same gene across species to regulate behaviourally similar tasks but in somewhat different ways (Lucas and Sokolowski, 2009).

1.2.3.2 The pleiotropic role of foraging in gene regulatory networks

Foraging and its orthalogs have been shown to influence multiple physiological and behavioural traits beyond foraging behaviour in many insect species (Reaume and Sokolowski, 2009, Hofmann et al., 2006, Mery et al., 2007, Kaun et al., 2007, Belay et al., 2007). Numerous associations with traits not linked directly to the foraging behavioural phenotype have been found in *D. melanogaster*, including; sensory habituation, sucrose responsiveness, stress tolerance, olfactory and visual learning and sleep patterns (Mery et al., 2007, Kaun et al., 2007, Belay et al., 2007). Similarly, mammalian orthologs *cGKI* and *cJKII* have been shown to be involved in nociception responses, learning and memory, and circadian rhythmicity (Reaume and Sokolowski, 2009, Hofmann et al., 2006).

The pleiotropic associations with *foraging* expression and its functional role as a cGMP-dependant kinase strongly suggests that it plays an important role within one or more gene regulatory networks (Hofmann et al., 2006). However, variation in the pattern of *foraging* expression seen across social insect species suggests that evolutionary changes to factors within the gene regulatory networks (GRNs) themselves are responsible for diversifying foraging behaviour rather than sequence changes to the protein itself (Robinson and Ben-Shahar, 2002), since foraging orthologues are highly conserved (Ingram et al., 2011, Ingram et al., 2005). One example can be seen in the association between foraging behaviour and phototaxis in honeybees (Ben-Shahar et al., 2003). *Amfor* expression is upregulated in the lamina of the optic lobes and the mushroom bodies of the brain (Lutz et al., 2012, Ben-Shahar et al., 2002), suggesting that gene regulatory networks involved in phototaxis also regulate foraging behaviour in honeybees (Ben-Shahar et al., 2003). Supporting studies revealed that nurses and foragers are negatively and

positively phototactic respectively (Menzel and Greggers, 1985, Southwick and Moritz, 1987). *Amfor* expression was upregulated in the positively phototactic "undertaker" workers (Ben-Shahar et al., 2003). Finally, young nurse bees treated with cGMP showed a clear transition from negative to positive phototaxis, supporting the association between *Amfor* expression, foraging behaviour and phototaxis (Ben-Shahar et al., 2003).

Recent studies of the differential expression of the harvester ant cGMP ortholog, *Pofor,* demonstrated that gene expression differed not only between individual workers but also temporally (Ingram et al., 2011, Ingram et al., 2005). It was shown that *Pofor* expression remained constant with workers that remained within the nest but fluctuated substantially within the foragers, with the peak levels of expression coinciding with mid-day when foragers are most active. Similarly, the consistent expression of *Pofor* in nest workers relates to their 'round the clock' locomotor activity (Ingram et al., 2009). These findings indicate a quantitative function for *Pofor*, where division of labour and task specificity is controlled by the quantity of protein present. Furthermore, the expression pattern of *Pofor* correlates with the pattern of foraging behaviour in different worker castes, demonstrating the potential for *Pofor* expression patterns in regulating behavioural plasticity (Ingram et al., 2011), although it must be reiterated that correlation does not equal causation and as of yet it is not known how cGMP expression directly regulates foraging behaviour. Furthermore, It is not yet known whether *Pofor* is expressed in different regions of the brain, or whether this discretion in expression is age related (Ingram et al., 2011).

However, behavioural plasticity has been linked to *foraging* expression at a specific location within the brain of the desert locus, *Schistocerca gregaria* (Lucas et al., 2010). Solitary locusts form large aggregations when food sources become scarce and undergo wide behavioural,

physiological and morphological changes to transist into a gregarious form (Pener and Simpson, 2009). Lucas *et al.* (2010) showed that PKG activity was greatly increased in the brain PI cells of gregarious locusts, which in turn correlated significantly with the aggregation behaviour. They hypothesised that due to the close proximity of the PI cells to the major neurosensory centres, multiple pathways of gene action are available in influencing behavioural expression. These include the role of PKG in response to environmental stress, circadian rhythmicity and phototaxis behaviours and the linkage between PKG and SERT (a serotonin transporter) activity (Lucas et al., 2010).

1.3 Aggression; a polygenic behavioural trait regulated by variation in genotypes and expression

Aggression is an interesting social trait, which has been shown to vary widely both within and between different eusocial insect species (Cournault and Peeters, 2012, Alaux et al., 2009b, Keller and Ross, 1998, Gill and Hammond, 2011a, Gill and Hammond, 2011b, Tibbetts and Reeve, 2000, Whitehouse and Jaffe, 1996). Aggression can arise between two individuals in a variety of different contexts and its purpose is often associated with the maintenance and enforcement of a eusocial organisation (Ratnieks and Wenseleers, 2008, Ratnieks and Helantera, 2009), reproductive dominance (Tibbetts and Reeve, 2000, Cournault and Peeters, 2012) or defending the nest from intruders (Alaux et al., 2009b, Whitehouse and Jaffe, 1996). It is known to be polygenic in nature, requiring the expressive input of many genes to complete the final phenotype (Nelson, 2005). Interestingly, variation in aggressive behaviour in social insects has been explained via heritable genotypes as seen in 'green beard' genes in *Solenopsis invicta* (Keller and Ross, 1998, Gardner and West, 2010) or via environmental cues which alter gene expression (Alaux et al.,

2009b, Rittschof and Robinson, 2013). Furthermore, as I will explain, variation in aggression may be explained via genes or gene expression that predispose the individual to more aggressive behaviour, or regulate sensitivity within the individuals to signals that provoke aggression.

1.3.1 Inherited heightened aggression and social environmental effects

Evidence in honeybees suggests that genes associated with variation in behavioural aggression can become fixed as heritable traits through gene regulatory mechanisms as well as being influenced through the social environment via changes in gene expression (Alaux et al., 2009b). Cross fostering of European honeybees (EHB) and highly aggressive Africanised honeybees (AHB) revealed that both the social environment and individual genotype played a significant role in the levels of aggression shown (Alaux et al., 2009b). Furthermore, AHBs reared within EHB colonies display significantly lower levels of aggression but are still significantly more aggressive than native EHBs and vice versa (Hunt et al., 2003, Guzman-Novoa et al., 2004). Interestingly, 30% of the variation in brain gene expression was associated with individual genotypic differences and a further 25% of the variation was explained by the social environment (Alaux et al., 2009b). Significant overlap of genes inherent within AHB's, EHB's exposed to alarm pheromone and old EHB's was also observed, supporting the polygenic nature of aggression (Alaux et al., 2009b). Old honeybees are known to exhibit much higher levels of aggression than young bees and so it was not surprising that shared genes were involved in behavioural aggression. Shared genes included *moody* and *Cyp6Q1*, which have been shown to be associated with aggressive behaviour in rodents (Soma et al., 2008) and *Drosophila* respectively (Drnevich et al., 2004). Taken together, these findings suggest that regulation of behavioural aggression may be controlled by a complex relationship between heritable genes that promote higher aggression

and social environmental factors that influence gene expression (Alaux et al., 2009b). Thus both the genotype and the social environment are both important in determining complex polygenic behavioural traits.

Furthermore, transcriptomal analysis also revealed a common robust molecular signature associated with aggression regardless of either inherited or environmental origin (Alaux et al., 2009b). Results showed that 10% of the genes commonly expressed by AHB were also up regulated in EHB when stimulated with alarm pheromone (Alaux et al., 2009b). Therefore, the AHB heritable phenotype was akin to an environmental response to alarm pheromone in EHBs (Alaux and Robinson, 2007), suggesting fixation of an ancestral plastic phenotype. Stimulation by alarm pheromone induces two temporally separate effects in individual bees (Alaux and Robinson, 2007). The first is an immediate short term aggressive response, which appears to be rapidly acted upon by transcriptional mechanisms and the second is a more long term sensitisation to alarm pheromone, which elicits a quicker response upon future contact (Alaux et al., 2009a, Robinson, 1987). Therefore, constant stimulation coupled with the long term sensitisation effects of alarm pheromone exposure could explain how behavioural aggression can become fixed as a heritable trait in AHBs through the action of a gene regulatory mechanism (Alaux et al., 2009b).

Indeed, Waddington's model of "genetic assimilation" explains how phenotypic evolution can precede assimilation of plastic responses to the environment (West-Eberhard, 2005, Waddington, 1942, Waddington, 1953). Changes in the expression of polygenic regulatory mechanisms have the potential to profoundly alter the phenotype, which may be selected for, ultimately leading to reproductive isolation and fixation (West-Eberhard, 2005). This idea may

hold true for the evolution of aggressive behaviour in honeybees and explain the variation seen between the European and Africanised subspecies.

1.3.2 Sensitivity to recognition signals and directed aggression

Variation in aggression need not necessarily be regulated by inherent tendencies to act aggressively but may also be regulated by variation in an individual's sensitivity to aggression inducing signals. An important social context for the application of aggression is in resolving conflict over reproduction. Many ant species are either polygynous (Bourke and Franks, 1995, Keller, 1995), containing multiple mated queens, or polyandrous, where a single queen is mated with multiple males (Stürup et al., 2014). In both cases, the within colony relatedness is reduced, which reduces the indirect benefits that workers receive from rearing queen-laid offspring. In theory, this situation should select for a gene that allows workers to recognise (via kin recognition signals) and preferentially direct their help towards closely related kin, a condition called nepotism. However, despite the fact that social insects are well known for their ability to detect nestmates from non-nest mates using blends of cuticular hydrocarbons (ChCs), there is as yet no conclusive evidence to support within colony kin discrimination through kin recognition signals (Holzer et al., 2006, Dani et al., 2004, Wensleers, 2007).

However, it has been suggested that 'green beard' genes might provide a heritable mechanism that allows nestmates to preferentially help closely related kin (Nonacs, 2011a, Gardner and West, 2010, Dawkins, 1976, Dawkins, 1982). Green beard genes are categorised by three major defining qualities. First, they cause a phenotypic effect. Second, they enable the bearer of the gene to recognise homologs in other individuals. Finally, individuals bearing the gene behave differently towards other individuals, which either bear or do not bear the same

green beard gene (Gardner and West, 2010, Dawkins, 1982, Dawkins, 1976). Four different categories of green beard genes can exist; facultative helping, facultative harming, obligate helping or obligate harming (Gardner and West, 2010). The facultative element suggests the individual has flexible control over its behaviour towards conspecifics either carrying the green beard gene or not. The obligate element describes the opposite, whereby the specific behaviour is constantly expressed to all conspecifics regardless of green beard status (Gardner and West, 2010). Despite robust discussion on the hypothetical action of green beard genes (Dawkins, 1982, Dawkins, 1976, Nonacs, 2011a, Gardner and West, 2010), the existence of a real example in social insects was not provided until 1998 when a seminal study conducted by Keller and Ross showed a direct relationship between the gene *Gp-9* and aggressive behaviour in *Solenopsis invicta*, which regulated variation in the number of reproducing queens tolerated within the colony (Keller and Ross, 1998, Ross and Keller, 1998).

These early studies focused on introducing recently reproductive female queens to polygynous colonies and then recording the aggressive behaviour they received from native workers (Ross and Keller, 1998, Keller and Ross, 1998). The results indicated that the *Gp-9* genotype predicted the level of worker aggression each queen received. Specifically, queens possessing the $Gp\text{-}9^{BB}$ allele were the singular recipients of aggressive behaviour from workers, which ultimately led to their deaths. Furthermore, studies on worker genotypes found that aggressive workers were highly likely to possess the *b* form allele (Ross and Keller, 1998, Keller and Ross, 1998). It was hypothesised that the *b* allele was acting as a "green beard", allowing bearers to recognise other bearers and directing aggression towards all non-bearers. Interestingly, queens receiving aggression from workers were always killed and so the *b* allele was able to

quickly spread within the colony, ultimately affecting the reproductive skew and the reproductive strategy (Ross and Keller, 1998, Keller and Ross, 1998). Crucially, heterozygous *Bb* workers were ruled out as being more aggressive than *BB* homozygotes since they did not alter their aggressive behaviour towards ant control queens from an alternative species (Keller and Ross, 1998). This meant that workers possessing alternative genotypes at the *Gp-9* locus were not naturally more aggressive, but instead conspecifics were capable of recognising allelic variation between one another and directing behavioural responses as dictated by the recipient's genotype at the *Gp-9* locus (Keller and Ross, 1998).

In support of the hypothesis that genotypic variation at the *Gp-9* locus affected chemosensory recognition of conspecific genotypes, it was discovered that the product of *Gp-9* was an odorant binding protein (Krieger and Ross, 2002). The product was found to be 153 amino acids long in its native form and is a member of a fairly diverse family of insect odorant binding proteins (Krieger and Ross, 2002), some of which have been shown to possess important functions within the peripheral chemosensillia (Xu, 2005). The structural formation of the b-like and B-like protein products differ in their affinity to these pheromones, which in turn determines the specificity on which worker tolerance towards queens is based (Gotzek and Ross, 2009, Krieger and Ross, 2002, Krieger, 2005).

The *Gp-9* locus appears to act as a facultative harming green beard, since allelic variation leads to a divergent phenotype, bearers can detect co-bearers and bearers help co-bearers by behaving aggressively towards non-bearers (Gardner and West, 2010). These findings demonstrate how variation in recognition and chemosensory traits can translate into complex patterns of aggression, which ultimately shape the future social organisation of the colony.

Considering the commonality of polygynous ant species in nature (Bourke and Franks, 1995), it is possible that many of these species evolved polygyny through rapid fixation of green beard genes at their recognition loci, a phenomenon that is difficult to detect in nature. A similar prediction explains the low aggression and low relatedness within unicolonial ant species (Nonacs, 2011a). Furthermore, although it is currently unknown exactly how prevalent green beards are in nature, a recent modelling study predicted that altruism through green beard chromodynamics is stable and implied that the green beard effect is potentially common in nature (Jansen and van Baalen, 2006) Regardless, the example of *Gp-9* in *S. invicta* demonstrates the potential for green beard genes to play important roles in the evolution and diversification of complex social phenotypes.

1.4 Supergenes can underpin variation in complex social phenotypes

Social organisation is a highly complex phenotype, which refers to the number of individuals in a social group, their behavioural and genetic relationships and their reproductive skew (Ross and Keller, 1995). Eusocial insects have been shown to display wide inter and intra species variation is all aspects of social organisation (Wilson, 1971, Sherman et al., 1995, Keller and Perrin, 1995, Bourke and Franks, 1995). Variation in queen number is a particularly interesting aspect of social organisation as it directly impacts upon within colony relatedness, correlates with ecological constraints and is often associated with a suite of additional traits (Ross and Keller, 1995, Keller, 1995, Purcell and Chapuisat, 2013, Chapuisat et al., 2004, Bourke and Franks, 1995, Bourke and Heinze, 1994, Keller, 1991, Boulay et al., 2014). In particular, traits associated with polymorphic social organisation might include: differences in colony founding and dispersal (Ross and Keller, 1995, DeHeer et al., 1999), morphological and physiological differences in queens (Keller and Ross, 1999, Keller and Ross, 1995) and differences in queen tolerance behaviour

(Keller and Ross, 1998). Understanding the causal mechanisms for variation in social organisation and why particular traits co-adapt can provide insights into how complex phenotypes intrinsically linked with sociality can evolve in response to environmental and social selection (Ross and Keller, 1995).

A significant challenge posed to the elaboration of social organisation is the necessity to adapt and combine multiple characteristics into a complex phenotype. To evolve a complex phenotype, two or more adapted traits must be selectively advantageous when inherited together (Thompson and Jiggins, 2014). However, recombination can impose a high selective cost to the formation of complex phenotypes as co-adapted alleles become broken up, resulting in substandard phenotypes (Thompson and Jiggins, 2014). Therefore, the fitness of co-adapted alleles in complex phenotypes can be increased with a genomic architecture that favours the co-inheritance of favourable allele combinations and represses the negative effects of recombination (Thompson and Jiggins, 2014).

Supergenes are suites of tightly linked genes that regulate the expression of multiple related traits. Their primary advantage is that co-adapted traits are tightly linked, which reduces the tendency for recombination to break up advantageous allele combinations (Thompson and Jiggins, 2014, Schwander et al., 2014). Supergenes have typically been associated with cases where a balanced polymorphism in a complex phenotype appears to have a simple genetic basis (Thompson and Jiggins, 2014). For example, the best known cases of supergenes are seen in the multiple independent evolutions of sex chromosomes, where a mutation occurs which causes the bearer to preferentially develop into a male or female (Charlesworth et al., 2005, Bachtrog et al., 2011). Alleles that are advantageous in one sex, but detrimental to the other sex (sexually

antagonistic), at associated loci lead to selection for decreased recombination between these sexually antagonistic genes and the sex determination locus (Kirkpatrick and Guerrero, 2014).

Tight linkage of co-adapted alleles and suppression of recombination can be achieved through a number of different ways. First, alleles located in close physical proximity on the chromosome to one another are far more likely to be inherited together. Second, certain regions of a chromosome are characterised by low recombination, such as the centromere and hetero-chromatic regions. Third, structural differences between homologous chromosomes can make cross-over impossible. For example, the supergene can be entirely absent in the wild type or the supergene can be inverted (Schwander et al., 2014).

Recently, supergenes have been shown to be key in maintaining a balanced polymorphism in social organisation in two ant species, *Solenopsis invicta* (Wang et al., 2013) and *Formica selysi* (Purcell et al., 2014). Populations of these two ant species can come in one of two social forms which differ in their social organisation, namely their tolerance for multiple reproductive queens. Colonies can be monogynous where only one reproductive queen is tolerated or polygynous where queen tolerance is higher, allowing multiple reproductive queens. Each social phenotype is further associated with a syndrome of additional co-varying biological traits, which have adapted to promote the success of the social phenotype. Colonies of *S. invicta* possessing alternate social phenotypes differ in their level of inter-colony aggression, colony founding, worker size, queen fecundity, mature queen odour and fat deposition (Lawson et al., 2012, Krieger and Ross, 2002, DeHeer et al., 1999, Keller and Ross, 1998, Keller and Ross, 1993, Keller and Ross, 1999, Keller and Ross, 1995). Similarly, colonies of *F. selysi* differ in queen size and queen dispersal, colony life span, colony size, the allocation to offspring and brood development

time (Purcell et al., 2014, Rosset and Chapuisat, 2006, Rosset and Chapuisat, 2007, Schwander et al., 2005). These traits are always inherited together with their associated social phenotype, strongly suggesting tight linkage within a suite of genes (Wang et al., 2013).

Studies revealed large non-recombining regions in both ant species that spanned significant portions of a single chromosome, which were termed social chromosomes (Purcell et al., 2014, Wang et al., 2013). In the case of *S. invicta*, the social chromosome was found to contain two chromosomal inversions totalling approximately 13Mbs (55% of the chromosome), which effectively nullify recombination between the two social genotypes (Wang et al., 2013). Furthermore, the largest non-recombining region (9Mb) contained genes that were differentially expressed between the two social forms (Wang et al., 2013). *F. selysi* was also found to possess a social chromosome with a large non-recombining region but the region was shown not to be homologous with the *S. invicta* social chromosome (Purcell et al., 2014). Both cases of the social chromosome draw parallels to sex chromosomes. First, similar to how the Y chromosome is only present in males, the social chromosome is only present in one of the two social organisations. Second, the *S. invicta* social chromosome showed an accumulation of mildly deleterious mutations and repetitive elements similar to Y sex chromosome evolution (Charlesworth et al., 2005, Wang et al., 2013) and the *F. selysi* social chromosome showed reduced polymorphism (Purcell et al., 2014).

Crucially, both examples of ant supergenes have evolved independently indicating the importance and general applicability of the supergene architecture in maintaining complex social phenotypic polymorphisms (Purcell et al., 2014). Furthermore, the fact that suppression of recombination in each supergene is achieved by one or more chromosomal inversions also

suggests that chromosomal rearrangements, as structural aspects, represent an important genetic mechanism for achieving suppressed recombination and linking genes together (Purcell et al., 2014). Additionally, supergenes are becoming increasingly associated with variation in complex phenotypes across diverse taxa, including; heterostyle in flowers (Gilmartin and Li, 2010), Batesian mimicry in *Heliconius* butterflies (Joron et al., 2011), plumage polymorphism in swallows (Thomas et al., 2008) and sex specific cryptic colouration in cichlid fish (Roberts et al., 2009). These examples further support the universal potential for supergenes to underpin and regulate variation in complex phenotypes, social or otherwise.

1.5 A possible genetic basis for worker aggression in the facultatively polygynous ant species, *Leptothorax acervorum*

In the facultatively polygynous ant, *Leptothorax acervorum*, geographically distinct populations display two fundamentally different social organisations (Gill et al., 2009, Hammond et al., 2006, Hammond et al., 2001, Trettin et al., 2011, Gill and Hammond, 2011b, Felke and Buschinger, 1999, Bourke et al., 1997). Colonies of *L. acervorum* on average contain multiple mated queens, which are each capable of reproduction, however, colonies may differ in their within colony reproductive skew depending on which population they are sampled from. Polygynous colonies, sampled from the UK and parts of Europe, contain multiple queens which all contribute equally towards colony reproduction and are characterised by low skew and low relatedness (Hammond et al., 2006, Bourke et al., 1997, Heinze et al., 1995). Alternatively, colonies sampled from populations in central Spain and Japan contain multiple mated queens but only one queen matures reproductively (Trettin et al., 2011, Gill and Hammond, 2011b, Gill et al., 2009, Ito, 2005, Felke and Buschinger, 1999). These functionally monogynous colonies are

therefore characterised by high skew and high relatedness (Trettin et al., 2011, Gill and Hammond, 2011b, Gill et al., 2009, Felke and Buschinger, 1999).

A recent study of the behaviour of functional monogynous populations found that the reproductive queen was predicted by low levels of worker aggression (Gill and Hammond, 2011a, Gill and Hammond, 2011b). The workers instead direct significant levels of aggression towards all the queens that ultimately fail to reproduce (Gill and Hammond, 2011a, Gill and Hammond, 2011b). A further study found that queen-queen aggression was also significantly high and was also strong enough to predict the reproductive outcome of all present queens (Trettin et al., 2011). Furthermore, ponerine ant workers have been shown to use aggression to maintain the social hierarchy of the colony and maintain high skew, thereby safeguarding their own inclusive fitness (Monnin and Ratnieks, 2001). Therefore aggression appears to be a fundamental trait in regulating the social organisation of colonies between different populations.

Interestingly, Gill et al. (2009) suggest that the observed difference in worker aggression between social phenotypes might be explained via a genetic basis. First, colonies sampled within the same population do not vary in social phenotype despite variation in local environment and colony organisation (i.e. queen-worker ratio, colony size, etc.) (Gill and Hammond, 2011b, Gill et al., 2009, Hammond et al., 2006). Second, colonies sampled from both FM and P social phenotypes do not switch from one to the other in response to environmental changes when maintained in a lab controlled common environment (Gill and Hammond, 2011a, Gill and Hammond, 2011b, Gill et al., 2009, Hammond et al., 2006). These lines of evidence strongly suggest a genetic component to explaining variation in social phenotype rather than social plasticity.

These findings in *L. acervorum* draw intriguing parallels with the social phenotypes of *S. invicta* and *F. selysi*. First, colonies show a marked polymorphism in social organisation, which has far reaching consequences on colony productivity and relatedness (Gill et al., 2009, Hammond et al., 2006, Bourke et al., 1997). Second, the aggression received by queens plays a key role in the social phenotype (Trettin et al., 2011, Gill and Hammond, 2011a, Gill and Hammond, 2011b). Third, each social phenotype forms a syndrome of correlated traits with some overlap between all three species (i.e. method of queen dispersal) (Felke and Buschinger, 1999, Franks et al., 1991). Although there are also key differences between *L. acervorum* and *S. invicta*/*F. selysi*, namely that *L. acervorum* colonies contain an average of >2 mated queens regardless of social phenotype, the similarities might suggest that one or more social chromosomes may explain the variation in social organisation. Furthermore, akin to the involvement of odorant binding proteins in the *S. invicta* social chromosome, variation in signalling and chemosensory traits may be responsible for regulating the targeted nature of worker-queen aggression (Trettin et al., 2011, Gill and Hammond, 2011a, Gill and Hammond, 2011b, Gill et al., 2009, Felke and Buschinger, 1999). One aim of this thesis is to investigate these possibilities by exploring the genomic architecture of the alternative social organisations in *L. acervorum*.

1.6 Conclusions and thesis directions

In this review, I have explored how variation in complex social traits can be underpinned by a plethora of genetic architectures. The variation within these architectures is so rich that complex phenotypes might be explained by something as simple as genotypic differences at a single locus, a complex and integrated supergene or a pleiotropic gene network, and anything in between. Therefore, it is clear from the examples I have explored that a full explanation of the

evolutionary processes involved in diversifying social phenotypes requires not only an in depth understanding of the phenotype itself but also its supporting genomic architecture. Only then can the complexities of genotypes to expressed social phenotypes be truly appreciated.

The aims of this thesis are twofold. First, I aim to gain a greater understanding of the way in which phenotypic variation in complex social behaviours can directly impact upon fundamental aspects of the social organisation of colonies. To this end, I explore the role of aggression as a behavioural mechanism involved in diversifying the polygynous and functionally monogynous social organisations present between colonies of *L. acervorum* (see 1.5). I also test the predictions of enforcement theory (Ratnieks and Wenseleers, 2008, Wenseleers and Ratnieks, 2006, Ratnieks et al., 2006) by investigating the impacts of worker aggression in each of the alternate social phenotypes. Polygynous species, like *L. acervorum*, are expected to require the enforcement of altruistic behaviour amongst reproductive individuals due to the colony relatedness dynamics of kin selection. First, I investigate the relationship between aggressive worker enforcement and queen reproduction in functionally monogynous colonies by removing worker aggression altogether. Second, , I test whether worker aggression is important for enforcing the high altruism seen amongst mated queens in functionally monogynous colonies by exposing polygynous queens to high worker aggression. Finally, I test the validity of recent findings that aggressive enforcement behaviour is a plastic trait that occurs within multiple queen colonies as a result of variation in colony structure (Trettin et al., 2011).

The second aim was to explore the underlining genomic architecture of *L. acervorum* in order to explain the apparent genetic basis of variation in social organisation. The recent advent and availability of powerful sequencing techniques have made exploring variation in fundamental

social traits more accessible than ever. These techniques hold great potential for bridging the gaps in our knowledge between evolutionary theory and the fundamental genetic mechanisms leading to expressed phenotypes (Fischman et al., 2011). Therefore, I conducted a comparative genome wide associated study (GWAS) to detect areas of divergence between polygynous and functionally monogynous colonies of *L. acervorum*, with the aim of exploring the underlining genomic architecture responsible for variation in social organisation.

Chapter 2

High reproductive skew is maintained in functionally monogynous colonies of the ant, *Leptothorax acervorum*, in the absence of aggressive worker enforcement

2.1 Introduction

Social groups consisting of closely related, non-clonal individuals can often come into conflict over many aspects of social living (Frank, 1995, Bourke and Franks, 1995, Queller and Strassmann, 1998, Ratnieks et al., 2006, Bourke, 2011). One important aspect is reproductive division of labour, which has facilitated the diversity and ecological success of eusocial species (Bourke and Franks, 1995, Wilson and Holldobler, 2005). However, it has been suggested that the elaboration of reproductive division of labour has also required the evolution of varied conflict resolution mechanisms, many of which are behavioural (Ratnieks and Wenseleers, 2008, Bourke, 2011, Bourke and Ratnieks, 1999, Wenseleers and Ratnieks, 2006b, Ratnieks et al., 2006). Therefore, understanding the behavioural mechanisms employed by social organisms in conflict resolution over reproduction can reveal fresh insights into the evolution and elaboration of eusociality (Fischman et al., 2011, Bourke, 2011, Bourke and Franks, 1995).

A key source of conflict shared by vertebrates and invertebrates alike is the partitioning of reproduction amongst individuals within the social group, a concept termed reproductive skew (Reeve and Keller, 1995, Vehrencamp, 1983, Nonacs and Hager, 2011, Clutton-Brock, 1998). Many eusocial species contain multiple queens (MQ) within the colony, which are mated and capable of reproducing individually (Keller, 1993, Bourke and Franks,

1995, Keller, 1995, Boulay et al., 2014, Ross and Carpenter, 1991). Within MQ colonies, there is a strong potential for conflict between reproductive queens competing directly over reproductive rights. For example, queens in many MQ ant species regulate skew by forming reproductive dominance hierarchies (Heinze and Lipski, 1990, Holldobler and Carlin, 1985, Heinze et al., 1997, Heinze et al., 1992, Heinze and Smith, 1990, Satoh and Ohkawara, 2008, Yamauchi et al., 2007).

Importantly, a conflict of interest over reproduction exists between queens and workers because individual interests between the two parties may not coincide (Oster et al., 1977, Trivers and Hare, 1976, Bourke, 1988). Skew theory assumes that queens are ultimately in control of determining the reproductive skew within a colony, a concept I refer to as the queen control hypothesis (Johnstone, 2000, Reeve et al., 1998, Keller and Reeve, 1994, Vehrencamp, 1983, Nonacs and Hager, 2011). However, workers may also play a deciding role in maintaining the reproductive skew of a colony, since their inclusive fitness is directly affected by their relatedness to all reproducing queens (Gill et al., 2009). This concept I refer to as the worker control hypothesis. Indeed, worker influence over reproductive skew amongst queens should not be that surprising as workers already have influence over other aspects of reproduction including; policing of worker eggs (Ratnieks and Visscher, 1989), aggression (Monnin and Ratnieks, 2001), colony sex ratio (Hastings et al., 1998, Sundström and Ratnieks, 1998) and female caste determination (Ratnieks and Helantera, 2009, Ratnieks and Wenseleers, 2008, Wenseleers and Ratnieks, 2006b, Wenseleers et al., 2003). For example, ponerine ant workers use aggression to maintain the social hierarchy of the colony and maintain high skew, thereby safeguarding their own inclusive fitness (Monnin and Ratnieks, 2001). These examples demonstrate how workers can directly influence skew through the expression of aggressive enforcement mechanisms to maintain their fitness interests.

The concept of enforcement was first proposed to explain the extreme levels of altruism seen in many eusocial species (Queller, 2011, Ratnieks and Wenseleers, 2008, Wenseleers and Ratnieks, 2006b). It was argued that relatedness values between workers and queens alone were insufficient to explain extreme altruism in species where workers can lay unfertilised male destined eggs. Under these circumstances, the workers are more related to their sons on average (50%, singly mated queen) than they are to their brothers (25%, singly mated queen) and so, as predicted by kin selection alone, large portions of the colony workforce (25%) are selected to reproduce selfishly (Ratnieks and Helantera, 2009). This would place a substantial cost on the colony as a whole, impacting inclusive fitness and colony efficiency (Ratnieks and Wenseleers, 2008, Wenseleers and Ratnieks, 2006b, Wenseleers et al., 2003). However, this is rarely the case and many diverse eusocial species show extreme levels of altruism by not reproducing despite low within colony relatedness (Ratnieks and Helantera, 2009).

Instead, strong enforcement from nest mates prevents selfish individuals from reproducing (Ratnieks and Wenseleers, 2008). In species where queens are multiply mated or colonies contain multiple reproducing queens, the evolution of enforcement behaviour can be explained through kin selection, as nephews are likely to be less related on average in comparison to brothers (Ratnieks and Wenseleers, 2008, Ratnieks, 1988). Therefore it is within the inclusive fitness interests of sisters to enforce altruistic behaviour by policing each other's selfish reproduction (Ratnieks et al., 2006, Wenseleers and Ratnieks, 2006b, Ratnieks and Helantera, 2009, Ratnieks and Wenseleers, 2008). Interestingly, species presenting the most extreme levels of altruism often have the lowest within colony relatedness and the most efficient enforcement (Ratnieks and Wenseleers, 2008, Wenseleers and Ratnieks, 2006b). This suggests that enforcement is necessary for maintaining altruism when overall colony relatedness is low. Therefore, there is great potential for enforcement behaviours to play a

role in regulating colony skew in MQ species by coercing reproductive individuals (queens) to behave altruistically.

Variation in skew can exist both within and between populations of MQ species (Gill et al., 2009, Bourke et al., 1997, Pamilo and Seppa, 1994, Ross, 1993). An excellent example of within species variation in reproductive skew can be found in the MQ ant species *Leptothorax acervorum*. Populations of *L. acervorum* are polymorphic in social organisation, which has substantial impacts upon colony relatedness and behaviour (Trettin et al., 2011, Gill and Hammond, 2011a, Gill and Hammond, 2011b, Gill et al., 2009, Hammond et al., 2006, Ito, 2005, Felke and Buschinger, 1999, Bourke et al., 1997, Bourke, 1994). In populations found in Spain and Japan, colonies commonly contain multiple queens which are inseminated and capable of reproducing but just one queen monopolises all reproduction (Gill et al., 2009, Felke and Buschinger, 1999, Gill and Hammond, 2011b, Ito, 2005). This social organisation is known as functional monogyny (FM) (Buschinger, 1968) and colonies are characterised by high skew, high relatedness and high rates of aggression between individuals (Gill and Hammond, 2011b, Gill et al., 2009).

Alternatively, some European populations of *L. acervorum* also contain multiple queens that are mated and capable of reproducing but are instead characterised by low skew and low relatedness (Hammond et al., 2006, Bourke et al., 1997, Hammond et al., 2001). This social phenotype is called polygyny (P) (Hammond et al., 2006). To date, very little aggression has been recorded in polygynous populations of *L. acervorum* between queens or workers (see Chapter 5, Bourke, 1991).

Within FM colonies, queen dominance over reproduction may be determined mechanistically via aggressive queen-queen (Q-Q) interactions, where queens establish a dominance hierarchy amongst themselves or through aggressive worker-queen (W-Q) interactions, where workers actively determine the dominant reproductive through aggressive

enforcement behaviour (Trettin et al., 2011, Gill and Hammond, 2011a, Gill and Hammond, 2011b, Gill et al., 2009). Currently, the evidence is confused as to which party, the queens or the workers, is in control of colony skew (Trettin et al., 2011, Gill and Hammond, 2011a, Gill and Hammond, 2011b, Gill et al., 2009). Frequent Q-Q aggression has been recorded in *L. acervorum* colonies from Alaska, the Spanish Pyrenees and Japan (Trettin et al., 2011, Ito, 2005, Heinze and Ortius, 1991). These studies suggest that queens establish a dominance hierarchy between themselves and that the future reproductive queen was predicted by low rate of Q-Q aggression. Indeed, queens in some *Lepthothorax* spp are known to behave aggressively towards one another and establish linear reproductive dominance hierarchies, which would support the queen control hypothesis in *L. acervorum* (Heinze and Smith, 1990, Ito, 2005, Heinze and Ortius, 1991).

Conversely, W-Q aggression was found to be very common amongst colonies of *L. acervorum* from two Spanish populations and has been shown to predict the future reproductive queen (Gill and Hammond, 2011b, Gill and Hammond, 2011a). In total, 99% of all recorded interactions with queens were W-Q aggression and Q-Q aggression contributed only a small fraction of the remaining 1% leading Gill and Hammond (2011) to support a worker control hypothesis over reproductive skew.

The observations of Gill and Hammond (2011a,b) are interesting because they challenge the long established concept of queen control over reproductive skew (Johnstone, 2000, Reeve et al., 1998, Keller and Reeve, 1994, Vehrencamp, 1983, Nonacs and Hager, 2011). It is therefore possible that W-Q aggression in FM colonies of *L. acervorum* acts as a mechanism of enforcement (hitherto referred as worker enforcement), which ultimately coerces queens to behave altruistically, preserving the high skew structure of the colony. Furthermore, through worker enforcement behaviour, workers can control the skew within the colony to meet their inclusive fitness benefits (Gill et al., 2009). In addition, the low skew

amongst P colonies may represent an example of colony structure in the absence of aggressive worker enforcement. Yet a further possibility is that both parties play an important role in determining skew (Reeve and Jeanne, 2003).

I aimed to investigate the worker control hypothesis over reproductive skew in FM colonies by testing the necessity of worker enforcement for maintaining high skew in FM *L. acervorum* colonies. My main hypothesis was that FM queens would not be prevented from reproducing equally in the absence of worker enforcement, which would lower colony skew. This relationship is predicted if skew is controlled by workers. Alternatively, if skew is not affected by the absence of worker enforcement then it is likely that queens are in control.

In order to investigate these hypotheses, I removed worker enforcement in multiply queened FM *L. acervorum* colonies and recorded the resulting reproductive skew in comparison to un-manipulated FM control colonies. Worker enforcement was removed by cross fostering FM queens collected from two separate Spanish populations with P workers from a UK population. I observed and compared the behaviour of workers and queens within these chimeric colonies to test for differences in worker and queen behaviour in the presence and absence of worker enforcement. Finally, I investigated the reproductive skew within all colonies by recording the ovarian development and egg production to determine whether FM queens in the absence of worker enforcement reproduce equally.

2.2 Methods

2.2.1 Colony collection, maintenance and composition

Fig 1: Sample population locations. (Images taken from Google maps).

2.2.1.1 Colony collection

L. acervorum colonies were collected from two functionally monogynous (FM) Spanish populations, Orihuela del Tremedal, Sierra de Albarracin (OT) and Valdelinares, Sierra de Gudar (V) in August 2013 (Fig 1). Polygynous (P) colonies were also collected from Santon Downham (SD), Thetford Forest in the UK in July 2013 to provide the P brood (Fig 1). All colonies were collected whole from decaying twigs found on the forest floor and brought back to the lab. Colonies were removed from their twigs 2-10 days after collection, censused for number of workers, queens and all brood, and provided with an artificial nest in the laboratory (see 2.2.1.2 and Fig 2).

2.2.1.2 Artificial nest box construction and colony maintenance

Artificial nests (see Fig 2) were modified from those used by Gill and Hammond, 2011. The nest was made from two transparent glass slides (2x3 inches) separated by 1mm thick

cardboard. The cardboard had a 30x37mm (1110mm^2) space cut-out to provide a nesting area, which had a nest entrance 10mm in length and 3-5mm in width. The thickness of the cardboard allowed for a single layer of individuals within the nesting area which facilitated clear observations of colony behaviour. Each nest was placed in a foraging arena with the vertical sides (18mm) coated in Fluon® to prevent individuals escaping. The base of each foraging area was layered with 2-5mm of plaster of Paris, which was regularly kept damp to prevent the nest box drying out. Damp cotton wool and a diet of honey solution (carbohydrate) and chopped-up meal worm (protein) were provided three times a week during the spring and summer periods. 10% honey solution was soaked into a small ball of cotton wool and left in the feeding tray to reduce the numbers of individuals drowning in excess honey water. Cotton balls were replaced three times a week to prevent honey fermentation soiling the nest box. Lab colonies were kept in a versatile environmental chamber (*Sanyo* MLR-351H), which provided flexible control over the temperature, light, and humidity regimes. See Table 1 for seasonal condition information.

2.2.1.3 Experimental colony selection

Ten MQ colonies were selected from both FM populations (OT=5; V=5) to be used as treatment colonies. Colonies contained 4.4 queens (range=2-9) queens and 25.5 workers (range=12-54) per colony on average (Table 2).

Finally, 10 P colonies from the SD population were selected to provide the treatment colony brood. Average brood per colony was; 15.1 large larvae (range=8-33), 0.36 pre pupae (Range= 0-2) and 18.8 worker pupae (11-31) (See Table 2).

Table 1: Seasonal conditions for all colonies. Photoperiod: N=night and D=day

Season	No. weeks	Hourly rhythm	Temp./°C	Photoperiod (N-D-D-D) (relative light intensity)	Humidity/%
Winter	4	12-1-10-1	0-5-10-5	0-1-2-1	60-60-60-60
Autumn /Spring	2/8	11-1-11-1	10-15-20-15	0-2-3-2	70-70-80-70
Summer	8	9-1-13-1	15-20-25-20	0-2-3-2	70-70-80-70

*Conditions were based on Gill and Hammond, 2011.

Ten MQ colonies from the FM populations (OT=5; V=5) were also selected to act as FM controls. They maintained their original composition, which at the beginning of the first summer period (Fig 5) had an average of 7.7 queens (range=2-18) and 44.5 workers (range=18-72) per colony.

2.2.2 Treatment colony setup: removing worker enforcement

2.2.2.1 The rationale

To test the worker enforcement hypothesis, it was essential to devise a method of removing worker aggression from FM colonies. One possible method was to mix ant workers from a P social organisation, which rarely behave aggressively towards reproductive queens (Chapter 5, Bourke, 1991), with queens from a FM social organisation, which commonly receive worker aggression (Trettin et al., 2014, Gill and Hammond, 2011a, Gill and Hammond, 2011b). Hypothetically, this would produce a hybrid colony containing non-aggressive P workers and FM queens and would provide the perfect basis for observing skew amongst FM queens in the absence of worker enforcement.

However, it was not possible to simply introduce P adult workers into colonies containing FM queens due to the fact that most ant species are able to distinguish nestmates from non-nestmates with high specificity (Martin and Drijfhout, 2009) and the detection of non-nestmates provokes an aggressive response in *L. acervorum* (pers.obs). Instead, cross fostering of P worker pupae could potentially overcome the barriers of nestmate recognition

as previously demonstrated in other ant species (Signorotti et al., 2014, van Zweden et al., 2010).

All ant colonies have a specific gestalt odour consisting of a blend of different cuticular hydrocarbons (CHCs), which are used for nest mate discrimination (van Wilgenburg et al., 2011, Martin and Drijfhout, 2009). However, nestmate discrimination of the pre-pupal stages of ant development is more difficult (Fouks et al., 2011). For this reason, social parasitism, and slave making in particular, amongst ants is very common (Guillem et al., 2014). *L. acervorum* is also socially parasitized by the slave making ant *Harpagoxenus sublaevis*, demonstrating that newly raided non-nest mate larvae are not discriminated against by the enslaved *L. acervorum* workers. Therefore, it was likely that FM *L. acervorum* colonies would accept P worker brood.

It was also important that newly emerged P workers did not behave aggressively towards FM queens due to non-nestmate recognition cues. However, previous studies suggest that newly emerged ant workers acquire the colony gestalt odour via close interactions with all colony individuals and behave as if they were in the natal colony (Signorotti et al., 2014, Blatrix and Sermage, 2005). Therefore, P worker aggression due to nestmate discrimination should be absent in cross fostered colonies.

2.2.2.2 Treatment colony nest box construction

The 10 treatment colonies (see 2.2.1.3) were constructed by first removing all individuals and separating the queens from the workers. Queens were marked by carefully tying 0.03mm copper wire around the petiole (Fig 3 and 4A). The wire 'whiskers' were then cut to different sizes so that each individual could be positively identified (Fig 3). This marking technique has been successfully used to make *L. acervorum* queens in previous studies (Trettin et al., 2011, Trettin et al., 2014, Kuehbandner et al., 2014). The technique is described in detail in Chapter 4, 4.2.2.

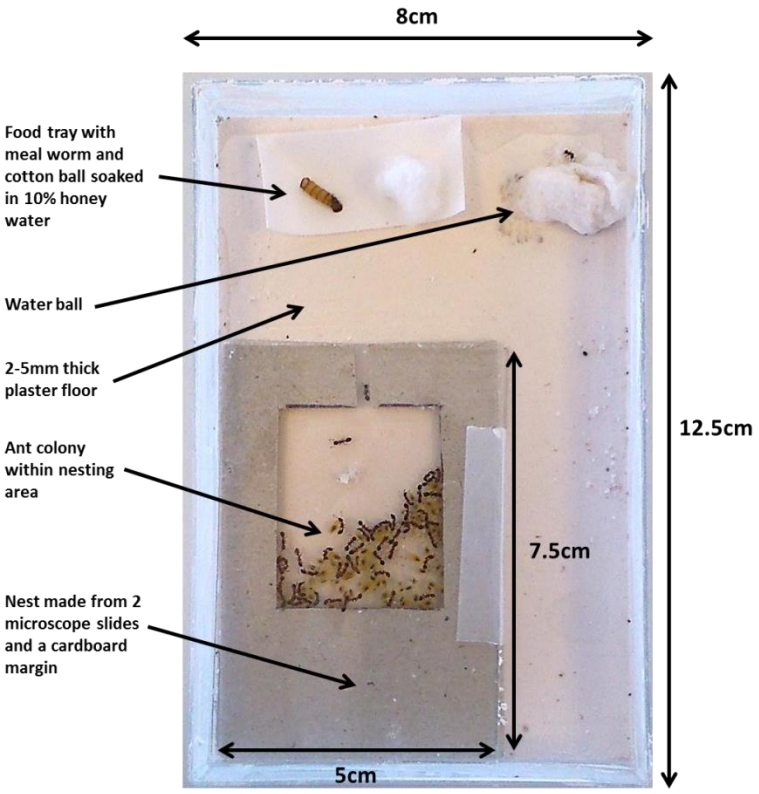


Fig 2: Artificial nest setup. The foraging area contains a moist water ball and a wet plaster surface to prevent the colony box from drying out.

All large larvae, pre-pupae and worker pupae were removed from their original colonies (see Table 2 for brood origins and numbers) and carefully transplanted into a newly constructed nest (Fig 2) with a pair of fine forceps and a fine horsehair paint brush.

It was crucial that larvae were handled carefully to limit damage. The nest was then sealed and left in the nest box.

Ten workers from the FM queens native colony were marked by removing one central leg (tarsal clipping) and introduced into the treatment colony nest boxes (Fig 4A). Tarsal clipping allowed the FM workers to be distinguished from all newly hatched P callow workers. When all brood had been transferred and all queens and workers had been marked, they were introduced into the nest box and left undisturbed to move into the artificial nest (Fig 4A).

All treatment colonies were maintained in summer conditions until all P worker pupae had emerged (Fig 4B). Following P worker emergence, all marked FM workers were removed leaving a colony consisting of queens from a FM colony and workers from a colony (see Table 2 and Fig 4B). Treatment colonies were then placed in autumn conditions for two weeks before overwintering (see Table 1 for environmental conditions and Fig 5 for experimental timeline). After overwintering in the lab, eight treatment colonies contained ≥2 queens with an average of 3.4 queens (range=2-6), and 11.9 workers (range=8-18) per colony (Table 2).

All queens from the FM control colonies were similarly marked with 0.03mm copper wire around the petiole to allow for individual queen identification. FM controls were treated to the same environmental conditions and seasonal time periods as the control colonies (see Table 1). After overwintering, there were an average of five queens (range=2-8) and 38.6 workers (range=21-50) per FM control nest.

Table 2: Treatment colony composition including FM queen and P worker origins during colony construction, before overwintering and after overwintering. Before overwintering refers to the last day of the autumn period (see Fig 5). After overwintering refers to the last day of the winter period (see Fig 5). The colony code is constructed from; queen population, worker population and unique colony number. The queen and worker codes are read as; population, year of collection, unique colony number. Brood consisted of large larvae (LL), pre pupae (PP) and worker pupae (WP).

			Colony Construction		Before overwintering		After Overwintering	
Treatment Colony ID	FM Queen ID	P Worker ID	FM Queen Number	Brood (LL-PP-WP)	FM Queen Number	P Worker Number	FM Queen Number	P Worker Number
VA.SD.04	VA.13.40	SD.13.32	5	11-0-11	5	9	4	9
VA.SD.05	VA.13.06	SD.13.20	4	10-0-26	4	5	1	0
VA.SD.06	VA.13.17	SD.13.01	6	14-0-19	6	15	5	18
VA.SD.07	VA.13.07	SD.13.08	4	3-0-17	4	10	1	16
VA.SD.08	VA.13.12	SD.13.13	4	22-0-13	4	9	3	13
OT.SD.01	OTB.13.08	SD.13.12	6	15-0-19	6	17	6	17
OT.SD.02	OTB.13.11	SD.13.26	2	20-2-17	2	8	2	8
OT.SD.03	OTD.13.08	SD.13.16	5	23-0-14	5	11	5	11
OT.SD.04	OTD.13.10	SD.13.37	3	8-0-24	3	21	3	16
OT.SD.05	OTA.13.16	SD.13.19	4	10-0-12	4	11	3	10

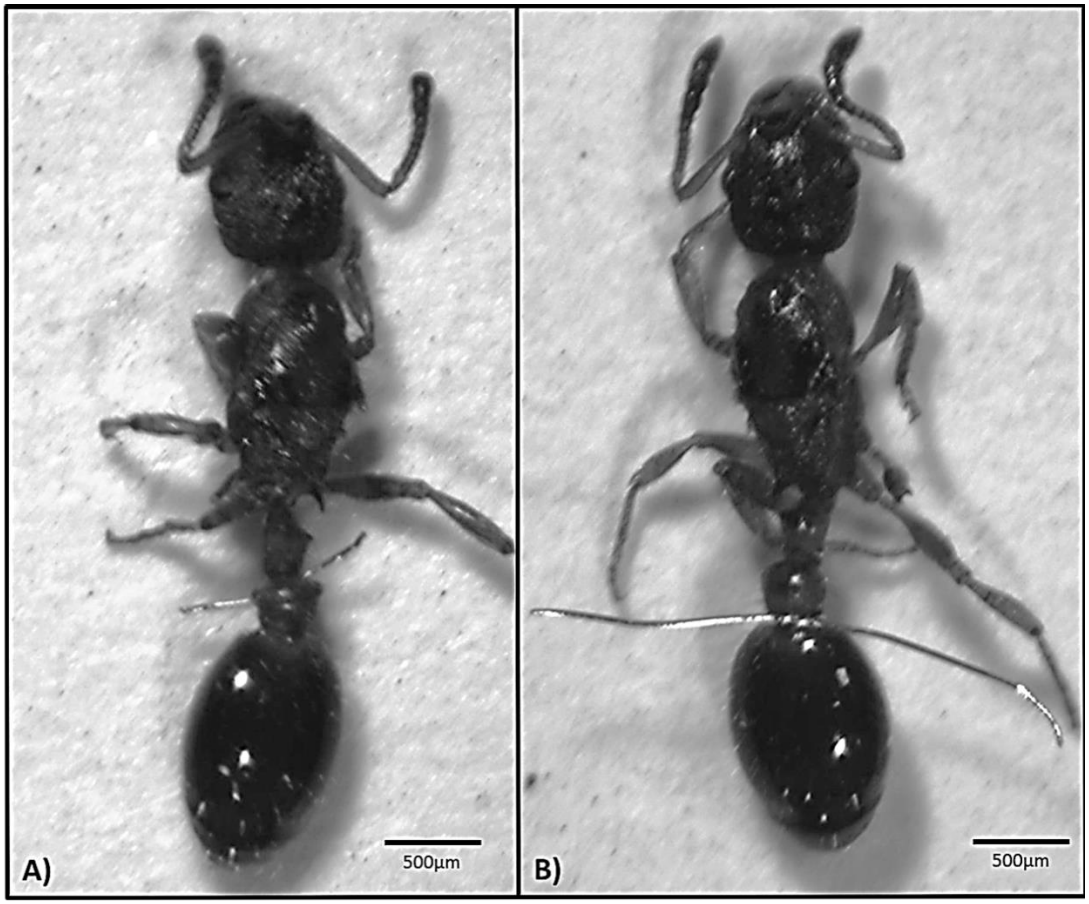


Fig 3: Examples of queen identification marking using wire. Wire was tied between the thorax and abdomen and the 'whiskers' were shorted to different lengths to allow easy identification. A) Example of wire tied between the petiole and post-petiole with short whiskers. B) Wire tired between the petiole and abdomen with long whiskers.

2.2.3 Behavioural observations and analysis

I observed aggressive and non-aggressive interactions received by individually marked queens in eight multiple queen (MQ) treatment colonies throughout the spring period. I then continued to observe and record all aggressive and non-aggressive interactions received by queens in five of the eight MQ treatment colonies (three were lost during the spring period due to queen death) and 10 MQ FM control colonies throughout the summer period. Colony behaviour was recorded from the start of spring until the end of summer (see Table 3).

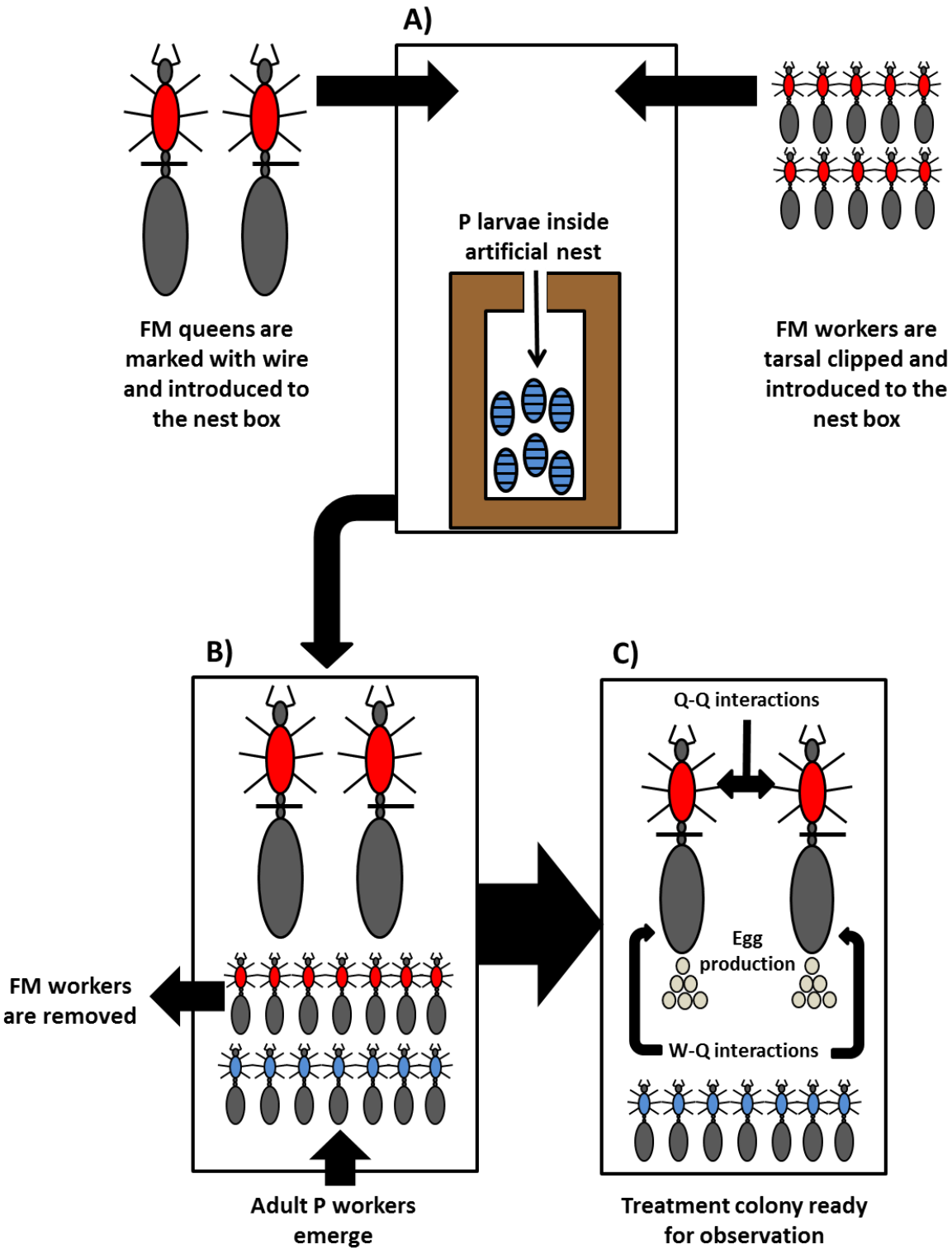


Fig 4: Flow diagram showing treatment colony construction. Treatment colonies containing functionally monogynous queens **(Red)** and polygynous workers **(Blue)** were established via a larval cross-fostering method. **A)** FM queens and workers are marked and introduced to P larvae. **B)** P adults emerge during summer conditions and FM workers are removed. **C)** Treatment colonies are ready for observations of behaviour and egg production.

2.2.3.1 Scan sampling

Treatment colonies were scan sampled three times a day (time points) for 1-5 days each week during the spring period (Fig 5). Following spring, both treatment and FM control colonies were scan sampled three times a day for 1-5 days each week during the summer period (Fig 5). Observations were stopped once a colony reached a single queen or less. During the scan sampling, each queen was observed for five seconds and all aggressive interactions during that time were recorded as either present (1) or absent (0). Aggressive interactions included biting, pulling, spreading and sting smearing and they were distinguished between queen-queen (Q-Q) and worker-queen (W-Q) interactions (see Table 4 for detailed descriptions of each behaviour). Grooming behaviours were not recorded.

Table 3: Summary of scan sampling observation days for treatment and FM control colonies during the spring and summer periods.

Spring Period	Average Obs Days Per Colony	Range
Treatment	23.3	18-24
Summer Period	**Average Obs Days Per Colony**	**Range**
Treatment	20.8	7-28
FM control	24.6	10-28

2.2.3.2 Video recording

Treatment and FM control colonies were filmed at random intervals for the first four weeks of the summer period during daytime hours (09.00-17.00) (Fig 5). In three FM control colonies (VA.13.01, VA.13.30 & OTA.13.05) recording was stopped prior to egg-laying as all but a single queen (the future reproductive) had either been evicted or had left the nest permanently. Behaviour was recorded using a focal queen approach. This meant the type of event (Table 4), number of events and duration of event were recorded for each individual queen in each recording (see 2.2.3.3). Each queen position was mapped at the beginning of the recording to aid the focal approach and each queen was identified based on the copper

wire identifier. Video recordings were restricted to the first four weeks of the summer period to maximise the chances of capturing aggressive behaviour. Queens that received no focal recording time were removed from the analysis.

Colony behaviour was recorded using a Logitech Carl Zeiss Tessar 1080p digital web camera (Model c920) connected to a laptop. Recordings were viewed in tandem with VAR© (Video Activity Recorder, Little Imp Company), which allowed the type, length and number of each behavioural event to be recorded accurately for each individual queen. Behavioural rates for each specific behavioural type were calculated by taking the total duration of the behaviour (seconds) / the total length of time a focal queen had been observed (hours) (Gill and Hammond, 2011a, Gill and Hammond, 2011b). Per capita rates were also calculated to correct for the disproportionate numbers of workers and queens per colony, meaning behavioural observations were not influenced by the total numbers of workers and queens (Gill and Hammond, 2011a, Gill and Hammond, 2011b).

2.2.3.3 Behavioural interactions

Four types of aggressive interactions were scored (see Table 4), each increasing in their degree of aggressiveness. The duration of a single bite was difficult to measure, and so each bite was standardised at one second per bite in order to calculate a rate (Gill and Hammond, 2011a, Gill and Hammond, 2011b). All forms of grooming behaviour were included under a single type 'grooming'. This included; W-Q grooming, Q-Q grooming, W-Q trophallaxis and W-W trophallaxis. Variation in observation times for each queen is explained by the fact that queens could leave and enter the nest. Queens outside of the nest could not be videoed (see Tables 5-6).

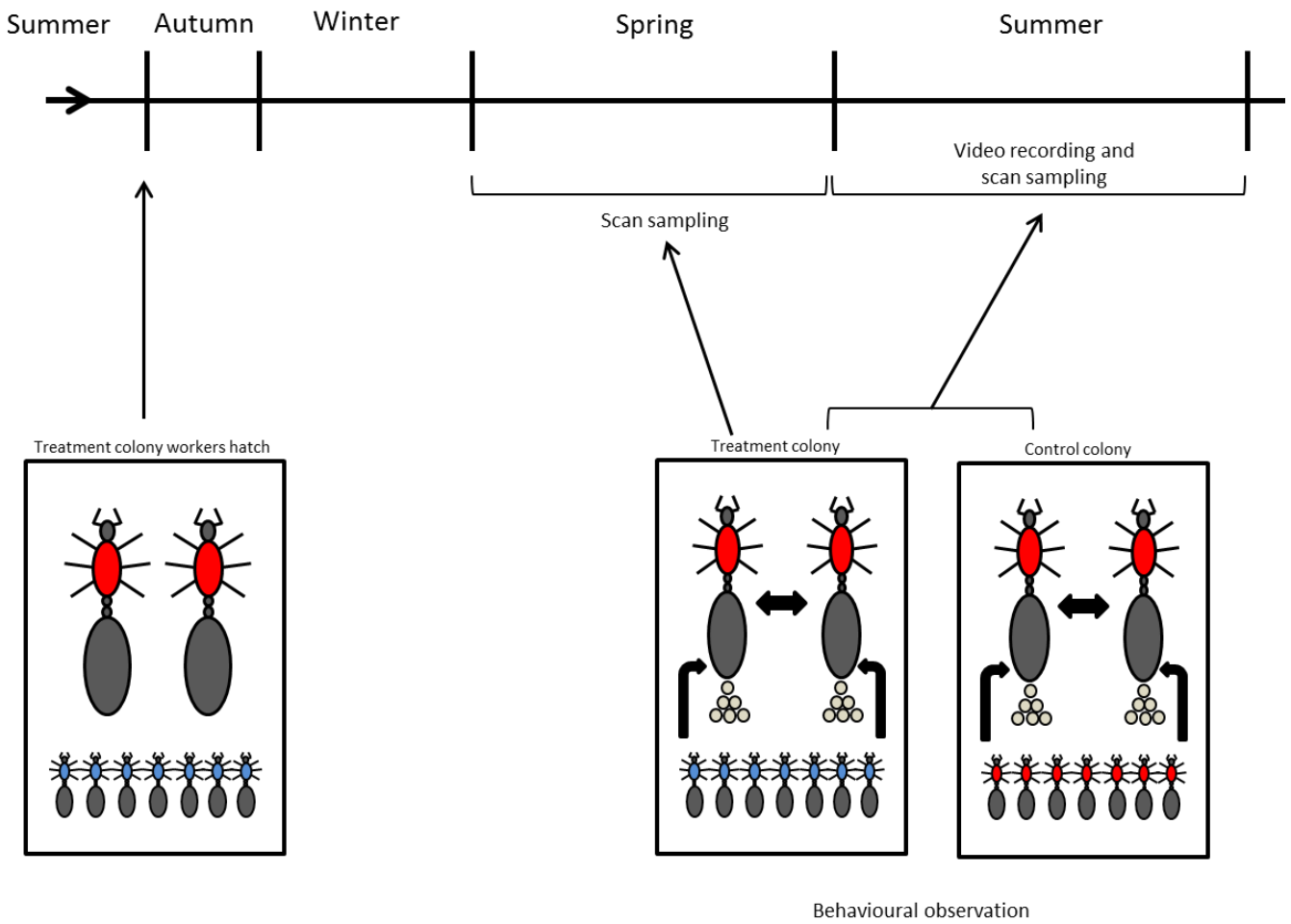


Fig 5: Experimental design showing the behavioural observation periods and sampling techniques. Treatment colonies (n=8) were observed via scan sampling from the onset of artificial spring. Treatment colonies (n=5) and FM control colonies (n=10) were observed via scan sampling and video recording techniques from the onset of artificial summer. FM queens shown in **red** and P workers shown in **blue**.

Table 4: Summary of behaviours recorded**.** Classification and description for each type of interaction recorded during video observation.

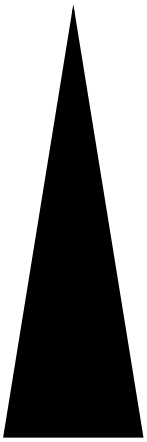
Type of aggression	Degree of aggressiveness	Definition
Grooming	0	A single individual is cleaned by multiple individuals. A single individual may also be fed by another individual (trophallaxis).
Single Bite	Low	A single individual bites another for ≤1 second.
Pulling		A single individual bites another individual usually on an appendage (i.e. legs, antennae, neck and petiole) and drags the attacked individual.
Spreading		Multiple individuals bite another individual's appendages and pull in opposite directions, completely immobilising the attacked individual. Prolonged spreading can lead to the loss of an appendage and/or death.
Sting Smearing	High	A single queen bites and holds another queen. When secure the aggressing queen pulls her abdomen round and smears the other individual's body with a secretion from her abdomen (location of the sting). Once complete, both queens break the hold and often worker aggression is witnessed directly afterwards between either/both queens.

Table modified from Gill, 2010 with permission.

2.2.4 Queen reproduction and mated state

At the end of the summer period, all remaining queens were removed from the colonies, snap frozen in LN_2 and stored at -80˚C. Similarly, all queens found dead during the experimental periods were snap frozen in LN_2 and stored at -80˚C. Mated status was initially determined by dissecting out and classifying the ovaries using the same criteria described in Gill et al (2009). Classification ranged from A (highly reproductive and actively laying eggs) to D (inactive and not laying). Ovaries that were currently active (A or B) showed relatively elongated ovarioles, many maturing eggs and an absence of trachioles. Inactive ovaries (C and

D) showed relatively small ovarioles with no large maturing eggs. It was also common to find dense clusters of trachea around the ovarioles in inactive ovaries.

Recent laying activity was evidenced by the presence of corpora lutea (Stille et al. 1991). If corpus lutea were observed in C ovaries, the queens were marked as 'reproductive' whereas ovaries that showed little to no evidence of activity (C and D with no corpora lutea present and small ovarioles) were marked as 'non-reproductive'. All queens snap frozen alive were dissected in 100µl RNAlater (Sigma-Aldrich, UK) to preserve RNA quality. Queens that were snap frozen dead were dissected in distilled water.

The total number of eggs present in each colony nest at the end of the summer period were recorded. Furthermore, the time (days) it took for the first egg to be laid within each colony was also recorded.

2.2.5 Statistical analyses

All continuous variable data sets were initially tested for normality via Kolmogorov–Smirnov test analysis. If the data were found to follow a normal distribution then appropriate parametric statistics were used to test hypotheses. If the data were not found to be normally distributed then non-parametric tests were used.

Statistic alpha (α) levels were Bonferroni corrected for repeat measures wherever necessary.

2.2.5.1 Behavioural frequency data from scan sampling

Binary frequency data for each of the four aggressive behaviours (Table 4) were recorded and analysed for differences between FM control and treatment groups using a linear mixed-effects model (LMM). The response variable was defined as the occurrence of aggression (1= aggressive event, 0= no aggressive event) towards a given queen, the independent variable was the treatment type the queen belonged to (FM control or

treatment). 'Colony code' (a coded ID specific to a given colony) was specified as a random effect variable to control for potential variation in individual colony aggressiveness.

All colonies were observed three times per day (time points) and all aggressive interactions for each queen were recorded in binary. All data for each aggression category (biting, pulling and spreading) at a particular time point were combined across all queens within a specific colony into a single binary value. This indicated whether an aggressive event had taken place within that colony at that time point. Therefore, each colony got three aggression binary data points per day of observation. Consequently, if two queens were present at a given time point and at least one queen received aggression the colony would receive a score of 1.

The scan data were analysed at the colony level rather than the queen level primarily to ascertain a more general idea of the frequency of aggression occurring within the treatment and control colonies as a whole. Furthermore, due to the fact that aggressive interactions are relatively rare, the scan sample data set was heavily 0 biased. Therefore, when the data were analysed at the queen level, the large bias in 0 scores meant there was very low power and the test was unable to distinguish higher frequencies of aggression between individual queens.

Data analysis was conducted using R (R_Core_Team, 2013), RStudio v0.98 (RStudio, 2012), the lme4 (Bates et al., 2014) package and the lmer module as described in Crawley [p.631] (2007). For binomial variables, generalised LMMs were conducted with the laplace approximation.

LMMs are particularly useful for analysing unbalanced or missing data than traditional analyses of variance (Baayen et al., 2008). Not all colonies were observed for the same

number of days as once the total queen number within a colony reached 1 or less, behaviour could no longer be observed. Therefore, the frequency data set was unbalanced.

2.2.5.2 Behavioural video data

The rates of Q-Q and W-Q aggression received by individual queens where compared both between and within the treatment and FM control groups using Mann-Whitney U tests. Additionally, the combined rates of aggression (Q-Q and W-Q interactions combined) were also compared between treatment and FM control colonies.

Individual behaviours were also compared between the treatment and FM control colonies, with Mann-Whitney tests. Some behaviour types were not observed in either sample group, therefore Wilcoxon's signed rank tests were used to test a sample group median against 0.

All behavioural rate data were also converted into per capita rates to correct for variation in queen and worker numbers. These per capita rates were used in exactly the same statistical analyses and the conclusions were used to support the conclusions of the equivalent uncorrected rates.

Binary logistic regression was also used to test if the future reproductive status of queens (reproductive queens coded 1, non-reproductive queens coded 0) could be predicted from rates of aggression. Prior to analyses the assumption that independent variables had a linear relationship with the logarithm of the dependent variable was tested, as described in Field [p.296] (2009). In all cases this assumption was met as there was no significant interaction term between the independent variable and its natural logarithm.

All statistical analyses on rate data were performed in Minitab version 16 and SPSS version 20.

2.2.5.3 Queen death and aggression

Binary logistic regression was also used to test if queen death could be predicted by the frequency of aggression. Queen death was scored as 1 and queens alive were scored as 0. As before, the assumption that independent variables had a linear relationship with the logarithm of the dependent variable was tested, as described in Field [p.296] (2009). In all cases this assumption was met.

2.2.5.4 Egg counts

Student's t-test analysis was used to test for differences in total egg numbers and the onset of egg laying between treatment and control colonies.

2.3 Results

2.3.1 Queen dissection, ovary classification and egg production

In total, 84.2% of the treatment queens were found to be mated and 86.4% of the FM control queens were mated. The proportions of mated queens were similar to those found in other studies (91%, Gill and Hammond, 2011a, 96%, Gill and Hammond, 2011b, 96%, Gill et al., 2009). Unmated queens were removed from the analysis entirely for two reasons. First, unmated queens may assume a worker like role in terms of behaviour and therefore provide an inappropriate representation of queen behaviour (Ito, 2005). Second, it is not fully understood if mated status can be detected by conspecific queens or workers, which could influence behavioural interactions (Ruel et al., 2013). Regardless, the removal of unmated queens from the analysis made no difference to the statistical outcomes.

All 24 colonies contained eggs at some point during the summer period. There was no significant difference in the final egg totals between the treatment and FM control colonies (Mean eggs per colony±(SE): Treatment= 8.60±3.09, FM control= 7.90±1.89, Student's T-test: $F_{(13)}$= 0.246, N_{treat} = 5, $N_{control}$ = 10, P= 0.628, see Fig 6A). Similarly, there was no significant difference in the number of days after the beginning of summer until the first egg was laid between the treatment and FM control (Mean days per colony±(SE):Treatment= 15±3.54, FM control=12.7±1.75, Student's T-test: $F_{(13)}$= 0.559, N_{treat} = 5, $N_{control}$ = 10, P= 0.52, Fig 6B).

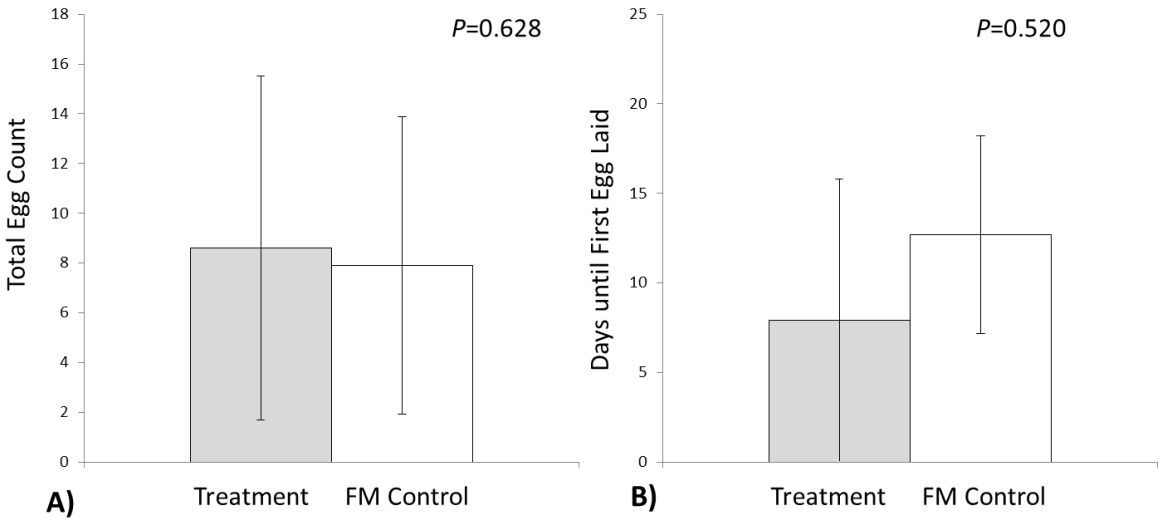


Fig 6: Mean final egg counts and days after summer until the first egg was laid for treatment and FM control colonies. A) Mean total eggs present in treatment (*N*=5) and control colonies (*N*=10). B) Mean number of days before the first egg was laid in treatment (*N*=5) and control colonies (*N*=10) starting from the summer period. Bars represent standard deviation. *P* values were calculated using a t-test.

2.3.2 Behavioural observations

2.3.2.1 Scan Sampling

The mean number of W-Q aggressive events per day was found to be significantly higher than the number of Q-Q aggressive events in the treatment group during the spring period (Mean events per day per colony±(SE):Q-Q = 0.004±0.003, W-Q=0.018±0.006, LMM: ß(s.e.)= 1.6559(0.8069), Z= 2.052, $N_{q\text{-}q}$=561, $N_{w\text{-}q}$=561, *P*=0.0402).

During the summer period Q-Q aggression was found to be significantly different between the treatment and FM control colonies with Q-Q aggression events being observed more frequently in the treatment group per day (Mean events per day per colony±(SE):Treatment = 0.034±0.010, FM Control=0.009±0.003, LMM: ß(s.e.)= -1.3482(0.6422), Z= 2.099, N_{treat}=348, $N_{control}$=699, *P*=0.0358, Fig 7). Conversely, the number of W-Q aggressive events was significantly different between treatment and FM control groups but with the control group having the greater number of observed events per day (Mean

events per day per colony±(SE): Treatment= 0.014±0.006, FM Control=0.066±0.009, LMM: ß(s.e.)= -1.8531(0.8832), Z=-2.099, N_{treat}=348, $N_{control}$=699, *P*=0.0359, Fig 7).

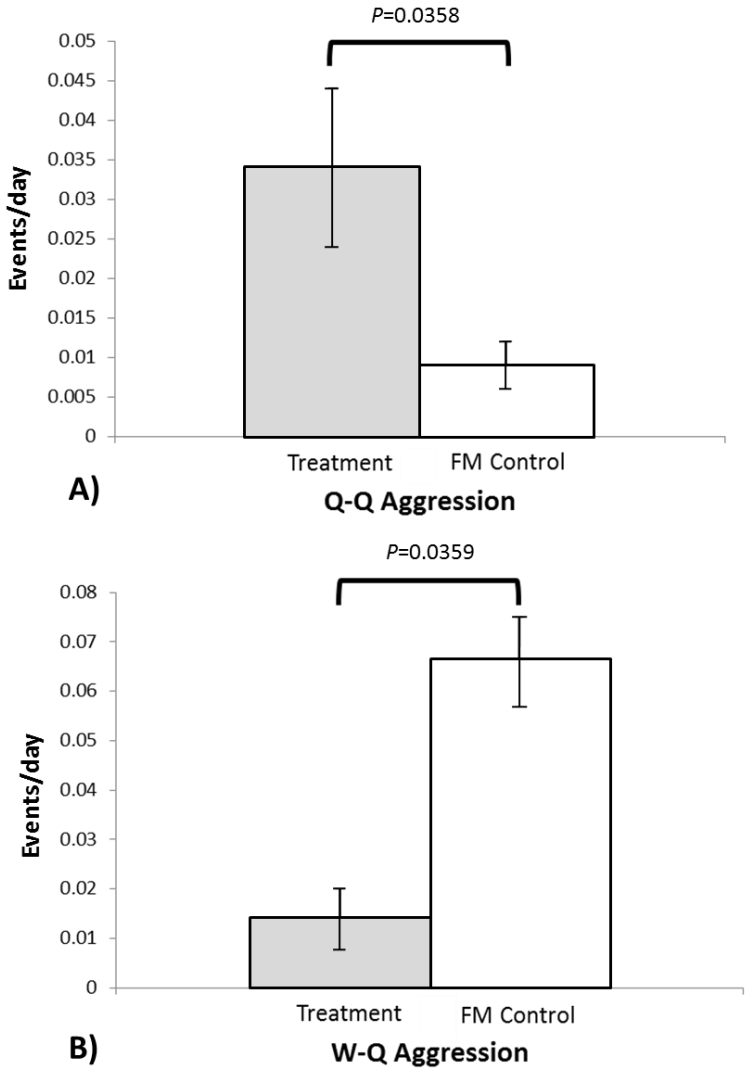


Fig 7: Mean aggressive events per day for treatment and FM control colonies. A) Q-Q aggressive events per day. B) W-Q aggressive events per day. Bars represent the mean average with standard error bars. *P* values were calculated using LMM analysis.

Comparisons of Q-Q aggression in the treatment group between the spring and summer periods revealed that there were significantly more Q-Q aggressive events per day during the summer period (Mean events per day per colony±(SE): Spring= 0.004±0.003, Summer=0.034±0.010, LMM: ß(s.e.)= 2.2000(0.8235), Z=2.672, N_{spring}=561, N_{summer}=348,

P=0.00755, Bonferroni corrected α=0.025). However, the number of W-Q aggressive events per day amongst the treatment colonies was not significantly different between the spring and summer periods (Mean events per day per colony±(SE): Spring= 0.018±0.006, Summer=0.014±0.006, LMM: ß(s.e.)= 0.1021(0.5005), Z=0.170, N_{spring}=561, N_{summer}=348, *P*=0.865, Bonferroni corrected α=0.025).

2.3.2.2 Video data

A total of 11.72 hours of individual queen behaviour was recorded for 16 treatment queens (mean per queen=43.94 minutes, range=20-60 minutes) and 18.65 hours for 50 FM control queens (mean per queen=22.38 minutes, range=0-40 minutes).

Rates of total aggression were not significantly different between queens from control or treatment colonies (see Table 7). However, the per capita rates (sec/min) of total aggression were significantly higher amongst the treatment queens compared to the FM control queens (see Table 8).

Rates of Q-Q aggression (sec/min) were found to be significantly higher amongst queens in the treatment colonies compared with the FM control colonies (see Table 7 and Fig 8). Furthermore, rates of W-Q aggression (sec/min) were found to be significantly higher in the FM control colonies (see Table 7 and Fig 9). These relationships were also true for per capita rates of aggression (see Table 8).

The rates of Q-Q aggression (sec/min) were significantly higher that the rates of W-Q aggression (sec/min) within the treatment colonies (see Table 7 and Fig 9). Conversely, the rates of Q-Q aggression (sec/min) were significantly lower than the rates of W-Q aggression (sec/min) within the FM control colonies (see Table 7 and Fig 9). These relationships were supported by the per capita rate data (see Table 8).

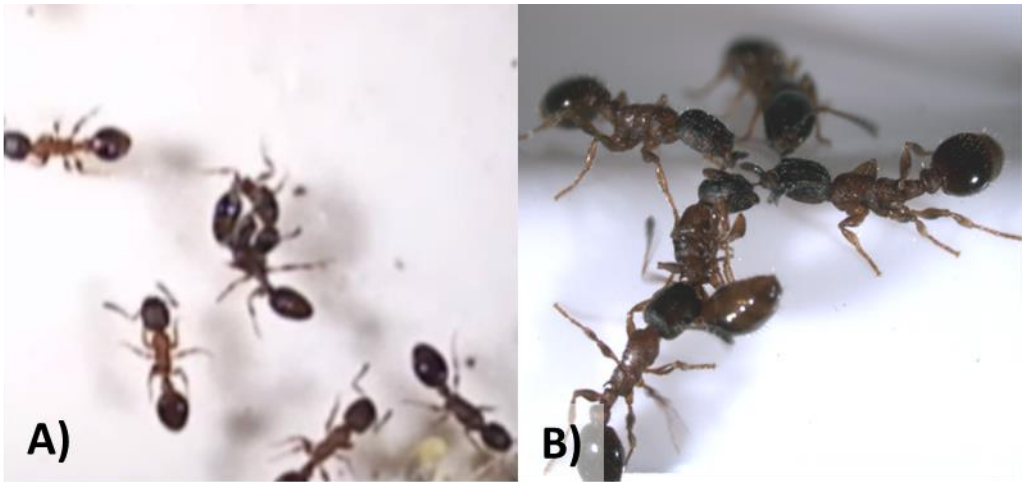


Fig 8: Examples of Q-Q and W-Q aggression. A) One queen smears another queen with chemicals secreted from the sting. B) Workers spreading a queen that later did not reproduce (the queens are in the centre of both images).

Analysis of rates of aggression from individual behaviours showed some differences between treatment and FM control colonies. Rates of Q-Q biting and Q-Q pulling (sec/min) were significantly higher in the treatment colonies compared to the FM control colonies (see Table 9). Rates of W-Q biting (sec/min) were significantly higher in FM control colonies compared to treatment colonies (see Table 9). Interestingly, rates of W-Q spreading (sec/min) were not significantly different between treatment and FM control colonies (see Table 9). Q-Q spreading was never observed amongst FM control colonies and W-Q spreading was never recorded amongst treatment colonies. Analysis of per capita rates did not alter the statistical outcomes (Table 10).

Q-Q sting smearing behaviour was observed 17 times across six different individual queens in three separate colonies within the treatment group (see Fig 8 for a visual example of sting smearing). Alternately, no sting smearing events were ever observed for any of the queens in the control colonies. In contrast to the findings of Gill et al (2011a), W-Q aggression was never observed prior to or preceding a sting smearing event in every case.

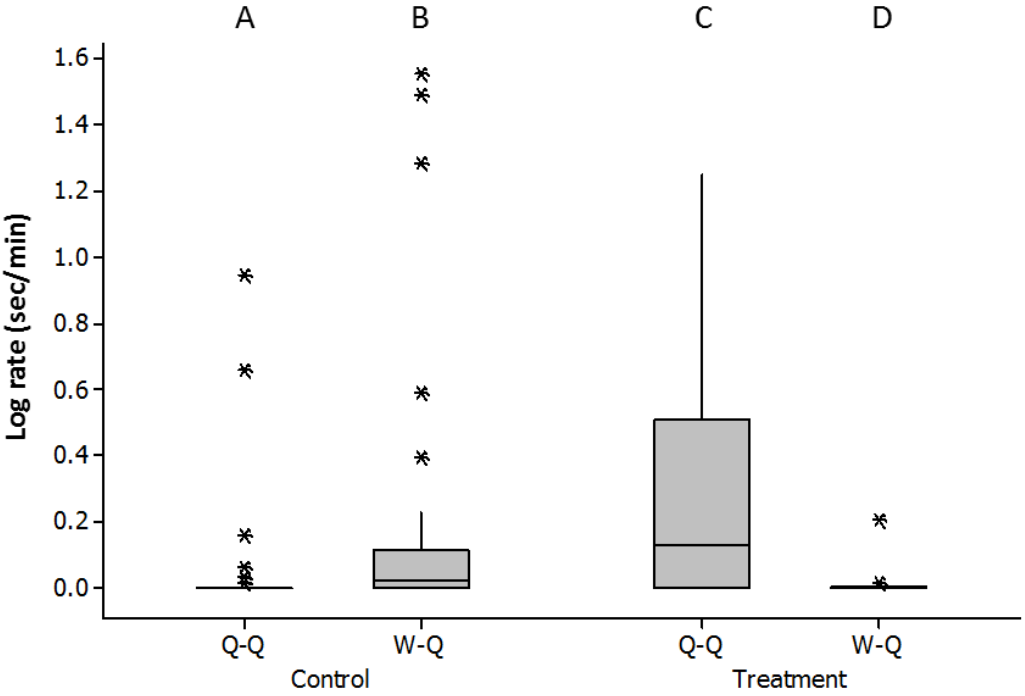


Fig 9: Rates of aggression (sec/min) received by individual queens in the control (*N*=39) and treatment (*N*=14) groups. For convenience of visualisation, rates of aggression were first standardised by adding 1 and then transformed by $\log_{10}$. Box plots show the median line, the quartiles (the box limits) and the whiskers (1.5 IQR). Outliers are marked with *. A vs B significant (*P*=0.0002), C vs D significant (*P*=0.012), A vs C significant (*P*=0.0005) and B vs D significant (*P*=0.0113). P values calculated with Mann-Whitney U tests. Bonferroni corrected α=0.0125.

2.3.3 Aggression as a predictor for future queen reproductive status

Unlike previous studies (Gill and Hammond, 2011a, Gill and Hammond, 2011b), the future reproductive status of queens could not be predicted by rate of aggression (sec/min) received regardless of origin (W-Q or Q-Q) or group (treatment or FM control) (Table 11).

Table 5: Summary of aggression in treatment colonies. Mated state, the rate of worker aggression received, and the total focal queen time observed for each queen in 5 treatment MQ colonies. The future reproductive status of each queen is classed as either reproductive (RQ) or non-reproductive (NRQ). The mated status is classed as mated (M), unmated (U), or undetermined (?). The rate (sec/min) of aggression is in **bold**.

Colony	Treatment Group Q-Q Aggression Queen reproductive status				
	RQ	NRQ_1	NRQ_2	NRQ_3	NRQ_4
OT.SD.01		M 0.03	U **0**		
OT.SD.04	M 16.8	M 0.1	U **0**		
VA.SD.04	M 2.37	M 2.2	M 1.54		
VA.SD.06	M **0**	M 0.77	M **0**	M **0**	M 0.66
VA.SD.08	M 0.03	M **0**	M **0**		
Colony	**Treatment Group W-Q Aggression** **Queen reproductive status**				
	RQ	**NRQ_1**	**NRQ_2**	**NRQ_3**	**NRQ_4**
OT.SD.01		M 0.03	U **0**		
OT.SD.04	M **0**	M **0**	U **0**		
VA.SD.04	M **0**	M **0**	M **0**		
VA.SD.06	M **0**	M **0**	M **0**	M **0**	M 0.6
VA.SD.08	M 0.03	M **0**	M 2.39		
Colony	**Total Focal Queen Observation Period (mins)**				
	RQ	**NRQ_1**	**NRQ_2**	**NRQ_3**	**NRQ_4**
OT.SD.01		29.97	20		
OT.SD.04	30	59.97	60		
VA.SD.04	39.28	53.73	11.07		
VA.SD.06	60	33.98	59.98	28.93	50
VA.SD.08	59.37	59.03	47.7		

Table 6: Summary of aggression in FM control colonies. Mated state, the rate of worker aggression received, and the total focal queen time observed for each queen in 10 control MQ colonies. The future reproductive status of each queen is classed as either reproductive (RQ) or non-reproductive (NRQ). The mated status is classed as mated (M), unmated (U), or undetermined (?). The rate (sec/min) of aggression is in **bold**.

FM Control Group Q-Q Aggression									
Colony	**Queen reproductive status**								
	RQ	**NRQ_1**	**NRQ_2**	**NRQ_3**	**NRQ_4**	**NRQ_5**	**NRQ_6**	**NRQ_7**	**NRQ_8**
OT.13.03	M **0**	M **0.13**	M **0**	M **0**					
OT.13.05		M **0**	M **0**	M **0.1**	M **0**	M **0**	M **0**	M **0**	M **0.3**
OT.13.17	M **3.54**	M **0**	M **0**	M **0**					
OT.13.21	M **0**	M **0**							
OT.13.40	M **0**	M **4.97**	M **0**	M **0**	M **0**	M **0**			
VA.13.01		M **0**	M **0**	M **0**	U **0**				
VA.13.09	M **0**	M **0**	U **0**						
VA.13.13		M **0**	M **0**	M **0**	M **0**	M **0**			
VA.13.30	M **0**	M **0**	M **0**						
VA.13.38	M **0**	M **0**	U **0**	U **0**					
FM Control Group W-Q Aggression									
Colony	**Queen reproductive status**								
	RQ	**NRQ_1**	**NRQ_2**	**NRQ_3**	**NRQ_4**	**NRQ_5**	**NRQ_6**	**NRQ_7**	**NRQ_8**
OT.13.03	M **0**	M **0.13**	M **0**	M **0**					
OT.13.05		M **0.03**	M **0**	M **0**	M **0.03**	M **0**	M **0.1**	M **0.13**	M **0**
OT.13.17	M **0.1**	M **0**	M **0.07**	M **0**					
OT.13.21	M **0**	M **0.7**							
OT.13.40	M **0**	M **0.07**	M **8.81**	M **0**	M **0.6**	M **0**			
VA.13.01		M **0.03**	M **20.02**	M **15.15**	U **12.28**				
VA.13.09	M **0.9**	M **0.07**	U **0**						
VA.13.13		M **0**	M **0.03**	M **0.03**	M **0.03**	M **0**			
VA.13.30	M **1.23**	M **0**	M **0.33**						
VA.13.38	M **0**	M **0.07**	U **0**	U **1.5**					

Colony	Total Focal Queen Observation Period (mins)								
	RQ	NRQ_1	NRQ_2	NRQ_3	NRQ_4	NRQ_5	NRQ_6	NRQ_7	NRQ_8
OT.13.03	40	40	40	40					
OT.13.05		19.9	10	20	10	9.7	10	13.47	20
OT.13.17	29.9	29.9	29.9	27.7					
OT.13.21	30	30							
OT.13.40	10	19	14.4	10	30	18.8			
VA.13.01		10	20	13.07	30				
VA.13.09	40	30.97	39.47						
VA.13.13		17.07	36.63	20	39.97	40			
VA.13.30	25.07	15.47	39.77						
VA.13.38	40	29.83	3.57	25.63					

Table 7: A summary of all behavioural rate (sec/min) data test statistics. Non-parametric test statistics carried out on behavioural rate data for the treatment and FM control groups. Mann-Whitney U tests and Wilcoxon's signed rank tests were performed for all data and the associated median (inter quartile range (IQR)), *N* (queen number), Z/W and *P* values for each test are reported below. Total aggression refers to the total addition of rates for Q-Q and W-Q aggression in both the treatment and control groups. * denotes significant Bonferroni corrected *P* values <0.0125.

Test	Median (IQR)	*N*	Z/W	*P*
Q-Q treatment	1.678 (1.034)	14	-2.514	0.012*
W-Q treatment	0.042 (0.037)	14		
Q-Q FM control	0.00 (0.000)	39	-3.739	0.0002*
W-Q FM control	0.05 (0.300)	39		
Q-Q treatment VS Q-Q control	Treatment: 1.678 (1.034) FM Control: 0.00 (0.000)	Treatment: 14 Control: 39	-3.516	0.0005*
W-Q treatment VS W-Q control	Treatment: 0.042 (0.037) FM Control: 0.05 (0.30)	Treatment: 14 Control: 39	-2.545	0.0113*
Total aggression	Treatment: 0.43 (2.240) FM Control: 0.07 (0.60)	Treatment: 14 Control: 39	-0.926	0.376

Table 8: A summary of all per capita behavioural rate (sec/min) data test statistics. Non-parametric test statistics carried out on per capita behavioural rate data for the treatment and FM control groups. Mann-Whitney U tests and Wilcoxon's signed rank tests were performed for all data and the associated median (inter quartile range (IQR)), *N* (queen number), Z/W and *P* values for each test are reported below. Total aggression refers to the total addition of rates for Q-Q and W-Q aggression in both the treatment and control groups. * denotes significant Bonferroni corrected *P* values <0.0125.

Test	Median (IQR)	*N*	Z/W	*P*
Q-Q treatment	0.112 (0.984)	14	-3.024	0.002*
W-Q treatment	0.000 (0.000)	14		
Q-Q FM control	0.00 (0.000)	39	-3.359	0.001*
W-Q FM control	0.0007 (0.0042)	39		
Q-Q treatment VS Q-Q FM control	Treatment: 0.112 (0.984) FM Control: 0.00 (0.000)	Treatment: 14 Control: 39	-3.668	0.0002*
W-Q treatment VS W-Q FM control	Treatment: 0.000 (0.000) FM Control: 0.0007 (0.0042)	Treatment: 14 Control: 39	-2.622	0.009*
Total aggression	Treatment: 0.52 (2.95) FM Control: 0.0009 (0.0166)	Treatment: 14 Control: 39	-2.532	0.011*

Table 9: Summary of rates of aggression (sec/min) between discrete behavioural types. Q-Q and W-Q rates are compared using Mann-Whitney U tests. Where the comparison sample contained 0 observations Wilcoxon's signed rank test was used to test if the sample median was significantly different from 0. The associated median (IQR), *N* (queen number), Z/W and *P* values for each test are reported below. * denotes significant *P* values <0.0125.

Q-Q Aggression	Median (IQR)	*N*	Z/W	*P*
Biting treatment	0.03 (0.320)	14	-3.190	0.001*
Biting FM control	0.00 (0.000)	39		
Pulling treatment	0.32 (1.710)	14	-3.313	0.001*
Pulling FM control	0.00 (0.000)	39		
Spreading treatment	0.00 (0.000)	14	1.0	1.0
Spreading FM control	Not-observed	39		
W-Q Aggression	**Median (IQR)**	**N**	**Z/W**	***P***
Biting treatment	0.00 (0.000)	14	-2.527	0.012*
Biting FM control	0.00 (0.067)	39		
Pulling treatment	0.00 (0.000)	14	-1.409	0.159
Pulling FM control	0.00 (0.000)	39		
Spreading treatment	Not-observed	14	15.0	0.058
Spreading FM control	0.00 (0.000)	39		

Table 10: Summary of per capita rates of aggression (sec/min) between discrete behavioural types. Q-Q and W-Q rates are compared using Mann-Whitney U tests. Where the comparison sample contained 0 observations Wilcoxon's signed rank test was used to test if the sample median was significantly different from 0. The associated median (IQR), *N* (queen number), Z/W and *P* values for each test are reported below. * denotes significant *P* values <0.0125.

Q-Q Aggression	**Median (IQR)**	***N***	**Z/W**	***P***
Biting treatment	0.03 (0.320)	14	-3.381	0.001*
Biting FM control	0.00 (0.000)	39		
Pulling treatment	0.32 (1.710)	14	-3.366	0.001*
Pulling FM control	0.00 (0.000)	39		
Spreading treatment	0.00 (0.000)	14	1.0	1.0
Spreading FM control	Not-observed	39		
W-Q Aggression	**Median (IQR)**	**N**	**Z/W**	***P***
Biting treatment	0.00 (0.000)	14	-2.342	0.019*
Biting FM control	0.00 (0.067)	39		
Pulling treatment	0.00 (0.000)	14	-1.380	0.167
Pulling FM control	0.00 (0.000)	39		
Spreading treatment	Not-observed	14	10.0	0.1
Spreading FM control	0.00 (0.000)	39		

2.3.4 Queen death and eviction

There was no difference in the proportion of dead/evicted queens between the treatment and control groups (Treatment: 63% (18 queens) versus Control: 54% (26 queens); Fisher's exact test: df=1, *P*= 0.58). However, queen eviction and death was found to be high across control and treatment groups. One treatment queen and three FM control queens found dead by the end of the experiment were the reproductive queens for their respective colonies. In the colonies where this happened, all other queens presented ovaries with small underdeveloped ovarioles and an absence of maturing eggs.

Table 11: Summary of all logistic regression test statistics. N_1= number of reproductive queens, N_2= number of non-reproductive queens. For both treatment and control groups, rates of Q-Q aggression and W-Q aggression (sec/min) as predictive variables were tested in isolation and in combination. All treatment and control queens were also tested together with combined rates of Q-Q and W-Q aggression (sec/min) as the predictive variable.

Treatment	**ß(s.e.)**	N_1	N_2	**Wald**	**d.f**	***P***
Q-Q Aggression	0.281(0.305)	4	10	0.853	1	0.356
W-Q Aggression	-4.771(10.552)	4	10	0.204	1	0.651
Q-Q and W-Q Aggression Combined	-0.272(0.287)	4	10	0.892	1	0.345
Control	**ß(s.e.)**	N_1	N_2	**Wald**	**d.f**	***P***
Q-Q Aggression	0.278(0.350)-0.166(0.276)	7	32	0.631	1	0.427
W-Q Aggression		7	32	0.363	1	0.547
Q-Q and W-Q Aggression Combined	0.077(0.151)	7	32	0.259	1	0.611
All Queens	**ß(s.e.)**	N_1	N_2	**Wald**	**d.f**	***P***
All Treatment and Control Queens. Q-Q and W-Q Aggression Combined	-0.04(0.072)	11	42	0.271	1	0.603

Six colonies (treatment =OT.SD.01, OT.SD.04 and VA.SD.04. FM control =OTA.13.05, VA.13.01, VA.13.30) were reduced to a single queen by the end of the summer study period. Only one colony (FM control colony VA.13.01) was rendered effectively queenless as the single surviving queen was unmated. No relationship between total rate of aggression and queen death was found within the treatment group (Median queens alive or evicted/dead per colony±IQR: Alive=0.067 (2.24). Dead/evicted: =0.42 (2.24), Mann-Whitney U test: Z= -0.334, N_{alive}=6, N_{dead}=10, *P*=0.78). Similarly, no relationship was found to exist between rate of aggression and queen death/eviction within the control group (Rate of aggression per queen: Alive=0.067 (0.901) sec/min. Dead/evicted=0.03 0.133 sec/min, Mann-Whitney U test: Z= -

0.349, N_{alive}=23, N_{dead}=27, P=0.74). Using per capita rates did not alter the statistical outcomes for either test.

2.3.5 Grooming

Rates of non-aggressive grooming behaviour (s/m) did not significantly differ between reproductive and non-reproductive queens in the treatment colonies (Median rate per queen±IQR: Reproductive= 3.17 (8.72) sec/min. Non-reproductive= 2.16 (4.56) sec/min, Mann-Whitney U test: Z= -0.424, N_{repro}=4, $N_{non\text{-}repro}$=10, P=0.724) or the control colonies (Median rate per queen±IQR: Reproductive= 1.50 (5.54) sec/min, Non-reproductive= 1.19 (3.012) sec/min, Mann-Whitney U test: Z= -0.990, N_{repro}=7, $N_{non\text{-}repro}$=32, P=0.332). Per capita rates did not alter the conclusions of the statistical analysis for either test.

There was also no significant difference between rates of grooming behaviour received on average by queens (both reproductive and non-reproductive) between the treatment and FM control groups (Median rate per queen±IQR: Treatment= 2.16 (4.60) sec/min. Control= 1.20 (3.988) sec/min, Mann-Whitney U test: Z= -0.757, N_{treat}=14, $N_{control}$=39, p=0.455). Once again, using per capita rates did not alter the statistical conclusions.

2.4.0 Discussion

This study provides evidence that the partitioning of aggression between queens and workers is altered in the presence or absence of aggressive worker enforcement in functionally monogynous (FM) colonies of the ant *L. acervorum*. Specifically, rates of Q-Q aggression were significantly higher and more frequent in treatment colonies where aggressive worker enforcement was absent in comparison to control colonies where worker enforcement was present. Conversely, rates of W-Q aggression were significantly higher amongst the FM control colonies, supporting previous findings (Gill and Hammond, 2011a, Gill and Hammond, 2011b). Furthermore, the total rates of aggression received by queens in the treatment and FM control colonies were not significantly different and when rates were corrected for worker and queen numbers it was found that total aggression in the treatment colonies was significantly higher. This suggests that worker enforcement is not solely responsible for maintaining FM social organisation but is nevertheless likely to play an important regulatory role in natural colonies as demonstrated by the higher W-Q aggression observed in control colonies. Finally, treatment colonies never contained more than one reproductive queen and there were no differences found in the total number of eggs produced or how quickly eggs were laid, indicating that high skew was maintained.

2.4.1 Queen-queen aggression persists in the absence of worker enforcement

The results of this study contradict my initial hypothesis that FM queens would reproduce equally (low skew) in the absence of worker enforcement and that colony organisation would switch to a 'P like' social phenotype. The initial hypothesis was based on previous findings that worker aggression prevented recipient queens from becoming reproductive and ultimately it was workers that maintained high skew, the worker control hypothesis (Gill and Hammond, 2011a, Gill and Hammond, 2011b).

Instead, my observations of frequent Q-Q aggression in the absence of worker enforcement lend support to the idea that queens form a rank order dominance hierarchy as suggested by Trettin et al. (2011), which in turn supports the queen control hypothesis over colony skew. Furthermore, per capita rates of total aggression were significantly higher in treatment colonies, emphasising the frequency of Q-Q aggression and suggests that queens must engage in more frequent bouts of aggression in the absence of worker enforcement. Additionally, treatment queen ovary dissections revealed that only a single reproductive queen was ever present in the colony and egg production between treatment and FM control colonies was not significantly different. Unfortunately, this study was unable to predict the mated status of individual queens via the per capita rates of aggression they received unlike previous studies (Trettin et al., 2011, Gill and Hammond, 2011a, Gill and Hammond, 2011b). It is likely that limited power in the logistic regression analysis due to a low sample size failed to detect a difference, especially amongst the treatment group. Regardless, the evidence suggests that only a single queen per treatment colony became reproductive and that high skew was maintained. Therefore, Q-Q aggression seems sufficient to maintain the FM social phenotype and high skew in the absence of worker enforcement.

The establishment of dominance hierarchies through Q-Q aggression has been well studied in many eusocial species including ants (Yamauchi et al., 2007, Heinze et al., 1992, Heinze and Lipski, 1990, Heinze and Smith, 1990, Holldobler and Carlin, 1985) and it has been argued that ultimate control over reproductive skew rests with the winner of these contests (Nonacs and Hager, 2011, Vehrencamp, 1983, Reeve et al., 1998, Emlen et al., 1998, Johnstone and Cant, 1999, Johnstone, 2000, Buston and Zink, 2009). Leptothoracine species are thought to be especially susceptible to the establishment of dominance hierarchies due to commonality of multiply queened (MQ) colonies and their relatively small colony size (Heinze et al., 1994). Previous studies on *L. acervorum* have reported aggressive Q-Q behaviour being the main predictive factor behind the functional monogyny seen in Spanish, Japanese and

Alaskan colonies (Trettin et al., 2011, Heinze and Ortius, 1991, Heinze and Smith, 1990, Ito, 2005) and a number of other MQ ant species have also reported strong Q-Q aggression leading to the formation of reproductive dominance hierarchies (Yamauchi et al., 2007, Heinze et al., 1992, Heinze and Smith, 1990, Heinze and Lipski, 1990, Holldobler and Carlin, 1985, Roseler, 1991, Lipski et al., 1992). In light of previous evidence and my own findings that Q-Q aggression is both high and frequent between FM queens in colonies without worker enforcement, it is likely that queens in FM *L. acervorum* colonies aggressively interact in order to establish a reproductive dominance hierarchy.

However, the establishment of dominance hierarchies and the maintenance of the FM social phenotype and high skew in *L. acervorum* appears to be more complex than simply resulting from aggressive Q-Q interactions and suggests an important role for workers. For example, I observed low Q-Q aggression in the un-manipulated FM control colonies and rates of W-Q aggression were significantly higher than the treatment colonies in comparison. Furthermore, Q-Q aggression was significantly higher in the treatment colonies compared to the control colonies, suggesting that Q-Q aggression is rare in the presence of worker enforcement. Similarly, the average frequency of W-Q aggression events per control colony was also significantly higher than the average frequency of Q-Q aggression events as demonstrated by the scan sampling data. Curiously, the scan sampling also revealed that significantly more W-Q aggressive events were present in the spring period in comparison to summer where Q-Q interactions were common. However, there was no significant difference in the number of treatment colony W-Q aggressive events between the spring and summer periods, suggesting that a small amount of W-Q aggression persists throughout the breeding season.

If Q-Q aggression is sufficient to establish a reproductive dominance hierarchy as my evidence shows, then an important question is: why are rates of W-Q aggression higher and more frequent in un-manipulated FM colonies?

2.4.2 Explaining worker aggression in FM colonies

The observations of frequent W-Q aggression within un-manipulated FM colonies in this study and others (Trettin et al., 2011, Gill and Hammond, 2011a, Gill and Hammond, 2011b) strongly suggests that worker enforcement plays an important role in regulating skew.

One possible explanation is that worker aggression acts as an enforcement behaviour that coerces queen altruism. Enforcement mechanisms, such as punishment and policing, have been shown to act as an important evolutionary mechanism for regulating the overall relatedness of the colony in many eusocial species (Ratnieks and Wenseleers, 2008, Wenseleers and Ratnieks, 2006b, Wenseleers and Ratnieks, 2006a, Saigo and Tsuchida, 2004). Where multiple queens are present in predominantly monandrous species, and only relatedness benefits are considered, it is within the inclusive fitness interests of the workers to punish selfishly behaving supernumerary queens as worker inclusive fitness decreases in the presence of multiple reproducers (Ratnieks and Wenseleers, 2008, Bourke, 2011, Monnin et al., 2002). In the case of the queenless ponerine ant species *Dinoponera quadriceps*, aggressive enforcement from low ranking workers towards a beta worker looking to usurp the alpha maintains the inclusive fitness of both the alpha worker and the low ranking workers (Monnin et al., 2002, Liebig et al., 1999).

Similarly, FM *L. acervorum* queens are likely to be monandrous and supernumerary queens are often full sisters (Gill et al., 2009, Hammond et al., 2006, Hammond et al., 2001). Therefore, based on relatedness benefits, the same selection pressures exist within FM colonies of *L. acervorum* with multiple queens and should promote the reproduction of only a single queen. Indeed, colony relatedness has been shown to be high in Spanish FM colonies of

L. acervorum and the dominant queen , which was rarely subjected to worker enforcement, was found to be the mother of the majority workforce in the majority of colonies sampled (Gill and Hammond, 2011a, Gill and Hammond, 2011b, Gill et al., 2009, Hammond et al., 2006). Additionally, skew models predict selection for FM when suitable sites for independent nesting are constrained (Keller and Reeve, 1994, Bourke and Heinze, 1994, Reeve and Keller, 2001), which seems likely for FM *L. acervorum* populations in the Spanish Sierra de Albarracin and the Sierra de Gudar (Braim and Hammond, personal observation, Trettin et al., 2011, Gill et al., 2009). Therefore, in situations where FM is selected in nature, worker enforcement may be beneficial to coerce other reproductive individuals to forgo reproduction and behave altruistically. Over time, this can become the primary mechanism for reproductive regulation amongst FM colonies in nature.

Unlike *D. quadriceps* however, many *L. acervorum* populations in Europe have the P social phenotype and do not display high rates of Q-Q or W-Q aggression (see Chapter 5, Bourke, 1991). Polygynous colonies are typically characterised with a low colony relatedness and low skew (Bourke et al., 1997, Hammond et al., 2006) and so perfectly demonstrate how colony relatedness is unable to be properly regulated in the absence of enforcement. Similarly, a lack of worker enforcement in queenless colonies of the honeybee show that 40% of workers lay eggs (Wenseleers and Ratnieks, 2006b). It has also been argued that low skew can be selected when ecological constraints are weak (Keller and Reeve, 1994), allowing for greater independent colony foundation, which is likely true of UK populations of *L. acervorum* (Bourke and Heinze, 1994, Franks et al., 1991). Furthermore, there is some evidence to suggest that larger colony size is associated with P social organisation (Boulay et al., 2014), although I have found no evidence that P colonies are larger than FM colonies in *L. acervorum* (see Chapter 5, Table 5)

Additionally, highly aggressive Q-Q interactions during dominance hierarchy formation may represent a serious risk to a potential reproductive queen. I found that 63% of the treatment queens were dead or permanently evicted by the end of summer. This is similar to colonies of *Leptothorax gredleri* where high queen mortality rate is common and colonies are frequently reduced to a single queen following bouts of aggressive behaviour between queens (Heinze et al., 1998, Heinze et al., 1992). Therefore worker enforcement may be necessary to relieve a dominant queen from risk of damage by continuing the aggressive enforcement of the dominance hierarchy. I found some evidence to support this idea, as one treatment colony lost the reproductive queen before the end of summer. However, three FM control colonies also lost the reproductive queen before the end of summer, presumably through aggressive interactions, highlighting a potential risk that workers may incorrectly direct their aggression, perhaps due to potentially indiscriminate queen smearing signals (discussed in 2.4.3, Gill and Hammond, 2011a).

2.4.3 Characterising Q-Q aggression

Q-Q interactions observed across treatment and FM control colonies were highly aggressive, which contradicts the findings of Trettin et al. (2011). Q-Q aggression commonly consisted of biting, pulling and sting smearing behaviours (See Table 9), which were identical to the equivalents (except for sting smearing) observed between workers and queens (This study, Trettin et al., 2011, Gill and Hammond, 2011a, Gill and Hammond, 2011b, Gill et al., 2009, Ito, 2005, Heinze and Ortius, 1991). The Q-Q aggressive behaviours observed by Trettin et al. (2011) largely consisted of mandibular threat displays and antennal boxing, which I considered to be less overt than the behaviours I recorded (see Table 4).

I am only able to speculate as to the cause of this discrepancy. It may be that overt aggression is required to prevent ovary maturation in queens regardless of the worker or queen origin. Therefore in colonies where worker enforcement is absent, queens must

continue to engage in highly aggressive interactions in order to maintain the dominance hierarchy and high skew. In un-manipulated FM colonies, queens may only need to engage in overtly aggressive interactions early in the breeding season to establish the dominance hierarchy. Following dominance establishment, queens may use a chemical signal to direct worker aggression towards subordinate queens to maintain enforcement and high skew. Therefore, it may be that highly overt Q-Q aggression is rare and difficult to detect.

Sting smearing behaviour was common amongst the treatment colonies, occurring in 60% of colonies a total of 17 times. Furthermore, W-Q aggression never occurred following a queen smearing event, queens continued to participate in aggression and multiple sting smears were often observed in the same video recording. Sting smearing was never observed between queens in the FM control colonies. Sting smearing is a common mechanism for establishing a dominance hierarchy in many ant species including Leptothoracines (Smith et al., 2012, Monnin and Ratnieks, 2001, Heinze et al., 1994, Heinze et al., 1992, Heinze et al., 1998). A queen will typically wrestle with a rival and attempt to bend the abdomen upwards and touch the rival queen with the gaster where secretions from the Dufour's gland are smeared (see Fig 7). These secretions are known to provoke workers into behaving aggressively towards the smeared recipient in many ant species, and have been observed in *Leptothorax acervorum* (albeit rarely) (Heinze et al., 1992, Hemelrijk et al., 2005, Gill and Hammond, 2011a, Monnin et al., 2002, Smith et al., 2012).

Secretory signals from the Dufour's glad have been shown to operate within a range of detection specificity amongst different ant species. Dufour's glad secretions are general in the queenless ant species *Dinoponera quadriceps* (Monnin et al., 2002), where secretions from non-native nests triggered aggression. They may be caste specific amongst high and low ranking gamergates in *Aphaenogater cockerelli* (Smith et al., 2012) as only the queen's secretions induce aggression. Finally, they may be colony specific in the closely related

Leptothorax gredleri (Heinze et al., 1998), where workers can distinguish queen secretions from other colonies and only respond aggressively to their own. The common use of sting smearing amongst dominance hierarchy forming ant species makes sting smearing a likely candidate for directing worker enforcement in *L. acervorum*. Additionally, the observation that workers did not respond to the sting smearing events in the treatment colonies suggests that Dufour's gland secretions are specific at either the colony, population or the social phenotype level.

A previous study found sting smearing to be an uncommon event in FM colonies with only 11 observations in three colonies (Gill and Hammond, 2011a). Furthermore, it was shown that worker aggression was directed at both the actor and the recipient directly after the sting smear had occurred. This led Gill and Hammond (2011a) to dismiss the role of sting smearing as an honest signal for queens to direct worker enforcement towards rivals. However, the high frequency of sting smearing events observed in this study amongst the treatment colonies and the instances of multiple sting smearing events strongly suggests that sting smearing behaviour does play an important role in FM social organisation. I hypothesise, based on the evidence discussed, that queen smearing events are important in directing worker aggression and for maintaining the reproductive hierarchy. Although the signal has been shown to be indiscriminate in *L. acervorum* (Gill and Hammond, 2011a), it may be possible that the future dominant queen is more successful at smearing her opponent without evoking worker aggression to herself similar to *L. gredleri* (Heinze et al., 1998). Additional studies are required to investigate this hypothesis.

Alternatively, sting smearing was only previously observed in *L. acervorum* colonies that had been experimentally manipulated (Gill and Hammond, 2011a). Therefore, it may be a possibility that perturbations to original colony structure might cause an increase in Q-Q sting smearing events. This may explain why sting smearing was common amongst the treatment

colonies as the colony structure had been extensively altered. Again, further studies are required to investigate this hypothesis.

2.4.4 Evidence supporting a genetic basis to polymorphic social organisation

This study provides further evidence that polymorphic social organisation in colonies of *L. acervorum* is likely to be genetic and not environmental in origin as has been proposed before (Gill and Hammond, 2011a, Gill and Hammond, 2011b, Gill et al., 2009). First, the high rates of Q-Q aggression observed within treatment colonies suggest that FM queens may possess an inherent behavioural trait allowing them to behave aggressively and participate in reproductive dominance hierarchies. Second, the possible specificity of Dufour's gland chemicals in sting smearing events between queens may also suggest that FM workers inherit a genetic predisposition to detect and act upon queen secretions (Monnin et al., 2002). Indeed, it is possible that FM workers are able to detect and act upon sting smearing secretions, whereas P workers have lost that trait. Third, differences in Q:W ratios between each colony used in this study are unlikely to explain the differences in rates of Q-Q, as has been suggested previously (Trettin et al., 2014). The analysis of per capita rates of aggression did not alter the statistical conclusions. Furthermore, I provide evidence in Chapter 5 that P colonies do not alter aggressive behaviour in response to Q:W ratio. That being said, I cannot rule out the possibility that Q:W ratio does have an effect on rates of aggression in FM colonies.

2.4.5 Removal of worker enforcement

Worker enforcement was successfully removed through the use of brood exchange within the treatment colonies. The introduction of P worker pupae from the UK into a nest containing Spanish FM workers and queens was successful, and allowed P workers to co-exist with FM queens once all FM workers had been removed. This is evidenced by the low

incidence of W-Q aggression within treatment colonies compared to the FM control colonies and the non-significance in rates of W-Q aggression between the treatment colonies and the UK P controls. Finally, as already discussed, P workers in treatment colonies were never observed to attack queens following a Q-Q sting smearing event. If sting smearing is an important feature in directing worker enforcement towards subordinate queens in FM colonies, then the lack of response from P workers further suggests that worker enforcement within the control colonies was absent. These observations strongly suggest that worker enforcement was absent within treatment colonies and that the brood exchange methodology was appropriate for addressing the initial hypothesis.

However, it is worth mentioning that the proportions of brood that were successfully raised to adulthood in cross fostered colonies was highly variable (see Table 2), indicating the difficulty of this method. As a result, I recommend only using large numbers of early stage worker pupae (newly emerged from the pre-pupa stage) as they seem more robust to handling and hatch in a short time (pers. obs).

2.4.6 Conclusions

I found that FM queens, in the absence of aggressive worker enforcement, continue to engage in aggressive behaviours with each other and the absence of worker enforcement did not alter colony skew. The fact that high skew was maintained in colonies without worker enforcement suggests that W-Q aggression is not necessary for regulating reproductive skew. Instead, it is more likely that queens control skew by engaging in aggressive Q-Q interactions, which allows them to maintain reproductive dominance, possibly through the establishment of reproductive dominance hierarchies. However, aggressive worker enforcement is likely to be important in regulating and maintaining colony skew, possibly through the detection of Dufour's glad chemicals secreted by the queens. Furthermore, I have provided additional evidence to show that the social polymorphism presented in *L. acervorum* is likely to have a

genetic basis. This study, therefore, provides useful insights into the roles that queens and workers play in complex aggressive interactions, which perform an essential role in regulating skew in FM colonies of *L. acervorum*.

Chapter 3

Vitellogenin expression between reproductive and non-reproductive queens of the ant species *Leptothorax acervorum*: a molecular marker for measuring reproduction

3.1 Introduction

Creating new molecular markers is essential for addressing interesting biological questions and is particularly important in non-modal systems. The ant species, *Leptothorax acervorum*, represents a useful emerging model for exploring differences in social organisation, reproductive skew and behaviour (Trettin et al., 2014, Trettin et al., 2011, Gill and Hammond, 2011a, Gill and Hammond, 2011b, Gill et al., 2009, Hammond et al., 2006, Hammond et al., 2003, Hammond et al., 2002). *L. acervorum* colonies commonly contain multiple mated queens (MQ) with the potential to reproduce and colonies can belong to one of two social phenotypes. The first social phenotype, polygyny (P), describes an organisation whereby all queens are able to reproduce equally and without antagonism from conspecific workers or queens (Hammond et al., 2006, Bourke et al., 1997, Bourke and Heinze, 1994). Alternatively, colonies may possess the social phenotype functional monogyny (FM), where only one queen reproduces and all other viable queens fail to become reproductive (Trettin et al., 2011, Gill and Hammond, 2011b, Gill et al., 2009, Ito, 2005, Felke and Buschinger, 1999). There are significant comparative differences in behaviour, reproductive skew and relatedness between the alternate social phenotypes (Trettin et al., 2011, Gill and Hammond, 2011b, Gill et al., 2009, Hammond et al., 2006, Ito, 2005, Felke and Buschinger, 1999, Bourke et al., 1997, Bourke and Heinze, 1994), which make *L. acervorum* an excellent model for investigating

many interesting socio-evolutionary questions such as variation in skew, social organisation and behaviour.

The partitioning of reproduction within a social group (reproductive skew) is a source of conflict between reproductive individuals (Reeve and Keller, 1995, Vehrencamp, 1983, Nonacs and Hager, 2011, Clutton-Brock, 1998). Therefore, investigating reproductive skew is fundamental to understanding the evolution of social organisation and dissecting the mechanisms responsible for regulating reproduction (Bourke, 2011). Consequently, the within species differences in social phenotype present between populations of *L. acervorum* make it an excellent model for understanding differences in skew (Gill et al., 2009).

Methods of measuring reproduction in *L. acervorum* have so far been restricted to physical egg counts (Hammond et al., 2006, Hammond and Keller, 2004, Hammond et al., 2003, Hammond et al., 2002) and ovary dissections (Gill et al., 2009). Physical egg counts are arguably the most useful measure of reproduction as egg production directly translates into colony productivity. However, without constant observation, it is difficult to determine with any certainty which queens are responsible for producing the eggs (although it is possible to genotype eggs using microsatellites to determine parentage (Hammond et al., 2002)) and there is no reliable way of detecting reproductive queens before eggs are laid. It can also be difficult to tell reproductive and trophic eggs apart and *L. acervorum* workers are capable of laying unfertilised eggs in the presence of queens (Ito, 2005, Bourke, 1991). Additionally, *L. acervorum* colonies have been reported to participate in high rates of oophagy (Hammond et al., 2006, Hammond and Keller, 2004, Hammond et al., 2003, Hammond et al., 2002, Bourke et al., 1997, Ito, 2005). Both worker laying and oophagy can lead to a biased measurement of reproduction.

Ovary dissections are effective at determining recent reproductive activity due to the physical presence of a full spermatheca, elongated ovarioles, developing eggs and corpora

lutea (Bourke, 1991, Gill et al., 2009). However, the method for dissecting ant queens is tricky and ovaries can be easily damaged beyond recognition during the process. Furthermore, ovary tissue begins to decompose very quickly (even within 24 hours), which makes dissection and recognition very difficult in queens that are found dead during an experiment.

Therefore, the development of effective molecular markers for measuring reproduction can prove very useful for testing various hypotheses regarding reproductive skew between polygynous and functionally monogynous colonies of *L. acervorum*. An excellent candidate for measuring reproduction in *L. acervorum* is vitellogenin.

Vitellogenin (gene=*vg,* protein=Vg) codes for an egg yolk storage precursor, which is important for the maturation of eggs in the ovary, and its expression is often associated with the activation of the ovaries in queens before reproduction (Tufail and Takeda, 2008, Engels, 1974, Azevedo et al., 2011, Chen et al., 2004, Amdam et al., 2006, Tufail et al., 2014). More specifically in insects, vitellogenin is synthesised by the fat body and released into the haemolymph where it travels to the ovaries to be stored as vitellin (Engels, 1974, Raikhel and Dhadialla, 1992, Tufail and Takeda, 2008, Tufail et al., 2014). The importation of Vg into the ovaries is achieved via receptor-mediated endocytosis and to date a number of different vertebrate and invertebrate Vg receptors have been characterised (Chen et al., 2004, Tufail and Takeda, 2008).

The *vg* sequence can vary considerably between species (Avarre et al., 2007). However, it does contain multiple highly conserved motifs which are present amongst many different taxa (Spieth et al., 1991, James et al., 1982, Morandin et al., 2014, Baker, 1988). One such domain, the C-terminal von Willebrand Factor type D domain (vWFD), is characterised by a GL/ICG amino acid sequence motif, which is conserved amongst insect species including ants (Tufail and Takeda, 2008). The specific function of the vWFD is unknown, however it is often inherited along with other domains which have been identified as key contributors to Vg

provisioning (Morandin et al., 2014, Roth et al., 2013). Therefore, the conserved nature of the vWFD amongst insect species makes it an excellent target for designing primers that will allow the amplification and measurement of *vg* gene expression.

Interestingly, multiple *vg* genes are often observed amongst different ant species (Morandin et al., 2014, Corona et al., 2013, Wurm et al., 2011). Furthermore, duplicate copies of *vg* have been shown to possess novel functions in regulating worker behaviour in two myrmicine ant species, *Solenopsis invicta* (Wurm et al., 2011) and *Pogonomyrmex barbatus* (Corona et al., 2013). It may be possible that *L. acervorum*, also a myrmicine species, possesses multiple copies of the *vg* gene. This meant that any *L. acervorum vg* sequences obtained needed to be functionally verified so as to determine the suitability as a marker for reproduction.

The aim of this study was to develop and establish *vg* expression as an effective marker for reproduction in *L. acervorum* queens to be used in future RT-qPCR analysis. First, genomic sequence information for *vg* in *L. acervorum* needed to be obtained and validated. Second, the likelihood of reproductive function for the acquired *vg* sequence was determined via phylogenetic comparison with other ant *vg* sequences. Third, primers for use in RT-qPCR analysis were designed to amplify *vg* and a stable housekeeping gene in *L. acervorum* queens. Finally, the hypothesis that *vg* is significantly up-regulated in reproductive queens compared with non-reproductive queens was tested via RT-qPCR analysis using the designed *vg* primers. This study represents the first successful use of RT-qPCR analysis in *L. acervorum* and adds a new molecular marker to the toolkit for testing future hypotheses.

3.2 Methodology

3.2.1 Identification of conserved GLCG motif in insect vitellogenins

Currently there is no *vg* sequence information for *L. acervorum*. However, recent evidence aligning Vg protein sequence data from three bee species resulted in the identification of the conserved GLCG motif associated with the vWFD domain (Li et al., 2010). To identify if the GLCG motif is conserved in ants and other insects, 24 Vg protein sequences were obtained from 15 separate insect species and aligned using Clustal 2.1 (Larkin et al., 2007). The alignment revealed 100% conservation of the GLCG across all aligned *vg* sequences (Fig 1). This allowed the *vg* nucleotide sequence corresponding to the translated GLCG motif to be located in four ant species (*Atta cephalotes, Pogonomyrmex barbatus, Camponotus floridanus* and *Harpegnathos saltator*) and aligned to determine the degenerate nucleotides (Fig 2). Degenerate primers were then designed using CODEHOP software (Rose et al., 2003) to accommodate the synonymous positions (Table 1).

```
Acep_VgA_1          LVSN-RYRNALRGLCGNYDSDPSTDFLTPQNCVMKMPEVFTATYALT
Aech_VgA-1_1        LASD-RYRNTLRGLCGNYDSDPSTDFLTPQNCLVRKPEVFTATYALT
Si_Vg2-RA_1         SASD-AYRNAVRGLCGNFDSRPNTDFVTPKNCLLTKPEEFAATYAMT
Si_Vg3-RA_1         YANGATYRNAIRGLCGNYDSRRDNDFLTPKNCLLTKPEEFAATYAMT
Pbar_VgA-1_1        QASN-KYRNAVRGLCGNYDMQSDNDFMTPKNCVLRKSEEFAATFALT
Lhum_VgA-1_1        ELSN-KYRNKVRGLCGNYDRELKNDFLTPDNCIAEDARKFVAAYTLT
Lhum_VgB-RA_1       SASS-KYRTAISGLCGNYDRQANNDFITPSNCMMQKSDEFIATYSLT
Lhum_VgC-RA_1       QVTK-SYRNAVRGLCGNYDKQANNDFLIPQNCILQKPEEFSATFALL
Lhum_VgD-RA_1       QMTQ-DYRNMVRGLCGNYDTQTSNDFLTPQNCVLQKPEEFSATFAVL
Si_Vg1_1            MISD-NYLNAVRGLCGNYDTQPNNDFIIPENCILTKAEEFAATYAMT
Si_Vg4-RA_1         QSSY-KYYNAVRGLCGNYDTRSNNDFISPKNCILTKPEEFAATYALT
Aech_VgB-RA_1       LVHD-KYLYTVRGLCGNYDMQSNNDFVTPKNCILTKPEEFAATYALT
Aech_VgC-RA_1       YVHD-KYLYTLRGLCGNYDMQSNNDFVTPKNCILQKPEEFAATYALT
Pbar_VgB-RA_1       EAHR-KYRNSIRGLCGNYDLRSDNDFIIPKNCVLTRPEEFTASYILI
Lhum_VgE-RA_1       EVSD-KYRNTIRGLCGNYDAQPNNDFITPGNCILKEYEEFSAMYAMT
Pnip_Vg_1           KASG-KYRSDIRGLCGNFDGEPDNDFTSPKDCVLLKPEEFAASYALT
Aros_Vg_1           ILNN-EYRDQIRGLCGTFNGEPATDFTAPQNCILKNPEHFAASYALT
Nvit_Vg1_1          QAGD-RYRDSVRGLCGNNDLEPENDQQTPRGCTLQKSEEFSATYALT
Ppup_Vg_1           KAGD-RYRNSVRGLCGNNDLEPENDQQTPRGCMLQKSDEFSATFALT
Nvit_Vg2_1          ETGE-RYRDSVRGLCGNNDGESMNDQQTPKGCLLQKPEEFSATYALT
Efor_Vg_1           KVGN-RYRDSVRGLCGNNDGESVDDQQTPQGYLIQNPLEFAATYALT
Amel_Vg_1           KASE-DYRYSVRGLCGNFDHDSTNDFVGPKNCLFRKPEHFVASYALI
Cflo_Vg-RA_1        RVAD-QYANEIRGLCGNYDSRPDNDFLSPQNCVAQKPEHFHAMYTLM
Hsal_Vg-RA_1        KASD-KYNGELRGLCGAKANIREHQLITPAGCYVEEPADVVELYAYD
                          *   : ****        :   * .        .   :
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Fig 1: Clustal alignment of 24 insect Vg protein sequences. Ant Vg sequences display complete conservation of the **GLCG** motif.

Table 1: Primer pairs designed for *L. acervorum*. **Bold and *** indicates primer pairs that produced a single product of expected size and were used in further qPCR analysis.

Primer Name	5'-3' Sequence	Purpose	Annealing Temperature
GLCG P1 (T)	CGYGGTCTYTGTGG	Cloning Vitellogenin	40˚C
GLCG P1 (C)	CGYGGTCTYTGCGG	Cloning Vitellogenin	40˚C
M13_M4 Oligo dT	GTTTTCCCAGTCACGACTTTTTTTTTTTTTTTT	Cloning Vitellogenin	65˚C
M13_M4 Reverse	GTTTTCCCAGTCACGAC	Cloning Vitellogenin	55˚C
Vit_1 Forward	CCAATCCAGATTTTACTCGAAAGC	qPCR	60˚C
Vit_1 Reverse	CACTTCGGTATTGTTGGATTACGC	qPCR	60˚C
Vit_2 Forward*	**AAGTCGCACGTTTTCCAAGTTC**	**qPCR**	**60˚C**
Vit_2 Reverse*	**CGGTATATCACTTCGGTATTGTTGG**	**qPCR**	**60˚C**
Vit_3 Forward	CAAGTCGCACGTTTTCCAAGTT	qPCR	60˚C
Vit_3 Reverse	TGCATATGATCGGTATATCACTTCG	qPCR	60˚C
ELF_1 Forward	CTTCCCCTTCAGGATGTGTACAAG	Expression Control	60˚C
ELF_1 Reverse	CGAAGGTAACAACCATACCAGGTT	Expression Control	60˚C
ELF_2 Forward*	**GAAGTTAAGTCCGTCGAAATGCAC**	**Expression Control**	**60˚C**
ELF_2 Reverse*	**GATACGTTCTTGACGTTGAAACCA**	**Expression Control**	**60˚C**
ELF_3 Forward	TACAAGATTGGTGGTATCGGAACA	Expression Control	60˚C
ELF_3 Reverse	CGAAGGTAACAACCATACCAGGTT	Expression Control	60˚C
RPS18_1 Forward	CACGAACATTGACGGTAACAGAA	Expression Control	60˚C
RPS18_1 Reverse	TCGGCCTTCTTCAAGACGATATTA	Expression Control	60˚C
RPS18_2 Forward*	**AATTGTCACTATCATGGCCAATCC**	**Expression Control**	**60˚C**
RPS18_2 Reverse*	**GATGTCCTTTTGCCTGTTAAGGAA**	**Expression Control**	**60˚C**
RPS18_3 Forward	CGAATCAACAGTGCTAATGTTCCA	Expression Control	60˚C
RPS18_3 Reverse	GATGTCCTTTTGCCTGTTAAGGAA	Expression Control	60˚C
UBIQ_1 Forward	CCCGATCAACAAAGACTGATTTTC	Expression Control	60˚C
UBIQ_1 Reverse	GGATATTGTAGTCGGACAAAGTTCG	Expression Control	60˚C
UBIQ_2 Forward*	**CCCGATCAACAAAGACTGATTTTC**	**Expression Control**	**60˚C**
UBIQ_2 Reverse*	**CCTCTAAGTCGAAGTACAAGGGGAAG**	**Expression Control**	**60˚C**

3.2.2 Amplification of *L.acervorum vg* using 3'RACE

RNA was collected and pooled from the whole bodies of 2x queens, 2x workers, 2x larvae and 2x eggs belonging to colonies NF10.4 and NF10.12 using a GenElute® Mammalian Total RNA Mini Preparation kit (Sigma-Aldrich, UK) following standard protocols. Colonies NF.10.4 and NF.10.12 were collected from Dawkins Bottom in the New Forest (3.2.5). All concentration and purity measurements for each RNA sample were estimated using a NanoDrop spectrophotometer (LabTech International). First strand cDNA was transcribed following a 3' RACE (Rapid Amplification of CDNA Ends) protocol using an M13_M4 oligo dT Primer (Table 1). The NanoDrop RNA concentration values were used to calculate a total sample volume of 17.75µl containing; 0.5 µg/µl of sample RNA, 0.5 µl (100mM) M13_M4 oligo dT primer and sterile water. The solutions were then incubated for 3 minutes at 70˚C to allow primer annealing. Following incubation, 5 µl MMLV Buffer *(Promega, UK)*, 1 µl MMLV Reverse Transcriptase *(Promega, UK)* and 1.25 µl dNTP's were added to each sample and subjected to PCR amplification. First strand cDNA PCR protocol was as follows; 2 minutes at 94˚C, 35 cycles of 94˚C for 30 secs, 55˚C for 1 min and 72˚C for 2 mins. Finally an extension step at 72˚C for 10 mins was included to finish off any remaining transcription.

Protein	**R**	**G**	**L/I**	**G**	**C**
Hsal	**cgc**	**ggt**	**ttg**	**tgc**	**ggc**
Cflor	**cgc**	**ggt**	**ctt**	**tgt**	**ggc**
Aceph	**cgt**	**ggt**	**ctc**	**tgt**	**ggc**
Pbarb	**cgc**	**ggt**	**ctc**	**tgc**	**ggc**

Fig 2: Alignment of ant *vg* nucleotide sequences. The conserved GLCG motif is highlighted in **Red**. Base pair mismatches are highlighted in **green**. Sequences origins were; *Harpegnathos saltator, Camponotus floridanus, Atta cephalotes and Pogonomyrmex barbatus.*

Second stand synthesis was achieved using two degenerate primers (see Table 2) designed to amplify a conserved motif of ant derived Vitellogenin (GLCG(T) and GLCG(C)) isolated from CLUSTAL alignment of insect *vg* sequences (Fig 1-2). 50µl reactions were made for each colony sample and primer set using the following mixture: 5 µl PCR Buffer (10x), 5 µl $MgCl_2$ (25mM), 0.8 µl dNTP's (10mM), 0.8 µl primer (10mM), 0.8 µl Jumpstart Taq (2.5 units/µl, Sigma-Aldrich, UK), 27.6 µl dH_2O and 10 µl single stranded cDNA template. The PCR conditions for second strand synthesis followed the first strand protocol described above. All PCR products were electrophoresed on a 1% agarose gel to check for single product amplification. PCR products amplified using GLCG (T) primers presented a single band (see Fig 3) so they were purified using a GeneElute PCR Cleanup kit (Sigma-Aldrich, UK) as per user instructions.

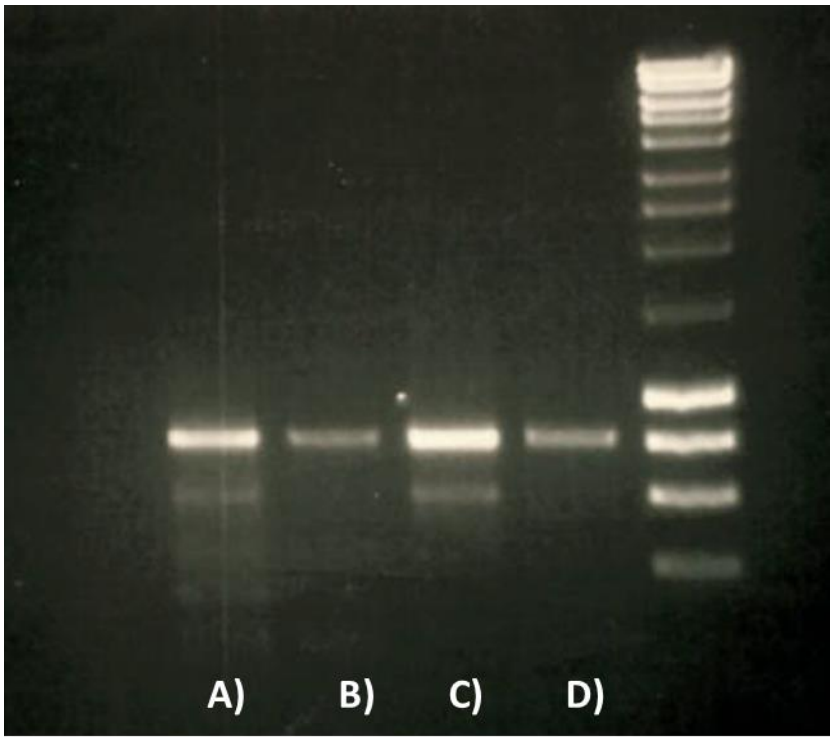


Fig 3: Gel electrophoresis of *vg* primer products resulting from 3' RACE. A) Primer GLCG P1 (T) with sample NF.10.12. B) Primer GLCG P1 (C) with sample NF.10.12. C) Primer GLCG P1 (T) with sample NF.10.04. D) Primer GLCG P1 (C) with sample NF.10.04. All samples had 2µg loading volume. The ladder is HyperLadder 1 (Bioline, UK).

3.2.2 *L.acervorum vg* cloning and Sanger sequencing

Purified *vg* cDNA samples were cloned using a pGEM T-Easy kit (*Promega*) following user protocols. Cloning efforts resulted in 11 successful inserts (10 resulting from sample NF.10.4 and 1 from sample NF.10.12). PCR confirmation was then carried out using the following 10µl reaction volume protocol for each sample: 1 µl PCR Buffer (10x), 0.4 µl $MgCl_2$ (25mM), 0.25 µl dNTP's (10mM), 0.5 µl primer (10mM), 0.1 µl Taq DNA polymerase (2.5 units/µl, *Bioline, UK*), 7.25 µl dH_2O and 50% of the bacterial colony transferred using a sterile pipette tip. The PCR schedule was as follows; 1 minute at 95˚C, 35 cycles of 95˚C for 15 secs, 55˚C for 15 secs and 72˚C for 30 secs and a final an extension step at 72˚C for 7 mins. PCR products for all samples were separated by agarose electrophoresis and six samples presented a single band at the expected size.

Samples presenting the expected *vg* insert were then prepared for Sanger sequencing using a GeneElute Plasmid Miniprep kit (Sigma-Aldrich, UK) following user protocols. All samples were then sent to Genome Enterprise Limited (Norwich) for forward and reverse Sanger sequencing.

3.2.2 Consensus construction and ant *vg* sequence confirmation

Initial quality control on the forward and reverse *vg* sequences involved trimming off vector associated and ambiguous nucleotides in Geneious 7.1.5, using the 'trim ends' function and by eye. The remaining high quality sequences were aligned using a 'global alignment with free end gaps' model included in Geneious 7.1.5. False duplicate nucleotides caused by software miss-calling were removed by eye, leaving a complete high quality sequence totalling 778bp. The consensus was subjected to a BLASTn alignment with 'somewhat similar' parameters against the nr database to search for similarity with other published vitellogenin sequences.

3.2.3 Ant *vg* phylogeny and confirmation of reproductive role

Recent studies have demonstrated the frequent duplication of *vg* genes in ant genomes and some duplicates have been shown to be associated with division of labour. To confirm that our *vg* sequence is likely to be involved in reproduction and not the division of labour, a phylogenetic tree was constructed using currently published ant *vg* genes and the *L. acervorum* consensus. Geneious Tree Builder was used to construct the phylogeny with the following model options; 1) global alignment with free end gaps, 2) cost matrix 65% similarity (5.0/04.0), 3) Jukes-Cantor genetic distance model, 4) Neighbor-Joining tree building method and 5) *Apis mellifera* was used for the outgroup.

3.2.4 *L. acervorum* qPCR housekeeper primer design and stability testing

Three housekeeping genes were selected in order to normalise *vg* expression data in the RT-qPCR analysis based on their known effectiveness in other social insects, *elongation factor 1α* (*elf*), *ubiquitin* (*ubiq*) and *ribosomal protein S18* (*rps18*). Sequence for *S. invicta elf* (ENA accession number: EFZ18275.1) was used to design qPCR primers. Predicted *rps18* sequence from the honeybee *Apis mellifera* was taken from the NCBI database (accession number: XM_625101) and BLASTn aligned against the *S. invicta* genome (http://hymenopteragenome.org/ant_genomes/). This provided a 357bp sequence which was used as the template for primer design. Finally, *S. invicta polyubiquitin-A-like* sequence (accession number: XM_011165906) was used as the template for designing primers to amplify *ubiq*. Primer3web v4.0.0 software (http://frodo.wi.mit.edu/, (Untergasser et al., 2012)) was used to design all housekeeping primers for use in RT-qPCR according to the following specifications: primer size 20-26bp, primer TM 50-65˚C, primer %GC 40-60 and product size 70-120bp. Three forward and reverse primer pairs were initially designed for each housekeeping gene. All primer sets were tested for a single amplicon of the expected size

via PCR amplification of test cDNA and gel electrophoresis. One housekeeping primer pair (see Table 1) successfully amplified a single product of expected amplicon size for each housekeeper gene.

Stable expression of housekeeping genes across all treatment groups is essential for generating reliable gene of interest expression data (Vandesompele et al., 2002). To determine the most stable housekeeping gene, qPCR analysis was carried out using cDNA constructed from 8x queens and 8x workers collected from OT, V, PF and SD (see 3.2.5 for ant population information, 3.2.1 for RNA extraction protocol, see 3.2.7 for qPCR protocol and see Appendix 1, Table 1 for queen and worker qPCR data). Expression stability for each housekeeping gene was calculated using NormFinder (Andersen et al., 2004) and the best housekeeper gene was chosen for all further qPCR analysis.

3.2.5 Collection, maintenance and composition for colonies containing laying and non-laying queens

L. acervorum colonies were collected in the UK from Dawkins Bottom (NFDB) and Dunce's Arch (NFDA) in the New Forest in April 2010. Additional UK colonies were collected from Harlestone Firs (NFHF) in Northampton in 2012. Colonies were also collected in Spain from the Parque Natural Valles Occidentales, Jaca (JA), Orihuela del Tremendal, Sierra de Albarracin (OT) and Valdelinares, Sierra de Gudar (V) in July 2013. See Appendix 1, Fig 1 for population locations. Complete colonies were retrieved from decaying twigs found on the forest floor and taken back to the lab. All colonies were provided with an artificial nest consisting of a clear plastic box (Dimensions: 12cm x 8cm x 2cm) with 5mm of plaster of Paris in the base (see Chapter 2 for full details on nest box construction). Colonies were censused within 10 days of collection in all cases and stored in a versatile environment chamber (*Sanyo*, Model: MLR-351H) under spring controlled conditions (hourly rhythm=11-1-11-1, Temp/°C=10-15-20-15, Photoperiod (Night-Day-Day-Day)= 0-2-3-2, humidity/%=70-70-80-70).

Table 2: Summary data for reproductive and non-reproductive queens.

Colony	Collection Site	Country of Origin	Reproductive Status
JA.13.01.Q1	Jaca (JA)	Spain	Reproductive
JA.13.04.Q1	Jaca (JA)	Spain	Reproductive
JA.13.06.Q1	Jaca (JA)	Spain	Reproductive
JA.13.06.Q5	Jaca (JA)	Spain	Reproductive
JA.13.06.Q6	Jaca (JA)	Spain	Reproductive
JA.13.06.Q7	Jaca (JA)	Spain	Reproductive
JA.13.06.Q8	Jaca (JA)	Spain	Reproductive
JA.13.06.Q9	Jaca (JA)	Spain	Reproductive
JA.13.06.Q13	Jaca (JA)	Spain	Reproductive
JA.13.06.Q14	Jaca (JA)	Spain	Reproductive
JA.13.09.Q1	Jaca (JA)	Spain	Reproductive
JA.13.11.Q1	Jaca (JA)	Spain	Reproductive
HF.12.5.Q2	Harlestone Firs (HF)	UK	Reproductive
HF.12.20.Q1	Harlestone Firs (HF)	UK	Reproductive
NFDA.12.7.Q1	Dunce's Arch (DA)	UK	Reproductive
NFDA.12.10.Q1	Dunce's Arch (DA)	UK	Reproductive
NFDA.12.11.Q1	Dunce's Arch (DA)	UK	Reproductive
NFDA.12.20.Q1	Dunce's Arch (DA)	UK	Reproductive
NFDB.12.4.Q1	Dawkins Bottom (DB)	UK	Reproductive
NFDB.12.5.Q1	Dawkins Bottom (DB)	UK	Reproductive
NFDB.12.10.Q1	Dawkins Bottom (DB)	UK	Reproductive
NFDB.12.12.Q1	Dawkins Bottom (DB)	UK	Reproductive
JA.13.03.Q1	Jaca (JA)	Spain	Non-reproductive
JA.13.06.Q12	Jaca (JA)	Spain	Non-reproductive
JA.13.06.Q11	Jaca (JA)	Spain	Non-reproductive
JA.13.14.Q2	Jaca (JA)	Spain	Non-reproductive
JA.13.14.Q1	Jaca (JA)	Spain	Non-reproductive
HF.12.13.Q1	Harlestone Firs (HF)	UK	Non-reproductive
HF.12.16.Q1	Harlestone Firs (HF)	UK	Non-reproductive
NFDA.12.4.Q1	Dunce's Arch (DA)	UK	Non-reproductive
NFDA.12.8.Q1	Dunce's Arch (DA)	UK	Non-reproductive
NFDB.12.5.Q2	Dawkins Bottom (DB)	UK	Non-reproductive
NFDB.12.8.Q1	Dawkins Bottom (DB)	UK	Non-reproductive
NFDB.12.9.Q1	Dawkins Bottom (DB)	UK	Non-reproductive
OT.13.02.Q8	Orihuela del Tremendal (OT)	Spain	Non-reproductive
OT.13.13.Q7	Orihuela del Tremendal (OT)	Spain	Non-reproductive
OT.13.16.Q3	Orihuela del Tremendal (OT)	Spain	Non-reproductive
VA.13.28.Q3	Valdelinares (V)	Spain	Non-reproductive
VA.13.39.Q2	Valdelinares (V)	Spain	Non-reproductive

Colonies were fed half a meal worm and 10% honey water twice a week (Monday and Friday) and the nests were kept moist by adding three drops of water to the plaster during feeding.

Twenty eight colonies containing 39 queens were selected from across all populations (Table 2). Six colonies from NFDA, six colonies from NFDB and four colonies from HF were selected from the UK populations. Seven colonies from JA, three colonies from OT and two colonies from VA were selected from the Spanish populations. All queens were removed from the nest, snap frozen in liquid nitrogen and stored at -80˚C.

3.2.6 Queen reproductive status

Queen ovaries were dissected in 300µl of RNAlater (Sigma, UK) and assigned a category score (A-D) based on the presence of a mated spermatheca, ovariole development, eggs and the presence/absence of corpus lutea following the criteria established in Gill et al. 2009. Queens with either an A-B ovary were classified as reproductive and queens with a C-D ovary were classified as non-reproductive (Fig 4). Twenty two queens were classified as reproductive and 17 were classified as non-reproductive (Table 2).

3.2.7 RT- qPCR analysis of *vg* expression

Total RNA was extracted from each queen using a GenElute® Mammalian Total RNA Mini Preparation kit (Sigma-Aldrich, UK) and DNase treated using DNase 1 Amplification Grade kit (Sigma-Aldrich, UK), as per user instructions. All samples were analysed for RNA concentration using a NanoDrop spectrophotometer (LabTech International). One microgram of DNase treated total RNA was electrophoresed on non-denaturing 1.5% (w/v) agarose gel to check for degradation before use in the RT-qPCR reaction. cDNA construction was performed using random primers 0.5µg/µl (Promega, UK), M-MLV reverse transcriptase 200u/µl (Promega, UK), M-MLV RT 5x buffer (Promega, UK), dNTP's 10mM and RNAse free water (Fisher Scientific, UK). cDNA was constructed using the following protocol: random primer ligation; step, 0.1ng/µl of sample RNA was made up to 17.75µl final volume using 0.2µl

random primers and RNAse free water and incubated at 70˚C for 5 minutes. Extension step; 5µl of M-MLV x5 buffer, 1µl M-MLV RT and 1.25µl of dNTPs were added to each sample and incubated at 42˚C.

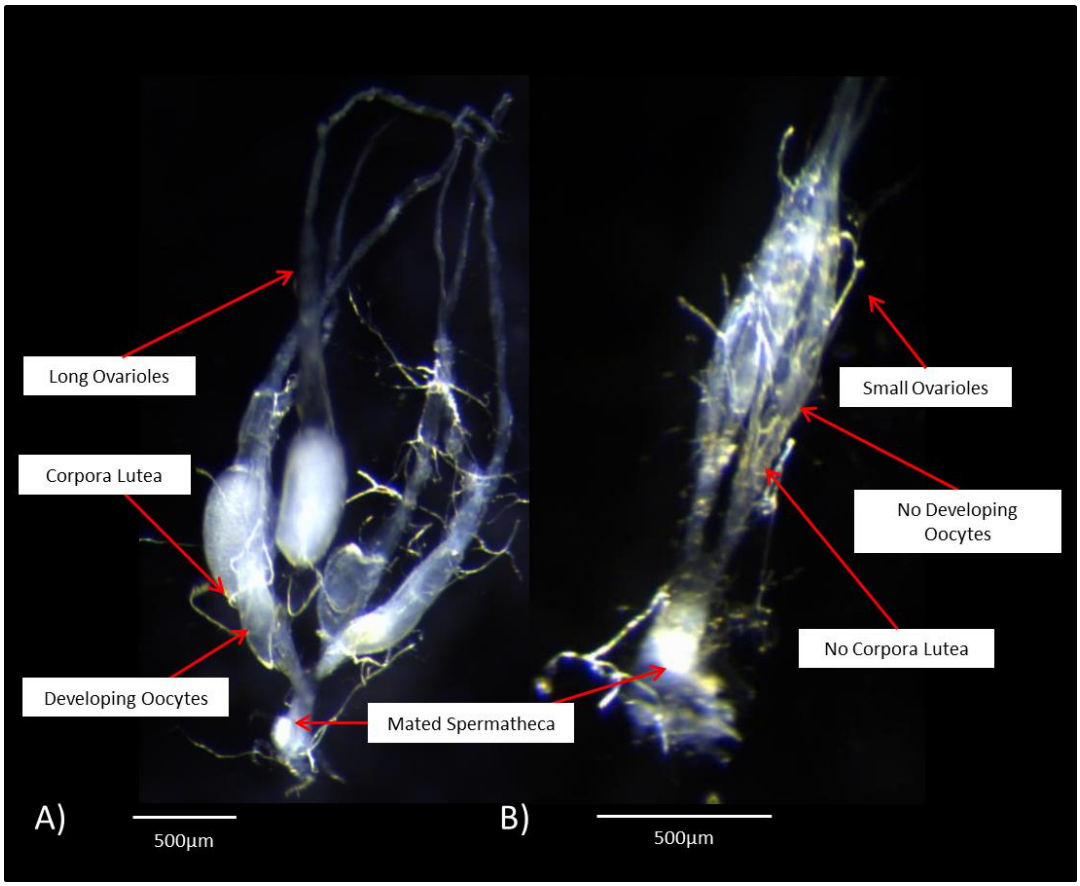


Fig 4: Examples of reproductive and non-reproductive queen ovary dissections. A) Reproductive ovary showing long developed ovarioles, corpora lutea and a mated spermathecal. B) A non-reproductive ovary with small ovarioles, no corpora lutea and a mated spermatheca.

RT-qPCR was conducted using the sample cDNA on a Chromo4 PTC-200 Peltier Thermal Cycler (BioRad) under the following cycling protocol: 95˚C for 3 mins, 40 cycles of 95˚C for 30 secs, 60˚C for 30 and 72˚C for 30 secs. A dissociation step at 60-95˚C was then performed to check that only one product per reaction had been amplified. No cDNA template (WATER) and no reverse transcriptase controls (NORT) were also included for each primer pair

and cDNA template respectively to detect possible contamination. Each reaction was performed in triplicate (three technical replicates) to control for run variation.

3.2.8 *vg* expression analysis

All *vg* expression analysis was conducted using the classic ΔΔCt method (Livak and Schmittgen, 2001). Briefly, the mean average Ct values of all technical replicates (3x per queen sample) per queen sample were used in the analysis. *vg* expression was normalised in all samples using the Ct values for the most stable housekeeping primer set as determined in sections 2.4 and 3.4. The non-reproductive queen samples were used as the control group when calculating relative expression and the mean average ΔCt from the control group was used for calculating ΔΔCt.

Additionally, the relative expression of the *vg* gene was calculated for the ΔCt values for both the control and treatment groups via the following calculation, $2^{-\Delta Ct}$. This calculated the expression difference between *vg* and the housekeeper in both the treatment and control sample groups. Expression differences between treatment and control groups were analysed using Mann-Whitney U tests to determine significance. Furthermore, the complete data set was subdivided into two data sets containing queens from Spain and the UK. These two new data sets were analysed for *vg* expression differences following the same methods described above.

3.3 Results

3.3.1 *L. acervorum vg* consensus construction and confirmation

Sanger sequence obtained from samples containing plasmids with the inserted *vg* sequence were constructed into a high quality 778bp consensus (see section 2.4). An initial BLASTn search with 'somewhat similar' parameters of the resulting consensus returned *S. invicta vg_2* and *vg_3* as the two highest scoring matches with 74% and 72% identity matching respectively. Furthermore, the top 10 similarity matches all belonged to ant species including; *Solenopsis invicta, Cerapachys biroi, Camponotus floridanus, Acromyrmex echinatior* and *Formica exsecta*. Therefore, it is highly likely that the consensus constructed relates to *L. acervorum vg*.

3.3.2 Homology of *L. acervorum vg* with other ant species

Phylogenetic reconstruction of all currently available ant *vg* sequences placed the *L. acervorum* consensus within the clade associated with queen reproduction as represented by five different *vg* sequences from four different ant species and not worker associated polyethism as has been shown in *S. invicta vg_3*, *vg_4* and *P. barbatus vg_2* (see Fig 5). This indicates that the *L. acervorum* consensus obtained is likely associated with queen reproduction and not worker division of labour, therefore making L. acervorum *vg* an appropriate reproductive marker. Phylogenetic reconstruction of ant *vg* sequences supported previously reported phylogenies (Morandin et al., 2014, Corona et al., 2013), which further supports my conclusion that the L. acervorum vg sequence obtained is within the correct clade. (Fig 5). Finally, the fact that *L. acervorum vg* shared a clade with *S. invicta vg_2* and *vg_3,* the closest sequenced ant relative available (Moreau, 2006), provided extra confidence in the *vg* consensus and its location within the clade containing other known queen expressed *vg* sequences (Fig5).

3.3.3 Housekeeper efficiency using NormFinder

The ELF_2, RPS18_2 and UBIQ_2 housekeeper gene sets (see Table 1) were tested for stability in NormFinder software using the cT data for all individuals. First, all qPCR runs returned cT data that were un-confounded with DNA contamination in either the NORT or WATER controls. Second, the mean average cT of all technical replicates (3x per queen sample) were calculated for each queen and used in NormFinder. Housekeeper stability analysis in NormFinder revealed ELF_2 to be the most stable housekeeper primer set, with a stability value (*M*) of 0.001 (the closer *M* is to 0 the more stable the gene expression). All further qPCR analyses were therefore normalised using *elf* expression.

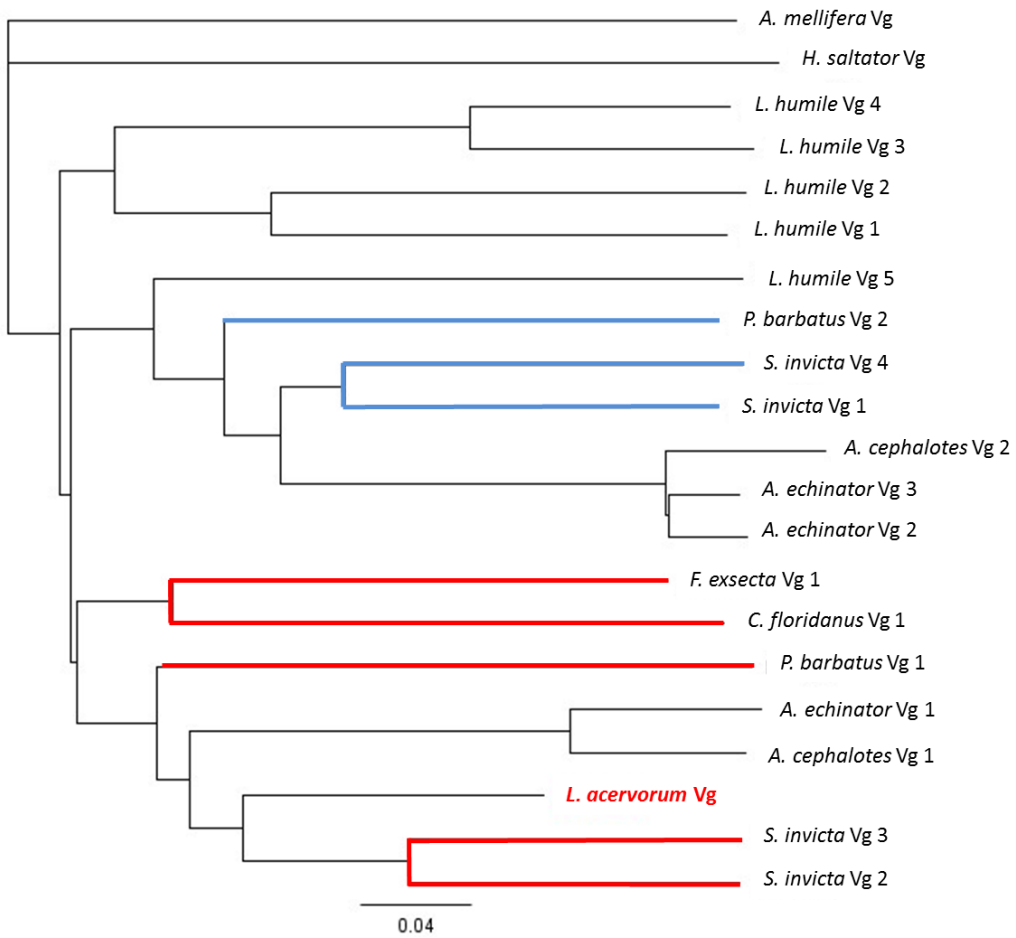


Fig 5: Phylogenetic tree of ant vitellogenin sequences. The *vg* consensus for *Leptothorax acervorum* is highlighted in **red text**. The **red branches** indicate vg genes that are known to be differentially expressed in queens and **blue branches** indicate vg genes that are known to be differentially expressed in workers. The scale bar is measured in amino acid substitutions per site.

3.3.4 *L. acervorum vg* expression between reproductive and non-reproductive queens

Considering all laying and non-laying queens together, *vg* expression was found to be significantly up-regulated in laying queens in comparison to non-laying queens. First, vg gene expression differences relative to elf expression (house keeper) calculated via the comparative equitation method ($2^{-\Delta Ct}$) were found to be significantly higher in reproductive queens compared to the non-reproductive queens (Median *vg* expression per reproductive queen±(IQR)= 12.78 (4.18-105.21). Mean *vg* expression per non-reproductive queen±(IQR)= 2.83 (0.53-52.9). Mann-Whitney U test: Z= 1.83, N_{repro}= 22, $N_{non\text{-}repro}$= 17, *P*= 0.034. See Fig 6). Second, relative gene expression analysis, as calculated via the classic ΔΔCt method, revealed a mean fold difference of 66.63 in vg expression amongst reproductive queens compared with non-reproductive queens (expression of 1).

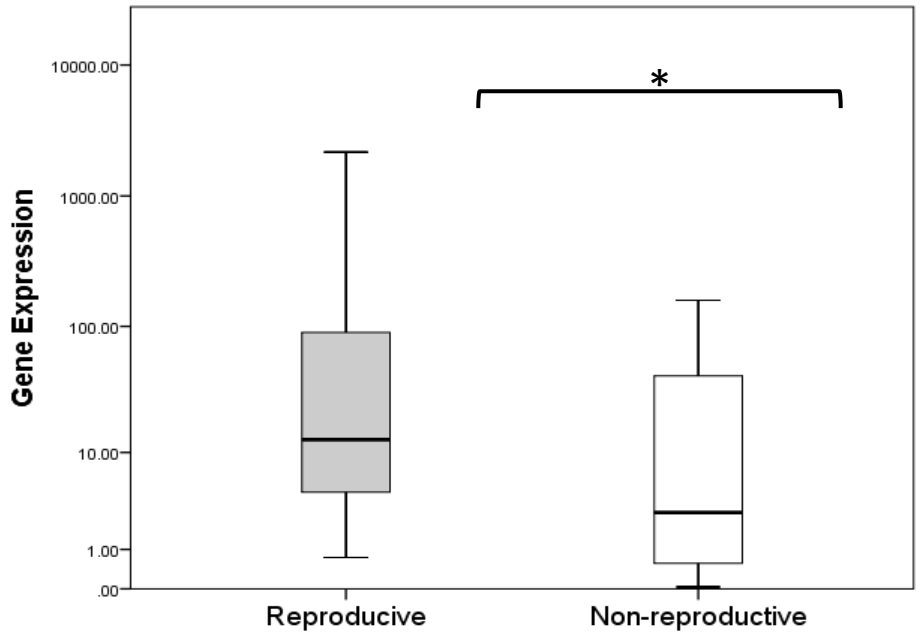


Fig 6: *vg* expression data for all reproductive and non-reproductive queens. Box and whisker chart shows the median and 1st and 3rd quartiles. Gene expression data are derived from the ΔCt values following the comparative quantitation method ($2^{-\Delta Ct}$). *vg* gene expression is significantly higher amongst reproductive queens compared to non-reproductive queens as determined by Mann-Whitney U analysis.

Despite the significant result, there was substantial variation in *vg* expression differences between reproductive and non-reproductive queens when the data were subdivided into Spanish and UK groups. Sub-division of the data into Spanish queens and UK queens yielded non-significant results (Spanish queens: Median *vg* expression per reproductive queen±(IQR)= 12.13 (5.32-18.5). Mean *vg* expression per non-reproductive queen±(IQR)= 5.79 (0.58-47.13). Mann-Whitney U test: Z= -0.857, N_{repro}= 12, $N_{non\text{-}repro}$= 10, *P*= 0.39. UK queens: Median *vg* expression per reproductive queen±(IQR)= 83.15 (2.81-1166.71). Mean *vg* expression per non-reproductive queen±(IQR)= 5.79 (0.58-47.13). Mann-Whitney U test: Z= -1.757, N_{repro}= 10, $N_{non\text{-}repro}$= 7, *P*= 0.079. See Fig 7). Relative gene expression analysis (ΔΔCt) found a mean fold difference of 17.26 in vg expression amongst reproductive queens compared with non-reproductive queens (expression of 1) from Spain and a mean fold difference of 155.51 amongst queens from the UK.

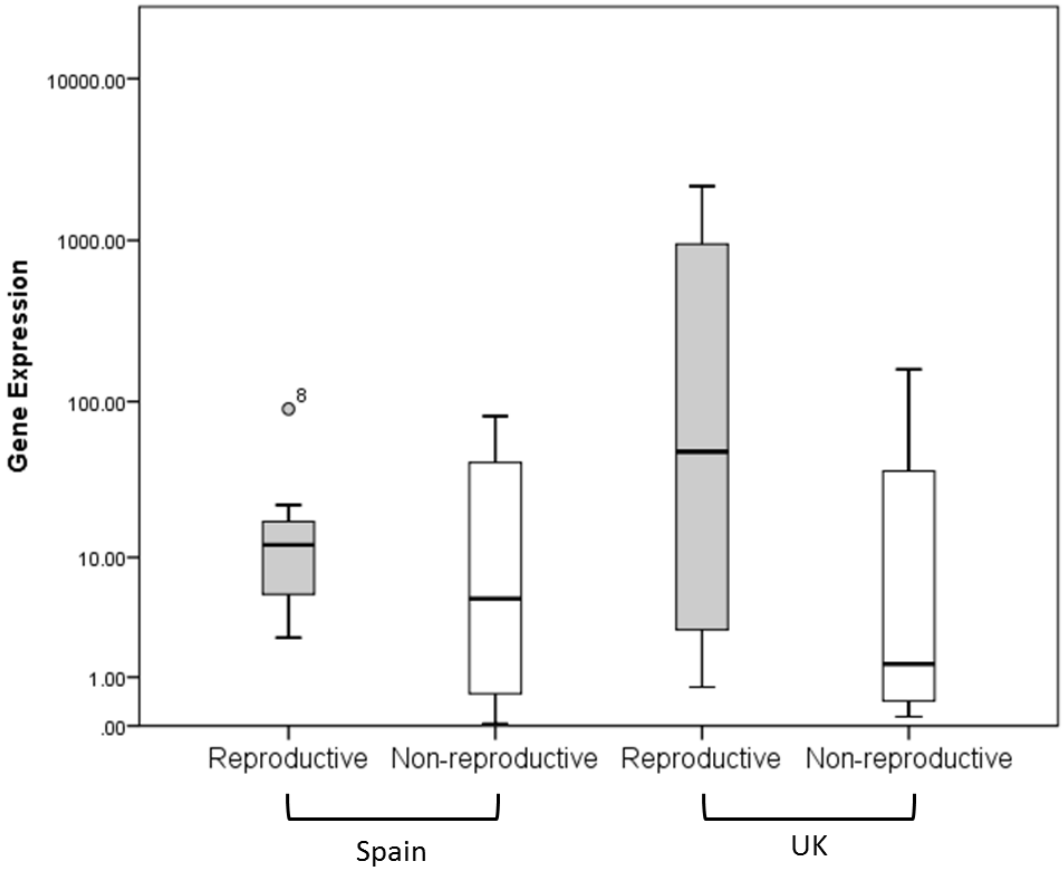


Fig 7: *vg* expression data for reproductive and non-reproductive queens from Spain and the UK. Box and whisker chart shows the median and 1st and 3rd quartiles. Gene expression data are derived from the ΔCt values following the comparative quantitation method ($2^{-\Delta Ct}$). *vg* gene expression is not significantly different between reproductive and non-reproductive queens from Spain or the UK as determined by Mann-Whitney analysis.

3.4 Discussion

I was able to successfully construct a high quality 778bp consensus relating to a copy of the *L. acervorum* vitellogenin gene (*vg*) and establish confidence in its role in queen reproduction. RT-qPCR analysis of *vg* expression in queens that had either developed or un-developed ovaries showed that *vg* was significantly up-regulated in reproductively active queens (see Fig 6). However, there was variation in *vg* expression amongst reproductive queens (see Fig 7), which suggests that up-regulation of *vg* might not be consistent with ovary development. Sub-dividing the reproductive and non-reproductive queens into separate Spanish and UK groups did not yield significant results (see Fig 7), suggesting that the instability of *vg* expression coupled with a smaller sample size may lead to non-significant results in future work. A possible explanation might be that *vg* expression is down-regulated more quickly than changes in ovary development/degeneration in response to ending the reproductive cycle. This is possible as insect *vg* expression is regulated by hormones, which are fast acting signals (Tufail et al., 2014). Therefore, the variation in *vg* expression found in this study might be explained if some of the queens were in the early stages of ovary degeneration, which might be difficult to tell based on ovary classification alone. Phylogenetic analysis of *L. acervorum vg* placed it in a clade with *S. invicta vg_2* and *vg_3* (Fig 5), which have both been shown to be expressed in reproductive queens (Wurm et al., 2011). Therefore, despite the variation, I am confident that *L. acervorum vg* expression is an appropriate measurement for queen reproduction and that the primers developed are effective in measuring *vg* expression through RT-qPCR analysis.

Using *vg* expression as a method of measuring queen reproduction has a number of advantages over physical egg counts and ovary dissections. First, it allows reproductive status to be determined without the need for delicate ovary dissections when compared with a non-reproductive control group. Similarly, measuring *vg* expression may help determine the

reproductive status of queens when ovaries are damaged during dissection. Second, *vg* expression could be used to determine the reproductivity of queens earlier in the breeding season when eggs have not yet been laid. Third, *vg* expression may react more rapidly to treatments (such as aggression, see Chapter 4) than ovary development/degeneration, which would allow impacts in reproductivity to be detected regardless of ovary classification. However, despite the advantages, it is strongly advised that queen reproductive status is determined via a combination of *vg* expression, ovary dissections and egg counts. Both methods mutually support each other resulting in a more confident and robust analysis.

I found no evidence to indicate multiple *vg* genes in *L. acervorum*, which has been shown to be a common feature amongst ant species, but I cannot rule out the possibility conclusively (Morandin et al., 2014, Corona et al., 2013, Wurm et al., 2011). Recent studies have shown closely related ant species to possess between one and five copies of the *vg* gene, with the most closely related species, *S. invicta*, possessing four copies (Wurm et al., 2011). The first duplication of *vg* in ants occurred after the divergence of the poneroid and formicoid clades making it a possibility that *L. acervorum* possesses at least two *vg* copies (Corona et al., 2013). Gene duplications can provide an important source of genomic material through which selection can act to co-opt gene copies to perform new novel functions (Force et al., 1999, Lynch and Force, 2000, Zhang, 2003). In *S. invicta* and *P. barbattus*, duplicate *vg* genes have been selected to perform novel function in regulating worker division of labour (Corona et al., 2013, Wurm et al., 2011). Furthermore, both species possess workers with high degree of variation in morphology allowing them to further specialise in labour tasks (Tschinkel et al., 2003, Johnson, 2000).

Despite the possibility of multiple *vg* copies in *L. acervorum*, duplicates can be quickly lost again (Morandin et al., 2014). This is the case for *Camponotus floridanus*, a formicine ant species, which only has a single copy that sits within the clade relating to vitellogenesis

function (see Fig 2). Unlike *S. invicta* and *P. barbattus*, *L. acervorum* is monomorphic and does not possess workers with morphological variation associated with division of labour. *L. acervorum* has no distinguishable nurses, foragers or soldiers (Kuehbandner et al., 2014), which suggests that any additional *vg* copies previously present may have degenerated rather that been co-opted into regulating worker division labour. Further work is therefore required to confirm the *vg* copy number in *L. acervorum*.

In conclusion, I was able to successfully sequence a copy of the *vg* gene in *L. acervorum* and design primers that were suitable for quantifying *vg* expression using RT-qPCR. Furthermore, I demonstrated that *vg* expression was significantly up-regulated in queens classified as reproductive based upon ovary dissections. However, substantial variation in *vg* expression was recorded amongst reproductive queens, which suggests that a combined approach of using *vg* expression, ovary dissections and egg production should be taken when assessing queen reproduction.

Chapter 4

Aggressive worker enforcement does not affect reproductive skew amongst polygynous colonies of the ant *Leptothorax acervorum*

4.1 Introduction

Social insect species can show remarkable variation in their social organisation (Keller, 1995, Bourke and Franks, 1995). Understanding the evolutionary mechanisms which promote the diversification of social organisation is crucial to explaining the evolution of eusociality, the most recent major transition in evolutionary history (Johnson and Linksvayer, 2010, Szathmary and Smith, 1995). Currently, there is a growing body of theory attempting to explain the evolutionary origins and elaboration of eusociality and many studies testing the predictions of kin selection and inclusive fitness theory have yielded interesting insights (Abbot et al., 2011, Birch and Okasha, 2015, Gardner et al., 2011, Bourke, 2011b, Hamilton, 1964, Gardner and West, 2014, Queller, 2011, Nonacs, 2011b, Nonacs, 2011a, Marshall, 2011, Nowak et al., 2010, Foster et al., 2006, Wilson and Holldobler, 2005, Linksvayer and Wade, 2005, Queller and Strassmann, 1998). However, the behavioural mechanisms maintaining and diversifying eusocial organisation are less well understood (Bourke, 2011a).

Behavioural mechanisms can be essential in maintaining an altruistic eusocial organisation (Ratnieks and Wenseleers, 2008). For example, the prevention of selfish reproduction and the coercion of altruism may require the use of enforcement behaviour (Ratnieks and Wenseleers, 2008, Wenseleers and Ratnieks, 2006b, Ratnieks et al., 2006). Selfish reproduction is expected when the personal relatedness benefits outweigh the colony inclusive fitness benefits. Indeed, many eusocial insect species possess workers that have the potential to lay unfertilised male eggs (Bourke, 1988). Given a colony headed by a singly mated queen, relatedness benefits should predict that a large proportion of workers should

produce sons (Ratnieks and Wenseleers, 2008). This is because sons are more related on average (0.5) than brothers (0.25). However, the extreme level of altruism and the lack of worker produced males observed in some species suggests that enforcement behaviour must play a key role in maintaining altruism (Ratnieks and Wenseleers, 2008, Wenseleers and Ratnieks, 2006b, Ratnieks et al., 2006, Wenseleers et al., 2004a, Wenseleers et al., 2004c, Hammond and Keller, 2004).

Recent evidence in the relatedness and egg laying frequencies of honey bees (*Apis mellifera*) showed that <0.1% of workers lay eggs in queen-right colonies. However, given an average relatedness of 0.3-0.275 between female offspring (Strassmann, 2001, Ratnieks, 1993, Ratnieks and Wenseleers, 2008), Hamilton's rule predicts that approximately 54% of workers should attempt to reproduce if individuals have freedom of choice (Ratnieks and Wenseleers, 2008, Wenseleers et al., 2004c). In reality, almost all of the workers within a honey bee colony behave altruistically by not attempting to reproduce individually. Indeed, the extreme level of altruism observed could only be explained by a family level relatedness of 1 if just the relatedness value alone was important. Conversely, when the queen was removed from the colony, worker reproduction quickly began with 40% of the workers reproducing, which is close to the predicted 54% based on relatedness benefits (Wenseleers and Ratnieks, 2006b), suggesting that an enforcement mechanism is important for maintaining worker reproductive altruism in queen-right colonies.

Worker policing provides an excellent example of enforcement and demonstrates how a behavioural mechanism can be essential in maintaining a social organisation. In honeybees, conspecific workers will eat worker-laid eggs upon discovery (worker policing) and are so efficient at this task that the 98% of workers will not even develop their ovaries or attempt to lay eggs (Ratnieks and Helantera, 2009). Producing eggs is a costly if there is a high likelihood that the egg will be eaten before it pupates. Therefore, worker policing establishes

a strong selective force acting to promote extreme levels of altruism and ultimately maintain the social organisation of the colony (Ratnieks and Helantera, 2009, Ratnieks and Wenseleers, 2008).

Worker policing has been shown to exist across a range of different ant, bee and wasp species and similarly, queen policing (the eating of worker-laid eggs by queens) is also considered to be a common behaviour amongst species with small colony sizes where queens can effectively police all workers (Ratnieks et al., 2006, Wenseleers and Ratnieks, 2006a, Ratnieks and Wenseleers, 2008). Importantly, a recent study looking at the effectiveness of worker policing and the frequency of worker reproduction across 10 species (wasps and honey bee) found that significantly fewer workers attempted to reproduce in colonies where worker policing was effective (Wenseleers and Ratnieks, 2006b), a pattern that supports enforcement as a significant selective mechanism.

Further examples of worker enforcement behaviours include female caste determination and aggression. In the case of female caste determination, kin selection predicts that if given the choice, 50% of all female larvae should develop into queens (Wenseleers et al., 2003) rather than workers because an individual is more closely related to her own offspring than to her sisters' (Bourke and Ratnieks, 1999). To prevent this unwanted bias in queen numbers, workers have to power to coerce larvae to develop into workers through provisioning and nursing behaviour (female caste determination). Honeybee queens are much larger than workers, and so queen destined larvae are reared in larger brood chambers (Ratnieks and Wenseleers, 2008). Therefore the construction of large brood chambers by workers acts to control the queen ratio of the colony. Queen larvae are also fed with royal jelly, an extra food provision containing the chemical royalactin, which triggers the development of a larva into a queen pupa (Kamakura, 2011). Absence of royal jelly means a larva will always develop into a worker. Since the workers are responsible for provisioning the

larvae within the colony, their provisioning behaviour has a strong influence over colony structure and is highly effective in coercing larvae to develop altruistically. Therefore workers have the power to directly affect colony structure.

Conversely, stingless bees from the genus *Melipona* rear queens that are the same size as workers (yet are morphologically distinct) in identical closed brood cells. This prevents workers from being able to influence the queen ratio within the colony and results in 20% excess queen production on average (Ratnieks, 2001, Wenseleers and Ratnieks, 2004, Bourke and Ratnieks, 1999). This is far higher than the number of queens necessary to head a swarm and so the excess are executed by workers because they neither work nor independently found new colonies (Wenseleers et al., 2004b). This over-production is a great cost to the colony, and neatly demonstrates how the absence of worker enforcement mechanisms leads to less altruism and more selfishness.

Finally, aggressive behaviours have been demonstrated in a number of ant and bee species to be effective in promoting altruism (Wenseleers and Ratnieks, 2006b, Monnin and Ratnieks, 2001, Kawabata and Tsuji, 2005, Lommelen et al., 2010, Cournault and Peeters, 2012, Visscher and Dukas, 1995, Liebig et al., 1999, Iwanishi et al., 2003, Hartmann et al., 2003). In the ponerine species *H. saltator* and *G. menadensis*, colonies are largely queenless and contain multiple reproducing gamergates (Monnin and Ratnieks, 2001). When colonies were experimentally divided to contain one half with all the gamergates and one orphan half with none, workers began within the orphan half begin to produce reproductive eggs. When the two halves were reintroduced, the newly reproductive workers were immediately attacked and immobilised. This behaviour resulted in the prevention of any further eggs being laid by any of the newly reproductive workers (Monnin and Ratnieks, 2001). Consequently, aggressive behaviour from nestmates acted as an enforcement mechanism preventing selfish reproduction and maintaining colony organisation.

There are many ant species which contain multiple mated (MQ) queens within the colony that are capable of reproducing (Gill et al., 2009, Kellner et al., 2007, Bourke and Franks, 1995, Keller, 1995). In many of these MQ species, enforcement mechanisms are largely absent, such as aggression (see Chapter 5, Bourke, 1991) and egg policing (Bourke, 1994), which allows all queens to selfishly reproduce and, because the queens are mated to unrelated males, lowers within-colony relatedness (Gill et al., 2009, Kellner et al., 2007, Hammond et al., 2006, Heinze et al., 2001, Bourke and Heinze, 1994, Bourke and Franks, 1995). MQ species with equal partitioning of reproduction (low reproductive skew) possess a social organisation called polygyny (P). Alternative social organisations exist within MQ species however. For example, functionally monogyny (FM) describes a social organisation where only a single queen reproduces and all other mated queens forgo their reproduction by not developing their ovaries (Buschinger, 1968), which results in a high skew and high within colony relatedness (Gill and Hammond, 2011b, Gill et al., 2009). Many MQ functionally monogynous colonies appear to regulate reproduction through the establishment of dominance hierarchies and aggressive enforcement behaviours (Lipski et al., 1992, Heinze et al., 1992, Heinze and Smith, 1990, Trettin et al., 2011, Gill and Hammond, 2011a, Gill and Hammond, 2011b, Ito, 2005). In many cases either the queens or the workers will administer high levels of aggression to other queens lower in the ranking order, which prevents them from reproducing (Trettin et al., 2011, Gill and Hammond, 2011a, Gill and Hammond, 2011b, Ito, 2005).

The common ant species, *Leptothorax acervorum,* is an MQ species which is polymorphic in social organisation (Felke and Buschinger, 1999, Trettin et al., 2011, Gill and Hammond, 2011b, Gill et al., 2009, Hammond et al., 2006, Ito, 2005, Bourke and Heinze, 1994, Bourke, 1994). Many populations throughout the UK and central Europe possess a P social phenotype, where colonies are characterised by low skew and low relatedness (Hammond et al., 2006, Hammond and Keller, 2004, Bourke and Heinze, 1994, Hammond et al., 2001, Heinze

et al., 1995a). However, two populations in central Spain (Trettin et al., 2011, Gill and Hammond, 2011a, Gill and Hammond, 2011b, Gill et al., 2009, Felke and Buschinger, 1999) and two populations in Japan (Gill, 2010, Ito, 2005) have been shown to possess the FM social phenotype. Importantly, aggressive enforcement behaviours have been observed to frequently occur in FM colonies, and in a number of studies, the rate of aggression received by a queen was sufficient to predict her future reproductive success (Trettin et al., 2011, Gill and Hammond, 2011a, Gill and Hammond, 2011b). Both queen-queen (Q-Q) aggression and worker-queen (W-Q) aggression have been reported as being involved in determining the future reproduce success of queens and so it is possible that aggressive behaviour is acting as an enforcement mechanism which prevents ovary development and coerces subordinate queens to act altruistically (Trettin et al., 2011, Gill and Hammond, 2011a, Gill and Hammond, 2011b). Conversely, aggression has not been reported to occur in P colonies (see Chapter 5, Bourke, 1991), which suggests that low skew, which characterises the P social phenotype, might be the result of a lack of enforcement behaviour.

Therefore, the variation in aggressive behaviour observed between P and FM populations of *L. acervorum* makes it an excellent model for exploring the importance of enforcement behaviours in maintaining high levels of reproductive altruism within social societies. In this study, I tested the hypothesis that P queens, which normally reproduce without receiving high levels of aggression, would be prevented from reproducing in the presence of aggressive worker enforcement behaviour. I tested this hypothesis by exposing P queens to regular treatments of worker aggression for a period of six weeks during the breeding season and observed whether exposure to worker enforcement altered queen reproduction and skew.

4.2 Methods

Fig 1: Sample locations for colonies collected in the New Forest, Southampton.

4.2.1 Colony collection, nest box construction and maintenance

Multiple queen (MQ) *L. acervorum* colonies were collected from three locations within the New Forest, Southampton, in April 2013. The collection sites included Hawk's Hill (HH), Dunce's Arch (DA) and Ivy Wood (IW) (Fig 1). Complete colonies were collected from hollow twigs, which were retrieved whole from the forest floor. All ant colonies were brought back to the lab where they were removed carefully within two days after collection.

All colonies were provided with an artificial nest consisting of a clear plastic box (Dimensions: 12cm x 8cm x 2cm) with 5mm of plaster of Paris in the base. Colonies were censused and stored in a versatile environment chamber (*Sanyo*, Model: MLR-351H) under the spring conditions (hourly rhythm=11-1-11-1, Temp/°C=10-15-20-15, Photoperiod (Night-Day-

Day-Day)= 0-2-3-2, humidity/%=70-70-80-70). All colonies were fed one half of a meal worm and 10% honey water three times a week (Monday, Wednesday and Friday) and the nests were kept moist by adding three drops of water to the plaster and a cotton ball during feeding. Nest boxes were kept clear by regularly changing the food trays and water balls. Ants within each colony box were left free to set up their brood wherever they liked, which was commonly within one of the corners of the case.

All colonies were assigned a unique identification tag which consisted of the location, the year collected and a number. Location identifiers included; HH= Hawks Hill, DA= Dunce's Arch and IW= Ivy Wood. The example identifier tag 'HH.13.01' identifies the 1st colony collected from Hawks Hill in the year 2013.

4.2.2 Colony selection and queen marking

Twelve MQ colonies containing ≥3 queens were selected for this study (see Table 1). Initially, all eggs were removed from the colonies to make it easier to record egg production during the experiment. The colonies were then divided equally into two separate colony fragments each containing an equal number of queens, workers and brood (see Fig 3A-C). These fragments were labelled as 'treatment' and 'control' colonies. Where queen numbers were not equal, the treatment colony received the greatest number of queens. Each colony fragment was given the additional identification tags 'T' and 'C' to represent 'treatment' and 'control' respectively. See Table 1 for colony information and Fig 3 for a diagrammatic overview of the experimental design.

Queens were marked by carefully tying 0.03mm copper wire between the petiole and post-petiole or petiole and abdomen in order to easily identify control queens from treatment queens Fig 2). Queens to be marked were immobilised head first in a block of polystyrene foam. This prevented their legs from moving but still allowed the petiole and abdomen to be exposed. Copper wire (0.03mm) was tied in a loop using fine dissection forceps and lassoed in

between the petiole and postpetiole of the pedicel. Finally the lasso was tightened and the remaining whiskers were shortened to differing lengths using fine dissection scissors (for examples of different lengths see Fig 2). Queens were marked to prevent misidentification with control queens. All queens within either the control or the treatment colony were marked (Fig 3B-C). Furthermore, an even number of control and treatment colonies were marked to control for any potential confounding marking effects (see Table 1). Queen marking using wire has been successfully demonstrated in previous studies using *L. acervorum* (Trettin et al., 2011, Trettin et al., 2014, Kuehbandner et al., 2014).

Table 1: Treatment and control colony fragment census at the beginning of the aggression assay.

Colony ID	Queens	Workers	Large Larvae	Small Larvae	Pre-pupae	Marked
HH.13.01.T	3	52	27	77	0	Y
HH.13.01.C	3	51	27	77	0	N
HH.13.02.T	2	57	34	47	0	N
HH.13.02.C	2	57	34	47	0	Y
HH.13.07.T	2	20	6	7	0	N
HH.13.07.C	2	20	6	6	0	Y
HH.13.06.T	4	102	33	72	0	Y
HH.13.06.C	3	102	33	71	0	N
DA.13.05.T	2	77	20	33	0	Y
DA.13.05.C	1	77	20	34	0	N
DA.13.04.T	6	92	83	180	0	N
DA.13.04.C	5	91	83	179	0	Y
DA.13.03.T	3	53	28	48	1	Y
DA.13.03.C	3	54	28	48	0	N
DA.13.16.T	3	59	15	31	6	N
DA.13.16.C	3	58	15	30	6	Y
DA.13.15.T	3	27	8	67	0	N
DA.13.15.C	2	27	8	69	0	Y
DA.13.10.T	6	67	11	29	1	N
DA.13.10.C	5	67	10	29	1	Y
DA.13.11.T	4	54	15	52	0	Y
DA.13.11.C	3	54	15	55	0	N
DA.13.13.T	2	76	15	29	0	Y
DA.13.13.C	1	76	15	29	0	N

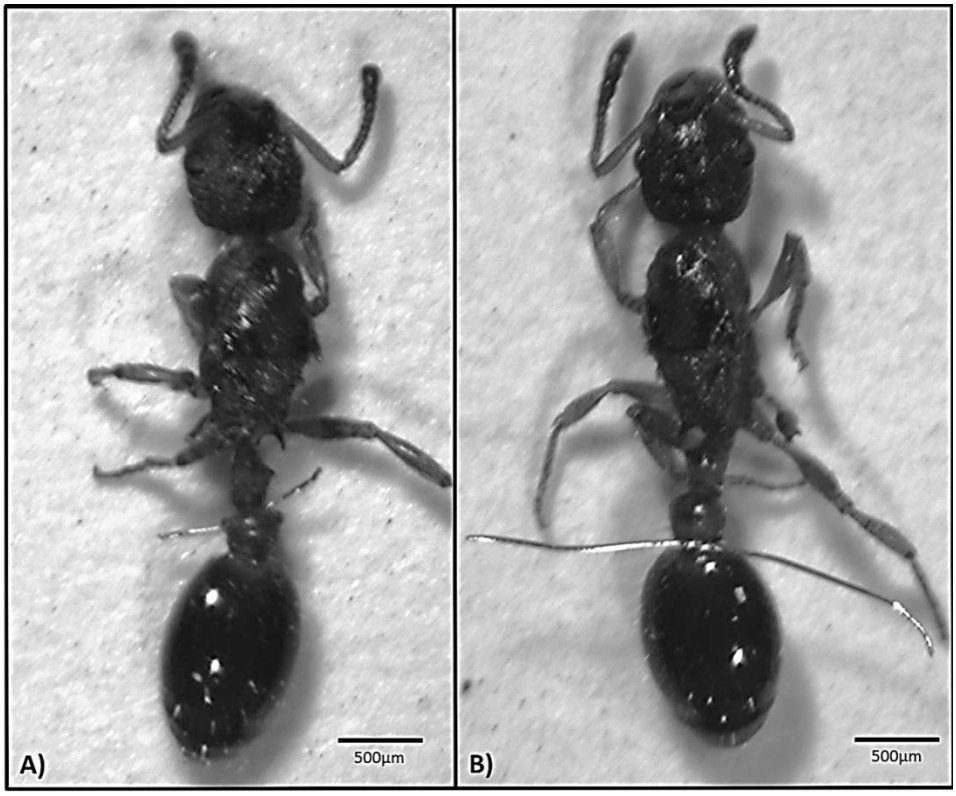


Fig 2: Examples of queen marking with wire. Wire was tied between the thorax and abdomen and the 'whiskers' were shorted to different lengths to allow easy identification. A) Example of wire tied between the petiole and post-petiole with short whiskers. B) Wire tired between the petiole and abdomen with long whiskers.

4.2.3 Worker aggression assay

Following colony division and queen marking, the colony fragments were given 24 hours to acclimatise into the new environment. With the colony fragments established and acclimatised the aggression treatment could begin (see Fig 3A-B).In total there were 20 treatment bouts which were conducted over a period of 42 days (six weeks). The first 28 days were conducted in spring conditions (hourly rhythm=11-1-11-1, Temp./°C=10-15-20-15, Photoperiod (Night-Day-Day-Day)= 0-2-3-2, humidity/%=70-70-80-70) and the final 14 days were conducted in summer conditions (hourly rhythm=9-1-13-1, Temp./°C=15-20-25-20, Photoperiod (Night-Day-Day-Day)= 0-2-3-2, humidity/%=70-70-80-70).

All queens within the treatment colony fragments were exposed to 5mins of worker aggression every Monday, Wednesday and Friday. During the aggression treatment, each

queen was gently removed from the treatment colony fragment and placed into a separate colony containing non-native workers (see Fig 3D). This 'non-native worker' colony contained no queens or brood and consisted only of workers from a colony which was non-native to all colonies involved in the experiment (Identified as HH.13.03).

When non-native workers encountered a queen belonging to different colony they attacked immediately by employing similar immobilisation behaviours to those observed in natural FM colonies (see Fig 4, Gill and Hammond, 2011a, Gill and Hammond, 2011b). Once the 5min period of treatment was over, workers were removed very carefully from each queen using two pairs of dissection forceps. It was important to grab the workers and queen by the petiole so as not to cause any further damage to either individual. Furthermore, grabbing attacking workers by the petiole with the forceps often resulted in the workers opening their mandibles and releasing the queen. Each treatment queen was then re-introduced into the original treatment colony (see Fig 3B).

All control queens were removed and placed into the treatment colony (Fig 3B and D) for a period of 5 mins. During this period, control queens were allowed to roam freely within the treatment colony and interact with the workers and brood. Control queens were observed down a microscope for the whole 5 min period and all aggressive worker-queen interactions during that time were recorded for each queen. Behaviours to be observed included, biting, pulling and spreading (Table 2). The number of each type of aggressive behaviour was recorded for each observation period. After the 5 min period, each control queen was carefully put back into the control colony.

Additionally, the total number of eggs per colony fragment was recorded at the beginning of each treatment day. Great care was taken not to damage any of the brood during this process by using a fine horsehair paint brush to move eggs and larvae.

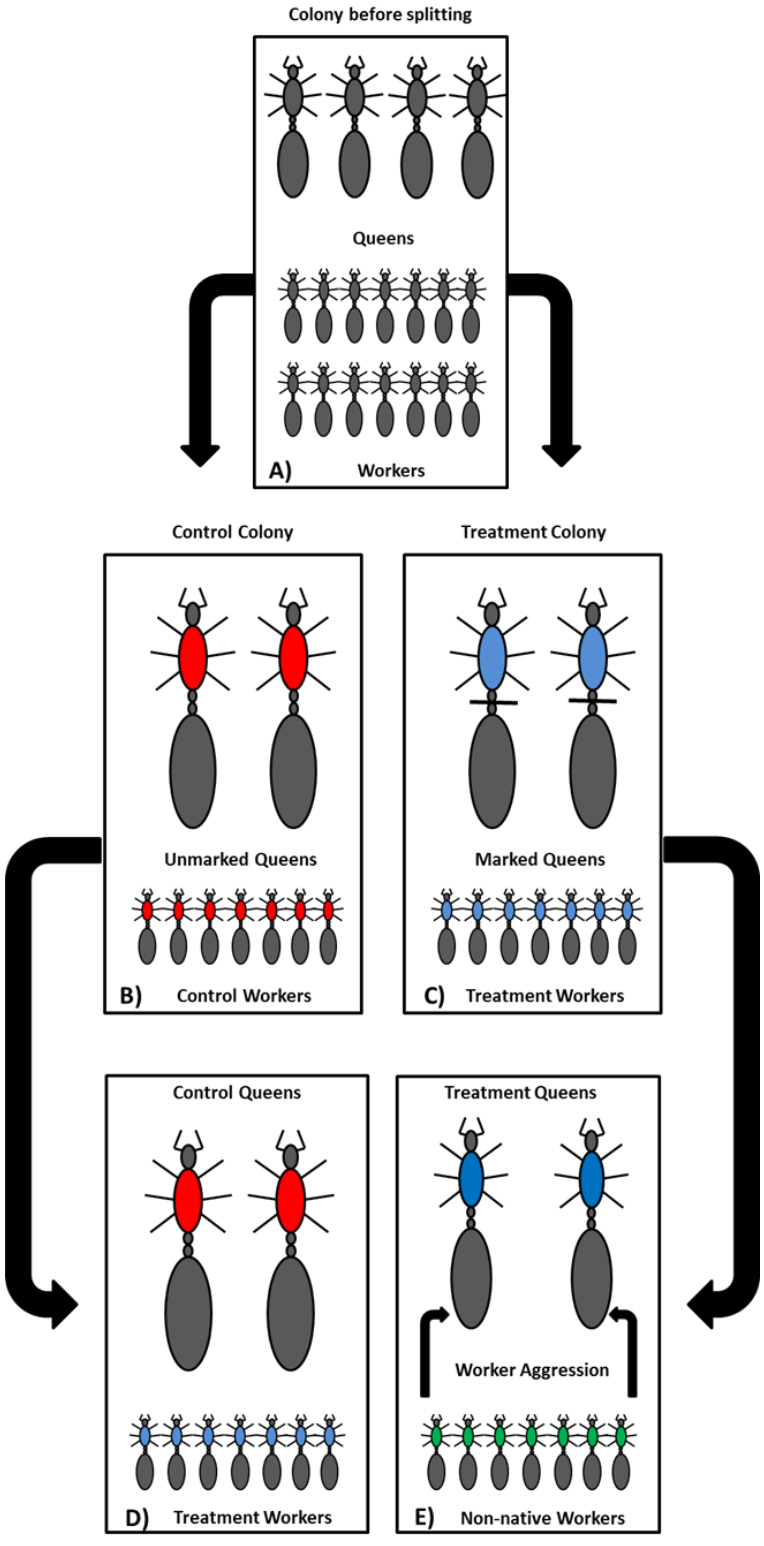


Fig 3: Worker aggression assay. **A)** Each colony was divided equally (queens, workers and brood) into **B)** a control colony (**red**) and **C)** a treatment colony (**blue**). **D)** Control queens were transferred to the treatment colony for 5 mins per bout. **E)** Treatment queens were exposed to non-native workers (**green**) for 5 mins per bout. All queens in either the treatment or control colony were marked with wire (Table 1 and Fig 2).

Fig 4: Examples of W-Q aggression. Two examples of natural W-Q aggression in Spanish FM colonies (the queen is in the centre of both images). The worker enforcement treatment aimed to replicate these types of aggressive behaviour. The figure was taken from Gill, 2010 with permission.

Table 2: Summary of behaviours recorded**.** Classification and description for each type of interaction recorded during observation periods.

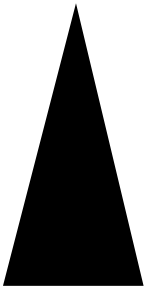
Type of aggression	Degree of aggressiveness	Definition
Single Bite		A single individual bites another for ≤1 second.
Pulling		A single individual bites another individual usually on an appendage (i.e. legs, antennae, neck and petiole) and drags the attacked individual.
Spreading		Multiple individuals bite another individual's appendages and pull in opposite directions, completely immobilising the attacked individual. Prolonged spreading can lead to the loss of an appendage and/or death.

Table modified from Gill, 2010 with permission.

4.2.3 Removal of dead queens during the worker aggression assay

It was essential to maintain pairwise comparisons between treatment and control queens to account for differences in reproductivity over time. Therefore, whenever a treatment or control queen died before the end of the experiment, a live queen from the corresponding control or treatment colony was randomly selected and immediately snap frozen in LN_2. This maintained equal numbers of queens between each colony fragment. An exception was made if a queen died in a fragment which was initially uneven in queen number (i.e. 3x treatment queens and 2x control queens). In these cases, a live queen was not removed from the alternative colony. At the end of the aggression assay, all live queens were snap frozen in LN_2immediately after the final treatment and stored at -80. All queens that were found dead during the experiment were immediately stored at -80.

4.2.4 Ovary dissections and classification

The reproductive status of all queens was determined via ovary dissection following the methodology established in Gill et al. (2009). Queens snap frozen alive were dissected in 100µl RNAlater (Sigma-Aldrich, UK) to preserve RNA quality and facilitate *vg* expression analysis. Dead queens were dissected in distilled water.

Mated status of queens was determined visually using a 32x microscope (Ceti Vari-zoom 10 binocular microscope) by the opaqueness of the spermatheca (mated=opaque, unmated=transparent). Queens were considered to be 'mated' if mated status could not be determined due to damage of the spermathecal. This is due to the likelihood that the queens were mated as shown in previous studies (Gill and Hammond, 2011b, Gill et al., 2009). Unmated queens were removed from all analysis. This resulted in a total of 60 queens being classified as mated, with 19 of those queens having damaged ovaries. The remaining 13 queens were unmated.

The reproductive status of each queen was determined using the criteria established in Gill et al (2009); A= relatively large elongated ovarioles containing large yolk filled eggs and corpora lutea; B= relatively short ovarioles with a small number of large yolk filled eggs; C= relatively short ovarioles with no large yolk filled eggs; and D= very short ovarioles with no eggs present. The presence or absence of tracheae were also recorded to help with determining reproductive status, as dense concentrations of trachea often indicate an inactive ovary (personal observation). If the ovary classification could not be determined due to damage then the queen was not considered in any further analysis.

4.2.5 RT-qPCR analysis of *vg* expression

Total RNA was extracted from each queen frozen alive using a GenElute® Mammalian Total RNA Mini Preparation kit (Sigma-Aldrich, UK) as per user instructions and DNase treated using DNase 1 Amplification Grade kit (Sigma-Aldrich, UK) as per user instructions. All samples were analysed for RNA concentration using a NanoDrop spectrophotometer (LabTech International) and 1 microgram of RNA was electrophoresed on non-denaturing 1.5% (w/v) agarose gel to check for degradation before use in RT-qPCR analysis. cDNA was constructed using the following protocol: Random primer ligation; step, 0.1µg of sample RNA was made up to 17.75µl final volume using 0.2µl random primers (0.5µg/µl, Promega, UK)and RNAse free water and incubated at 70˚C for 5 minutes. Extension step; 5µl of M-MLV x5 buffer (Promega, UK), 1µl M-MLV RT (Promega, UK) and 1.25µl of dNTPs (10mM) were added to each sample and incubated at 42˚C.

RT-qPCR was conducted using a Chromo4 PTC-200 Peltier Thermal Cycler (BioRad) under the following cycling protocol: 95˚C for 3 mins, 40 cycles of 95˚C for 30 secs, 60˚C for 30 and 72˚C for 30 secs. A dissociation step at 60-95˚C was used to check that only one product per reaction had been amplified. No cDNA template (WATER) and no reverse transcriptase controls (NORT) were included for each primer pair and cDNA sample respectively to detect

possible contamination. Each reaction was performed in triplicate (three technical replicates) to control for run variation. *vg* expression was normalised in all samples using the housekeeper ELF_2 primer set (see Chapter 3 Table 1).

4.2.6 Statistical methods

All continuous variable data sets were initially tested for normality using Kolmogorov–Smirnov analysis. If the data were found to follow a normal distribution then appropriate parametric statistics were used to test hypotheses. If the data were not found to be normally distributed then non-parametric tests were used instead. All data analysis was conducted in Minitab 17 and SPSS 22 statistical software packages unless otherwise stated.

4.2.6.1 Egg productivity

Total egg production and numbers of living queens were recorded at each treatment bout (Monday, Wednesday and Friday) throughout the aggression assay. Total egg counts between bouts within many of the colony fragments fluctuated, therefore it was necessary to devise a productivity score at each bout to account for these fluctuations. Productivity was calculated as follows:

$$P_x = E_i - E_j$$

P_x: Total productivity at treatment bout x

E_i: Total egg count at current bout

E_j: Total egg count at previous bout

The productivity values at each sample bout were then used to calculate a rate of productivity per queen per sample bout by dividing the productivity by the number of viable queens. The number of eggs produced by each mated queen was considered to be even, mirroring skew reported in natural P UK colonies (Hammond et al., 2006, Bourke et al., 1997). Total productivity per queen per bout (P_q) was calculated as follows:

$$P_q = \frac{\Sigma\left(\frac{P_x}{Q_x}\right)}{B}$$

P_q: productivity per queen per sample bout

P_x: Total productivity at treatment bout x

Q_x: number of mated queens at bout x

B: total number of bouts

The sample mean P_q values for control and treatment colony fragments were then compared using a paired Student's t-test.

Additionally, the number of days between the beginning of the experiment and the first egg laid were recorded to test whether treatment queens began reproduction later than the control queens. Student's T test analysis was used to test for differences in the onset of egg laying.

4.2.6.2 Ovary classification frequency distribution

Chi-square tests were used to analyse the frequency distributions of different ovary classifications between the control and treatment colony fragments. I performed multiple chi-square tests each relating to different sub categories of the data set including: total data, living queens only, all queens present in the spring conditions and all queens present in the

summer conditions. Significance was corrected for multiple testing via Bonferroni correction to α=0.016.

4.2.6.3 *vg* expression analysis

The expression of *vg* was compared between control and treatment queens using a relative expression model corrected for PCR amplification efficiency using REST software (Pfaffl et al., 2002). Gene expression data were calculated for each queen by averaging the cT for each technical replicate. Only full pairwise treatment and control queen combinations were used in the analysis to account for *vg* expression differences associated with time (due to queens dying before the end of the experiment, see 2.3). This meant that the data set was populated with equal numbers of treatment and control queens frozen on day X and day Y and so on. Furthermore, *vg* expression data were used providing that only a single gene product had been amplified and all NORT and water samples were uncontaminated.

The control queen samples were used as the control group and their relative *vg* expression was compared with the treatment queen samples to determine whether *vg* expression was significantly different using the pairwise randomisation test feature of REST (Pfaffl et al., 2002).

4.3 Results

4.3.1 Ovary dissections

At the end of the aggression assay, 50 queens had been snap frozen alive and 23 queens had been snap frozen after being discovered dead. Nine queens found dead could not be dissected because of desiccation and were removed from the analysis. In total, 80% of living queens were mated (40/50) and 78.6% of dead queens were mated (11/14) (see Table 3). Unfortunately, one colony (DA.13.05) had no mated control queens and so was removed from all further analysis. Ovary classification was successful for 50 queens frozen alive and 14 queens frozen dead, which included 23 control queens and 28 treatments queens (see Table 3).

The ovary classification data for all queen ovaries revealed that 52.9% of queens were classified as C, which indicated that the majority of queens were no longer laying eggs by the end of the experiment. Similarly, the majority classification for both the control and treatment queens was C (see Table 4).

Table 3: Ovary classification data for all mated queens.

Ovary Classification	Total count from all queens (n=51)	% from all queens (n=51)	Total count from living queens (n=40)	% from all living queens (n=40)	Total count from dead queens (n=11)	% from all dead queens (n=11)
A	5	9.8	2	5	3	27.3
B	11	21.6	11	27.5	0	0
C	27	52.9	25	62.5	2	18.2
D	8	15.7	2	5	6	54.5

Table 4: Ovary classification data for control and treatment queens.

Ovary Classification	Total count from all control queens (n=23)	% from all control queens (n=23)	Total count from all treatment queens (n=27)	% from all treatment queens (n=27)
A	1	4.3	3	11.1
B	8	34.8	3	11.1
C	12	52.2	15	55.6
D	2	8.7	6	22.2

Ovary classification frequencies were not found to be significantly different between the treatment and control queens when all queens were considered together (Chi-square test: X^2= 5.32, df= 3, *P*= 0.150, Table 5). Similarly, ovary classification frequencies did not significantly differ between pairwise comparisons of treatment and control queens (Chi-square test: X^2= 5.639, df= 3, *P*= 0.356, Table 5). Finally, ovary classification frequencies did were not statistically different between the treatment and control queens that were still alive by the end of the experiment (Chi-square test: X^2= 1.932, df= 3, *P*= 0.587, see Table 5).

Table 5: Ovary classification frequency data. Ovary classification frequency data for all control and treatment queens used in chi-square analysis.

Treatment Type	**Ovary classification for all queens**			
(*N*)	**A**	**B**	**C**	**D**
Control (23)	1	8	12	2
Treatment (27)	3	3	15	6
Treatment Type	**Ovary classification for all pairwise queen comparisons**			
(*N*)	**A**	**B**	**C**	**D**
Control (19)	1	8	9	1
Treatment (20)	3	2	13	2
Treatment Type	**Ovary classification for all living queens**			
(*N*)	**A**	**B**	**C**	**D**
Control (22)	1	8	12	1
Treatment (17)	1	3	13	1

4.3.2 Egg productivity

All colony fragments produced at least one egg during the course of the assay. Comparisons of mean egg productivity per queen per treatment bout (P_q) between treatment and control colonies were found to be non-significant (Mean P_q per colony±(SE): Treatment= 0.541(0.087), Control=0.597(0.131). Paired Student's t-test: t=-0.69, SE=0.69, *N*= 11, *P*=0.489, see Fig 5).

Furthermore, the initiation of egg laying by treatment queens was not significantly later than control queens (Mean days per colony±(SE): Treatment= 10.09(3.46), Control=13.45(4.05). Paired Student's t-test: t=0.89, SE=0.69, *N*= 11, *P*=0.394, Fig 6).

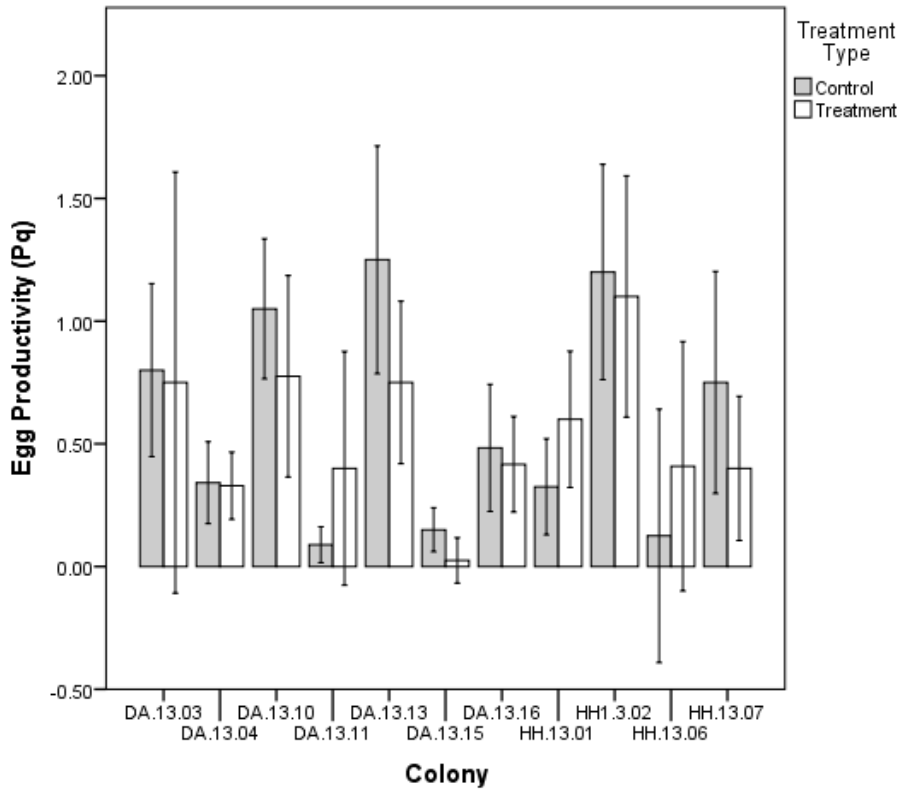


Fig 5: Mean egg productivity between treatment and control colonies. Egg productivity represents the mean number of eggs laid per queen per observation day (P_q). SE bars are included.

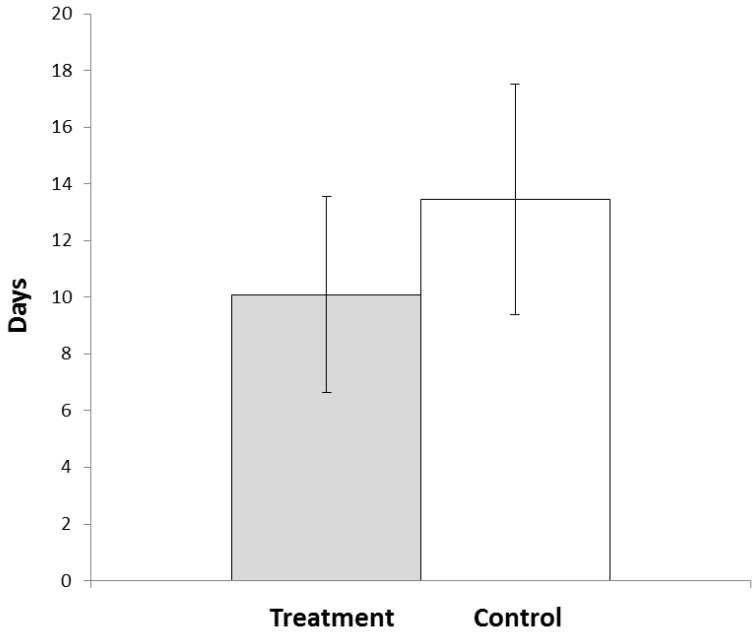


Fig 6: Mean number of days until the first egg laid in treatment and control colonies. SE bars are included.

4.3.3 *vg* expression and RT-qPCR analysis

Twelve treatment and control queen pairs were tested for *vg* expression from seven of the colonies. The vg gene expression differences relative to elf expression (house keeper) calculated via the comparative equitation method ($2^{-\Delta Ct}$) were not found to be significantly different between treatment and control queens (Median *vg* expression per treatment queen±(IQR)= 0.069 (0.02-0.14). Median *vg* expression per non-reproductive queen±(IQR)= 0.037 (0.02-0.18). Mann-Whitney U test: Z= -0.43, $N_{treatment}$= 12, $N_{control}$= 12, *P*= 0.664. See Fig 7). Second, relative gene expression analysis, as calculated via the classic ΔΔCt method, revealed a mean fold difference of 0.93 in *vg* expression amongst treatment queens compared with the control queens (expression of 1).

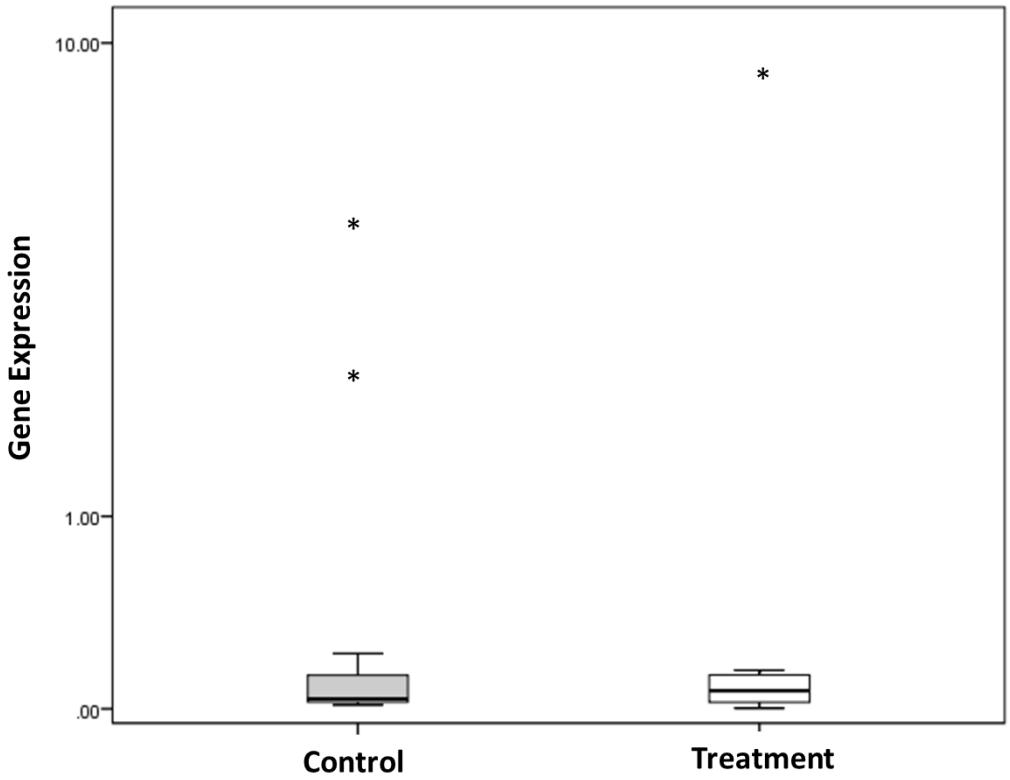


Fig 7: *vg* expression data for all control and treatment queens. Box and whisker chart shows the median and 1st and 3rd quartiles. Gene expression data are derived from the ΔCt values following the comparative quantitation method ($2^{-\Delta Ct}$). *vg* gene expression was not significantly higher amongst control queens compared to treatment queens as determined by Mann-Whitney U analysis.

4.3.4 Control queen aggression

Control queens were never observed to engage in aggressive interactions (see Table 2 for behavioural descriptions) with either queens or workers while occupying the treatment nest box. Anecdotally, queens tended to explore the nest box until they found the brood pile. Following discovery, they often settled into the brood pile and ceased to explore (pers. obs).

4.4 Discussion

I found no evidence to support the hypothesis that queens from a UK polygynous (P) population altered their reproductive behaviour in response to regular bouts of aggressive worker enforcement. First, ovary dissections revealed no difference in the number of different ovary classifications between treatment and control queens. Second, there was no significant difference in the egg productivity (eggs laid/queen/bout) or the initiation of egg laying between treatment and control queens. Finally, comparative *vg* expression was not significantly different between treatment and control queens. These findings suggest that skew was not affected amongst P colonies from a UK population in response to aggressive worker enforcement.

The aim of this study was to test the universal applicability of enforcement behaviours in maintaining high levels of altruism within social systems (Ratnieks and Wenseleers, 2008, Wenseleers and Ratnieks, 2006a, Wenseleers and Ratnieks, 2006b, Ratnieks et al., 2006, Wenseleers et al., 2004c, Wenseleers et al., 2004a, Kawabata and Tsuji, 2005, Iwanishi et al., 2003, Hartmann et al., 2003, Monnin and Ratnieks, 2001, Liebig et al., 1999) by observing whether aggressive worker behaviour enforced reproductive altruism amongst queens in P colonies of *L. acervorum*. The data I collected do not support this hypothesis as P queen reproduction was not affected by aggressive worker enforcement. Furthermore, these findings contrast with the strong support that aggressive enforcement is important in maintaining queen altruism and skew in FM *L. acervorum* colonies (see Chapter 2, Trettin et al., 2011, Gill and Hammond, 2011a, Gill and Hammond, 2011b, Ito, 2005). Therefore, it is necessary to address the possibilities why aggressive worker enforcement did not affect queen reproduction in P queens.

It is possible that the worker enforcement treatment was insufficient to have an effect on queen reproduction. Previous studies have demonstrated that FM queens receive an

average of 7 mins of aggression per hour (Gill and Hammond, 2011b), which is far higher than the 5 mins of aggressive treatment three times per week received by polygynous queens in this study. Unfortunately, more aggressive treatments were not possible as pilot studies resulted in high queen mortality when the length or frequency of aggression per day was increased. Furthermore, 78.3% of the queens that died during the course of this study were treatment queens, highlighting the difficulty of treating queens to repeated exposure of worker aggression. The lack of response from P treatment queens to worker aggression suggests that P queens may possess a tolerance threshold for worker aggression. In other words, for aggressive worker enforcement to have an effect on reproduction, the rate of enforcement may need to exceed a threshold, which the worker aggression treatment used in my study may not have achieved.

However, a recent study investigating the flexibility of social phenotype in *L. acervorum* showed that very low rates of aggression (median= 2.9 attacks per queen in 13.3hrs of observation) were sufficient to cause ovary regression in P queens from a population in Reichswald Germany (Trettin et al., 2014). This suggests that the threshold for aggressive worker enforcement to have an effect on reproduction in P queens is low and that my aggression treatment, which produced higher rates of aggression than those observed in P queens from the Reichswald population, should have been sufficient to have had an effect (Trettin et al., 2014).

Interestingly, closer inspection of reported colony relatedness values from the Reichswald population suggests that the social phenotype might not be fully polygynous (Heinze et al., 1995b). Relatedness estimates between queens (Relatedness ± SE= 0.63±0.057) and workers (Relatedness ± SE= 0.46±0.04) from the Reichswald population predicted a smaller proportion of mother queens (Predicted=1.5-2.9) in relation to the true number of inseminated queens present (Mean=3.2), which is indicative of a more 'FM like' social

organisation. This suggests that if ovary development is dependent on a threshold of aggression being met in queens, then the threshold amongst queens from the Reichswald population is likely to be very low, which would be expected from FM colonies where aggression amongst individuals is common (see Chapter 2, Trettin et al., 2011, Gill and Hammond, 2011a, Gill and Hammond, 2011b, Ito, 2005). Therefore, despite the comparatively low rate of aggression used in this study, it is likely to have been sufficient cause an effect if UK P colonies were comparable to the supposed P colonies from Reichswald Germany.

Another potential explanation for my findings is that P *L. acervorum* queens may have been selected for reduced sensitivity to aggressive enforcement and are therefore not prevented from laying when they receive aggressive behaviour from workers. This is an expected consequence under kin selection, as females are generally more related to their own offspring and so can be selected to evade enforcement (Ratnieks and Helantera, 2009). For example, some colonies of *A. mellifera* contain 'anarchistic' workers, which are able to lay eggs that are not detected by worker policing (Beekman and Oldroyd, 2003). Furthermore, there may be a fitness benefit to having multiple reproducing queens and a larger colony size when ecological constraints are relaxed (Boulay et al., 2014), which could select for a reduction in aggressive behaviour between queens and workers, an increased sensitivity to aggression, or both in P *L. acervorum* colonies.

A further explanation for the ineffectiveness of aggressive enforcement in P *L. acervorum* queens, which is not mutually exclusive, is that the social phenotype is determined by a suite of linked genes. Therefore sensitivity to aggressive enforcement behaviour may be genetically absent in P queens compared to FM queens. There are multiple lines of evidence to support a genetic basis for the social phenotype. First, P and FM colonies do not alter their social phenotype in response to lab controlled environmental conditions (Gill et al., 2009). Second, there is no recorded variation in social phenotype within different populations of *L.*

acervorum, where all colonies within a population are either P or FM (Trettin et al., 2011, Gill and Hammond, 2011b, Hammond et al., 2006, Ito, 2005, Felke and Buschinger, 1999, Bourke et al., 1997, Hammond et al., 2001). Third, removing worker enforcement from FM colonies does not cause them to switch to a P social phenotype (see Chapter 2). Finally, genetic differences associated with the social phenotype are present between P and FM colonies of *L.acervorum ,* suggesting that at least one social region underpins the polymorphic social phenotype (see Chapter 6).

In conclusion, I found no evidence to suggest that aggressive worker enforcement affects queen reproduction, and therefore colony skew, in P colonies of *L. acervorum* from a UK population. It is possible that the aggression treatment used in this study was not sufficient to have an effect of queen reproduction. However, an alternative explanation might be that differences in the genomic architecture between social phenotypes may be responsible for the loss of sensitivity to aggressive enforcement in P queens. Furthermore, the absence of an effect of aggressive enforcement in P queens does not rule out its importance in regulating skew in FM colonies.

Chapter 5

Multiple-queen colonies of the ant *Leptothorax acervorum* from a UK population do not show plasticity in colony social organisation

5.1 Introduction

Animal societies show wide variation in the organisation of individuals and explaining the mechanisms involved in maintaining variation is a key goal in understanding the evolution and elaboration of eusociality (Bourke, 2011, Fischman et al., 2011). Furthermore, understanding the extent to which social traits are plastic or inherited is important for understanding the evolution of complex social phenotypes (Thompson and Jiggins, 2014, Schwander et al., 2014, Purcell et al., 2014, Wang et al., 2013, Woodard et al., 2011, Hartfelder and Engels, 1998, Hughes et al., 2003, Evans and Wheeler, 2001, Herbers and Banschbach, 1999).

The partitioning of reproduction among group members (reproductive skew) is a key aspect of eusocial society and acts as a potent source of conflict between reproductive individuals in many species (Frank, 1995, Ratnieks et al., 2006, Vehrencamp, 1983b, Keller and Reeve, 1994, Clutton-Brock, 1998, Nonacs and Hager, 2011). For the majority of ant species studied that have multiple queens (MQ), reproduction is shared more or less equally between all present reproductive queens (low skew) with little to no conflict, forming a social organisation known as polygyny (P) which is characterised by low colony relatedness (Reeve and Keller, 2001, Keller and Reeve, 1994, Keller, 1995, Keller, 1993, Bourke and Franks, 1995, Hammond et al., 2006, Bourke et al., 1997). However, a number of MQ ant species have been identified to possess an alternative social organisation, functional monogyny (FM) (Buschinger, 1968, Buschinger, 1979, Buschinger et al., 1980, Heinze et al., 1993, Heinze et al.,

1992, Lipski et al., 1992, Heinze and Smith, 1990, Buschinger and Francoeur, 1991, Gill et al., 2009, Ito, 2005, Ito, 1990, Felke and Buschinger, 1999, Seppa et al., 1995). Species with the FM phenotype also maintain multiple queens that are mated and have reproductive potential, but crucially only one queen reproduces, resulting in colonies with high skew and high relatedness (Gill and Hammond, 2011b, Gill et al., 2009). Skew is therefore a fundamental aspect of the social phenotype in all MQ eusocial species and has wide reaching effects on colony structure and relatedness. Importantly, social organisation and skew can vary greatly between species but appear to be relatively robust within species and populations (Field et al., 1998, Reeve et al., 2000, Fournier and Keller, 2001, Seppa et al., 2002, Sumner et al., 2002, Hannonen and Sundstrom, 2003, Nonacs et al., 2004, Liebert and Starks, 2006).

Although rare amongst MQ ant colonies (Bourke and Franks, 1995), FM has been reported to exist in specific populations of the MQ species, *Leptothorax acervorum* in populations from central Spain and Japan (Trettin et al., 2011, Gill and Hammond, 2011a, Gill and Hammond, 2011b, Gill et al., 2009, Ito, 2005, Felke and Buschinger, 1999, Seppa et al., 1995, Ito, 1990). This is interesting as populations from the UK and central Europe possess the P social phenotype (Hammond et al., 2006, Hammond et al., 2001, Bourke et al., 1997, Heinze et al., 1995, Chan and Bourke, 1994, Bourke, 1993, Stille et al., 1991, Bourke, 1991, Douwes et al., 1987). There is a substantial theoretical work attempting to explain variation in skew with particular focus on how ecological constraints to solitary nest founding can encourage the re-adoption of queens (Johnstone, 2000, Reeve et al., 1998, Vehrencamp, 1983a, Vehrencamp, 1983b, Emlen, 1982). However, it is unclear whether variation in social organisation and skew is due to plasticity, as assumed by optimal skew models (Kokko, 2003), or genomic architecture, as demonstrated by the recent discovery of social chromosomes in *S. invicta* (Wang et al., 2013) and *F. selysi* (Purcell et al., 2014).

The observed inflexibility of the social phenotype expressed by both P and FM colonies of *L. acervorum* suggests that variation in social organisation is underpinned by a genetic basis and is not behaviourally plastic (Gill and Hammond, 2011b, Gill et al., 2009). Studies of nestmate relatedness and ovary status in colonies of *L. acervorum* have revealed no evidence of variation in social phenotype between colonies of a given population (Heinze et al., 1995, Hammond et al., 2006, Douwes et al., 1987, Bourke et al., 1997, Chan and Bourke, 1994, Stille et al., 1991, Gill and Hammond, 2011b, Gill et al., 2009, Hammond et al., 2001). Furthermore, colonies of *L. acervorum* collected from a well-studied P population from the UK and a FM population from Spain were shown to maintain their respective social phenotypes despite common environmental conditions (Gill et al., 2009).

Interestingly, genomic architecture has been recently shown to play a fundamental role in determining the social organisation of two ant species that also socially polymorphic. Colonies of the ant species *Solenopsis invicta* and *Formica selysi* are present in either a monogynous or a polygynous social phenotype, which differ in their queen number tolerance (Chapuisat et al., 2004, Purcell and Chapuisat, 2013, Keller and Ross, 1998, Gotzek and Ross, 2009, Wang et al., 2013, DeHeer et al., 1999, Keller and Ross, 1993). Each social phenotype is further associated with a syndrome of additional co-varying biological traits, which have co-adapted to facilitate the success of the social phenotype. These include differences in the level of inter-colony aggression, colony founding, worker size, queen fecundity, colony size, colony life span, mature queen odour and fat deposition (Lawson et al., 2012, Krieger and Ross, 2002, DeHeer et al., 1999, Keller and Ross, 1998, Keller and Ross, 1993, Keller and Ross, 1999, Keller and Ross, 1995, Purcell et al., 2014, Rosset and Chapuisat, 2007, Rosset and Chapuisat, 2006, Schwander et al., 2005).

Recent studies comparing the genomic architecture of the two social forms have revealed the presence of social chromosomes, which consist of large suites of genes linked together to

form a single heritable unit. Furthermore, investigations have revealed the importance of suppression of recombination in linking large suits of genes together (Thompson and Jiggins, 2014, Schwander et al., 2014), which prevents maladaptive social phenotypes from forming. Unlike *S. invicta* and *F. selysi*, the alternate social organisations present in *L. acervorum* do not possess a large suite of correlated phenotypes. However, there is still strong potential for genetic factors to play a role in determining the social phenotype in *L. acervorum* as colonies from different social phenotypes have been shown to vary in queen tolerance and nest founding strategy (Trettin et al., 2011, Gill and Hammond, 2011b, Gill et al., 2009, Hammond et al., 2006, Felke and Buschinger, 1999, Bourke et al., 1997, Franks et al., 1991, Bourke, 1991). Furthermore, in Chapter 6, I provide evidence that a large contiguous chromosomal region is associated with differences in the social phenotype/organisation in *L. acervorum*.

Despite the evidence supporting a fixed genetic basis for expression of social phenotype in *L. acervorum*, a recent study suggested that the social phenotype is plastic and can be altered with the manipulation of certain ecological constraints, namely queen:worker (Q:W) ratio, in colonies from the German Reichswald population, which were previously reported as polygynous (Trettin et al., 2014). Trettin et al. (2014) showed that over a period of ten days, P colonies altered their behaviour to resemble that of a FM colony and that crucially, ovary development regressed for all queens bar one when Q:W ratios were high, suggesting colony skew was affected. Trettin et al. (2014) therefore argued that the social phenotype is a behaviourally plastic response to environmental constraints rather than an evolved genetic trait, which supports the assumptions of optimal skew models (Kokko, 2003).

Clearly, the contrast between the lines of evidence supporting either a fixed genetic or socially plastic basis for social organisation in colonies of *L. acervorum* warrants further investigation. If variation in social phenotype is explained through a plastic basis then colonies sampled from a UK P population should demonstrate the same social plasticity when colony

Q:W ratios are naturally high. Therefore, the population should display variation in the proportions of colony social phenotypes that reflect the Q:W ratios. Furthermore, experimental manipulation of P colonies from the same population should also mimic the same plasticity seen in the Reichswald population as demonstrated by Trettin et al. (2014).

The aim of this study was to test the plasticity of the P social phenotype amongst colonies of *L. acervorum* from Santon Downham in the UK. First, I observed behaviour in natural colonies that had not had their Q:W ratio experimentally manipulated in order to test whether natural variation in Q:W ratio affected colony aggression. Second, I observed the behaviour of UK colonies with manipulated Q:W ratios using a similar methodology to Trettin et al. (2014) in order to test the hypothesis that social phenotype can alter in the presence of high Q:W ratios compared to low Q:W ratios. Finally, I dissected the ovaries of all participant queens to determine their reproductive activity and ascertain whether Q:W ratio had an effect on colony skew.

5.2 Methods

Fig 1: Sample site locations in Santon Downham, Thetford Forest, Norfolk. Shown are the sample locations for SDA.13 and SDB.13 colonies.

5.2.1 Colony Collection, census and laboratory conditions

L. acervorum colonies were collected whole from two locations (A and B) within Thetford Forest, Santon Downham (SD), Norfolk, on 4th July 2014 (see Fig 1). Colonies were found in decaying twigs lying on the forest floor and returned to the lab. All colonies were removed from their twigs within two days after collection, provided with an artificial (see Fig 2) nest and censused for numbers of queens, workers and brood within two days of collection.

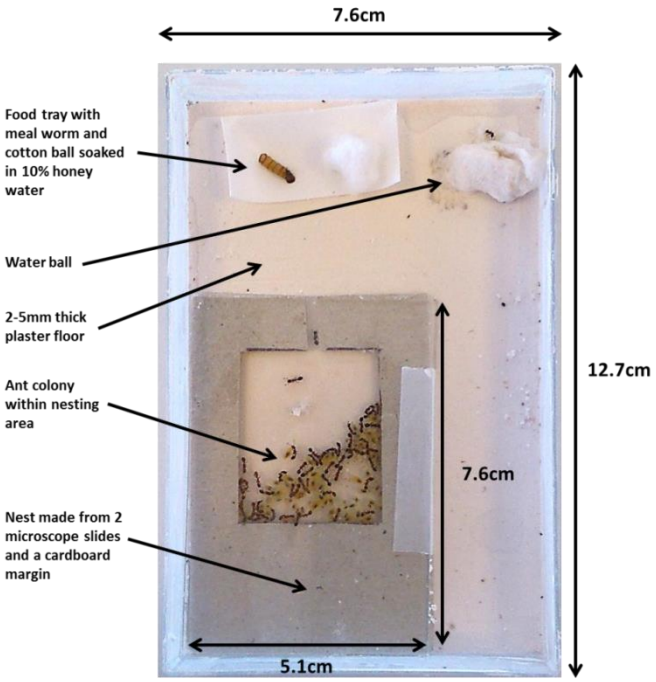


Fig 2: Nest box setup with dimensions.

Artificial nests (see Fig 2) were modified from those used by Gill and Hammond, 2011. The nest was made from two transparent glass slides (5.1x7.6cm) separated by 1mm thick cardboard. The cardboard had a 30x37mm (11.1cm^2) space cut-out to provide a nesting area, which had a nest entrance 10mm in length and 3-5mm in width. The thickness of the cardboard allowed for a single layer of individuals within the nesting area which encouraged clearer and more efficient observations of colony behaviour. Each nest was placed in a foraging arena (transparent container: 7.6x12.7cm) with the vertical sides (18mm) coated in Fluon® to prevent individuals escaping. The base of each foraging area was layered with 2-5mm of plaster of Paris, which was kept damp by adding 2-3 drops of water. Damp cotton wool and a diet of 10% honey solution and chopped-up meal worm were provided three times a week during the observation period. 10% honey solution was soaked into a small ball of cotton wool and left in the feeding tray to minimise the numbers of individuals drowning in excess honey water. These cotton balls were replaced three times a week to prevent the

honey fermenting. It was important that worker mortality was kept to a minimum to prevent Q:W ratios becoming biased, so great care was taken during feeding to prevent workers from drowning in excess liquid.

Lab colonies were kept in an environmental chamber (*Sanyo* MLR-351H) set to spring conditions (hourly rhythm=11-1-11-1, Temp./°C=10-15-20-15, Photoperiod (Night-Day-Day-Day)= 0-2-3-2, humidity/%=70-70-80-70). The conditions within the chamber did not change throughout the experimental period.

A total of 39 complete colonies were collected. Eighteen of these colonies contained multiple queens (MQ, queen number range: 2-12, median 4.5), which made them suitable for behavioural observation. All queens were marked with 0.03mm copper wire tied carefully around the petiole following the protocol detailed in Chapter 4 section 4.2.2 and other studies (Trettin et al., 2014, Trettin et al., 2011, Kuehbandner et al., 2014). Wire 'whiskers' of different lengths were cut in order to identify individual queens during behavioural observation. For colonies containing ≥3 queens, multiple whiskers were necessary. These were always tied between the thorax and petiole and the post-petiole and abdomen.

All colonies were allowed to acclimatise to their new nest, wire-marking and environment for a two week period before beginning the first phase of behavioural observation.

5.2.2 Behavioural observation of un-manipulated colonies

14 un-manipulated MQ colonies were selected for observation (queen number range per colony: 2-9, median: 3.5). The four colonies which possessed ≥10 queens were omitted from the observation due to difficulties associated with distinguishing large numbers of different queens from colonies that also contain many workers in video footage. Queen:worker (Q:W) ratios were calculated for each colony and showed a diverse range of

values (Q:W ratio range: 0.011-1, median: 0.0869, see Table 1). The Q:W ratios of the omitted colonies were distributed throughout the total range of W:Q ratios (see Table 1) and so their omission was unlikely to alter the analysis or conclusions.

All colonies were observed for a total of 10 minutes per day for 10 days starting on 22nd July and finishing 3rd August (days were consecutive apart from two occasions where a gap of 1 and 2 days respectively occurred between recordings). A 10 day period was chosen to mimic the observation period used in Trettin etal, 2014. Colonies were either observed during the morning (between 9-12am) or the afternoon (between 1-5pm) following a random sequence (5x days in the morning and 5x days in the afternoon were maintained) to control for any potential confounding differences in behaviour relating to time of day. Colony behaviour was recorded using a Logitech 1080p digital web camera connected to a laptop. Video recording software was used in tandem with Logitech webcam software v2.1 to make clear high definition recordings.

All colonies were inspected before each recording and queen and worker deaths were recorded. Dead queens were immediately snap frozen in LN_2 and placed in -80^{o}C for future dissection. Worker deaths were recorded to keep track of changing Q:W ratios.

5.2.3 Behavioural observation of manipulated colonies

Sixteen polygynous colonies were selected to test the hypothesis that experimentally manipulated Q:W ratio within colonies increases the rate of Q-Q aggression and/or W-Q aggression. Colonies were selected after the observation period from the un-manipulated colonies and, unlike the first observation period, colonies with ≥10 queens were included in the sample (see Table 2). This was because Q:W ratio manipulation was restricted by the number of workers available within a colony. Therefore high Q:W ratios could be more easily constructed from colonies with many queens and low Q:W ratios could be constructed from colonies containing fewer queens.

Selected colonies were sorted into two treatment groups containing eight colonies each; high Q:W ratio and low Q:W ratio. Colonies in the high Q:W ratio treatment group were manipulated to contain 3x workers for every queen to give a controlled Q:W ratio of 0.333. Conversely, colonies in the low Q:W ratio treatment group were manipulated to contain 10x workers per queen, which fixed the Q:W ratio at 0.1. Additionally, total brood numbers within all colonies (in both treatment groups) were fixed at a brood:worker (B:W) ratio of 0.5 (1x brood item for every 2x workers). This controlled for any potential confounding effects B:W ratio might have on worker or queen behaviour. There was no statistical difference between the numbers of queens per colony in the high ratio or low ratio treatment groups (Student's t test: mean high ratio=6.75, SD=3.49 mean low ratio=4, SD=2.78, N_{high}=8, N_{low}=8, t=-1.74, P=0.28).

Table 1: Summary of Q:W ratio information for un-manipulated MQ colonies. The colony ID is constructed as follows: SD(A or B)=Santon Downham collection site A or B, the year or collection=2014 and the colony number. Colonies marked in **Bold** were not observed during the first observation period.

Colony	Queen total	Worker total	Queens per worker ratio (Q:W)
SDB.14.06	3	3	1
SDB.14.12	5	20	0.25
SDA.14.09	3	13	0.230
SDA.14.01	**10**	**50**	**0.20**
SDA.14.11	5	36	0.138
SDB.14.01	7	55	0.127
SDB.14.19	**10**	**86**	**0.116**
SDB.14.03	9	89	0.101
SDB.14.15	6	64	0.093
SDA.14.04	**10**	**114**	**0.088**
SDB.14.27	**12**	**139**	**0.086**
SDB.14.02	2	25	0.08
SDB.14.25	3	41	0.073
SDB.14.10	2	35	0.057
SDB.14.14	4	89	0.044
SDB.14.22	2	48	0.041
SDB.14.11	2	54	0.037
SDA.14.07	4	115	0.034
SDB.14.08	1	50	0.02
SDA.14.08	1	92	0.010

Following Q:W ratio manipulation, all colonies were allowed to re-acclimatise to their nest boxes in spring conditions (see 5.2.1) for a period of nine days before recording could begin. To make our study comparable to that of Trettin etal (2014), colony recordings were started on 12th August and continued for 10 days (days were consecutive except for three instances where a gap of 1 day, 1 day and 5 days occurred respectively) until 28th August. Recordings were made using the same equipment as described in section 5.2.2 and the time of day was controlled for by randomly determining the sequence of 5x morning and 5x afternoon recording periods.

Table 2: Summary Q:W ratio information for all observed manipulated colonies.

Colony	Queen total	Worker total	Queens per worker ratio (Q:W)
SDB.14.14	3	30	0.1
SDA.14.07	4	40	0.1
SDB.14.11	2	20	0.1
SDB.14.22	2	20	0.1
SDB.14.10	2	20	0.1
SDB.14.03	6	60	0.1
SDB.14.25	3	30	0.1
SDA.14.04	10	100	0.1
SDB.14.27	12	36	0.3
SDB.14.01	5	15	0.3
SDB.14.15	6	18	0.3
SDB.14.19	10	30	0.3
SDA.14.11	5	15	0.3
SDA.14.09	2	6	0.3
SDA.14.01	10	30	0.3
SDB.14.12	4	12	0.3

5.2.4 Video analysis and behavioural scoring

All recordings from both the un-manipulated and manipulated sample groups were viewed and analysed using with VAR© (Video Activity Recorder, Little Imp Company), which allowed the type, length and number of each behavioural event to be recorded accurately for each individual queen. Four types of aggressive interactions were scored: 1) a single bite; 2) pulling; 3) spreading and 4) sting-smearing. Each behaviour was considered to be an increase in the degree of aggressiveness (see Table 3). To account for the rapidity of a single bite, each bite event was assigned a duration of 1 second in order to calculate a rate (Gill and Hammond,

2011a, Gill and Hammond, 2011b). All aggressive interactions were scored for queens and workers separately. Grooming behaviour was distinguished between queen grooming and worker grooming. Variation in observation times for each queen is explained by queens leaving and entering the nest as queens outside of the nest could not be videoed.

5.2.5 Behavioural observation analysis

All data sets were initially tested for a normal distribution using Kolmogorov–Smirnov analysis. The outcome of this test dictated whether parametric statistics were appropriate for further analysis. All statistical analyses were performed using a combination of SPSS 22, Minitab 17, R 3.2.0 (R_Core_Team, 2013) and RStudio (RStudio, 2012) software packages. All graphical figures were produced in Excel 2010, R 3.2.0 and R studio.

5.2.5.1 Behavioural analysis of un-manipulated colonies

I aimed to test the hypothesis that high Q:W colony ratios correlate with high rates of aggressive interactions, as observed by Trettin et al. (2014). I tested this prediction using three different analyses.

First, behavioural rates for each specific behavioural type were calculated for each queen by taking the total duration of the behaviour (seconds) / the total length of time a focal queen had been observed (minutes). Per capita rates of behaviour were then calculated for worker number (with Q-W interactions) and queen number (with Q-Q interactions) to correct for potential differences in aggression rates due to variation in both worker and queen numbers (Gill and Hammond, 2011a, Gill and Hammond, 2011b). Spearman's correlation analysis was used to detect relationships between per capita rate of behaviour and Q:W ratio.

Second, all colonies were grouped into high and low categories using a Q:W ratio threshold value of >0.1 for high and <0.1 for low. Mann-Whitney-Wilcoxon tests were used to test for significance between the medians of the high and low sample groups.

Finally, binary logistic regression was also used to test if the future reproductive status of queens (reproductive queens coded 1, non-reproductive queens coded 0) could be predicted from rates of aggression and grooming. Prior to analyses the assumption that independent variables had a linear relationship with the logarithm of the dependent variable was tested, as described in (Field, 2009). In all cases this assumption was met as there was no significant interaction term between the independent variable and its natural logarithm.

5.2.5.2 Behavioural analysis of manipulated high and low Q:W ratio treatment groups

Rates of behaviour (secs/min) for each behavioural type were calculated for each queen in exactly the same way as described in section 5.2.5.1. Similarly, rates of behaviour were corrected for queen and worker bias by calculating per capita rates of behaviour for queen and worker interactions respectively (Gill and Hammond, 2011a, Gill and Hammond, 2011b). Transformation of per capita behavioural rate data to fit a normal distribution could not be achieved through transformation and so non-parametric tests were used for all further analysis. Per capita behavioural rate data were always used in further analysis.

Comparisons of medians for a given behavioural type between high and low ratio groups were achieved using Mann-Whitney-Wilcoxon tests.

Finally, binary logistic regression was also used to test if rates of aggression and grooming could predict the future reproductive status of queens. Exactly as explained above, all assumptions were tested and met before binary logistic analysis was performed.

5.2.6 Queen ovary dissections

Queen reproductive status was determined via ovary dissection following the methodology established in Gill et al. (2009). All queens were dissected in distilled water.

Mated status of queens was determined visually using a 32x microscope (Ceti Vari-zoom 10 binocular microscope) by the opaqueness of the spermatheca (mated=opaque, unmated=transparent). 85 queens were found to be mated due to presence of an opaque spermatheca. Queens were considered to be 'mated' if mated status could not be determined due to damage of the spermatheca. This is due to the likelihood that the queens were mated as shown in previous studies (Gill and Hammond, 2011b, Gill et al., 2009). In total, the mated status of six queens could not be determined due to a damaged spermatheca. Finally, six queens were found to be unmated queens and were removed from all analyses.

The reproductive status of each queen was determined using the criteria established in Gill et al (2009); A= relatively large elongated ovarioles containing large yolk filled eggs and corpora lutea; B= relatively short ovarioles with a small number of large yolk filled eggs; C= relatively short ovarioles with no large yolk filled eggs; and D= very short ovarioles with no eggs present. Queens classified as either A or B were considered to be reproductive and queens classified as either C or D were considered to be non-reproductive. Degenerating ovaries (C or D) with corpora lutea were also classified as reproductive, as the presence of corpora lutea indicates recent reproductive activity (Stille et al., 1991). If the ovary classification could not be determined due to damage then the queen was not considered in any further analysis.

5.2.7 Analysis of published Q:W ratios from FM and P populations

Trettin et al. (2014) based their social plasticity hypothesis on observations of high Q:W ratios in high skew FM *L. acervorum* populations. However, the conclusion that FM colonies are characterised by higher Q:W ratios in comparison to P colonies was based on the data of only two FM studies and FM colony information from other populations were omitted. Therefore, I collected additional personal and published data for five FM and two P

populations and calculated the Q:W ratios. Median Q:W ratios were then compared between FM and P populations using Mann-Whitney U analysis.

Table 3: Summary of behaviours observed. Classification and description for each type of interaction recorded during video observation.

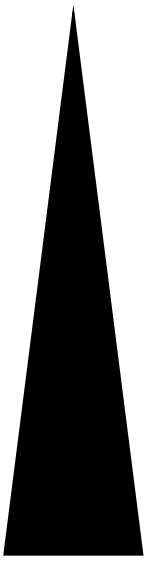
Type of behaviour	Degree of aggressiveness	Definition
Grooming	0	A single individual is cleaned by one or multiple individuals. A single individual may also be fed by another individual (trophallaxis).
Mandible Threat	Low	A single individual spreads her mandibles wide open and draws back her antennae towards the queen.
Antennal Boxing		A single individual repeatedly clubs the queen with her antennae. The behaviour is vigorous and direct.
Single Bite		A single individual bites another for ≤1 second.
Pulling		A single individual bites another individual usually on an appendage (i.e. legs, antennae, neck and petiole) and drags the attacked individual.
Spreading		Multiple individuals bite another individual's appendages and pull in opposite directions, completely immobilising the attacked individual. Prolonged spreading can lead to the loss of an appendage and/or death.
Sting Smearing	High	A single queen bites and holds another queen. When secure the aggressing queen pulls her abdomen round and smears the other individual's body with a secretion from her abdomen (location of the sting). Once complete, both queens break the hold and often worker aggression is witnessed directly afterwards between either/both queens.

Table modified from Gill, (2010) with permission.

5.3 Results

5.3.1 Queen reproductive status

From the 97 queens used in the study, 87.6% were found to be mated and 5.82% (*N*=6) were unmated. Unmated queens may behave differently to mated queens and conspecifics may in turn alter their behaviour accordingly (Ito, 2005, Gill and Hammond, 2011a) and so to eliminate this potential confounding factor, unmated queens were removed from the analysis. This meant that colonies SDB.14.02 and SDB.14.14 could no longer be used for analysis as the presence of unmated queens effectively made the colony monogynous. This effectively reduced the number of observed MQ colonies in the un-manipulated sample to 13 and the number of manipulated colonies to 15.

The remaining 5.82% (*N*=6) of the queens could not be classified due to decomposition of the ovary. Queens that possessed ovaries too decomposed to classify were considered to be mated due to the high likelihood that they were and included in all analysis except the binary logistic regression where exact determination of mated status was necessary. Removing data linked to queens with decomposed ovaries did not alter the result. Due to decomposed ovaries, two additional colonies, SDB.14.11 and SDB.14.14 had to be omitted from binary logistic regression analysis.

From 85 mated queens, 66 (65.9%) were classified as recently reproductive. There were three reproductive queens per colony on average (n=18, range=1-10) and the number of reproductive queens did not significantly differ between colonies with a high W:Q ratio (≥0.1 in un-manipulated colonies) or a low Q:W ratio (<0.1 in un-manipulated colonies) (median queens in high colonies=3, median queens in low colonies=2; Mann-Whitney: U=29.5, N_{high}=8, N_{low}=10, *P*=0.339, see table 5). However, only five queens were classified with an A or B ovarian status.

5.3.2 Un-manipulated colony behaviour

Observation of 13 (see section 5.3.1) un-manipulated MQ colonies revealed a strong bias towards W:Q interactions and did not present the levels of aggressive behaviour previously reported in high skew FM populations of *L. acervorum* (Trettin et al., 2011, Gill and Hammond, 2011a, Gill and Hammond, 2011b, Ito, 2005). The 14 un-manipulated colonies were observed for a total of 100 mins each over the course of 10 days, which resulted in 64.57 hrs of queen behaviour distributed over 45 resident queens (mean average= 1.43 hrs per queen, range= 10-100 mins, *N*=45 (corrected for mated status and observation time)). W-Q interactions accounted for the vast majority of all observed behaviour (97.97%) and covered both aggressive and non-aggressive categories (see Table 3-4). Q-Q interactions accounted for the remaining 2.02% of observed behaviour and were only ever socio-positive (see Fig 4).

5.3.2.1 Aggression

Aggressive behaviours only accounted for 0.24% of the total rate (sec/min) of all behaviours observed and only occurred between workers and queens (W-Q aggression, see Table 4). Specifically, there were 19 occurrences of biting and five occurrences of pulling behaviours between workers and queens but not the highly aggressive spreading behaviour commonly shown in FM high skew colonies (Gill and Hammond, 2011a, Gill and Hammond, 2011b). I did not observe Q-Q or W-Q antennal boxing or threat display. Spearman's Rho correlation analysis found no significant relationship between per capita rates (sec/min) of W-Q biting and Q:W ratio (Spearman's Rho correlation: *N*=45, d.f=43, r_s=-0.255, *P*=0.091, see Fig 3). However, a boarder-line significant negative relationship was observed between per capita rate (sec/min) of W-Q pulling and Q:W ratio (Spearman's Rho correlation: *N*=45, d.f=43, r_s=-0.313, *P*=0.036, see Fig 3), although this is likely due to a single data point representing a single colony where the average rate of W-Q pulling was relatively high. Finally, the combined per capita rates (sec/min) of W-Q biting and W-Q pulling also revealed a significant negative

relationship with Q:W ratio (Spearman's Rho correlation: N=45, d.f=43, r_s=-0.318, P=0.033, Table 4).

Furthermore, binary logistic regression analysis found that per capita rates (sec/min) of biting behaviour were not able to predict future reproductive status of queens (logistic regression: β(s.e)= 443.535 (1347.105), Wald=0.108, d.f=1, P=0.742, Table 4). Per capita rates (sec/min) of pulling behaviour also failed to predict the future reproductive status of queens (logistic regression: β(s.e)= 39658.956 (13648497.47), Wald=0.000, d.f=1, P=0.998, Table 4).

Per capita rates of aggression (sec/min) were also compared between colonies with a low and high Q:W ratio. Based on the distinctions of Trettin et al. 2014 and on previously reported average Q:W ratios for high and low skew populations (see Table 5), all colonies with a Q:W ratio of ≥0.1 were considered to have a high Q:W ratio and all colonies with <0.1 were considered to have a low Q:W ratio. Comparisons of per capita rates of W-Q biting were not found to differ significantly between high and low Q:W ratios (Median rate per queen (Range): high ratio= 0.00 (0-0.002) sec/min, low ratio= 0.00 (0-0.0004) sec/min, Mann-Whitney-Wilcoxon: W=226, N_{high}=20, N_{low}=25, P=0.395, Table 4). As pulling behaviour was not observed amongst queens in high Q:W ratio colonies, rates of pulling behaviour in the low Q:W ratio colonies were tested for significance against a median of 0. Consequently, pulling behaviour was not found to be significant (Median rate per queen (Range): low ratio= 0.00 (0-0.005) sec/min, Wilcoxon's signed-rank test: Z=10, N_{low}=25, P=0.10, Table 4).

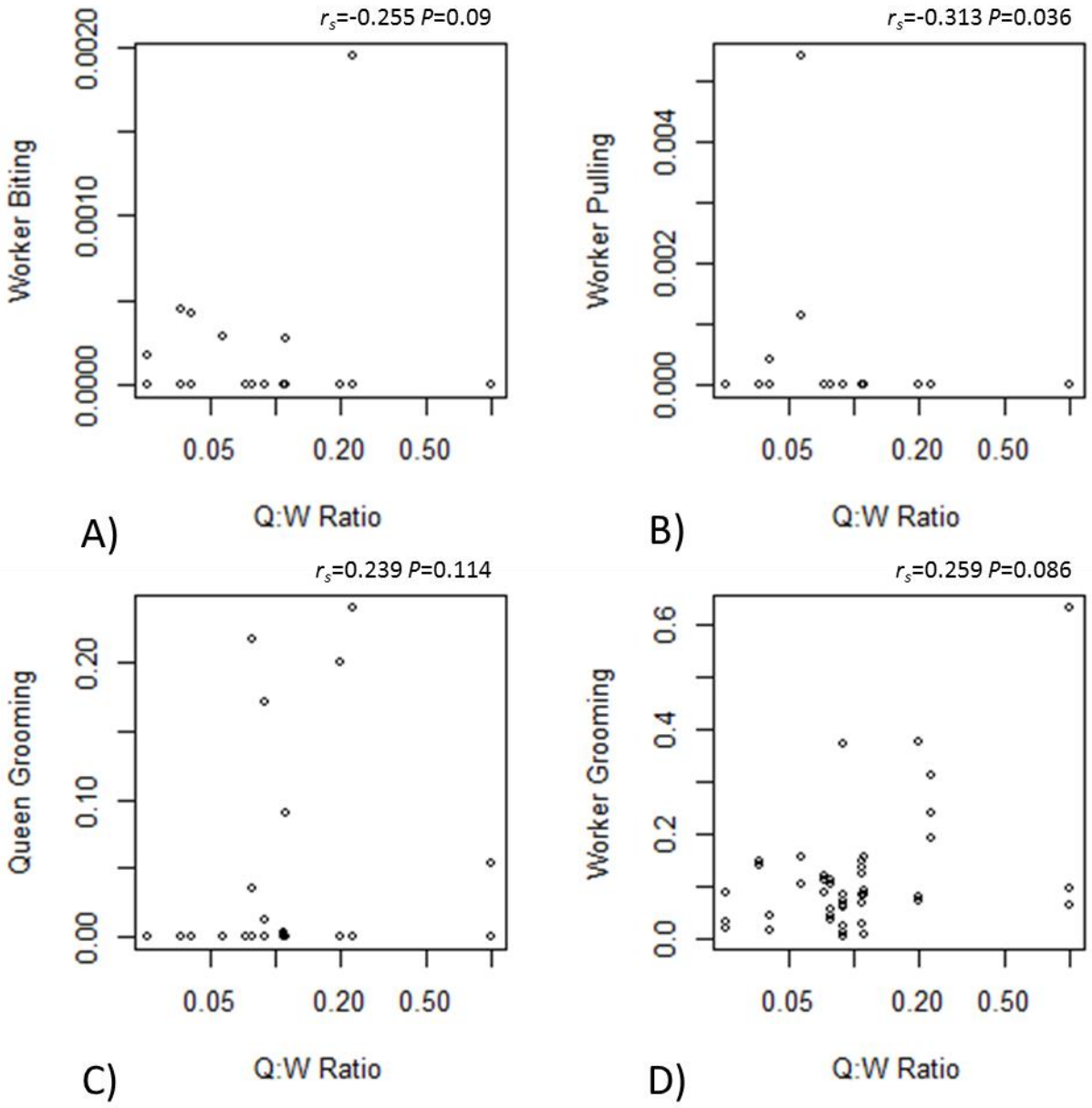


Fig 3: Correlations between Q:W ratio and per capita rates of behaviour. **A)** *per capita* rates of W-Q biting, non-significant correlation. **B)** *per capita* rates of W-Q pulling, significant correlation. **C)** *per capita* rates of Q-Q grooming, non-significant correlation. **D)** *per capita* rates of W-Q grooming, non-significant correlation. The x-axis for each plot has been $\log_{10}$ transformed. All rates of behaviour are measured in secs/min. See Table 4 for summary statistics.

5.3.2.2 Socio-positive grooming

Q-Q and W-Q socio-positive grooming behaviours were by far the most common behaviours observed in un-manipulated polygynous colonies, accounting for 95.43% of all behavioural observations. Per capita rates of W-Q grooming were significantly higher than per capita rates of Q-Q grooming (Median rate per queen (±IQR): Q-Q_{groom}= 0.00 (0.00) sec/min, W-Q_{groom} = 0.083 (0.08) sec/min, Mann-Whitney-Wilcoxon: W=209, $N_{Q\text{-}Q}$=45, $N_{W\text{-}Q}$=45, *P*=<0.001, see fig 3 and Table 4).

Previous studies have shown grooming to reliably predict the future reproductive queen in high skew FM colonies (Gill and Hammond, 2011b, Trettin et al., 2011). Therefore Spearman's Rho correlations were calculated to test the hypothesis that lower Q:W ratios might yield greater rates of grooming. No significant relationship was found between per capita rates (sec/min) of W-Q grooming and Q:W ratio (Spearman's Rho correlation: *N*=45, d.f=43, r_s=0.259, *P*=0.086, see Fig 3). Furthermore, there was no significant relationship between per capita rates of Q-Q grooming and Q:W ratio (Spearman's Rho correlation: *N*=45, d.f=43, r_s=0.239, *P*=0.114, see Fig 3 and Table 4).

Binary logistic regression analysis showed that rates of W-Q grooming behaviour (sec/min) were not able to predict future reproductive status of queens (logistic regression: β(s.e)= 3.340 (4.130), Wald=0.654, d.f=1, *P*=0.419). Similarly, rates of Q-Q grooming (sec/min) also could not predict the future reproductive status of queens (logistic regression: β(s.e)= -3.956 (5.671), Wald=0.487, d.f=1, *P*=0.485, Table 4).

Similarly to the aggression analysis, per capita rates of grooming behaviour were compared between colonies with a high Q:W ratio and colonies with a low Q:W ratio. Interestingly, per capita rates of W-Q grooming were found to differ significantly between high and low Q:W ratio sample groups, albeit at a borderline significance (Median rate per

queen (±IQR): high ratio= 0.093 (0.094) sec/min, low ratio= 0.073 (0.076) sec/min, Mann-Whitney-Wilcoxon: W=336, N_{high}=20, N_{low}=25, P=0.05, Table 4). Per capita rates of Q-Q grooming were not significantly different between high and low Q:W ratio sample groups (Median rate per queen (±IQR): high ratio= 0.000 (0.002) sec/min, low ratio= 0.000 (0.000) sec/min, Mann-Whitney-Wilcoxon: W=284, N_{high}=20, N_{low}=25, P=0.293, Table 4).

5.3.3 Manipulated colony behaviour

Sixteen manipulated MQ colonies were observed for a total of 100 mins for a total of 10 days, which resulted in 123.2 hrs of queen behaviour (mean average= 1.43 hrs per queen, range= 5.6-100mins, N=80). Unfortunately, colony B.14 (W:Q ratio=0.1) had to be removed from the analysis due to the presence of unmated queens, which effectively made the colony monogynous. Furthermore, four additional queens had to be omitted from the analysis due to unmated status. One queen was removed from the high W:Q ratio colony SDA.14.04 and one queen was removed from colonies SDB.14.15, SDB.14.19 and SDA.14.11. This did not significantly alter the W:Q ratios for either group (High ratio: 1 sample Wilcoxon signed rank test: test median Q:W ratio=0.333, calculated median Q:W ratio= 0.317, N=8, W=15.0, P=0.726. Low ratio: Wilcoxon signed rank test: test median Q:W ratio=0.1, calculated median Q:W ratio= 0.1, N=8, W=0.0, P=1.0, Table 4).

Similarly to un-manipulated colonies, W-Q interactions accounted for the majority of all observed behaviour (94.39%) and covered both aggressive and socio-positive categories (see Tables 3-4). Q-Q interactions accounted for the remaining 5.60% of observed behaviour and were never aggressive.

5.3.3.1 Aggression

Aggressive W-Q interactions were even more infrequent amongst manipulated colonies than they were between un-manipulated colonies (un-manipulated colonies= 19 instances of biting and 5 instances of pulling) with only nine instances of biting and no pulling

behaviour observed. Furthermore, antennal boxing and mandible threat display behaviours were entirely absent.

W-Q per capita rates of biting behaviour were not found to be significantly different between high and low Q:W ratios (Median rate per queen (Range): high ratio= 0.00 (0-0.0003) sec/min, low ratio= 0.00 (0-0.001) sec/min, Mann-Whitney-Wilcoxon: W=773, N_{high}=51, N_{low}=29, *P*=0.47, Table 4). Furthermore, per capita rates (sec/min) of worker biting were not sufficient to predict the future reproductive status of queens (logistic regression: β(s.e)= 1068.082 (2060.27), Wald=0.269, d.f=1, *P*=0.604, Table 4).

5.3.3.2 Socio-positive grooming

Similarly to un-manipulated colony observations, Q-Q and W-Q socio-positive grooming behaviours constituted the majority of all observed behaviour, accounting for 97.8% of all behavioural observations. Per capita rates of W-Q grooming were significantly higher than per capita rates of Q-Q grooming (Median rate per queen (±IQR): Q-Q_{groom}=0.000 (0.025) sec/min, W-Q_{groom}=0.071 (0.110) sec/min, Mann-Whitney-Wilcoxon: W=1048.5, $N_{Q\text{-}Q}$=80, $N_{W\text{-}Q}$=80, *P*=<0.001, Table 4).

Per capita rates of W-Q grooming were not found to differ significantly between high and low Q:W ratio sample groups (Median rate per queen (±IQR): high ratio=0.070 (0.116) sec/min, low ratio=0.074 (0.119) sec/min, Mann-Whitney-Wilcoxon: W=818, N_{high}=51, N_{low}=29, *P*=0.44). Interestingly, per capita rates of Q-Q grooming were significantly higher in high Q:W ratio sample groups compared with low Q:W ratio sample groups (Median rate per queen (±IQR): high ratio=0.004 (0.038) sec/min, low ratio=0.000 (0.000) sec/min, Mann-Whitney-Wilcoxon: W=517, N_{high}=51, N_{low}=29, *P*=0.01, Table 4).

Binary logistic regression analysis showed that there was no relationship between reproductive status of queens and per capita rates (sec/min) of W-Q grooming behaviour (logistic regression: β(s.e)= 1.294 (2.469), Wald=0.275, d.f=1, *P*=0.60). Similarly, rates of Q-Q

grooming also could not predict the future reproductive status of queens (logistic regression: β(s.e)= -4.466 (4.670), Wald=0.915, d.f=1, *P*=0.339, Table 4).

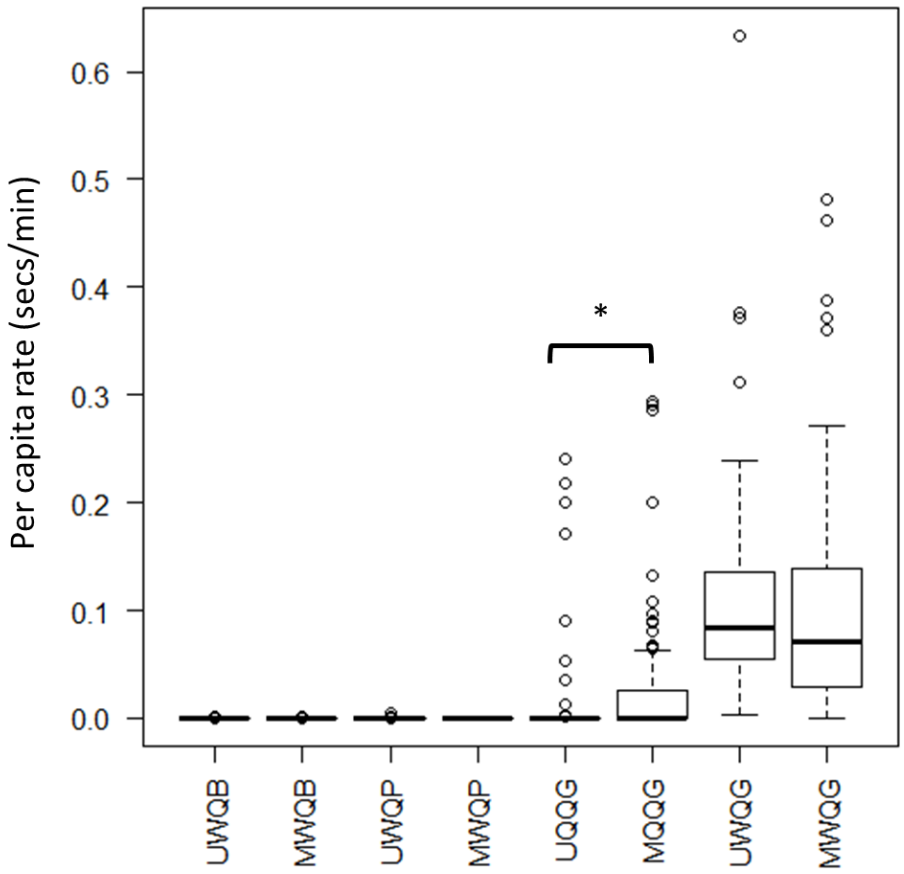


Fig 4: Per capita rates of observed aggressive and socio-positive behaviour. UWQB= un-manipulated W-Q biting. MWQB= manipulated W-Q biting. UWQP= un-manipulated W-Q pulling. MWQP= manipulated W-Q pulling. UQQG= un-manipulated Q-Q grooming. MQQG= manipulated Q-Q grooming. UWQG= un-manipulated W-Q grooming. MWQG= manipulated W-Q grooming. * indicates significance at p=<0.05, see Table 4.

5.3.4 Rate of behaviour comparisons between un-manipulated and manipulated colonies

Taken together and without regard for Q:W ratio, per capita rates of W-Q biting (sec/min) were not found to differ significantly between un-manipulated and manipulated

colonies (Median rate per queen (Range): un-manipulated= 0.00 (0-0.002) sec/min, manipulated= 0.00 (0-0.001) sec/min, Mann-Whitney-Wilcoxon: W =1654.5, N_{manip}=80, $N_{unmanip}$=45, *P*=0.16, Fig 4 and Table 4). Since W-Q pulling behaviour was not observed amongst the manipulated colonies, per capita rates of W-Q pulling from un-manipulated colonies were compared against a median of 0 (Median rate per queen (±IQR): low ratio= 0 (0.00) sec/min, Wilcoxon's signed-rank test: W=6, *N*=45, *P*=0.18, Fig 4and Table 4). These findings also suggest that W-Q rates of aggression do not change over time, which contrasts with the findings of Trettin et al. (2014) where Q-Q aggression was higher amongst colonies observed in September than in July.

As expected, per capita rates of Q-Q grooming were found to differ significantly between manipulated and un-manipulated colonies, where rates were higher amongst manipulated colonies (Median rate per queen (±IQR): un-manipulated= 0.000 (0.000) sec/min, manipulated= 0.000 (0.025) sec/min, Mann-Whitney-Wilcoxon: W =2174.5, N_{manip}=80, $N_{unmanip}$=45, *P*=0.026, Fig 4 and Table 4). However, there was no significant difference found in comparisons of per capita rates of W-Q grooming between manipulated and un-manipulated colonies (Median rate per queen (±IQR): un-manipulated= 0.084 (0.080) sec/min, manipulated= 0.0710 (0.109) sec/min, Mann-Whitney-Wilcoxon: W =1603.5, N_{manip}=80, $N_{unmanip}$=45, *P*=0.18, Fig 4 and Table 4).

5.3.5 Variation in Q:W ratios in all reported FM populations

The additional FM populations showed wide variation in median average Q:W ratios (see Table 5) and comparisons of median Q:W ratios between P and FM populations were not significantly different (Median Q:W ratio (IQR): P= 0.07 (0.036), FM= 0.17 (0.146), Mann-Whitney-Wilcoxon: W =40, N_P=7, N_{FM}=6, *P*=0.225).

Table 4: Summary statistics for all un-manipulated and manipulated colony analysis. *N*=sample number, *P*= *P*-value. Significant *P*-values are highlighted in **bold**. Combined aggression represents all rates of aggression (biting and pulling) combined.

Un-manipulated Colony Statistics						
Behaviour	**Comparison**	**Significance Test**	**Average (Variation)**	**Test Statistic**	***N***	***P***
W-Q Biting	Per capita rate (sec/min) and Q:W ratio	Spearman's Rho Correlation	Median (±IQR): 0.00 (0.00)	d.f=43, r_s=-0.255	45	0.091
W-Q Pulling	Per capita rate (sec/min) and Q:W ratio	Spearman's Rho Correlation	Median (±IQR): 0.00 (0.00)	d.f=43, r_s=-0.331	45	**0.036**
Combined Aggression	Per capita rate (sec/min) and Q:W ratio	Spearman's Rho Correlation	Median (±IQR): 0.00 (0.00)	d.f=43, r_s=-0.318	45	**0.033**
Q-Q Grooming	Per capita rate (sec/min) and Q:W ratio	Spearman's Rho Correlation	Median (±IQR): 0.00 (0.00)	d.f=43, r_s=0.239	45	0.114
W-Q Grooming	Per capita rate (sec/min) and Q:W ratio	Spearman's Rho Correlation	Median (±IQR): 0.08 (0.08)	d.f=43, r_s=0.259	45	0.086
W-Q Biting	High VS low W:Q ratio	Mann-Whitney-Wilcoxon	Median (±IQR): high ratio= 0.00 (0.00), low ratio= 0.00 (0.00)	W=226	N_{high}=20 N_{low}=25	0.395
Q-Q Grooming	High VS low W:Q ratio	Mann-Whitney-Wilcoxon	Median (±IQR): high ratio= 0.000 (0.002), low ratio= 0.000 (0.000)	W=284	N_{high}=20 N_{low}=25	0.293
W-Q Grooming	High VS low W:Q ratio	Mann-Whitney-Wilcoxon	Median (±IQR): high ratio= 0.093 (0.094), low ratio= 0.073 (0.076)	W=336	N_{high}=20 N_{low}=25	**0.05**
Grooming	Per capita rate (sec/min) Q-Q VS W-Q	Mann-Whitney-Wilcoxon	Median (±IQR): $Q\text{-}Q_{groom}$= 0.00 (0.00), $W\text{-}Q_{groom}$ = 0.083 (0.08)	W=209	$N_{Q\text{-}Q}$=45 $N_{W\text{-}Q}$=45	**<0.001**
W-Q Biting	Per capita rate (sec/min) and reproductive status	Binary Logistic Regression	β(s.e)= 443.535 (1347.105)	Wald=0.108, d.f=1	45	0.742
W-Q Pulling	Per capita rate (sec/min) and reproductive status	Binary Logistic Regression	β(s.e)= 39658.956 (13648497.47)	Wald=0.000, d.f=1	45	0.998
Q-Q Grooming	Per capita rate (sec/min) and reproductive status	Binary Logistic Regression	β(s.e)= -3.956 (5.671)	Wald=0.487, d.f=1	45	0.485
W-Q Grooming	Per capita rate (sec/min) and reproductive status	Binary Logistic Regression	β(s.e)= 3.340 (4.130)	Wald=0.654, d.f=1	45	0.419

Manipulated Colony Statistics						
Behaviour	**Comparison**	**Significance Test**	**Average (Variation)**	**Test Statistic**	***N***	***P***
W-Q Biting	High VS low W:Q ratio	Mann-Whitney-Wilcoxon	Median (±IQR): high ratio= 0.000 (0.000), low ratio= 0.000 (0.000)	W=773	N_{high}=51 N_{low}=29	0.47
Q-Q Grooming	High VS low W:Q ratio	Mann-Whitney-Wilcoxon	Median (±IQR): high ratio=0.004 (0.038), low ratio=0.000 (0.000)	W=517	N_{high}=51 N_{low}=29	**0.01**
W-Q Grooming	High VS low W:Q ratio	Mann-Whitney-Wilcoxon	Median (±IQR): high ratio=0.070 (0.116), low ratio=0.074 (0.119)	W=818	N_{high}=51 N_{low}=29	0.44
Grooming	Per capita rate (sec/min) Q-Q VS W-Q	Mann-Whitney-Wilcoxon	Median (±IQR): Q-Q_{groom}=0.000 (0.025), W-Q_{groom}=0.071 (0.110)	W=1048.5	$N_{Q\text{-}Q}$=80 $N_{W\text{-}Q}$=80	**<0.001**
W-Q Biting	Per capita rate (sec/min) and reproductive status	Binary Logistic Regression	β(s.e)= 1068.082 (2060.27)	Wald=0.269, d.f=1	80	0.604
Q-Q Grooming	Per capita rate (sec/min) and reproductive status	Binary Logistic Regression	β(s.e)= -4.466 (4.670)	Wald=0.915, d.f=1	80	0.339
W-Q Grooming	Per capita rate (sec/min) and reproductive status	Binary Logistic Regression	β(s.e)= 1.294 (2.469)	Wald=0.275, d.f=1	80	0.60
Un-manipulated VS Manipulated Colony Statistics						
Behaviour	**Comparison**	**Significance Test**	**Average (Variation)**	**Test Statistic**	***N***	***P***
W-Q Biting	Total Per capita rates (sec/min)	Mann-Whitney-Wilcoxon	Median (±IQR): un-manipulated= 0.000 (0.000), manipulated= 0.000 (0.000)	W =1654.5	N_{manip}=80$N_{unmanip}$=45	0.16
W-Q Pulling	Total Per capita rates (sec/min)	Wilcoxon Signed Rank	Median (±IQR): low ratio= 0 (0.00)	W=6	45	0.18
Q-Q Grooming	Total Per capita rates (sec/min)	Mann-Whitney-Wilcoxon	Median (±IQR): un-manipulated= 0.000 (0.000), manipulated= 0.000 (0.025)	W =2174.5	N_{manip}=8 $N_{unmanip}$=45	**0.026**
W-Q Grooming	Total Per capita rates (sec/min)	Mann-Whitney-Wilcoxon	Median (±IQR): un-manipulated= 0.084 (0.080), manipulated= 0.0710 (0.109)	W =1603.5	N_{manip}=80, $N_{unmanip}$=45	0.18

Table 5: Summary data for MQ *L.acervorum* colonies from P and FM populations. Populations in **Bold** are in addition to those originally referenced by Trettin et al (2014). * indicates mean±SE. References in **Bold** are unpublished personal data with the year of collection. SD= Santon Downham, UK. NF=New Forest, UK. OT= Orihuela del Tremedal, Spain. V= Valdelinares, Spain.

Population	Colonies (*n*)	Queens/Colony	Workers/Colony	Q:W Ratio	Social Organisation Reproductive Skew	Reference/collection
Germany	35	3.0 (2.5, 5.5)	86.0 (57.0, 120.0)	0.049 (0.030, 0.103)	P low skew	(Heinze et al., 1995)
UK	5	8.0 (7.0, 11.0)	127.0 (95.0, 151.0)	0.070 (0.046, 0.084)	P low skew	(Bourke, 1991)
UK (SD)	31	4.0 (3.0, 5.5)	62.0 (42.0, 121.5)	0.059 (0.041, 0.103)	P low skew	(Chan et al., 1999)
UK (NF)	25	3.0 (2.0, 7.0)	48.0 (25.0, 68.0)	0.095 (0.063, 0.135	P low skew	(Chan et al., 1999)
UK (Aberfoyle)	13	10.0 (5.0, 20.0)	174.0 (68.0, 514.0)	0.077 (0.049, 0.095)	P low skew	(Chan et al., 1999)
UK (NF)	20	4 (2, 7.5)	45 (30.75, 62.25)	0.118 (0.054, 0.193)	P low skew	**(Braim and Hammond, 2014)**
UK (SD)	6	3(2.3, 3)	46.8(32.3, 54.3)	0.069(0.059, 0.087)	P low skew	(Friend and Bourke, 2012)
Japan	4	5.5 (5.0, 6.3)	32.5 (27.5, 39.0)	0.192 (0.151, 0.223)	FM high skew	(Ito, 2005)
Spain	50	14.5 (6.0, 21.8)	54.5 (32.0, 77.3)	0.219 (0.125, 0.337)	FM high skew	(Felke and Buschinger, 1999)
Spain (OT)	60	9.2 ± 0.99*	105.6 ± 8.42*	0.087	FM high skew	(Gill et al., 2009)
Spain (OT)	40	6.1 ± 0.84*	41.9 ± 4.31*	0.146	FM high skew	(Gill et al., 2009)
Spain (OT and V)	22	3.1(2.0, 4.0)	77.3(47.0, 103.0)	0.048(0.029, 0.069)	FM high skew	(Gill and Hammond, 2011b)
Spain (OT and V)	33	3.5(2.0, 4.0)	74.9(43.0, 91.0)	0.068(0.027, 0.087)	FM high skew	(Gill and Hammond, 2011a)
Spain (OT)	35	5.1(2.0, 6.0)	41.1(29.0, 50.0)	0.151(0.048, 0.197)	FM high skew	**(Braim and Hammond, 2013)**
Spain (V)	25	7.1(3.0, 10.0)	42.9(26.0, 59.0)	0.206(0.065, 0.278)	FM high skew	**(Braim and Hammond, 2013)**

5.4 Discussion

I investigated the plasticity of the polymorphic social phenotype in *Leptothorax acervorum* by testing whether polygynous (P) colonies can respond to increased environmental constraints by switching to a functionally monogynous (FM) social phenotype. I found no evidence that increasing Q:W ratios in colonies with the P social phenotype from the UK affected queen-queen (Q-Q) behaviour or altered the social phenotype (see Figs 3-4 and Table 4). Instead, the evidence showed that behaviours of all types (socio-positive and socio-negative) remained largely unchanged. Interestingly, aggressive Q-Q interactions were never observed in either the natural un-manipulated or the experimentally manipulated colonies and the only aggressive behaviours to be observed in either experiment were between workers and queens. These findings do not support the social plasticity hypothesis established by Trettin et al (2014) and I found no evidence to support their conclusions that P colonies are able to switch to a FM social organisation in response to environmental constraints and colony composition. This supports previous studies demonstrating that social organisation is invariable within discrete populations and does not change in response to different environmental conditions (Gill et al., 2009). The discrepancy in the results presented in this study and those of Trettin et al. (2014) is intriguing and therefore requires further explanation.

First, all colonies studied by Trettin et al. were experimentally manipulated and none of the colonies represented the natural state. Before colonies were divided into 'stress' treatment groups, they were equalised to contain only 40 workers and 30 brood items, which is not representative of natural colonies in either FM or P populations (see Table 5) and changing group dynamics may initiate new behavioural types (Richardson et al., 2011). To avoid unnecessary manipulation, I collected colonies whole, calculated their natural Q:W ratios and observed their behaviour without any prior standardisation. Through my

observations of these natural colonies, I found no evidence of Q-Q aggression and W-Q aggression was infrequent (see Fig 3). Queens did not engage in bouts of antennal boxing, mandible threat displays or any of the more aggressive behaviours reported previously (Trettin et al., 2011, Ito, 2005, Heinze and Ortius, 1991, Heinze and Smith, 1990). Interestingly, a borderline significant negative correlation between W-Q aggression and Q:W ratio was observed within the un-manipulated colonies, potentially suggesting that W-Q aggression is associated with lower Q:W ratios (see Fig 3). However, the borderline significance suggests that rates of W-Q aggression are unlikely to be associated with Q:W ratio. Additionally, rates of aggression were compared between the high and low Q:W ratio groups and no significant differences were found (see Table 4).

Having shown no evidence of aggressive Q-Q behaviour in un-manipulated colonies, I also tested the hypothesis with experimentally standardised Q:W ratios similar to those set by Trettin et al. (2014). Once again, I did not observe aggressive Q-Q interactions of any kind and rates of W-Q aggression were low and non-significant between high and low ratio groups (see Table 4). Furthermore, I did not observe a difference in rates of aggression towards queens between different time periods (comparisons between un-manipulated and manipulated colonies, Table 4), which contradicts the findings of Trettin et al. (2014) where observations of queens in September showed a significantly greater rate of aggression compared to observations of queens in July.

Unfortunately, I was not able to sample queen ovarian development at the end of the first experimental observation period (un-manipulated colony observation) and when queen ovaries were dissected at the end of the second observation period in August, it was clear that the majority had finished egg laying and begun to degenerate their ovaries in preparation for hibernation. Regardless, I observed no difference in the number of queen ovaries presenting evidence of recent reproductive activity (see 5.3.6) between the high and low Q:W ratio

groups. Considering that the 15 colonies used in the second experimental period with a naturally high Q:W ratio were found to contain three laying queens on average suggests that high Q:W ratio in naturally occurring polygynous populations does not affect the reproductively of queens and by extension affect skew.

Second, it may be possible that variation in social plasticity exists in specific populations. Interestingly, close inspection of relatedness data for the Reichswald population suggests that reproductive skew may be much higher than expected for a P social phenotype (Heinze et al., 1995). Relatedness estimates between queens (Relatedness ± SE= 0.63±0.057) and workers (Relatedness ± SE= 0.46±0.04) within the Reichswald population predicted a smaller proportion of mother queens (Predicted=1.5-2.9) in relation to the true number of inseminated queens present (Mean=3.2). Furthermore, average relatedness estimates between newly produced queens in multiply queened colonies indicated that they were full sisters, as is found in high skew FM colonies (Gill and Hammond, 2011a, Gill and Hammond, 2011b, Heinze et al., 1995). Finally, the average relatedness of workers in MQ colonies was higher than that found on average in colonies from the UK (Reichswald=0.46±0.04, Santon Downham=0.278±0.026) (Hammond et al., 2001). Although the Reichswald population is certainly not representative of a fully FM phenotype, it does possess certain aspects that question the assumed P phenotype. The Q-Q aggression observed by Trettin et al. (2014) and the relatedness quirks mentioned above all share similarities with fully FM colonies from Spain (Gill and Hammond, 2011b, Gill et al., 2009). However, other important aspects of FM colonies, such as high worker relatedness, a single reproductive queen per colony and high W-Q aggression are absent. I would suggest that the Reichswald population is not a true representation of a fully P social phenotype nor is it fully FM. Instead, there seems to be variation within its social structure that makes it a poor representative for studies of the P social phenotype. With further study, the Reichswald population may reveal a 'missing link' between the P and FM social phenotypes, which may be plastic and not genetically fixed.

Finally, Trettin et al. (2014) collected colony structure and Q:W ratio data for P and FM colonies previously published and claimed that FM colonies naturally have a higher Q:W ratio. Unfortunately, they did not include other published colony census data, which clearly demonstrated that Q:W ratios between P and FM were not significantly different (see Table 5 and section 5.3.5). A more complete appraisal of available data clearly shows that Q:W ratio varies within and between P and FM populations. Furthermore, the median average Q:W ratios were not significantly different between P and FM populations. Therefore the assumption that FM colonies are characterised by high Q:W ratios is not supported, which in turn suggests that alternate social phenotypes in the majority of sampled *L. acervorum* populations are fixed and not plastic.

My observations do not support the idea that the P social phenotype in colonies sampled from Santon Downham is a flexible trait that can be altered by changing environmental constraints/Q:W ratio. Instead, the lack of aggression directed towards queens from both queens and workers in UK P colonies regardless of Q:W ratio is compatible with an inflexible genetic basis determining social organisation. Previous work comparing P and FM colonies of *L. acervorum* in a shared environment with no food restraints revealed the likelihood for a genetic basis for social organisation as social phenotype was not observed to change between colonies (P→FM or FM→P) (Gill and Hammond, 2011b, Gill et al., 2009). Furthermore, if social organisation is naturally plastic then mixed proportions of the two social organisations within populations are expected to reflect naturally occurring variation in colony Q:W ratios. However, this is not the case in either P populations in Europe or in FM populations in Spain and Japan, where there seems to be no within population variation in social phenotype (Trettin et al., 2011, Gill and Hammond, 2011b, Gill et al., 2009, Hammond et al., 2006, Bourke et al., 1997, Heinze et al., 1995).

Recently, a genetic basis for within species differences in social organisation has been demonstrated for two myrmicine species, *S. invicta* and *F. selysi* (Purcell et al., 2014, Wang et al., 2013). Both species possess large non-recombining chromosomal regions, termed social chromosomes, which link large numbers of genes together that might be functionally involved in determining the social phenotype (Purcell et al., 2014, Wang et al., 2013, Linksvayer et al., 2013, Libbrecht et al., 2013, Schwander et al., 2014). Both examples clearly demonstrate that genomic architecture can underpin divergent social phenotypes. Additionally, in Chapter 6, I detected a large contiguous chromosomal region (the social region), which was associated with difference in the social phenotypes in *L. acervorum* and possessed some similarities to the social chromosomes of *S. invicta* and *F. selysi*. Therefore, there is strong potential for genetic factors such as genomic architecture in determining the social phenotype in populations of *L. acervorum*.

In conclusion, I found no evidence to indicate that the social phenotype present in *L. acervorum* colonies from a UK population is flexible and can be manipulated by changing Q:W ratios. Specifically, I showed that P colonies from the UK show natural diversity in colony Q:W ratio and that this diversity does not correlate with colony social organisation. P colonies never engaged in Q-Q aggression and W-Q aggression contributed only a small minority of all behaviours observed regardless of Q:W ratio. Furthermore, each colony possessed an average of three reproductive queens regardless of natural Q:W ratio, indicating that skew was not affected by Q:W ratio manipulations. It is likely that P and FM social phenotypes are fixed in at least the UK and Spanish populations, likely through genetic factors, and that the Reichswald population represents an intriguing exception, which may possess a degree of social plasticity. Further study into the social phenotype of the Reichswald population is required to properly address this hypothesis.

Chapter 6

A large genomic region is associated with polymorphic social organisation in *Leptothorax acervorum*

6.1 Introduction

Eusociality is, barring the evolution of complex language in humans, the most recent major transitions in evolutionary history (Szathmary and Smith, 1995). Eusocial organisms show a wide variety of complex social phenotypes that have allowed them to be ecologically successful (Wilson and Holldobler, 2005b, Hölldobler and Wilson, 1990). Eusociality has evolved independently across taxa at least 24 times (Bourke, 2011a) and 12 times amongst insects (Wilson and Holldobler, 2005a). Furthermore, many complex social phenotypes involved in the elaboration of eusociality have also evolved independently multiple times. For example, fungus farming evolved independently in ants and termites (Mueller and Gerardo, 2002) and there have been multiple independent origins of slave-making behaviour in ants (Beibl et al., 2005). Therefore, understanding the genomic architecture underpinning variation in complex social phenotypes is a key focus in evolutionary biology (Bourke, 2011a, Bourke and Franks, 1995, Wang et al., 2008).

The independent evolution of eusociality raises the question as to the extent these complex social phenotypes share a common genetic foundation (Kapheim et al., 2015). Furthermore, this question also applies to the elaboration of social organisation since the evolution of eusociality that has led to the wide diversity of social phenotypes we see today

(Hölldobler and Wilson, 1990). By studying the underlying architecture – both behavioural and genetic - between different social phenotypes we can understand how variation in complex phenotypes has evolved.

Social organisation is a fundamental aspect of eusociality, and involves a number of complex traits including; group size and composition, the behavioural and genetic relationships between individuals and reproductive skew (Ross and Keller, 1995). However, considerable variation exists between the social organisations of eusocial species (Gadau et al., 2009), and in some cases social organisation differs within the same species (Ross and Keller, 1995, Gill et al., 2009, Soucy and Danforth, 2002). There is currently a wealth of theory available that explains the evolution of many important aspects of social organisation (Linksvayer and Wade, 2005, Marshall, 2011, Gardner et al., 2011, Bourke, 2011b, Nowak et al., 2010, Hamilton, 1964, Queller and Strassmann, 1998, Wilson and Holldobler, 2005a, Khila and Abouheif, 2010). However, the genetic basis underpinning variation within social traits is poorly understood (Fischman et al., 2011, Linksvayer and Wade, 2005, Smith et al., 2008, Ross and Keller, 1995).

Recent studies investigating complex social behaviours have revealed valuable insights into the diversity of genomic architecture underpinning various complex traits (Thompson and Jiggins, 2014, Schwander et al., 2014, Rueppell, 2014, Purcell et al., 2014, Wang et al., 2013). Two key genomic architectures facilitating the diversification of complex traits are supergenes (Thompson and Jiggins, 2014, Schwander et al., 2014) and pleiotropic gene regulatory networks (Rueppell, 2014). Both systems have been shown to play important roles in facilitating the diversification of complex behavioural phenotypes in social insects (Rueppell, 2014, Purcell et al., 2014, Wang et al., 2013) as well as other taxa (Gilmartin and Li, 2010, Joron et al., 2006, Jones et al., 2012, Thomas et al., 2008).

A good example of a pleiotropic gene network underlining genomic architecture is the pollen hoarding syndrome (Rueppell, 2014, Page et al., 2012). The pollen hoarding syndrome in the honeybee (*Apis meliffera*) is a highly complex social phenotype involving the behavioural, physiological and neurological integration of thousands of different larvae, foragers and nurses (Page et al., 2012, Rueppell, 2014). High and low pollen hoarding bee colonies differ in a range of traits at the individual and colony level, including variation in; foraging initiation, pollen load and preference, sucrose sensitivity and recruitment of foragers to pollen sources. Overall, high pollen hoarding bees are more responsive to pollen foraging stimuli, perform more pollen foraging behaviours and are more responsive to signalling cues (Page et al., 2012).

14 different QTLs have been associated with pollen hoarding behaviour and 12 have been mapped to the honeybee genome (Page et al., 2012, Graham et al., 2011, Hunt et al., 1995, Ruppell et al., 2004, Rueppell et al., 2006, Rueppell et al., 2004, Page et al., 2000, Wang et al., 2009, Rueppell et al., 2011). These QTLs range in size (1.8-16.5Mb) and are distributed throughout the genome across multiple chromosomes (CHR: 1, 2, 3, 4, 5, 6, 11 and 13). Investigations into the identified QTL's have revealed candidate genes involved in the insulin-insulin like (ILS) pathway (Hunt et al., 2007), metabolic regulation (Page et al., 2012, Rhea et al., 2010), foraging behaviour (Schulz and Robinson, 2001) and reproduction (Page et al., 2012, Graham et al., 2011, Sasaki and Harano, 2007, Sasaki and Nagao, 2002, Thompson et al., 2007, Whitfield et al., 2006). Furthermore, identified QTLs were found to be significantly associated with differences in worker ovary size and the hormonal dynamics of juvenile hormone and vitellogenin (Whitfield et al., 2006, Tsuruda et al., 2008, Amdam et al., 2007, Amdam and Page, 2010, Guidugli et al., 2005, Ihle et al., 2010). Taken together, variation in the pollen hoarding syndrome is underpinned by a network of pleotropic genetic elements distributed across multiple chromosomes (Page et al., 2012, Rueppell, 2014).

Amdam and Page (2010) argue that ancestral reproductive gene networks can act as a scaffold for diversifying worker associated division of labour (the reproductive ground plan hypothesis (RGPH)). Natural selection is expected to remove the functionality of reproductive gene networks in the worker caste to minimise reproductive conflict with the queens (Khila and Abouheif, 2008). However, selection may instead operate upon ancestral reproductive gene networks to co-opt them to perform an alternative function, such as division of labour in honeybees (Amdam and Page, 2010). Therefore, natural selection can co-opt a previously essential ancestral gene network and diversify it to perform a new but equally essential social function. The strength of selection and the pleotropic nature of the genes involved in these networks can be enough to promote their co-inheritance and overcome the constraints associated with gene covariance due to the likelihood of recombination breaking up favourable gene combinations (Agrawal and Stinchcombe, 2009). This is especially remarkable in the case of the PHS considering the exceptionally high level of recombination that occurs within the honeybee genome (Ross et al., 2015, Beye et al., 2006), suggesting that even in an environment of high recombination, gene covariance can evolve when selection is strong enough (although it is questionable whether the strength of selection that led to the PHS line is realistic in nature) .

An alternative genomic mechanism for promoting the diversification of social phenotypes is the supergene. The exact definition of a super gene was recently published as, “a genetic architecture involving multiple linked functional genetic elements that allow switching between discrete, complex phenotypes maintained in stable polymorphism” (Thompson and Jiggins, 2014). Supergenes are inherited as a single unit and can exercise tight control over complex phenotypes (Schwander et al., 2014) such as heterostyly (Gilmartin and Li, 2010), mimicry (Jones et al., 2012, Joron et al., 2006, Joron et al., 2011) and social organisation (Purcell et al., 2014, Wang et al., 2013). Recently, supergenes have been shown to underlie fundamental differences in the social organisation of two ant species, *Solenopsis*

invicta and *Formica selysi* which share a similar but independently evolved social organisation (Purcell et al., 2014, Wang et al., 2013). Populations of these two ant species can come in one of two social forms which differ in their tolerance for reproductive queens. Colonies can be monogynous where only one reproductive queen is tolerated or polygynous where queen tolerance is higher, allowing multiple reproductive queens. Each social organisation is further associated with a syndrome of additional co-varying biological traits. Colonies of *S. invicta* possessing alternate social phenotypes differ in their level of inter-colony aggression, colony founding, worker size, queen fecundity, mature queen odour and fat deposition (Lawson et al., 2012, Krieger and Ross, 2002, DeHeer et al., 1999, Keller and Ross, 1998, Keller and Ross, 1993, Keller and Ross, 1999, Keller and Ross, 1995). Similarly, colonies of *F. selysi* differ in queen size and queen dispersal, colony life span, colony size, the allocation to offspring and brood development time (Purcell et al., 2014, Rosset and Chapuisat, 2006, Rosset and Chapuisat, 2007, Schwander et al., 2005). These traits are always inherited together with their associated social phenotype.

Studies revealed large non-recombining regions in both ant species that spanned significant portions of a single chromosome, which were termed social chromosomes (Purcell et al., 2014, Wang et al., 2013). These social chromosomes possess similar characteristics to sex chromosomes (Wang et al., 2013, Bachtrog et al., 2011, Charlesworth et al., 2005) including, suppression of recombination at the locus containing the antagonistic allele and the recruitment of additional alleles that are beneficial to a particular sex (or social organisation in the case of the social chromosomes). In the case of *S. invicta*, the social chromosome was found to contain two chromosomal inversions totalling approximately 13Mbs (55% of the chromosome), which effectively nullify recombination between the two social genotypes (Wang et al., 2013). Furthermore, the largest non-recombining region (9Mb) contained genes that were differentially expressed between the two social forms. *F. selysi* also possesses a social chromosome with a large non-recombining region but the region is not homologous

with the *S. invicta* social chromosome. This strongly suggests that the supergene architecture is convergent, and that similar mechanisms led to the evolution of these supergenes, but the two supergenes do not share a common gene set. This is the key shared mechanism facilitating the evolution of gene suites responsible for transitions in social organisation in ants (Purcell et al., 2014).

The structure of supergenes provides great advantages over pleiotropic gene regulatory networks to building complex phenotypes (Schwander et al., 2014). Through tight linkage, alleles with complementary functions are inherited together without being broken up by recombination, which would lead to intermediate maladaptive phenotypes (Thompson and Jiggins, 2014). Furthermore, strong selection for the suppression of recombination can quickly promote the translocation of additional loci and expand the supergene further. Expansion in this way can eventually encompass the whole chromosome as it has with sex chromosomes (Bachtrog et al., 2011, Charlesworth et al., 2005).

The common ant *Leptothorax acervorum* also possesses variation in its social phenotype (Trettin et al., 2014, Friend and Bourke, 2012, Trettin et al., 2011, Gill and Hammond, 2011a, Gill and Hammond, 2011b, Gill et al., 2009, Hammond et al., 2006, Ito, 2005, Hammond et al., 2001, Felke and Buschinger, 1999, Bourke et al., 1997, Heinze et al., 1995b, Heinze et al., 1994, Heinze and Ortius, 1991, Ito, 1990). Populations collected from the UK, Germany, Alaska and Northern Spain display a polygynous social phenotype (P), where multiple reproductive queens are tolerated and reproduce equally (Friend and Bourke, 2012, Hammond et al., 2006, Bourke et al., 1997, Heinze et al., 1995b, Heinze et al., 1994, Bourke, 1994, Heinze and Ortius, 1991). Alternatively, two populations from Central Spain and two populations from Japan possess an alternative social phenotype known as functionally monogyny (FM) (Trettin et al., 2011, Gill and Hammond, 2011a, Gill and Hammond, 2011b, Gill et al., 2009, Felke and Buschinger, 1999, Ito, 1990, Gill, 2010). Within FM colonies, multiple

reproductive queens are maintained but only a single queen matures to full reproductive status. The supernumerary queens are seemingly prevented from reproducing mechanistically via high rates of queen and worker aggression (Trettin et al., 2011, Gill and Hammond, 2011a, Gill and Hammond, 2011b). In addition to differences in the rates of aggression received by mated queens, colonies possessing alternate social phenotypes also differ in their reproduction and mode of colony founding (Felke and Buschinger, 1999, Franks et al., 1991). P queens have been observed to mate in a swarm and on the ground, where they call males using pheromones (Franks et al., 1991). Following mating, P queens can then found a new colony or return to the natal nest (Felke and Buschinger, 1999, Heinze et al., 1995b, Bourke, 1994). In comparison, FM queens are likely to found new colonies via budding (Felke and Buschinger, 1999).

Current evidence suggests that a heritable genomic component underpins variation in the social phenotype of *L. acervorum* colonies (Gill et al., 2009). First, colonies sampled within the same population always display a common social phenotype despite variation in local environment and colony organisation (Gill and Hammond, 2011b, Gill et al., 2009, Hammond et al., 2006). Second, colonies sampled from both FM and P social phenotypes do not switch from one to the other in response to environmental changes (Gill and Hammond, 2011a, Gill and Hammond, 2011b, Gill et al., 2009, Hammond et al., 2006). If colonies from both FM and P populations are kept in a common controlled environment, they maintain their original social phenotype in all recorded cases. These lines of evidence suggest a genetic component to explaining differences in social phenotype rather than social plasticity. However, there are key differences between the *L. acervorum* social phenotype compared to the social polymorphisms in *F. selysi* and *S. invicta*. *F. selysi* and *S. invicta* colonies contain either multiple mated queens (polygynous) or a single mated queen (monogynous) (Keller and Ross, 1999, Chapuisat et al., 2004), whereas *L. acervorum* colonies commonly contain multiple mated queens regardless of the social phenotype, but vary in within colony skew (Gill et al.,

2009, Hammond et al., 2006, Bourke et al., 1997). However, there is some evidence that the social phenotype is plastic in a population from Reichswald Germany as queens were shown to engage in aggressive dominance behaviour when colony structure was manipulated to reflect ecological constraints and colony relatedness is generally higher than expected for a P social phenotype (Trettin et al., 2014, Heinze et al., 1995b). This could indicate that different populations may vary in their degrees of plasticity in social phenotype from inherited inflexibility to fully plastic. Either way, *L. acervorum* represents another excellent model for investigating the impacts of genomic architecture in creating variation in complex social phenotypes.

I set out to achieve three aims. First, to establish whether divergent SNP markers exist between populations with alternate social phenotypes. The discovery of SNP markers associated with different social phenotypes (social markers) would support the hypothesis that variation in social phenotype between the sampled populations is underpinned by genetics. Second, it was important to determine whether social markers are clustered together in tight linkage or distributed throughout the genome. Markers clustering in one or a few locations would indicate the formation of a supergene similar to *S. invicta* and *F. selysi*, whereas distribution throughout the genome would suggest a pleotropic gene network similar to the pollen hoarding syndrome. Finally, to determine a list of candidate genes closely associated with social markers, which might provide insights into role of genes within the social phenotype.

I investigated these three aims via a genome wide associated (GWAS) study using restriction associated DNA sequencing (RADseq) (Etter et al., 2011, Baird et al., 2008). I sampled five populations of *L. acervorum* representing each social phenotype (2x FM and 3x P) and differentiated social SNP markers from neutral population structure using an extreme F_{ST} outlier method (Westram et al., 2014, Renaut et al., 2012).

6.2 Methods

6.2.1 Experimental rationale

My main aim was to investigate the role of genomic architecture underpinning variation in social organisation by associating SNP markers with two different social phenotypes present in populations of *L. acervorum*. The fundamental rationale was that genetic markers underlying differences between the social phenotypes would be highly differentiated as measured by F_{ST} (a measure of population differentiation) (Lewontin and Krakauer, 1973). Because social phenotypes are restricted to particular populations it is essential that underlying genetic differences between populations – population genetic structure – is taken in to consideration. To do this, it was essential that >1 population representing each social phenotype was sampled so that markers associated with social phenotype could be differentiated from neutral population structure (see Fig 1 Westram et al., 2014). Furthermore, the current understanding of *L. acervorum* population structure indicates that genetic variation is high within populations but there is little genetic structure among populations (Foitzik et al., 2009, Heinze et al., 1995a), making it a good model to detect SNPs that do show high divergence between the different social phenotypes.

To differentiate markers associated with social phenotype (social markers) from neutral population structure, I combined the calculation of pairwise F_{ST} between multiple populations with a conservative F_{ST} outlier threshold (Westram et al., 2014, Renaut et al., 2012, Aguilar, 2006). I defined social markers as those SNPs with high threshold F_{ST} between all populations with alternative social phenotypes and low threshold F_{ST} between populations sharing the same social phenotype (see Fig 1). Determining the low and high thresholds is somewhat arbitrary (Westram et al., 2014, Renaut et al., 2012) but my aim was to obtain conservative qualitative results. The high value was set at the 95% quantile (so the top 5% highest F_{ST} values), in line with other studies (Westram et al., 2014, Renaut et al., 2012). The

low value should reflect F_{ST} values caused solely by genetic drift and gene flow, however, determining the point at which an F_{ST} becomes an outlier, and so potentially subject to selection, is notoriously difficult (Stinchcombe and Hoekstra, 2008, Storz, 2005, Via, 2012). Therefore I decided to use a conservative cut-off of the 60% quantile, below which we considered markers to be neutral to selection. If the above method is effective in discovering social markers, tests for low F_{ST} values between social phenotypes should yield fewer, if any, markers. This hypothesis was tested using the same filtering criteria.

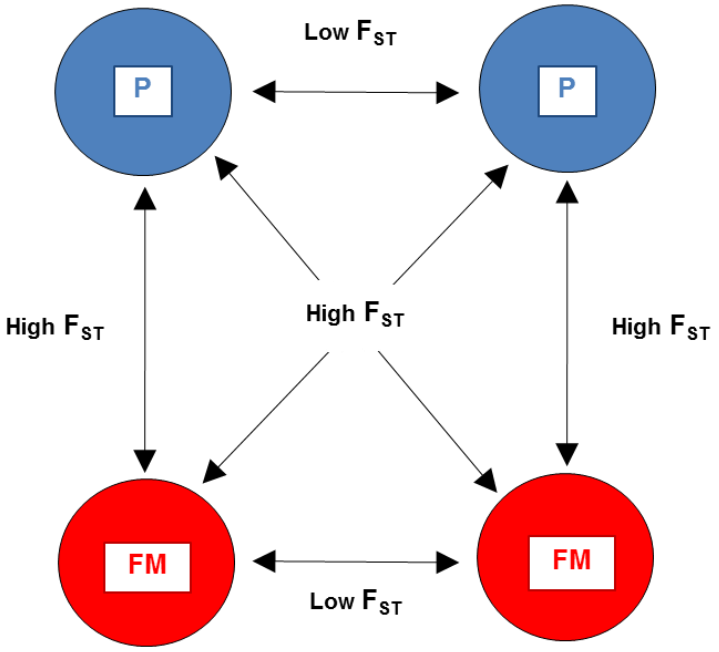


Fig 1: Approach for detecting 'social markers' (F_{ST} outliers associated with social phenotype). P= population with polygynous social phenotype. FM= population with functional monogyny social phenotype. Arrows represent pairwise F_{ST} comparisons. High F_{ST} was set at ≥95% quantile and low F_{ST} was set at ≤60% quantile. See 2.6 for details.

6.2.2 Ant collection and population sampling

L. acervorum colonies were collected from three Spanish populations, Orihuela del Tremedal, Sierra de Albarracin (OT), Valdelinares, Sierra de Gudar (V) and the Refugi Pla de la Font, Sierra del Pago near Espot (PF) in June 2013 (see Fig 2). All Spanish population locations

were isolated from one another on the top of mountains with altitudes ranging from of 1,500-2,000m above sea level. Colonies were also collected from two UK populations, Dawkins Bottom in the New Forest (NF) and Santon Downham in Thetford Forest (SD) in April and July 2013 respectively (see Fig 2 for population location details). OT and V populations are known to possess the FM phenotype (Trettin et al., 2011, Gill and Hammond, 2011b, Gill et al., 2009) and PF (see 6.3.1), NF (Chan et al., 1999) and SD (Bourke et al., 1997, Hammond et al., 2006, Hammond et al., 2001) are known to possess the P phenotype.

Colonies were collected whole inside decaying twigs and were removed from their twigs 2-13 days after collection and given an artificial nest (see Chapter 2 for figure and details of nest box construction). Once in artificial nests, each colony was censused for number of workers, queens and brood. Each colony was provided with damp cotton wool and a diet of honey solution and chopped-up meal worm twice a week and kept in spring conditions (hourly rhythm=11-1-11-1, Temp./°C=10-15-20-15, Photoperiod (Night-Day-Day-Day)= 0-2-3-2, humidity/%=70-70-80-70).

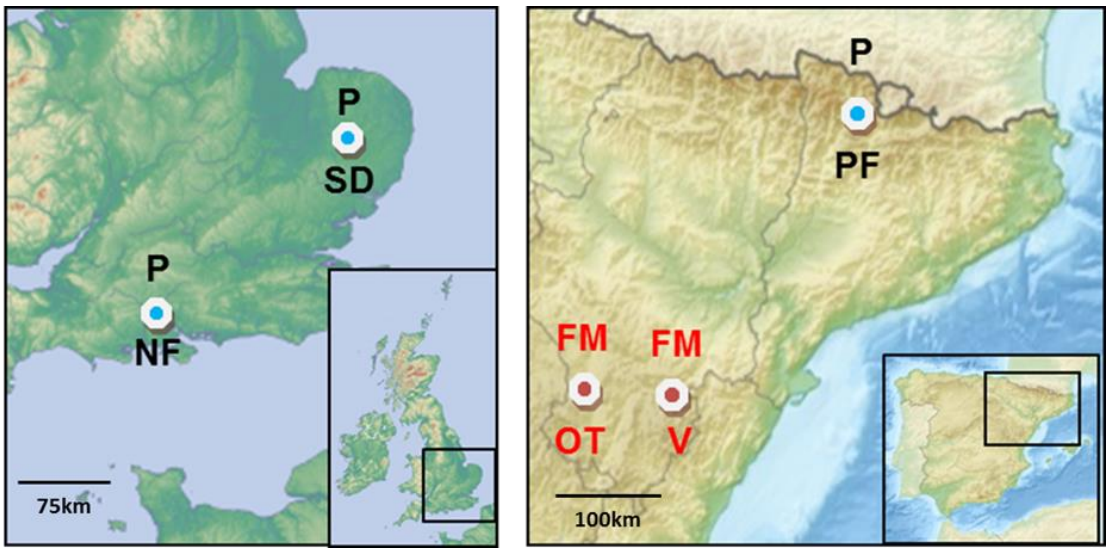


Fig 2: Geographical locations of populations sampled. UK: the New Forest (NF) and Santon Downham (SD). Spain: Orihuela de Tremendal (OT), Valdelinares (V) and Refugi Pla de la Font (PF). The social phenotype of each population is also included. P= polygyny and FM= functional monogyny.

6.2.3 Confirming the social phenotype of the PF population using siblingship analysis

To confirm the P social phenotype of the PF population, eight workers and eight larvae were sampled from nine multiply queened PF colonies (dealate queen average=4.6, range=2-16) collected in June 2011 in order to conduct siblingship analysis. Each individual was genotyped at four polymorphic microsatellite loci using markers and protocols previously established in Gill et al., 2009. Allele sizes were calculated based upon a liz 500 reference using an Applied Biosystems 3130xl Genetic Analyzer and genotyping was performed using Geneious 8 software. Only workers and larvae that were successfully typed at ≥3 loci were used in further analysis.

Siblingship analysis was conducted using COLONY 2.0.5.8 (Jones and Wang, 2010), which grouped the individuals from each colony into fullsibling families. Queens were assumed to be singly mated (Hammond et al., 2001) and the level of allelic dropout and genotyping errors were set to 0.005 for each microsatellite (Gill et al., 2009).

Colony relatedness estimates for both the worker and larvae population sample groups were calculated using the kinship pairwise relatedness estimator model available in KINGROUP v2 software (Goodnight and Queller, 1999, Queller and Goodnight, 1989).

6.2.4 DNA extraction and population pooling

Genomic DNA was sequenced from pools containing multiple individuals (n=30) from the five sampled populations (Fig 2). Using a pooled sequencing approach, we were able to include a large number of individuals for a more accurate representation of allele frequencies within the population at the expense of individual genomic information (Ferretti et al., 2013, Gautier et al., 2013). It also allowed us to sequence many individuals for a lower cost (Zhu et al., 2012, Futschik and Schlotterer, 2010, Gautier et al., 2013). A single ant worker was

randomly selected from 30 separate colonies per population for DNA extraction. Using 30 individuals from 30 different colonies provided a good estimate of variation in allele frequencies within each population pool, which was crucial for marker discovery (Gautier et al., 2013). DNA samples were extracted from the whole bodies of individual ant workers by homogenising using a sterile plastic pestle and extracting genomic DNA with a Qiagen Gentra Puregene kit (Qiagen, Hildern, Germany) using a mouse tail protocol. Resulting DNA samples were quantified using a NanoDrop spectrophotometer (LabTech International) and DNA integrity assessed using gel electrophoresis. Only samples with a single, clear band of high molecular weight DNA were selected for pooling.

Finally, each individual contributed 500ng of genomic DNA to its population pool. Individual ant samples varied in their DNA concentrations (Mean ng/µl (Range ng/µl): OT=33.5 (9.6-84.52), V=48.97 (7.13-123.93), PF=52.1 (27.4-124.0), NF=39.1 (13.54-100.0), SD=36.8 (6.1-99.1), so it was important to carefully calculate and pipette a volume containing exactly 500ng from each individual sample. It was crucial that each individual contributed equal quantities of DNA to the pool as unequal contributions can cause a biased sequencing effort and an unfair representation of the sampled population and it is not possible to determine if each individual within the pool was sequenced equally (Zhu et al., 2012, Gautier et al., 2013). Furthermore, 30 pooled individuals should be sufficient to capture the majority of the markers within the population and mitigate any bias in sequencing effort caused by accidental deviations in individual DNA contributions (Zhu et al., 2012, Gautier et al., 2013).

Once each population pool was complete, DNA quality was checked via gel electrophoresis and following the appearance of a clear band for each pool, they were then sent for library construction and RAD sequencing to the Edinburgh Genomics facility and the University of Edinburgh.(Ferretti et al., 2013, Gautier et al., 2013, Zhu et al., 2012, Futschik and Schlotterer, 2010)

6.2.5 Library construction and paired-end RAD sequencing

To generate a large number of RADtags for marker discovery, the frequently cutting EcoR1 restriction enzyme was chosen. There is currently no information available about the genome of *L. acervorum*, however a recent summary was published detailing the features of the seven ant genomes that are available (Gadau et al., 2012). Using this study as a guide, we roughly estimated the genome of *L. acervorum* to have 35% GC content and a genome size of 300Mb. Using the RADcounter V4 tool (https://www.wiki.ed.ac.uk/display/RADSequencing/Home), EcoR1 was predicted to produce 102,502 cut sites and 205,003 RADtags (see Table 1), providing an excellent basis for SNP marker discovery.

RAD library preparation, paired-end (PE) RAD sequencing and bioinformatic analyses were carried out by the NERC/NBAF Facility at Edinburgh Genomics, the University of Edinburgh, funded by the NERC/NBAF grant NBAF627, following the methodologies in Baird et al. 2008 and Etter et al. 2011. Three libraries for each population pool were created to give three technical replicates. Briefly, genomic DNA from each population pool was digested using the 6-base cutting enzyme EcoR1 and a specialised P1 adapter containing the forward primer site, the Illumina sequencing primer site and a unique 8 base-pair barcode sequence was ligated to the digested fragments. All P1 adapter ligated fragments from each population pool were then combined into a single sample and randomly sheared by sonication. P2 adapters, containing a divergent 'Y' sequence and an Illumina sequencing primer site were ligated to the fragments followed by PCR enrichment of all fragments containing both a P1 and a P2 adapter. Paired-end sequencing of 100 bp lengths was then performed on a Illumina HiSeq 2000 sequencer.

Table 1: Predicted numbers of cut sites and RADtags expected when digesting genomic DNA from *L. acervorum* using the restriction enzyme EcoR1. GC content was estimated at 35% and total genome size was estimated at 300Mb based on similar values in sequenced myrmicine species. The * indicates the cut location. Output taken from RADcounter V4.

Cut site	G*AATTC
Site frequency	0.000342
Sites/Mb	342
Number of sites in genome	102,502
Number of tags	205,003

6.2.6 Quality control and de novo consensus RADtag assembly

Raw reads were initially reviewed using fastQC to check for overall quality. Raw reads were then de-multiplexed using the *process_radtags* program from the STACKS package (Catchen et al., 2013). The sequencing effort for the NF population was unsuccessful in generating a high number of unique sequence reads and NF libraries contained ~10% of the total reads of all other populations (see Table 2). The reasons for the poor sequencing of the NF population pool are unknown, however as the NF libraries were of insufficient quality they were not considered in any further analysis.

The *process_radtags* program was used to check each read for the integrity of the barcode and the cut-site sequences and to make minor corrections to both where incomplete. To do this, *process_radtags* assessed the quality of the read within a sliding window (size=15% of total read length) and discarded reads where the quality within the window fell below a phred 10 score. *Process_radtags* was also set to correct erroneous barcodes and restriction enzyme cut sites where ≤2 bps were different from the list of recognised barcodes/cut site recognition sequence. The barcodes on average were 6bp different (range 4-8bp) from one another (see Appendix 1 Table 5). Whole reads missing a recognised barcode or cut site were removed.

PCR duplicates have been shown to bias the average GC content of population reads (Benjamini and Speed, 2012) and so these duplicates were removed using the *clone_filter*

program also available in STACKS. Briefly, the *clone_filter* program took paired-end data and reduced all exactly matching pairs of reads to just one. Non-PCR duplicates are highly unlikely to be identical at read2 due to random sheering.

Finally, all reads were quality end-trimmed using the FastX tool kit (Hannon, 2014) to remove bases that fell below a quality phred score of 20, while retaining only reads that remained at least 20bps long. This effectively trimmed the tail ends of reads, which are typically of low quality (see Appendix 2 Fig 1).

A *de novo* consensus assembly was constructed from RADtags using both the STACKS and RADmapper pipelines (Catchen et al., 2013, Catchen et al., 2011, Cezard, 2014). The STACKS package can only handle read1 sequences and so the initial *de novo* consensus was constructed using STACKS. First, the *ustacks* program was used to collapse the read1 sequences for each population into unique stacks which were then compared to form a set of loci and a list of SNPs. The default settings for *ustacks* were used allowing a unique stack to be created with a minimum coverage of 2 reads (stack depth) and a maximum nucleotide distance between stacks of 2 variable nucleotides (using a k-mer search algorithm of distance 2). Following the establishment of unique population stacks, a catalogue was created using *cstacks* to create a set of consensus loci where alleles (unique stacks) were merged at loci across all populations. Once again, the default settings were used for the *cstacks* program, which took the OT population as the initialiser and then merged in V, PF and SD populations in turn. Stacks were merged into a single consensus loci using the same k-mer algorithm found in *ustacks*. This merged stacks that differed by a maximum of 2 nucleotides together at each locus.

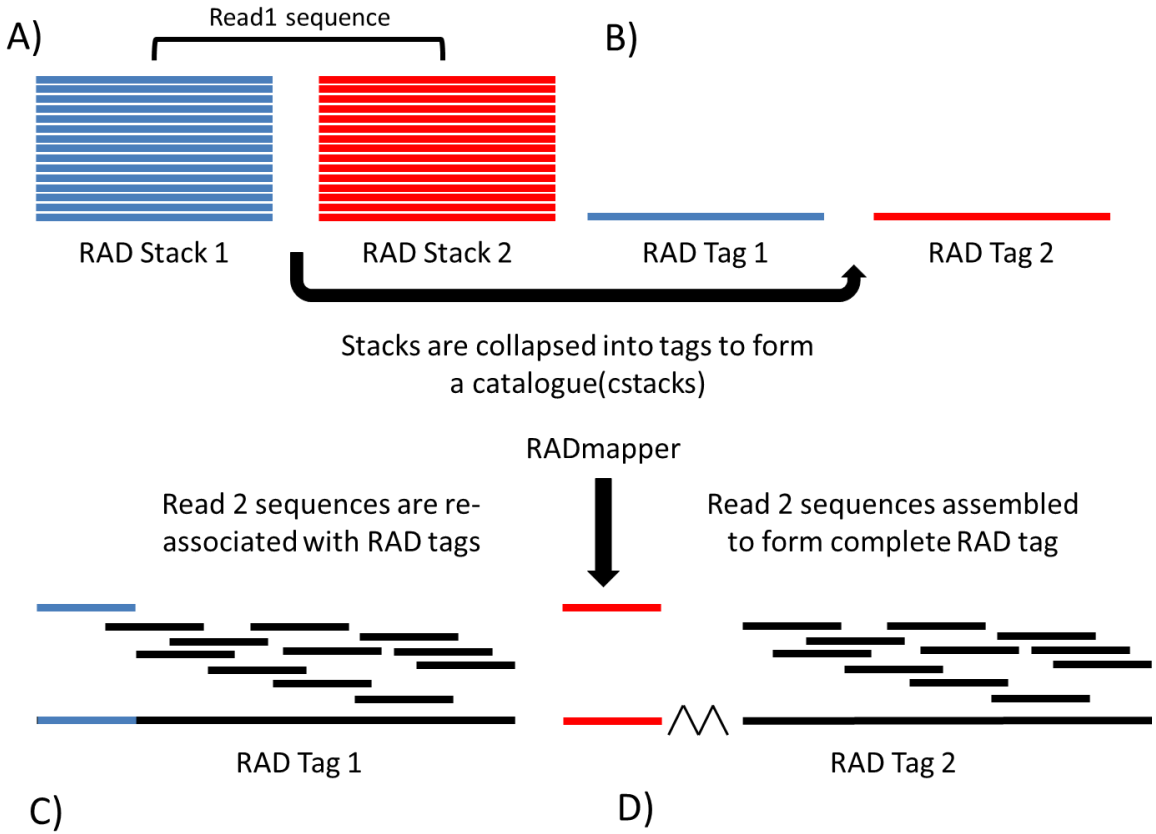


Fig 3: Consensus RAD tag construction using paired-end sequences. STACKS and RADmapper programs were used to create paired-end RADtag consensus loci. **A)** Initially, STACKS was used to assemble read 1 sequences into stacks, **B)** which were then collapsed to form a catalogue of RADtags. **C)** RADmapper then gathered read 2 sequences associated with each stack and assembled them into complete RAD tags before entering them into a catalogue. **D)** Where read 1 and read 2 sequences did not assemble together, the RAD tag was recorded as two consensus sequences.

The RADmapper package (Cezard, 2014) was then used to match the read2 sequences up with their corresponding read1 consensus loci (constructed in *cstacks*) and assemble them into complete consensus contigs. Due to random shearing and size selection, the read2 sequence assemblies did not always overlap with the read1 consensus. Where sufficient overlap was available between the read1 and read2 assemblies, they were combined to form one complete RAD tag.

Alternatively, non-overlapping read1 and read2 sequences were kept as separate consensuses forming a non-complete RADtag. All alignments contained with the RADmapper scripts were performed using BWA (Li and Durbin, 2009) on default settings in conjunction with SAMtools (Li et al., 2009). Execution of the RADmapper pipeline resulted in the assembly of read 1 and read 2 into consensus RADtags and SNP calling. See Fig 3 for a detailed schematic of paired-end RAD tag construction.

6.2.7 SNP calling, pooled F_{ST} calculations and outlier detection using Popoolation 2

6.2.7.1 Popoolation 2: SNP calling and FST calculation

Read1 sequences from each population pool were mapped to the *de novo* genome using BWA aligner with a seed length >100, allowing the complete read to be mapped. Only 1 gap was permitted and a mismatch probability score of 0.001 was specified (*bwa aln*: -o 1, -n 0.01, -l 200, -d 12, -e 12). SAMtools was then used to create the corresponding population SAM, BAM and mpileup files required as an input for the Popoolation2 pipeline.

Read1 sequences were used to maintain a consistently high coverage, to enable genuine SNPs to be distinguished confidently from sequencing error. High coverage is achieved with the read1 sequences because they form 'stacks' that are anchored by the EcoR1

cut site(Catchen et al., 2013) . Read2 sequences have a lower and less consistent coverage due to the random shearing step and so were not used in my analysis (Davey et al., 2013).

High quality SNPs were called using Popoolation2 (Kofler et al., 2011) with a minor allele threshold of 20, a minimum per population coverage of 20 and a maximum per population coverage of 200 to remove sequencing errors and uninformative SNPs. Popoolation2 also calculated pairwise comparisons of F_{ST} at each SNP between each of the four populations using the classical approach (Hartl and Clark, 2007). F_{ST} was calculated at each individual SNP by restricting the sliding window to a single base position and supressing all non-informative bases. Finally, all RAD tags containing ≥2 SNPs were removed to minimise the possibility of false SNP calling due to sequencing error (Etter et al., 2011). It was critical to establish a conservative and robust final list of SNPs so that the outlier analysis would not be compromised.

6.2.7.2 Outlier analysis

Population F_{ST} values for all SNPs were screened for extreme outliers associated with social phenotype (see Fig 1 and 2.1). Briefly, all SNPs were screened for 'low' F_{ST} (≤60% quantile) in the FM vs FM and P vs P population comparisons and 'high' F_{ST} (≥95% quantile) in all FM vs P population comparisons. (Westram et al., 2014, Renaut et al., 2012). Filtering for outlier F_{ST} was carried out in R using a custom script (Appendix 2, R script: social marker filtering).

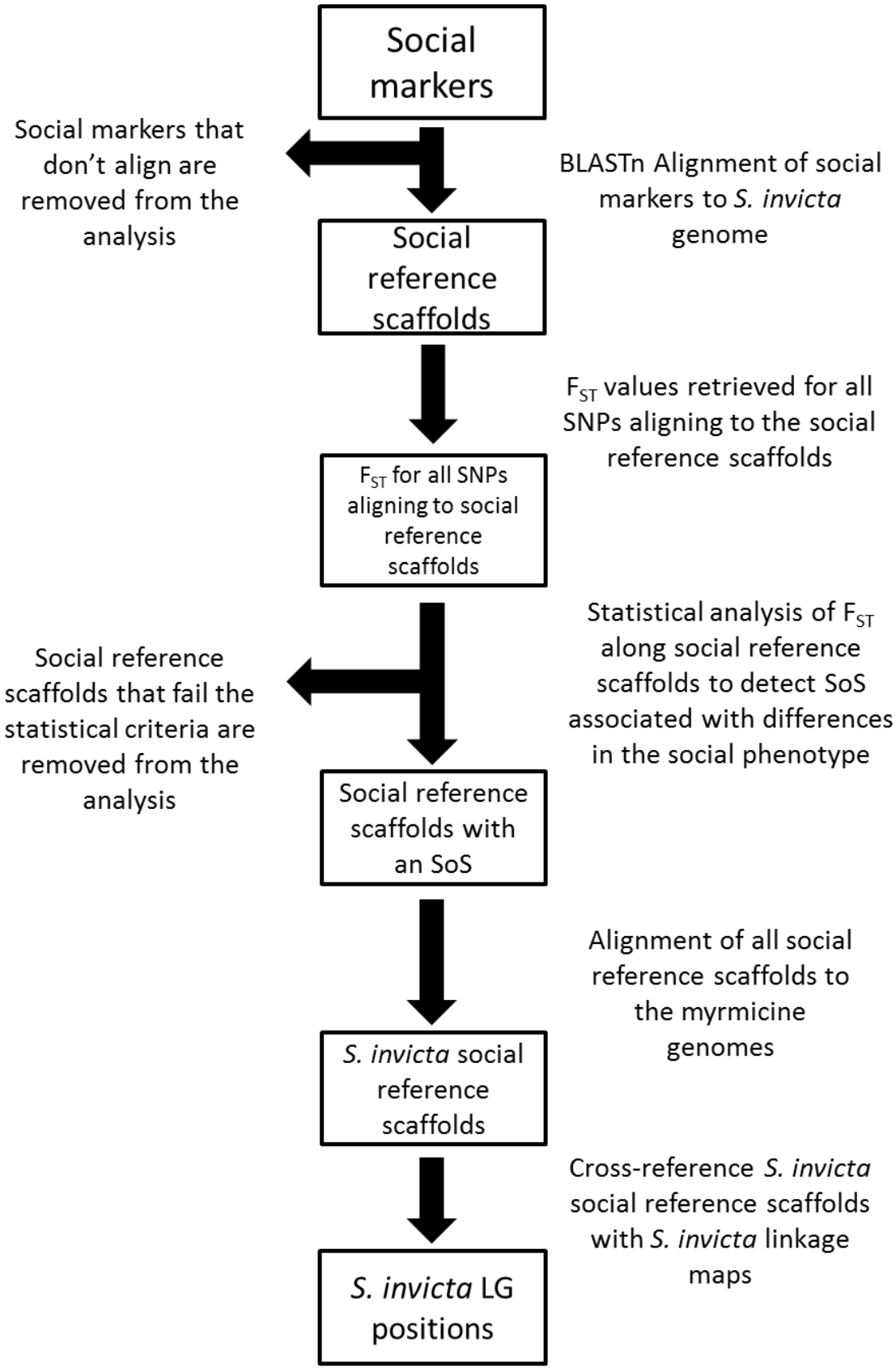


Fig 4: Flow diagram for social marker analysis. Social reference scaffolds refer to ant genome scaffold sequences that contained ≥1 *L. acervorum* aligned social markers.

6.2.8 Characterising the distribution of social markers throughout the genome of *L. acervorum*

6.2.8.1 Detecting signatures of selection (SoS) associated with social markers

Natural selection can change the amount of population differentiation between different populations of a single species (Nielsen, 2005, Akey et al., 2002, Oleksyk et al., 2010). A hypothetical example in this study would be if a given locus is under positive selection in a FM population but not in a P population then a comparison between the two is expected to have a high degree of population differentiation, as measured by F_{ST}. Furthermore, neutral linked loci are also expected to hitchhike along with the locus experiencing selection, therefore a proportion of the region is expected to display elevated F_{ST} (Oleksyk et al., 2010, Akey et al., 2002). These identified regions undergoing positive selection are considered to display a signature of selection (SoS) and it was important to search for SoS associated with difference in the social phenotype.

First, *L. acervorum* social marker sequences were aligned via BLAST to the four available myrmicine reference genomes (local BLASTn: returned a single hit for each SNP marker with the lowest e-value and 70% match to query identity. Minimum e-value threshold= $\leq 1.0^{-8}$). Reference scaffolds that contained ≥1 BLAST aligned social SNP markers were defined as social reference scaffolds. The social reference scaffolds detected were considered to be the most likely regions of the genome to be experiencing positive selection due to the fact that they contained at least one highly divergent social SNP marker.

Identifying regions under positive selection was achieved statistically by comparing the average F_{ST} of SNP markers along a social reference scaffold to the genome-wide average (Akey et al., 2002). First, all SNP markers were aligned to each of the four myrmicine ant genomes and filtered for a confident match to a scaffold ID (the social reference scaffold, see Fig 4, local BLASTn: returned a single hit for each SNP marker with the lowest e-value and 70%

match to query identity. Minimum e-value threshold= ≤ 1.0^{-8}). Only social reference scaffolds with ≥10 SNP marker hits were retained. Third, median F_{ST} for all SNPs present on a social reference scaffold was tested statistically to detect a SoS (see Fig 4). An SoS was considered to be associated with the social phenotype when the median pairwise F_{ST} for all within social phenotype comparisons (P vs P and FM vs FM) was not significantly different from the genome-wide median and the median pairwise F_{ST} for all between social phenotype comparisons (P vs FM) was significantly higher than the genome-wide median. Each pair-wise test was carried out using a one-sample Wilcoxon signed-rank tests with the test median being the genome-wide median F_{ST}. Because of multiple tests, the P-values were corrected using false discovery rate analysis (FDR) as described in Benjamini and Hochberg (1995) using a freely available excel spreadsheet (accessed from: https://dianakornbrot.wordpress.com/false-discovery-benjamini-hochberg/). If a social marker was associated with an SoS on different social references scaffolds in at least two genomes then it was considered to be a SoS associated with a difference in social phenotype.

To visualise SoS, F_{ST} for all SNP markers within the social reference were plotted by base position along the scaffold to create Manhattan plots. The Manhattan plots allowed a qualitative means of detecting regions of generally elevated F_{ST} (SoS) by revealing SNP markers with concordantly elevated F_{ST}, but that were not identified as social markers by the conservative criteria used to identify social markers (see 6.2.7.2).

Due to the pooled nature of the population samples, constructing a linkage map for *L. acervorum* was not possible. Therefore, the available linkage maps for *S. invicta* were considered appropriate substitutes for three reasons. First, *S. invicta* is the most closely related ant species to *L. acervorum* with a sequenced genome (Moreau, 2006, see Fig 5). Second, myrmicine genomes exhibit an average 74% synteny (Simola et al., 2013), suggesting that the gene orders between *S. invicta* and *L. acervorum* should be broadly comparable.

Third, the karyotype number for *S. invicta* (2n=16) is similar to *L. acervorum* (2n=13) (Hauschteckjungen and Jungen, 1983), which indicates that chromosomal arrangements should be broadly comparable.

All scaffolds containing an SoS were mapped to the *S. invicta* reference genome via local BLASTn (see Fig 4, local BLASTn: returned a single hit for each SNP marker with the lowest e-value and 70% match to query identity. Minimum e-value threshold= ≤ 1.0^{-8}). BLASTn alignments produced a list of *S. invicta* social reference scaffolds associated with an SoS, which could then be cross-referenced with the linkage map to determine the chromosomal positions (see Fig 4).

6.2.9 GO term retrieval using blast2go

Genes found within social reference scaffolds that showed a SoS were used in GO analysis using the program Blast2GO V3.0 (Conesa et al., 2005). The *S.invicta* gene list was used wherever possible to maximise to maximise gene comparability with *L.acervorum* due to recent heritage (Fig 4). Gene lists were initially aligned using BLASTx against the nr database with the following parameters; e-value threshold= ≤1.0^{-5}, number of hits returned=10. GO terms were retrieved for each gene with the interpro scan, mapping and annotation features with default settings. All GO terms were submitted to REVIGO for general GO category summarisation and interpretation (Supek et al., 2011). Treemaps of the GO term summaries were created in REVIGO and sized in proportion to the 'uniqueness' score calculated for each term, allowing the rarer terms to be visualised.

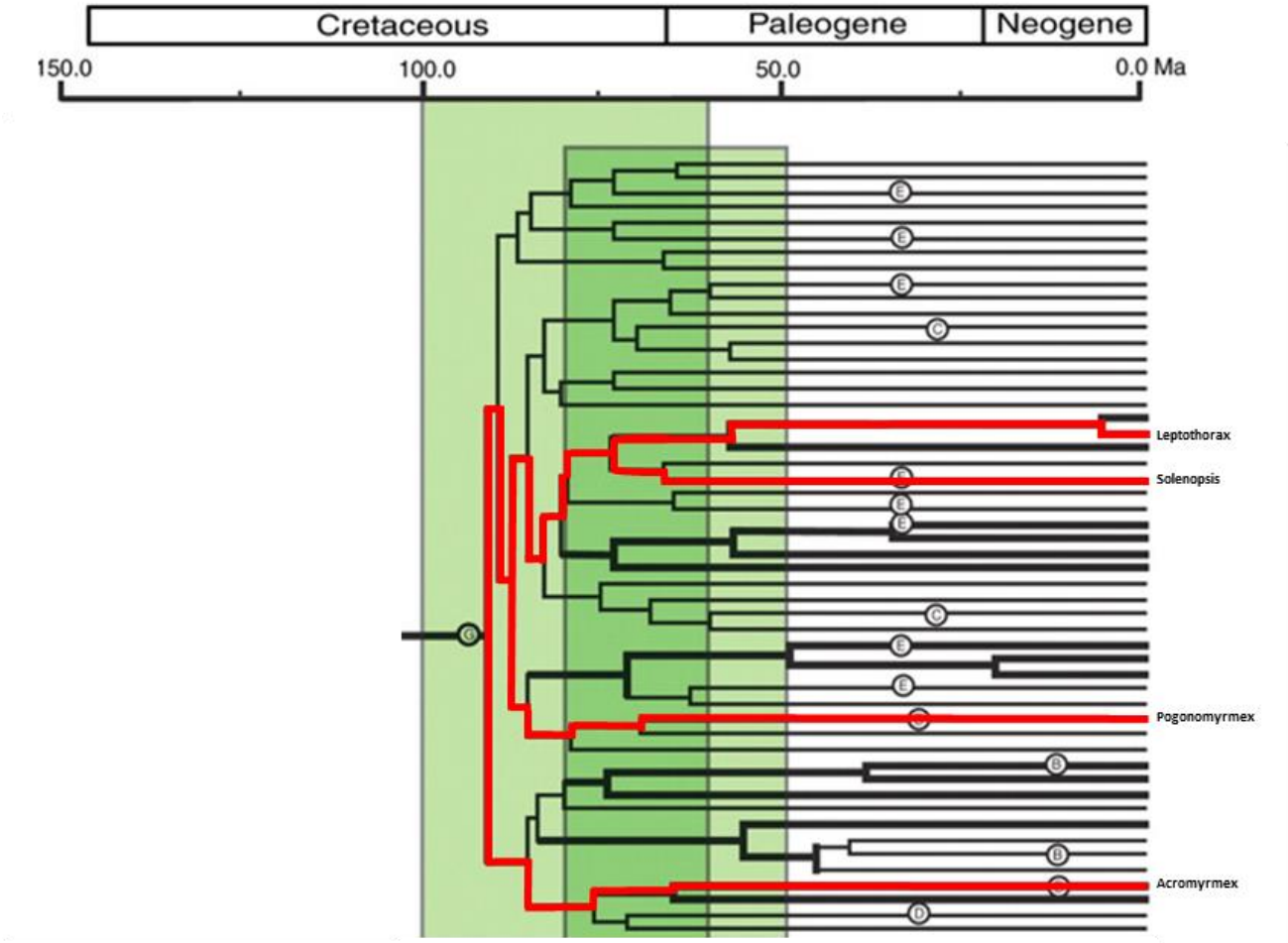


Fig 5: Phylogeny of myrmicine ant species. Red lines show the phylogenetic relationships between *Leptothorax, Solenopsis, Pogonomyrmex* and *Atta cephalotes*. The phylogeny confirms that *Solenopsis invicta* is the closest sequences relative to *Leptothorax acervorum*. However, all four species diverged from a common ancestor ~75mya, suggesting broad comparability. Figure has been modified from Moreau, 2006.

6.3 Results

6.3.1 Siblingship analysis of the PF population

In all nine colonies, workers belonged to ≥2 fullsibling families and in eight of the colonies larvae belonged to ≥2 fullsibling families (workers: mean average=5.4, range=4-7, larvae: mean average=4.0, range=1-6). All worker samples were successfully genotyped at ≥3 loci except for colony PF.13.03.w4, which was omitted from the analysis. Four samples of larvae from three colonies failed to be genotyped at ≥3 loci, which were similarly omitted from further analysis (see Appendix 1 Tables 1-4 for genotyping, siblingship and relatedness data).

Within colony estimates of larval and worker relatedness were low (workers: mean colony relatedness: r=0.18, SD=0.27, larvae: mean colony relatedness: r=0.19, SD=0.30). Furthermore, average colony relatedness was significantly different from 0.75 in both the worker and larvae population samples (Worker one sample Student's t-test: mean=0.18, SD=0.27, d.f=8 t=-10.84, *P*=<0.000. Larvae one sample Student's t-test: mean=0.19, SD=0.30, d.f=8, t=-12.99, *P*=<0.000).

Estimates of worker and larvae relatedness were combined into one data set and compared against the mean relatedness previously reported for 'old workers' from SD (mean relatedness=0.278, see Hammond et al., 2001) using a one sample Student's t-test. Mean PF worker/larvae relatedness was significantly lower than the mean relatedness previously reported (one sample Student's t-test: mean=0.18, SD=0.27, d.f=8 t=-2.78, *P*=0.013).

These results clearly demonstrate that multiple fullsibling families are present within all of the PF colonies and that group relatedness is significantly lower than expected, which

strongly indicates a polygynous social phenotype (Hammond et al., 2006, Bourke et al., 1997, Gill and Hammond, 2011b, Gill et al., 2009).

6.3.2 Raw reads, demultiplexing and QC

Analysis with STACKS recovered 119,604,767 unique read pairs distributed over five populations with three libraries each. Read quality (average Phred score per base) was good for all libraries (see Appendix 2 Fig 1 for examples of population library read quality) and reads contained an average GC% of 39-40.

Following initial visualisation of all population libraries in FastQC, the library containing the highest number of unique read pairs for each population was selected for low quality base trimming (see Table 2). On average, 88.9% (range=85.1-92.2) of the reads were retained for each population and read quality was greatly improved (see Table 2 and Appendix 2 Fig 1 for population read quality).

Table 2: Total read 1 sequence numbers for each population library following QC. The library with the highest total read 1 sequences for each population was selected for quality trimming. Programs used for raw read QC are listed. Selected libraries have been highlighted in **red**.

		Total Read 1 Sequences				
Pragram (Package)		OT	V	PF	SD	NF
process_radtags and clone_filter (STACKS)	Library 1	9676910	**10023781**	9156351	**12540262**	837184
	Library 2	11439315	8279344	9729443	2420313	946045
	Library 3	**11678965**	9614929	**10221924**	11730428	**1309573**
fastx_trimmer (FastX toolkit)		9933502	8551349	8900998	10818424	1198663

6.3.3 *De novo* consensus assembly and coverage analysis

Assembly of read pair sequences resulted in 146,679 unique RAD tags, which was ~ 50,000 less than expected based upon the predictions of RADcounter (see Table 1, methods 2.4). 97.06% of RAD tags (*N*=142,364) were present in each population with ≥5x coverage. Mean (±SE) coverage per RAD tag for each population was: OT=144.6x (0.50), V=122.7x (0.45),

PF=128.8x (0.49) and SD=116.5x (0.45). Furthermore, plotting the frequency distribution of RAD tag coverage per population showed that on average, 93% of RAD tags fell within a coverage bracket of 10-250x (OT=89.53%, V=93.06%, PF=94.36%, SD=95.06%, see Appendix 2 Fig 2).

Rad tag loci either composed of 1 contig (merged: *N*=110,461) or ≥1 contig (un-merged: *N*=36,218, mean±SD=1.49±2.58) due to the assembly of read1 and read2. The average contig length (mean±SD) was 370.12±91.44bps across all merged and un-merged contigs. There were 107,363 merged RAD tags in total, possessing an average length of 396.33±72.67bps (mean±sd).

RAD tags were filtered for presence and absence amongst populations with a shared phenotype or country of origin. Overall, 97.72% of RAD tags were shared by all four populations. 0.004% of RAD tags were shared between FM populations but were absent in P populations. Conversely, 0.22% of RAD tags were present amongst P populations but absent amongst FM populations. There were no RAD tags unique to the UK population (SD) and 0.63% RAD tags were shared between Spanish populations (OT, V and PF) but absent in the UK population. These findings show there is minimal population structure between geographically isolated populations of *L. acervorum* (Foitzik et al., 2009).

6.3.4 SNP calling, F_{ST} calculation and outlier detection

6.3.4.1 SNP data and pairwise F_{ST} analysis

In total, 22,693 consensus RAD tags containing a single high quality SNP were retrieved for use in F_{ST} analysis (see Methods 6.2.7.1). Pairwise F_{ST} for each population comparison was generally low and median averages were always <0.1 with the highest being 0.056 between OT and PF, and the lowest 0.020 between OT and V (see Table 3). Furthermore, only 1.26% (range=0.01-1.95 %) of SNPs per pairwise comparison had an F_{ST}

score of ≥0.5. As expected due to geographic proximity (~100Km apart) and social phenotype, pairwise F_{ST} for OT vs V was the lowest. Similarly, pairwise F_{ST} for PF vs SD was the second lowest, likely reflecting the shared social phenotype.

Table 3: Median pairwise F_{ST} data. Median ± IQR.

	OT	V	PF
V	0.020±0.049		
PF	0.056±0.131	0.053±0.126	
SD	0.040±0.096	0.040±0.090	0.032±0.081

Overall, the distributions of pairwise F_{ST} at each SNP were heavily biased towards low F_{ST}, the majority of SNPs having a pairwise F_{ST} of ≤0.04 (Range=0.002-0.056, see Fig 6). The OT vs V comparison yielded the lowest F_{ST} distribution overall indicating that population structure between Spanish FM populations is low. Tellingly, the comparison between PF and SD had a similar distribution to all FM vs P comparisons, suggesting that population structure, albeit low, was present (Fig 6).

6.3.4.2 F_{ST} outlier detection and social marker calling

Outlier filtering (see 6.2.1 and 6.2.7.2 for criteria) produced a list of 121 (0.53% of the total number of SNP markers) social markers. The social marker filtering criteria was found to be effective at detecting SNP outliers associated with a difference in social phenotype since all tests for low F_{ST} values between social phenotypes yielded zero in all comparisons bar one which yielded 1 marker (see Table 4). This strongly indicates that social markers were successfully detected and are not false positives that are not the result of population structure (see 6.2.1).

Table 4: Social marker filtering criteria. Social markers were filtered for FST ≤60% quantile for the FM comparisons (OT vs V) and the P comparisons (PF vs SD), and FST ≥95% quantile for all FM vs P comparisons. The social marker filtering criteria is marked in bold. All control comparisons were calculated to test whether for low FST values (≤60% quantile) between social phenotypes produced fewer markers.

	≤60%	**≤60%**	**≥95%**	**≥95%**	**≥95%**	**≥95%**	**Total markers**
Social marker	**OT vs V**	**PF vs SD**	**OT vs PF**	**OT vs SD**	**V vs PF**	**V vs SD**	**121**
Control	V vs SD	OT vs PF	OT vs V	PF vs SD	V vs PF	OT vs SD	1
Control	OT vs SD	V vs PF	OT vs V	PF vs SD	OT vs PF	V vs SD	0
Control	OT vs PF	OT vs SD	OT vs V	PF vs SD	V vs PF	V vs SD	0
Control	V vs PF	V vs SD	OT vs V	PF vs SD	OT vs PF	OT vs SD	0
Control	PF vs SD	OT vs PF	OT vs V	OT vs SD	V vs PF	V vs SD	0
Control	OT vs V	OT vs PF	PF vs SD	OT vs SD	V vs PF	V vs SD	0
Control	OT vs V	OT vs SD	PF vs SD	OT vs PF	V vs PF	V vs SD	0
Control	OT vs V	V vs PF	PF vs SD	OT vs PF	OT vs SD	V vs SD	0
Control	OT vs V	V vs SD	PF vs SD	OT vs PF	OT vs SD	V vs PF	0
Control	PF vs SD	OT vs SD	V vs SD	OT vs PF	V vs PF	V vs SD	0
Control	PF vs SD	V vs PF	PF vs SD	OT vs PF	OT vs SD	V vs SD	0
Control	PF vs SD	V vs SD	PF vs SD	OT vs PF	OT vs SD	V vs PF	0
Control	OT vs PF	V vs PF	OT vs V	PF vs SD	V vs SD	OT vs SD	0
Control	OT vs SD	V vs SD	OT vs V	PF vs SD	OT vs PF	V vs PF	0

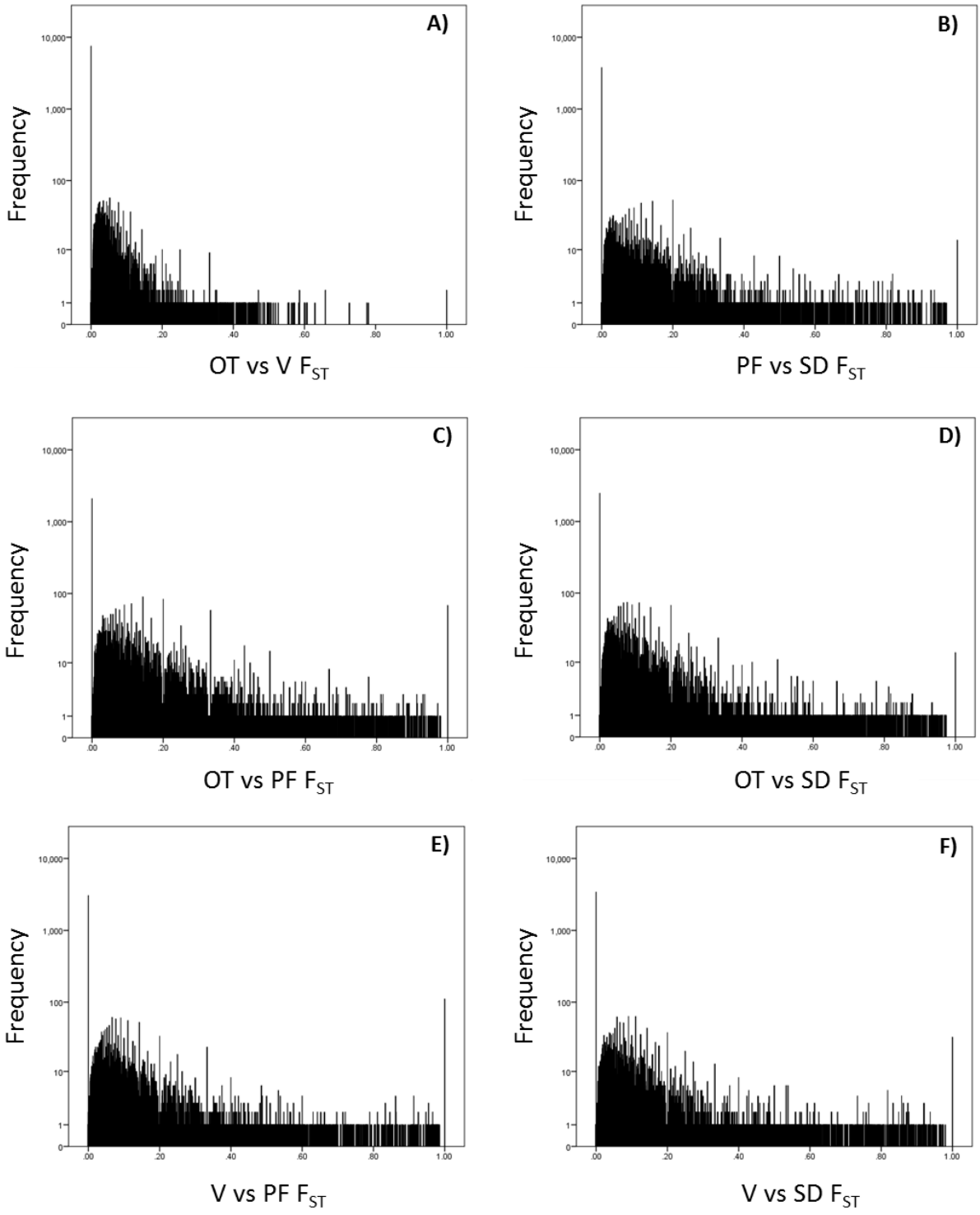


Fig 6 Frequency distributions of pairwise F_{ST} per SNP. The frequency is the number of SNPs with a given pairwise F_{ST}. Pairwise F_{ST} between the two Spanish FM populations (A) was low for the majority of SNPs compared to all other comparisons, which had a broader distribution of SNP pairwise F_{ST} (B-F).

6.3.5 Mapping social markers to the myrmicine genomes, detecting SoS and the *S. invicta* linkage map

6.3.5.1 Mapping social markers to the four myrmicine genomes

The 121 social markers were aligned to the four available myrmicine ant genomes via a local BLASTn search (returning hits with an e-value ≤ 1.0^{-8} and 70% query coverage), which resulted in 85 total hits representing 35 different social markers (28.9% of total markers) distributed across all four genomes (see Table 5). Individually, 25 social markers aligned to *A. cephalotes*, 24 social markers aligned to *A. echinatior*, 17 social markers aligned to the *P. barbatus* and 19 social markers aligned to *S. invicta* (see Table 5). The majority of the social markers (85.7%) aligned to ≥2 species, with eight social markers aligning to all four genomes, and ten uniquely aligning to a single species (see Table 5). Social markers aligned to 60 social reference scaffolds, with 10, 18, 18 and 14 scaffolds belonging to *S. invicta*, *A. echinatior*, *A. cephalotes* and *P. barbatus* respectively (see Table 5). Social reference scaffolds contained 1.5 social markers on average (range=1-5).

6.3.5.2 Statistical analysis and detection of SoS associated with the social phenotype

In total, 12 social reference scaffolds from all four of the genomes passed the statistical criteria for an SoS associated with the social phenotype (see 6.2.8.1 for statistical criteria) and contained 12 unique social markers (see Table 6). Furthermore, consensus_113656 displayed a significant SoS in all four genomes.

Aligning all non-*S. invicta* social reference scaffolds with an SoS to the *S. invicta* genome via BLASTn (returning a single hit with an e-value ≤ 1.0^{-8} and 70% query coverage) produced a list of three scaffolds; Si_gnF.scaffold03952, Si_gnF.scaffold03327 and Si_gnF.scaffold02536 (see Table 6 and Figs 7-9).

Table 5: Social markers that map to myrmicine genomes.

	Social reference scaffold ID			
Social marker	*S.invicta*	*A.cephalotes*	*A.echinator*	*P.barbatus*
consensus_101572				gnl|Pbar_1.0|scf7180000350268
consensus_102042	Si_gnF.scaffold00335	gnl|Acep_1.0|scaffold00007	gnl|Aech_2.0|scaffold56	gnl|Pbar_1.0|scf7180000350074
consensus_105502	Si_gnF.scaffold00514			
consensus_107934		gnl|Acep_1.0|scaffold00001		
consensus_113656	Si_gnF.scaffold03952	gnl|Acep_1.0|scaffold00055	gnl|Aech_2.0|scaffold18	gnl|Pbar_1.0|scf7180000350268
consensus_116770	Si_gnF.scaffold05424			
consensus_120326	Si_gnF.scaffold03327	gnl|Acep_1.0|scaffold00065	gnl|Aech_2.0|scaffold425	gnl|Pbar_1.0|scf7180000350316
consensus_120416	Si_gnF.scaffold00514	gnl|Acep_1.0|scaffold00013	gnl|Aech_2.0|scaffold646	gnl|Pbar_1.0|scf7180000350335
consensus_123464	Si_gnF.scaffold00514			
consensus_1283		gnl|Acep_1.0|scaffold00075	gnl|Aech_2.0|scaffold456	
consensus_176425			gnl|Aech_2.0|scaffold135	
consensus_18150	Si_gnF.scaffold04395	gnl|Acep_1.0|scaffold00002	gnl|Aech_2.0|scaffold192	gnl|Pbar_1.0|scf7180000350023
consensus_1896			gnl|Aech_2.0|scaffold456	gnl|Pbar_1.0|scf7180000350268
consensus_20001		gnl|Acep_1.0|scaffold00009		
consensus_24817	Si_gnF.scaffold02189	gnl|Acep_1.0|scaffold00005		gnl|Pbar_1.0|scf7180000350334
consensus_265		gnl|Acep_1.0|scaffold00081	gnl|Aech_2.0|scaffold220	
consensus_32352	Si_gnF.scaffold02536	gnl|Acep_1.0|scaffold00007	gnl|Aech_2.0|scaffold319	
consensus_3747		gnl|Acep_1.0|scaffold00050	gnl|Aech_2.0|scaffold1	gnl|Pbar_1.0|scf7180000350321
consensus_39709	Si_gnF.scaffold00514			
consensus_42764		gnl|Acep_1.0|scaffold00025	gnl|Aech_2.0|scaffold531	gnl|Pbar_1.0|scf7180000350249
consensus_43780		gnl|Acep_1.0|scaffold00009		
consensus_45830		gnl|Acep_1.0|scaffold00010	gnl|Aech_2.0|scaffold64	
consensus_48060	Si_gnF.scaffold01962	gnl|Acep_1.0|scaffold00009	gnl|Aech_2.0|scaffold586	
consensus_50671	Si_gnF.scaffold00741	gnl|Acep_1.0|scaffold00069	gnl|Aech_2.0|scaffold520	
consensus_51448			gnl|Aech_2.0|scaffold480	gnl|Pbar_1.0|scf7180000350337
consensus_5302	Si_gnF.scaffold02536	gnl|Acep_1.0|scaffold00007	gnl|Aech_2.0|scaffold480	gnl|Pbar_1.0|scf7180000350337
consensus_56788		gnl|Acep_1.0|scaffold00097	gnl|Aech_2.0|scaffold750	gnl|Pbar_1.0|scf7180000350188
consensus_61625	Si_gnF.scaffold02536	gnl|Acep_1.0|scaffold00007	gnl|Aech_2.0|scaffold480	gnl|Pbar_1.0|scf7180000350337
consensus_62922		gnl|Acep_1.0|scaffold00004		gnl|Pbar_1.0|scf7180000350284
consensus_75387	Si_gnF.scaffold03327		gnl|Aech_2.0|scaffold425	gnl|Pbar_1.0|scf7180000349967
consensus_75820	Si_gnF.scaffold03952	gnl|Acep_1.0|scaffold00007	gnl|Aech_2.0|scaffold91	
consensus_82796	Si_gnF.scaffold03952	gnl|Acep_1.0|scaffold00079	gnl|Aech_2.0|scaffold390	gnl|Pbar_1.0|scf7180000350316
consensus_83087		gnl|Acep_1.0|scaffold00026	gnl|Aech_2.0|scaffold425	
consensus_83869	Si_gnF.scaffold03327	gnl|Acep_1.0|scaffold00065		
consensus_99917			gnl|Aech_2.0|scaffold1	

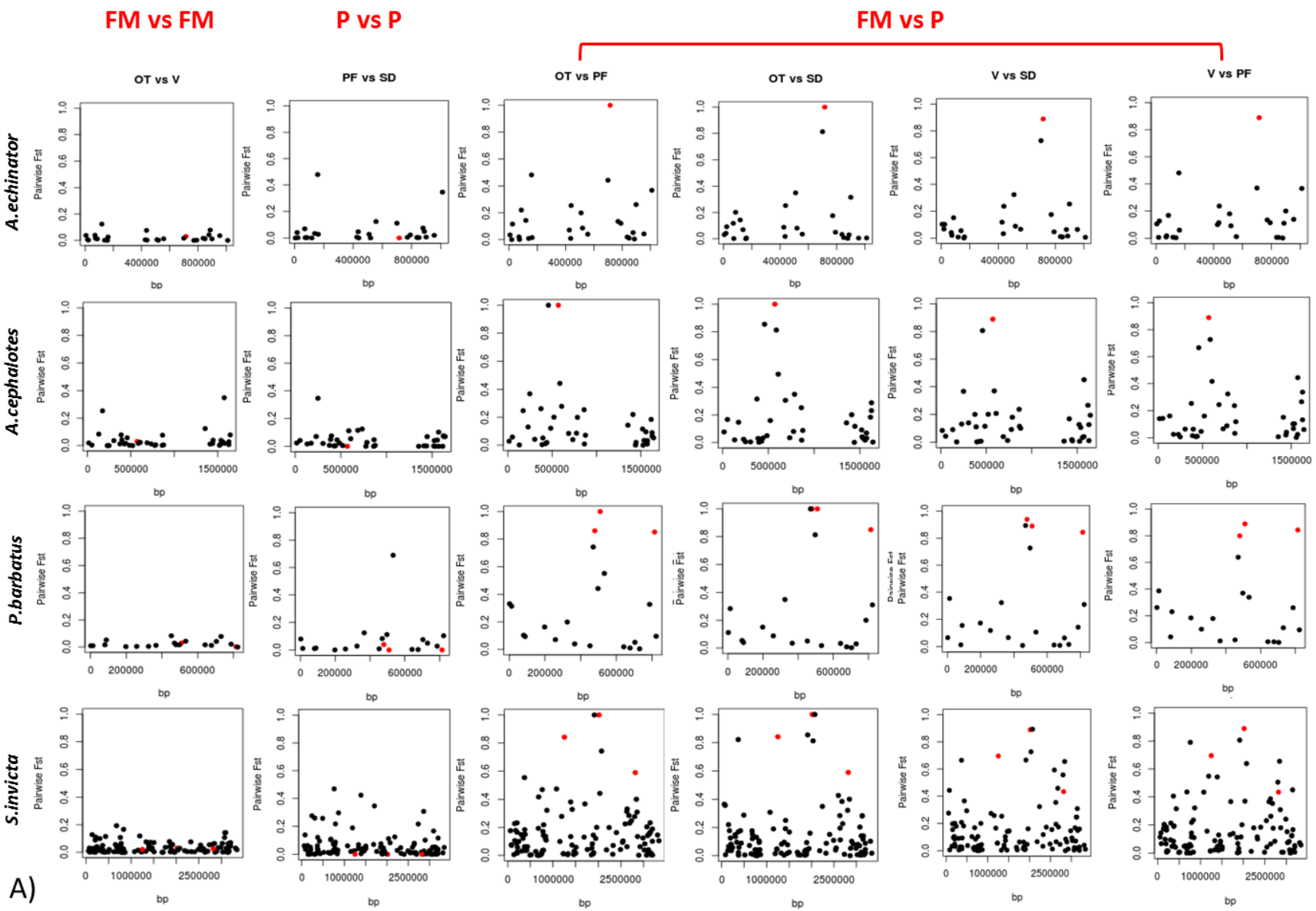
Table 6: Scaffolds containing an SoS that aligned to the *S. invicta* genome. All scaffolds containing an SoS were aligned to the *S. invicta* genome via BLASTn (returning a single hit with an e-value ≤ 1.0^{-8} and 70% query coverage). All scaffolds were found to align to three different *S. invicta* scaffolds. Scaffold IDs in **bold red** contained an SoS in *S. invicta*. Grey shading highlights all social markers that mapped to a specific *S. invicta* scaffold.

Social marker	*A. echinatior* scaffold	*P. barbatus* scaffold	*A. cephalotes* scaffold	*S.invicta* scaffold
consensus_120326	gnl|Aech_2.0|scaffold425			**Si_gnF.scaffold03327**
consensus_75387	gnl|Aech_2.0|scaffold425	gnl|Pbar_1.0|scf7180000349967		**Si_gnF.scaffold03327**
consensus_83869	gnl|Aech_2.0|scaffold425			**Si_gnF.scaffold03327**
consensus_113656	gnl|Aech_2.0|scaffold18	gnl|Pbar_1.0|scf7180000350268	gnl|Acep_1.0|scaffold00055	**Si_gnF.scaffold03952**
consensus_1896	gnl|Aech_2.0|scaffold456	gnl|Pbar_1.0|scf7180000350268		Si_gnF.scaffold03952
consensus_101572		gnl|Pbar_1.0|scf7180000350268		Si_gnF.scaffold03952
consensus_82796				**Si_gnF.scaffold03952**
consensus_75820				**Si_gnF.scaffold03952**
consensus_1283	gnl|Aech_2.0|scaffold456		gnl|Acep_1.0|scaffold00075	Si_gnF.scaffold03952
consensus_51448	gnl|Aech_2.0|scaffold480	gnl|Pbar_1.0|scf7180000350337		Si_gnF.scaffold02536
consensus_5302	gnl|Aech_2.0|scaffold480	gnl|Pbar_1.0|scf7180000350337		**Si_gnF.scaffold02536**
consensus_61625	gnl|Aech_2.0|scaffold480	gnl|Pbar_1.0|scf7180000350337		**Si_gnF.scaffold02536**

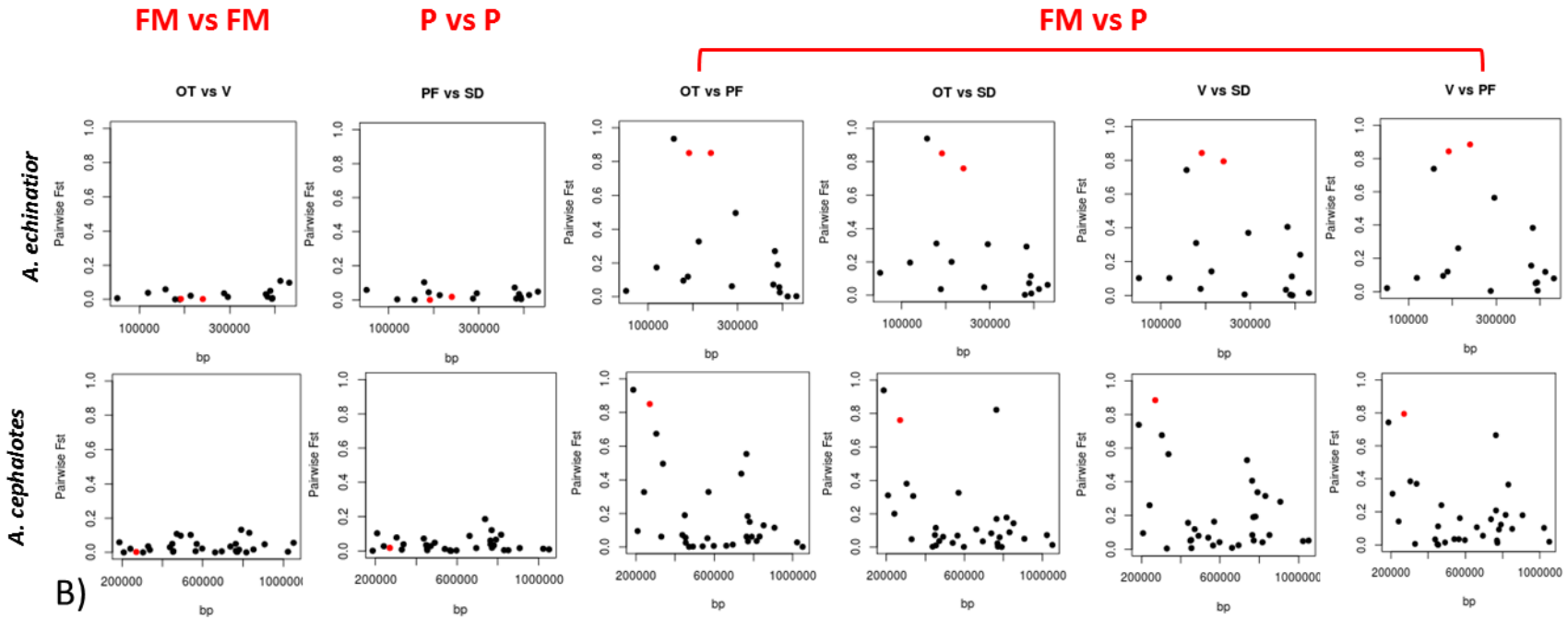
6.3.5.3 Manhattan plots of F_{ST}

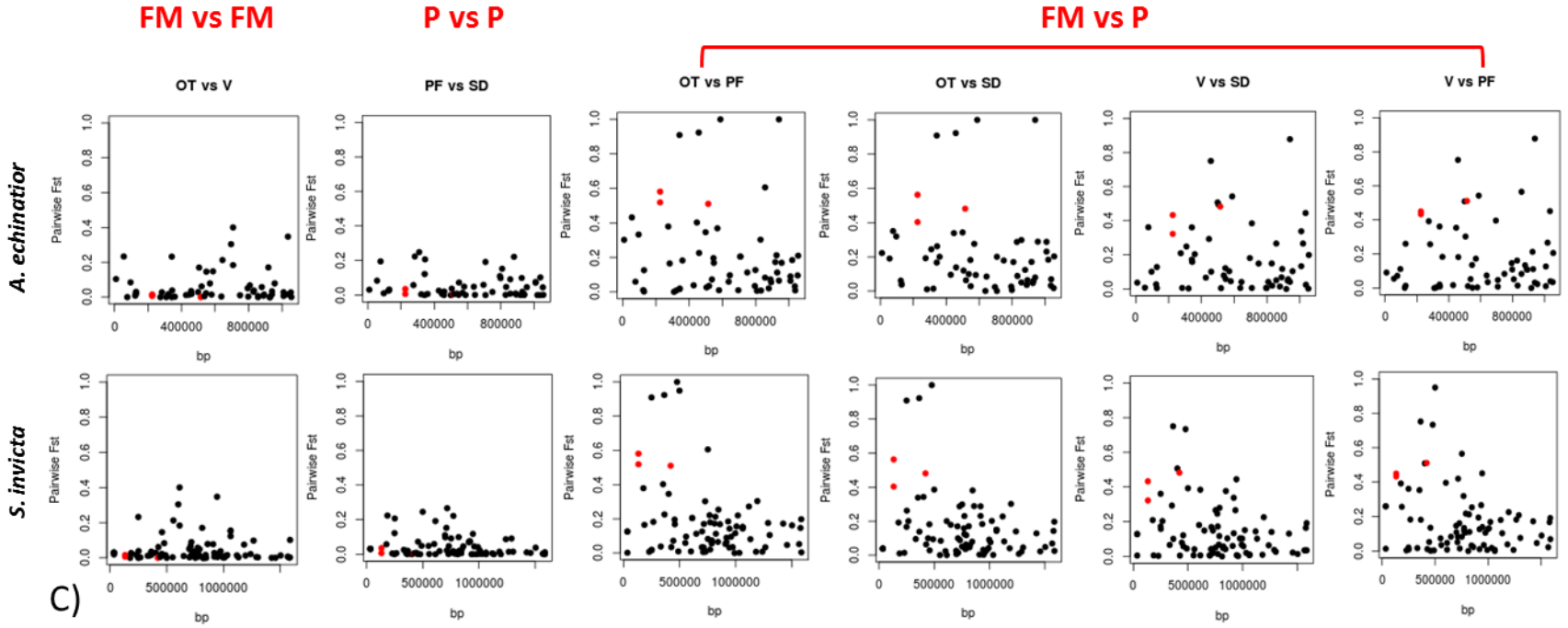
The Manhattan plots displayed in Fig 7 clearly show a pattern of elevated F_{ST} in all cross social phenotype comparisons (FM vs V). In contrast, the within social phenotype comparisons (FM vs FM and P vs P) show a consistent pattern of low F_{ST}. Furthermore, the position of the social SNP markers (red) indicates that they are situated near the top of a rising peak of F_{ST}, which is expected as population differentiation is strongest at the region containing the gene under selection and decreases further up and down stream of the region (Oleksyk et al., 2010, Akey et al., 2002).

Manhattan plots were also made for three of the four largest *S. invicta* scaffolds (Si_gnF.scaffold01122, Si_gnF.scaffold02694 and Si_gnF.scaffold02797.) to demonstrate the distribution of F_{ST} amongst neutral non-social markers (Fig 8). Each scaffold aligned to separate LGs in the linkage map LG14, 5 and 4 respectively). The Manhattan plots in Fig 8 demonstrate that F_{ST} was more or less evenly distributed within each pairwise population comparison and shows no obvious SoS.


FM vs FM
P vs P
FM vs P
OT vs V
PF vs SD
OT vs PF
OT vs SD
V vs SD
V vs PF
A.echinator
A.cephalotes
P.barbatus
S.invicta
Pairwise Fst
bp
0
400000
800000
500000
1500000
200000
600000
1000000
2500000

A)


FM vs FM
P vs P
FM vs P
OT vs V
PF vs SD
OT vs PF
OT vs SD
V vs SD
V vs PF
A. echinatior
A. cephalotes
Pairwise Fst
bp
B)


FM vs FM
P vs P
FM vs P
OT vs V
PF vs SD
OT vs PF
OT vs SD
V vs SD
V vs PF
A. echinatior
S. invicta
Pairwise Fst
bp
C)

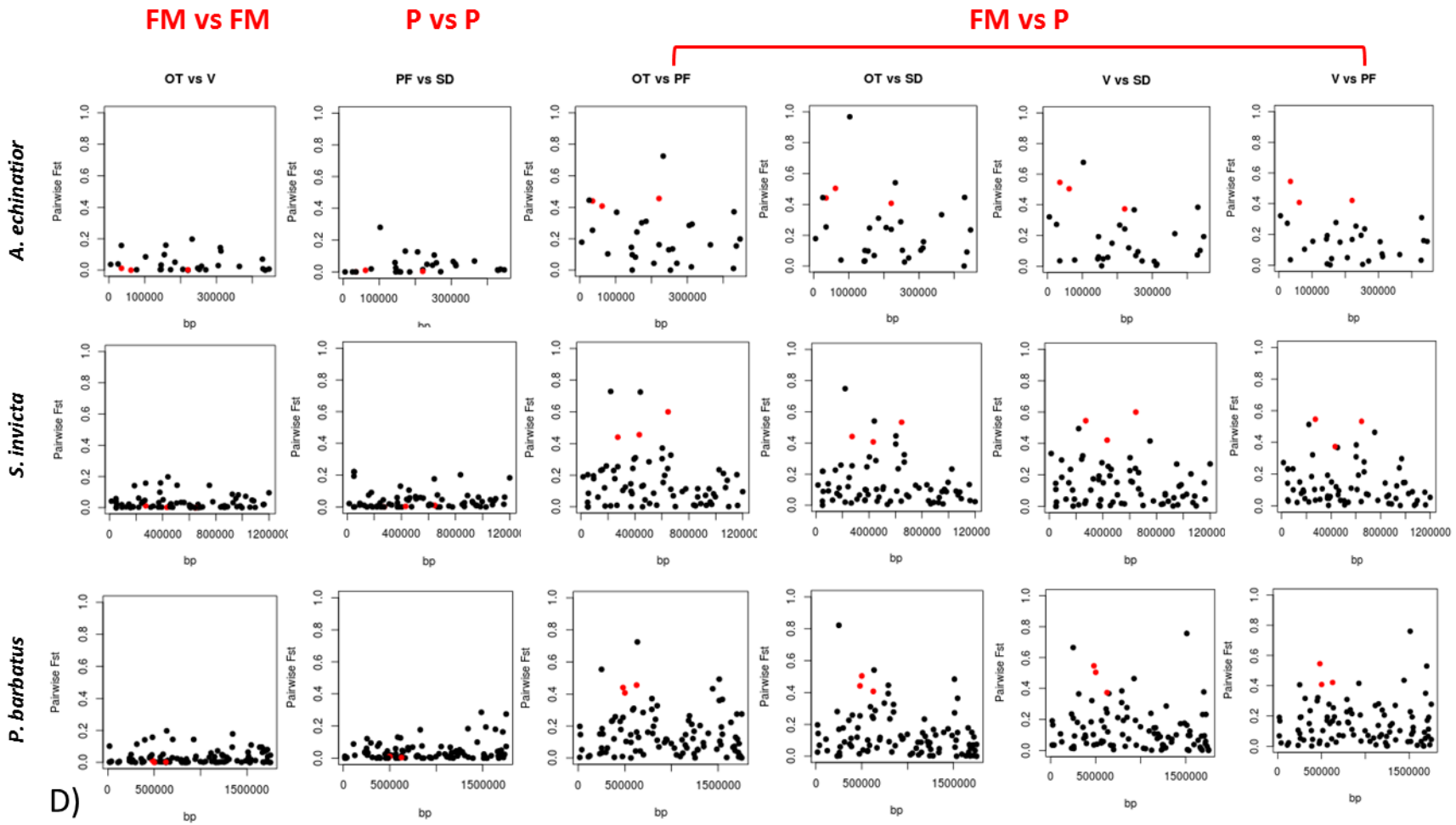


Fig 7: Manhattan plots of pairwise F_{ST} for social reference scaffolds. A-B) Social reference scaffolds containing the seven social markers which map to *S. invicta* scaffold Si_gnF.scaffold03952 (Table6). C) Social reference scaffolds containing the three social markers that mapped to the *S. invicta* scaffold Si_gnF.scaffold03327 (Table6). D) Social reference scaffolds containing the nine social markers which map to *S. invicta* scaffold Si_gnF.scaffold02536 (Table6). The population comparisons are displayed in columns and each row represents a different myrmicine species social reference scaffold. Social marker SNPs are in **red** and neutral SNPs are in black. Social phenotype comparisons are highlighted in **red**. A clear SoS is present as each P vs FM comparison displayed elevated F_{ST} compared to the P vs P and FM vs FM comparisons. See Table 6 for social reference scaffold information and Table 7-9 for statistical analysis and Fig 7 for positional information.

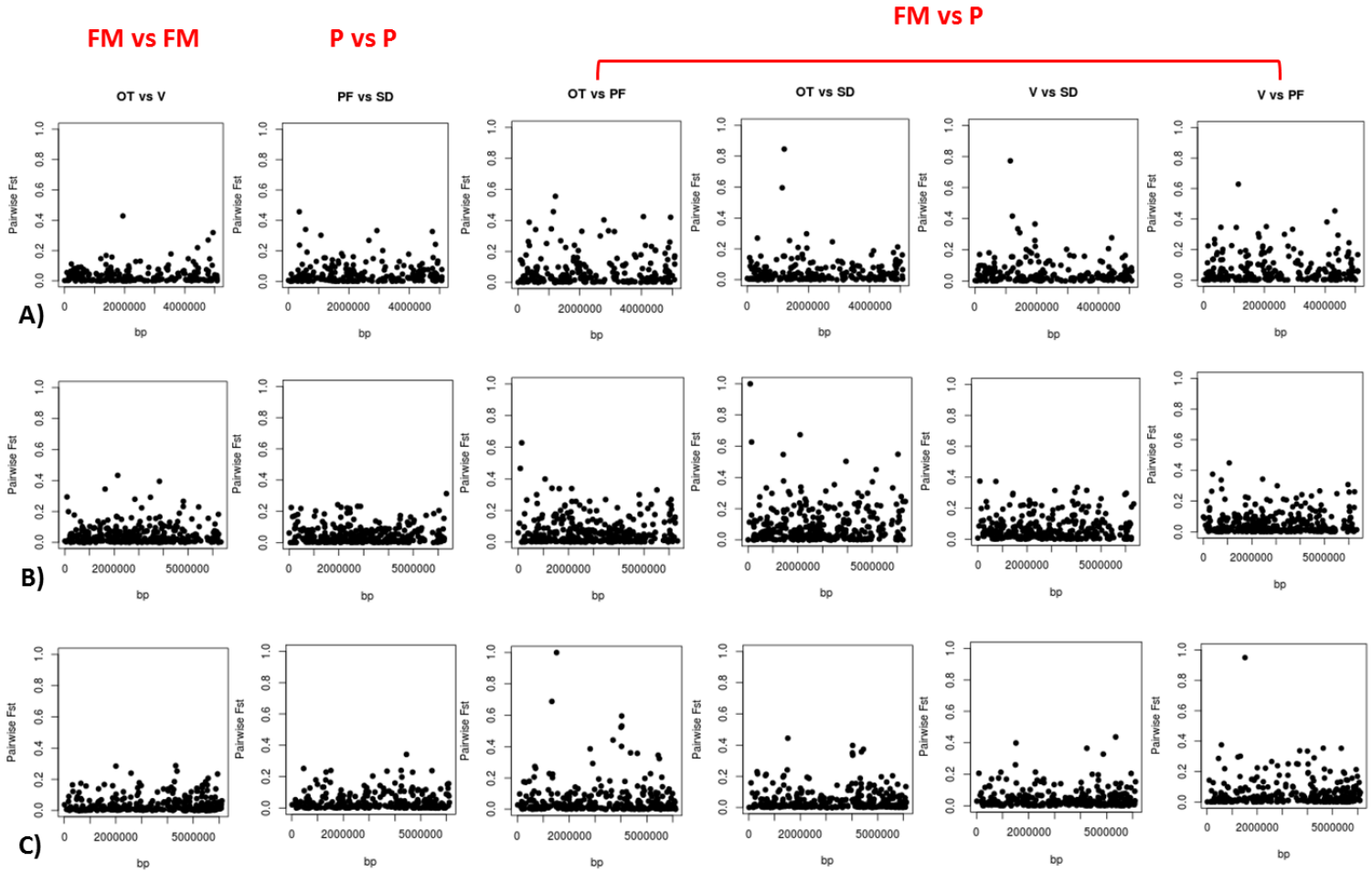


Fig 8: Manhattan plots of pairwise F_{ST} for *L. acervorum* SNP markers aligned to the three largest *S. invicta* scaffolds. These scaffolds did not contain any social markers and were plotted give a visual demonstration of the distribution of F_{ST} along neutral scaffolds. **A)** Si_gnF.scaffold01122 located on LG14. **B)** Si_gnF.scaffold02694 located on LG5. **C)** Si_gnF.scaffold02797 located on LG4. Social phenotype comparisons are highlighted in **red**.

Table 7: Statistical analysis for SoS detection on *A. echinatior* social reference scaffolds. One-sample Kruskal-Wallis tests were used to detect SoS associated with the social phenotype following an established criteria (see 2.8.1). Social markers highlighted in **red** passed the statistical criteria and were considered to contain an SoS associated with the social phenotype. Shown above each pairwise population comparison are the genome-wide F_{ST} medians for each pairwise population comparison. *N*= number of SNPs mapping to the social reference scaffold. M= sample median. W=Kruskal-Wallis. *P*= *P*-value. *P*-values were corrected for multiple testing using FDR analysis. Where *P*-values 0.000 they were <0.001.Grey shading highlights different population comparisons.

A.echinatior	Genome-wide medians	Median=0.02 OT vs V				Median=0.056 OT vs PF				Median=0.04 OT vs SD				Median=0.053 V vs PF				Median=0.04 V vs SD				Median=0.032 PF vs SD			
social marker consensus	social scaffold	*N*	M	W	*P*	*N*	M	W	*P*	*N*	M	W	*P*	*N*	M	W	*P*	*N*	M	W	*P*	*N*	M	W	*P*
consensus_18150	gnl|Aech_2.0|scaffold192	235	0.03	15823	0.061	235	0.11	20298	0.009	235	0.04	14248	0.697	235	0.10	20779	0.025	235	0.04	14511	0.502	235	0.09	21896	0.042
consensus_265	gnl|Aech_2.0|scaffold220	112	0.02	3623	0.182	112	0.06	3231	0.838	112	0.07	4524	0.017	112	0.05	3012	0.634	112	0.06	4105	0.028	112	0.04	3982	0.024
consensus_102042	gnl|Aech_2.0|scaffold56	101	0.02	2865	0.327	101	0.14	4614	0.009	101	0.09	4293	0.018	101	0.15	4803	0.026	101	0.10	4333	0.034	101	0.05	3548	0.039
consensus_50671	gnl|Aech_2.0|scaffold520	99	0.03	3326	0.002	99	0.11	3543	0.010	99	0.08	3626	0.018	99	0.10	3640	0.026	99	0.08	3769	0.035	99	0.05	3188	0.030
consensus_176425	gnl|Aech_2.0|scaffold135	89	0.03	2638	0.007	89	0.08	2454	0.055	89	0.04	2154	0.519	89	0.08	2599	0.012	89	0.05	2363	0.106	89	0.06	2805	0.042
consensus_120326	**gnl|Aech_2.0|scaffold425**	**60**	**0.03**	**1180**	**0.053**	**60**	**0.16**	**1513**	**0.010**	**60**	**0.17**	**1695**	**0.019**	**60**	**0.14**	**1484**	**0.027**	**60**	**0.14**	**1552**	**0.035**	**60**	**0.04**	**1007**	**0.457**
consensus_75387	**gnl|Aech_2.0|scaffold425**	**60**	**0.03**	**1180**	**0.051**	**60**	**0.16**	**1513**	**0.011**	**60**	**0.17**	**1695**	**0.019**	**60**	**0.14**	**1484**	**0.027**	**60**	**0.14**	**1552**	**0.036**	**60**	**0.04**	**1007**	**0.457**
consensus_83869	**gnl|Aech_2.0|scaffold425**	**60**	**0.03**	**1180**	**0.051**	**60**	**0.16**	**1513**	**0.011**	**60**	**0.17**	**1695**	**0.019**	**60**	**0.14**	**1484**	**0.028**	**60**	**0.14**	**1552**	**0.036**	**60**	**0.04**	**1007**	**0.457**
consensus_42764	gnl|Aech_2.0|scaffold531	50	0.04	843	0.045	50	0.06	677	0.888	50	0.05	808	0.159	50	0.05	657	0.931	50	0.05	808	0.140	50	0.04	778	0.238
consensus_45830	gnl|Aech_2.0|scaffold64	76	0.03	2106	-0.002	76	0.04	1206	0.172	76	0.05	1871	0.015	76	0.04	1234	0.286	76	0.05	1877	0.005	76	0.03	1356	0.536
consensus_51448	**gnl|Aech_2.0|scaffold480**	**30**	**0.03**	**280**	**0.330**	**30**	**0.21**	**435**	**0.012**	**30**	**0.22**	**447**	**0.020**	**30**	**0.16**	**409**	**0.029**	**30**	**0.16**	**422**	**0.037**	**30**	**0.03**	**211**	**0.621**
consensus_5302	**gnl|Aech_2.0|scaffold480**	**30**	**0.03**	**280**	**0.330**	**30**	**0.21**	**435**	**0.013**	**30**	**0.22**	**447**	**0.021**	**30**	**0.16**	**409**	**0.029**	**30**	**0.16**	**422**	**0.038**	**30**	**0.03**	**211**	**0.620**
consensus_61625	**gnl|Aech_2.0|scaffold480**	**30**	**0.03**	**280**	**0.329**	**30**	**0.21**	**435**	**0.013**	**30**	**0.22**	**447**	**0.021**	**30**	**0.16**	**409**	**0.030**	**30**	**0.16**	**422**	**0.038**	**30**	**0.03**	**211**	**0.620**
consensus_113656	**gnl|Aech_2.0|scaffold18**	**48**	**0.01**	**477**	**0.252**	**48**	**0.12**	**950**	**0.013**	**48**	**0.11**	**1002**	**0.022**	**48**	**0.10**	**968**	**0.030**	**48**	**0.09**	**1005**	**0.038**	**48**	**0.04**	**627**	**0.646**
consensus_48060	gnl|Aech_2.0|scaffold586	24	0.03	168	0.612	24	0.12	201	0.135	24	0.08	183	0.331	24	0.11	263	-0.029	24	0.09	200	0.119	24	0.06	191	0.200
consensus_120416	gnl|Aech_2.0|scaffold646	22	0.04	208	0.003	22	0.23	227	-0.013	22	0.12	226	-0.021	22	0.17	183	0.038	22	0.10	158	0.275	22	0.11	198	0.026
consensus_99917	gnl|Aech_2.0|scaffold1	41	0.03	584	0.041	41	0.06	534	0.168	41	0.06	582	0.027	41	0.06	538	0.135	41	0.06	606	0.016	41	0.04	588	0.006
consensus_3747	gnl|Aech_2.0|scaffold1	41	0.03	584	0.041	41	0.06	534	0.167	41	0.06	582	0.027	41	0.06	538	0.135	41	0.06	606	0.017	41	0.04	588	0.006
consensus_32352	gnl|Aech_2.0|scaffold319	20	0.01	87	0.507	20	0.07	139	0.196	20	0.07	161	0.015	20	0.10	0	0.038	20	0.12	174	0.029	20	0.02	78	0.275
consensus_1896	**gnl|Aech_2.0|scaffold456**	**38**	**0.02**	**461**	**0.185**	**38**	**0.16**	**636**	**0.015**	**38**	**0.14**	**601**	**0.023**	**38**	**0.16**	**687**	**0.032**	**38**	**0.17**	**647**	**0.040**	**38**	**0.03**	**317**	**0.393**
consensus_1283	**gnl|Aech_2.0|scaffold456**	**38**	**0.02**	**461**	**0.185**	**38**	**0.16**	**636**	**0.016**	**38**	**0.14**	**601**	**0.023**	**38**	**0.16**	**687**	**0.032**	**38**	**0.17**	**647**	**0.041**	**38**	**0.03**	**317**	**0.393**
consensus_82796	gnl|Aech_2.0|scaffold390	16	0.04	94	0.179	16	0.10	94	0.171	16	0.11	116	0.010	16	0.07	94	0.154	16	0.09	102	0.042	16	0.03	66	0.889
consensus_56788	gnl|Aech_2.0|scaffold750	14	0.03	63	0.522	14	0.14	71	0.242	14	0.09	63	0.505	14	0.12	64	0.457	14	0.07	70	0.245	14	0.03	54	0.900
consensus_75820	gnl|Aech_2.0|scaffold91	12	0.03	50	0.402	12	0.27	76	-0.013	12	0.08	66	0.013	12	0.30	76	0.029	12	0.09	68	0.017	12	0.06	50	0.360

Table 8: Statistical analysis for SoS detection on *P. barbatus* social reference scaffolds. One-sample Kruskal-Wallis tests were used to detect SoS associated with the social phenotype following an established criteria (see 2.8.1). Social markers highlighted in **red** passed the statistical criteria and were considered to contain an SoS associated with the social phenotype. Shown above each pairwise population comparison are the genome-wide F_{ST} medians for each pairwise population comparison. *N*= number of SNPs mapping to the social reference scaffold. M= sample median. W=Kruskal-Wallis. *P*= *P*-value. *P*-values were corrected for multiple testing using FDR analysis. Where *P*-values 0.000 they were <0.001.Grey shading highlights different population comparisons.

P.barbatus	Genome-wide medians	Median=0.02				Median=0.056				Median=0.04				Median=0.053				Median=0.04				Median=0.032			
		OT vs V				OT vs PF				OT vs SD				V vs PF				V vs SD				PF vs SD			
social marker consensus	social scaffold	*N*	M	W	*P*	*N*	M	W	*P*	*N*	M	W	*P*	*N*	M	W	*P*	*N*	M	W	*P*	*N*	M	W	*P*
consensus_24817	gnl|Pbar_1.0|scf7180000350334	112	0.02	3601.5	0.205	112	0.07	3906	0.010	112	0.04	3419	0.443	112	0.06	3817	0.033	112	0.05	3450	0.373	112	0.04	3451	0.364
consensus_62922	gnl|Pbar_1.0|scf7180000350284	110	0.04	4261	0.000	110	0.10	4469	0.000	110	0.09	4726	0.000	110	0.11	4559	0.000	110	0.08	4639	0.000	110	0.04	3399	0.259
consensus_120416	gnl|Pbar_1.0|scf7180000350335	104	0.02	3304	0.062	104	0.13	3965	0.000	104	0.06	3483	0.000	104	0.12	4010	0.000	104	0.06	3253	0.055	104	0.08	4159	0.000
consensus_51448	**gnl|Pbar_1.0|scf7180000350337**	**98**	**0.02**	**2579**	**0.586**	**98**	**0.14**	**4170**	**0.000**	**98**	**0.12**	**4278**	**0.000**	**98**	**0.13**	**4137**	**0.000**	**98**	**0.12**	**4238**	**0.000**	**98**	**0.03**	**2257**	**0.508**
consensus_5302	**gnl|Pbar_1.0|scf7180000350337**	**98**	**0.02**	**2579**	**0.586**	**98**	**0.14**	**4170**	**0.000**	**98**	**0.12**	**4278**	**0.000**	**98**	**0.13**	**4137**	**0.000**	**98**	**0.12**	**4238**	**0.000**	**98**	**0.03**	**2257**	**0.508**
consensus_61625	**gnl|Pbar_1.0|scf7180000350337**	**98**	**0.02**	**2579**	**0.585**	**98**	**0.14**	**4170**	**0.000**	**98**	**0.12**	**4278**	**0.000**	**98**	**0.13**	**4137**	**0.000**	**98**	**0.12**	**4238**	**0.000**	**98**	**0.03**	**2257**	**0.507**
consensus_3747	gnl|Pbar_1.0|scf7180000350321	88	0.04	2722	0.000	88	0.07	2293	0.148	88	0.05	2243	0.217	88	0.07	2480	0.002	88	0.05	2448	0.005	88	0.04	2217	0.237
consensus_18150	gnl|Pbar_1.0|scf7180000350023	87	0.03	2268	0.131	87	0.11	2896	0.000	87	0.04	1866	0.920	87	0.10	2659	0.000	87	0.05	2379	0.012	87	0.07	3009	0.000
consensus_102042	gnl|Pbar_1.0|scf7180000350074	72	0.03	1529	0.225	72	0.14	2197	0.000	72	0.10	2193	0.000	72	0.15	2376	0.000	72	0.10	2256	0.000	72	0.04	1583	0.086
consensus_120326	gnl|Pbar_1.0|scf7180000350316	71	0.03	1760	0.001	71	0.10	1903	0.000	71	0.09	2158	0.000	71	0.09	1851	0.000	71	0.10	2080	0.000	71	0.03	1190	0.441
consensus_82796	gnl|Pbar_1.0|scf7180000350316	71	0.03	1760	0.001	71	0.10	1903	0.000	71	0.09	2158	0.000	71	0.09	1851	0.000	71	0.10	2080	0.000	71	0.03	1190	0.441
consensus_56788	gnl|Pbar_1.0|scf7180000350188	43	0.02	509	0.662	43	0.09	616	0.071	43	0.06	549	0.339	43	0.08	619	0.048	43	0.04	489	0.813	43	0.05	573	0.182
consensus_42764	gnl|Pbar_1.0|scf7180000350249	32	0.05	390	0.013	32	0.06	310	0.589	32	0.05	303	0.671	32	0.06	310	0.573	32	0.04	300	0.694	32	0.03	222	0.252
consensus_113656	**gnl|Pbar_1.0|scf7180000350268**	**21**	**0.02**	**113**	**0.938**	**21**	**0.25**	**200**	**0.000**	**21**	**0.19**	**198**	**0.000**	**21**	**0.22**	**206**	**0.000**	**21**	**0.21**	**203**	**0.000**	**21**	**0.04**	**133**	**0.506**
consensus_1896	**gnl|Pbar_1.0|scf7180000350268**	**21**	**0.02**	**113**	**0.938**	**21**	**0.25**	**200**	**0.000**	**21**	**0.19**	**198**	**0.000**	**21**	**0.22**	**206**	**0.000**	**21**	**0.21**	**203**	**0.000**	**21**	**0.04**	**133**	**0.506**
consensus_101572	**gnl|Pbar_1.0|scf7180000350268**	**21**	**0.02**	**113**	**0.937**	**21**	**0.25**	**200**	**0.000**	**21**	**0.19**	**198**	**0.000**	**21**	**0.22**	**206**	**0.000**	**21**	**0.21**	**203**	**0.000**	**21**	**0.04**	**133**	**0.505**
consensus_75387	**gnl|Pbar_1.0|scf7180000349967**	**14**	**0.02**	**59**	**0.698**	**14**	**0.21**	**91**	**0.000**	**14**	**0.19**	**101**	**0.000**	**14**	**0.20**	**84**	**0.019**	**14**	**0.17**	**98**	**0.000**	**14**	**0.04**	**56**	**0.801**

Table 9: Statistical analysis for SoS detection on *S. invicta* social reference scaffolds. One-sample Kruskal-Wallis tests were used to detect SoS associated with the social phenotype following an established criteria (see 2.8.1). Social markers highlighted in **red** passed the statistical criteria and were considered to contain an SoS associated with the social phenotype. Shown above each pairwise population comparison are the genome-wide F_{ST} medians for each pairwise population comparison. *N*= number of SNPs mapping to the social reference scaffold. M= sample median. W=Kruskal-Wallis. *P*= *P*-value. *P*-values were corrected for multiple testing using FDR analysis. Where *P*-values 0.000 they were <0.001. Grey shading highlights different population comparisons.

S.invicta	Genome-wide medians		Median=0.02			Median=0.056				Median=0.04				Median=0.053				Median=0.04				Median=0.032			
			OT vs V			OT vs PF				OT vs SD				V vs PF				V vs SD				PF vs SD			
social marker consensus	social scaffold	*N*	M	W	*P*	*N*	M	W	*P*	*N*	M	W	*P*	*N*	M	W	*P*	*N*	M	W	*P*	*N*	M	W	*P*
consensus_48060	Si_gnF.scaffold01962	138	0.02	5542	0.113	138	0.08	5963	0.004	138	0.08	7087	0.000	138	0.09	6787	0.000	138	0.08	6986	0.000	138	0.08	6817	0.000
consensus_105502	Si_gnF.scaffold00514	123	0.03	4731	0.020	123	0.10	5307	0.000	123	0.04	3970	0.675	123	0.09	4898	0.000	123	0.04	3608	0.572	123	0.06	5540	0.000
consensus_120416	Si_gnF.scaffold00514	123	0.03	4731	0.020	123	0.10	5307	0.000	123	0.04	3970	0.675	123	0.09	4898	0.000	123	0.04	3608	0.571	123	0.06	5540	0.000
consensus_123464	Si_gnF.scaffold00514	123	0.03	4731	0.019	123	0.10	5307	0.000	123	0.04	3970	0.675	123	0.09	4898	0.000	123	0.04	3608	0.571	123	0.06	5540	0.000
consensus_39709	Si_gnF.scaffold00514	123	0.03	4731	0.019	123	0.10	5307	0.000	123	0.04	3970	0.674	123	0.09	4898	0.000	123	0.04	3608	0.570	123	0.06	5540	0.000
consensus_82796	**Si_gnF.scaffold03952**	**117**	**0.03**	**4207**	**0.160**	**117**	**0.12**	**5492**	**0.000**	**117**	**0.10**	**5542**	**0.000**	**117**	**0.12**	**5504**	**0.000**	**117**	**0.10**	**5423**	**0.000**	**117**	**0.04**	**3935**	**0.145**
consensus_113656	**Si_gnF.scaffold03952**	**117**	**0.03**	**4207**	**0.160**	**117**	**0.12**	**5492**	**0.000**	**117**	**0.10**	**5542**	**0.000**	**117**	**0.12**	**5504**	**0.000**	**117**	**0.10**	**5423**	**0.000**	**117**	**0.04**	**3935**	**0.144**
consensus_75820	**Si_gnF.scaffold03952**	**117**	**0.03**	**4207**	**0.159**	**117**	**0.12**	**5492**	**0.000**	**117**	**0.10**	**5542**	**0.000**	**117**	**0.12**	**5504**	**0.000**	**117**	**0.10**	**5423**	**0.000**	**117**	**0.04**	**3935**	**0.144**
consensus_50671	Si_gnF.scaffold00741	102	0.03	3207.5	0.049	102	0.10	3527	0.000	102	0.08	3916	0.000	102	0.10	3903	0.000	102	0.08	3894	0.000	102	0.05	3290	0.000
consensus_120326	**Si_gnF.scaffold03327**	**83**	**0.03**	**2009**	**0.224**	**83**	**0.13**	**2962**	**0.000**	**83**	**0.12**	**3053**	**0.000**	**83**	**0.14**	**2938**	**0.000**	**83**	**0.11**	**2887**	**0.000**	**83**	**0.03**	**1580**	**0.415**
consensus_75387	**Si_gnF.scaffold03327**	**83**	**0.03**	**2009**	**0.223**	**83**	**0.13**	**2962**	**0.000**	**83**	**0.12**	**3053**	**0.000**	**83**	**0.14**	**2938**	**0.000**	**83**	**0.11**	**2887**	**0.000**	**83**	**0.03**	**1580**	**0.415**
consensus_83869	**Si_gnF.scaffold03327**	**83**	**0.03**	**2009**	**0.223**	**83**	**0.13**	**2962**	**0.000**	**83**	**0.12**	**3053**	**0.000**	**83**	**0.14**	**2938**	**0.000**	**83**	**0.11**	**2887**	**0.000**	**83**	**0.03**	**1580**	**0.414**
consensus_102042	Si_gnF.scaffold00335	78	0.02	1588	0.809	78	0.15	2677	0.000	78	0.09	2427	0.000	78	0.14	2685	0.000	78	0.08	2446	0.000	78	0.04	2001	0.000
consensus_32352	**Si_gnF.scaffold02536**	**72**	**0.02**	**1447**	**0.451**	**72**	**0.13**	**2194**	**0.000**	**72**	**0.11**	**2361**	**0.000**	**72**	**0.13**	**2101**	**0.000**	**72**	**0.11**	**2302**	**0.000**	**72**	**0.03**	**1189**	**0.437**
consensus_5302	**Si_gnF.scaffold02536**	**72**	**0.02**	**1447**	**0.450**	**72**	**0.13**	**2194**	**0.000**	**72**	**0.11**	**2361**	**0.000**	**72**	**0.13**	**2101**	**0.000**	**72**	**0.11**	**2302**	**0.000**	**72**	**0.03**	**1189**	**0.437**
consensus_61625	**Si_gnF.scaffold02536**	**72**	**0.02**	**1447**	**0.450**	**72**	**0.13**	**2194**	**0.000**	**72**	**0.11**	**2361**	**0.000**	**72**	**0.13**	**2101**	**0.000**	**72**	**0.11**	**2302**	**0.000**	**72**	**0.03**	**1189**	**0.436**
consensus_18150	Si_gnF.scaffold04395	59	0.03	1171	0.024	59	0.11	1364	0.000	59	0.04	909	0.835	59	0.12	1344	0.000	59	0.05	1062	0.142	59	0.06	1296	0.000
consensus_24817	Si_gnF.scaffold02189	36	0.03	432	0.114	36	0.11	467	0.020	36	0.05	386	0.384	36	0.08	442	0.055	36	0.02	240	0.105	36	0.07	509	0.000
consensus_116770	Si_gnF.scaffold05424	35	0.07	497.5	0.000	35	0.14	520	0.000	35	0.10	544	0.000	35	0.07	382	0.243	35	0.06	453	0.000	35	0.03	314	0.943

Table 10: Statistical analysis for SoS detection on *A. cephalotes* social reference scaffolds. One-sample Kruskal-Wallis tests were used to detect SoS associated with the social phenotype following an established criteria (see 2.8.1). Social markers highlighted in **red** passed the statistical criteria and were considered to contain an SoS associated with the social phenotype. Shown above each pairwise population comparison are the genome-wide F_{ST} medians for each pairwise population comparison. *N*= number of SNPs mapping to the social reference scaffold. M= sample median. W=Kruskal-Wallis. *P*= *P*-value. *P*-values were corrected for multiple testing using FDR analysis. Where *P*-values 0.000 they were <0.001. Grey shading highlights different population comparisons.

A. cephalotes		Median=0.02				Median=0.056				Median=0.04				Median=0.053				Median=0.04				Median=0.032			
		OT_V				OT_PF				OT_SD				V_PF				V_SD				PF_SD			
social marker consensus	social scaffold	*N*	M	W	*P*	*N*	M	W	*P*	*N*	M	W	*P*	*N*	M	W	*P*	*N*	M	W	*P*	*N*	M	W	*P*
consensus_107934	gnl|Acep_1.0|scaffold00001	694	0.02906	149671	0.000	694	0.07318	150233	0.009	694	0.06868	170002	0.017	694	0.06273	137575	0.024	694	0.05554	154840	0.034	694	0.04023	139904	0.042
consensus_18150	gnl|Acep_1.0|scaffold00002	369	0.02489	39676	0.006	369	0.09988	47128	0.009	369	0.04696	37962	0.032	369	0.09729	46989	0.026	369	0.04417	36536	0.207	369	0.07195	51429	0.042
consensus_62922	gnl|Acep_1.0|scaffold00004	392	0.03147	49623	0.001	392	0.09054	53502	0.009	392	0.0735	54866.5	0.018	392	0.09538	54924	0.026	392	0.07651	56293	0.034	392	0.03864	43726	0.023
consensus_24817	gnl|Acep_1.0|scaffold00005	813	0.027	218632	0.001	813	0.058	191876	0.010	813	0.045	193735	0.018	813	0.055	191019	0.026	813	0.045	197895	0.035	813	0.04	207616	0.043
consensus_102042	gnl|Acep_1.0|scaffold00007	222	0.02187	13127	0.432	222	0.1482	21472	0.010	222	0.1002	21030	0.018	222	0.1454	21610	0.027	222	0.1008	20391	0.035	222	0.04106	14872	0.024
consensus_32352	gnl|Acep_1.0|scaffold00007	222	0.02187	13127	0.432	222	0.1482	21472	0.010	222	0.1002	21030	0.019	222	0.1454	21610	0.027	222	0.1008	20391	0.035	222	0.04106	14872	0.025
consensus_5302	gnl|Acep_1.0|scaffold00007	222	0.02187	13127	0.432	222	0.1482	21472	0.011	222	0.1002	21030	0.019	222	0.1454	21610	0.027	222	0.1008	20391	0.036	222	0.04106	14872	0.025
consensus_61625	gnl|Acep_1.0|scaffold00007	222	0.02187	13127	0.431	222	0.1482	21472	0.011	222	0.1002	21030	0.019	222	0.1454	21610	0.028	222	0.1008	20391	0.036	222	0.04106	14872	0.025
consensus_75820	gnl|Acep_1.0|scaffold00007	222	0.02187	13127	0.431	222	0.1482	21472	0.011	222	0.1002	21030	0.020	222	0.1454	21610	0.028	222	0.1008	20391	0.036	222	0.04106	14872	0.026
consensus_20001	gnl|Acep_1.0|scaffold00009	296	0.02411	24981	0.039	296	0.07532	26537	0.010	296	0.06539	29640	0.020	296	0.08241	30069	0.028	296	0.0694	29808	0.037	296	0.07042	31163	0.045
consensus_43780	gnl|Acep_1.0|scaffold00009	296	0.02411	24981	0.038	296	0.07532	26537	0.010	296	0.06539	29640	0.020	296	0.08241	30069	0.029	296	0.0694	29808	0.037	296	0.07042	31163	0.045
consensus_48060	gnl|Acep_1.0|scaffold00009	296	0.02411	24981	0.038	296	0.07532	26537	0.010	296	0.06539	29640	0.021	296	0.08241	30069	0.029	296	0.0694	29808	0.037	296	0.07042	31163	0.046
consensus_45830	gnl|Acep_1.0|scaffold00010	405	0.027	54681	0.004	405	0.044	38578	0.270	405	0.042	45644	0.033	405	0.048	42869	0.426	405	0.048	50817	0.038	405	0.034	45884	0.003
consensus_120416	gnl|Acep_1.0|scaffold00013	236	0.02469	16441	0.014	236	0.1095	20798	0.013	236	0.04843	15665	0.088	236	0.1053	20250	0.030	236	0.04787	15760	0.053	236	0.07412	20995	0.046
consensus_42764	gnl|Acep_1.0|scaffold00025	149	0.03442	7527	0.005	149	0.05114	5322	0.514	149	0.04205	5850	0.704	149	0.05105	5515	0.753	149	0.03912	5571	0.826	149	0.02757	5049	0.203
consensus_83087	gnl|Acep_1.0|scaffold00026	32	0.0352	385	0.235	32	0.1032	353	0.084	32	0.06558	382	0.006	32	0.09951	415	0.025	32	0.04294	276	0.791	32	0.06111	309	0.358
consensus_3747	gnl|Acep_1.0|scaffold00050	16	0.01303	59	0.654	16	0.08695	100	0.089	16	0.06795	108	0.019	16	0.07209	82	0.454	16	0.0671	100	0.064	16	0.02849	59	0.613
consensus_113656	**gnl|Acep_1.0|scaffold00055**	**39**	**0.02187**	**425**	**0.624**	**39**	**0.1095**	**567**	**0.000**	**39**	**0.1182**	**628**	**0.022**	**39**	**0.1148**	**645**	**0.031**	**39**	**0.1299**	**664**	**0.039**	**39**	**0.03571**	**437**	**0.468**
consensus_120326	gnl|Acep_1.0|scaffold00065	59	0.03829	1225	0.004	59	0.1821	1505	0.015	59	0.1708	1603	0.023	59	0.1607	1445	0.031	59	0.1332	1475	0.040	59	0.04891	1103	0.053
consensus_83869	gnl|Acep_1.0|scaffold00065	59	0.03829	1225	0.003	59	0.1821	1505	0.015	59	0.1708	1603	0.023	59	0.1607	1445	0.032	59	0.1332	1475	0.040	59	0.04891	1103	0.053
consensus_50671	gnl|Acep_1.0|scaffold00069	58	0.03136	1200	0.001	58	0.1042	1243	0.012	58	0.09391	1369	0.024	58	0.09178	1163	0.015	58	0.08764	1343	0.040	58	0.05637	1111	0.001
consensus_1283	**gnl|Acep_1.0|scaffold00075**	**32**	**0.02785**	**329**	**0.221**	**32**	**0.1104**	**376**	**0.021**	**32**	**0.1041**	**432**	**0.022**	**32**	**0.1613**	**434**	**0.030**	**32**	**0.1345**	**456**	**0.041**	**32**	**0.0333**	**275**	**0.795**
consensus_82796	gnl|Acep_1.0|scaffold00079	36	0.02685	418	0.176	36	0.1422	542	0.015	36	0.08157	491	0.011	36	0.109	503	0.025	36	0.05239	382	0.405	36	0.04944	409	0.277
consensus_265	gnl|Acep_1.0|scaffold00081	19	0.01883	93	0.944	19	0.08188	127	0.189	19	0.1258	169	0.022	19	0.1068	124	0.218	19	0.1249	179	0.040	19	0.07955	165	0.045
consensus_56788	gnl|Acep_1.0|scaffold00097	11	0.02468	39	0.917	11	0.2648	52	0.083	11	0.1125	49	0.143	11	0.189	59	0.010	11	0.09855	54	0.026	11	0.07434	38	0.639

6.3.6.3 Aligning social reference scaffolds to the *S. invicta* linkage map to determine their genomic distribution

Cross-referencing the three social reference scaffolds Si_gnF.scaffold03952, Si_gnF.scaffold03327 and Si_gnF.scaffold02536 with the *S. invicta* linkage map revealed their positions directly next to one another on linkage group 2 (LG2) (see Fig 9, Wang et al., 2013). In terms of recombination distance (cM), the region spanned 69.524cM, which represented roughly 54% of the total cM of LG2. Furthermore, the combined length in bps for all three scaffolds comes to ~6.2Mb, although in reality gaps of unknown length exist between each scaffold. I refer to this as the 'social region' (Fig 9).

6.3.7 Gene content and GO analysis for Si_ gnF.scaffold03952

To investigate the genes and their functional groups within the social region, I decided to use Si_gnFscaffold03952, the social reference scaffold with the most aligned social markers (6) and also the largest (3,360,376bp long) as an example.

A total of 189 genes were present on Si_gnFscaffold03952 (Wurm et al. 2011) and Blast2GO retrieved 1581 GO terms for 123 (65.1%) of the genes. Figs 10 and 11 display the GO term clusters within the biological processes and molecular functions hierarchies respectively. GO clusters covered a diverse range of biological processes and molecular functions (see Figs 10-11). However, GO terms that may indicate involvement with the social phenotype included factors involved in signal transduction; G-protein activity, odorant binding protein activity, steroid hormone receptor and neuropeptide activity, and methylation; methionine adenosyltransferase activity.

It was not appropriate to identify any specific genes as being the targets of selection in this study as neutral genes hitchhike within a region of linkage disequilibrium (Nielsen, 2005). However, it was possible to identify the genes closest to the social markers. Social marker consensus_113656 was located 10,505bps downstream of an unannotated hypothetical

protein (gene ID=SI2.2.0_10628) and 45,342bps upstream of a pyroglutamylated rfamide peptide receptor (gene ID=SI2.2.0_03956), which is a member of a G-protein receptor group linked to balancing nutrient metabolism in insects and feeding behaviour in nematodes (Cuenda, 2000).

Additionally, social marker consensus_1896 was located 3,860bps downstream of a choline o-acetyltransferase (gene ID=SI2.2.0_068413) and 5,0142bps upstream of mitogen-activated protein kinase kinase kinase 4 (gene ID=SI2.2.0_01144). Choline o-acetyltransferase is an enzyme responsible for the synthesis of the neurotransmitter acetylcholine. In insects, acetylcholine is highly abundant in the brain and targets nAChR receptors, which are thought to play important roles in chemical communication, olfactory learning and memory behaviour in bees (Gauthier, 2010, Pisa et al., 2015, Rabhi et al., 2014) and aggression behaviour in ants (Barbieri et al., 2013). Mitogen-activated protein kinase kinase kinase 4, is a member of the CMGC kinase family, which play key roles in cellular stress responses (Cuenda, 2000).

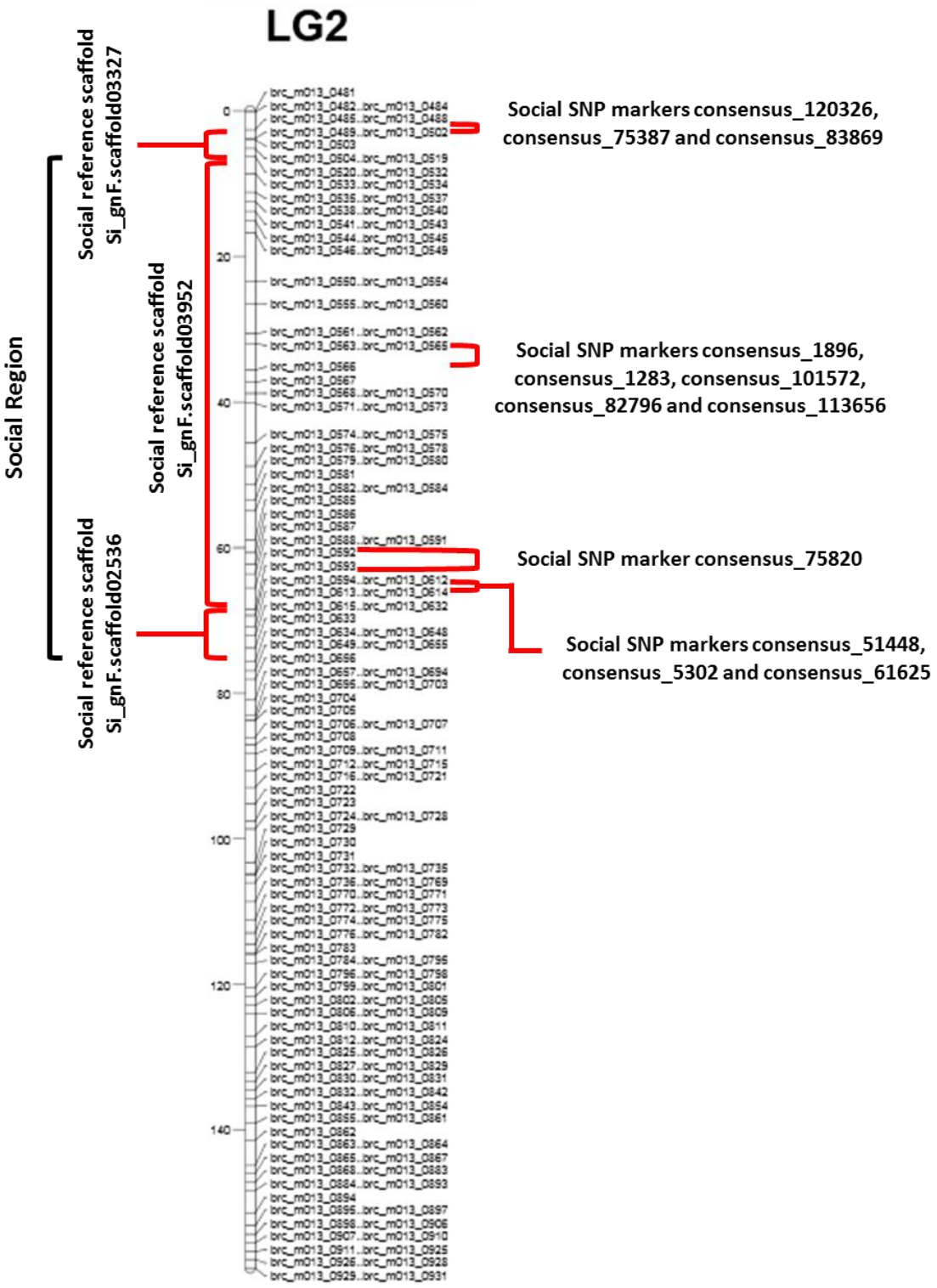


Fig 9: Alignment of social region to LG2. Position of *L. acervorum* social markers and social reference scaffolds on LG2 of the *S. invicta* linkage map. Twelve social markers (labelled right) aligned to the three *S. invicta* social reference scaffolds (labelled left), which formed the 'social region'. Numbers on the left indicate recombination distance in cM. Identifiers on the right (brc_m013_X) relate to *S. invicta* SNP markers used in the linkage map construction. The total region covered by the social reference scaffolds is 54% of the total cM. The linkage map modified from Wang et al. (2013).

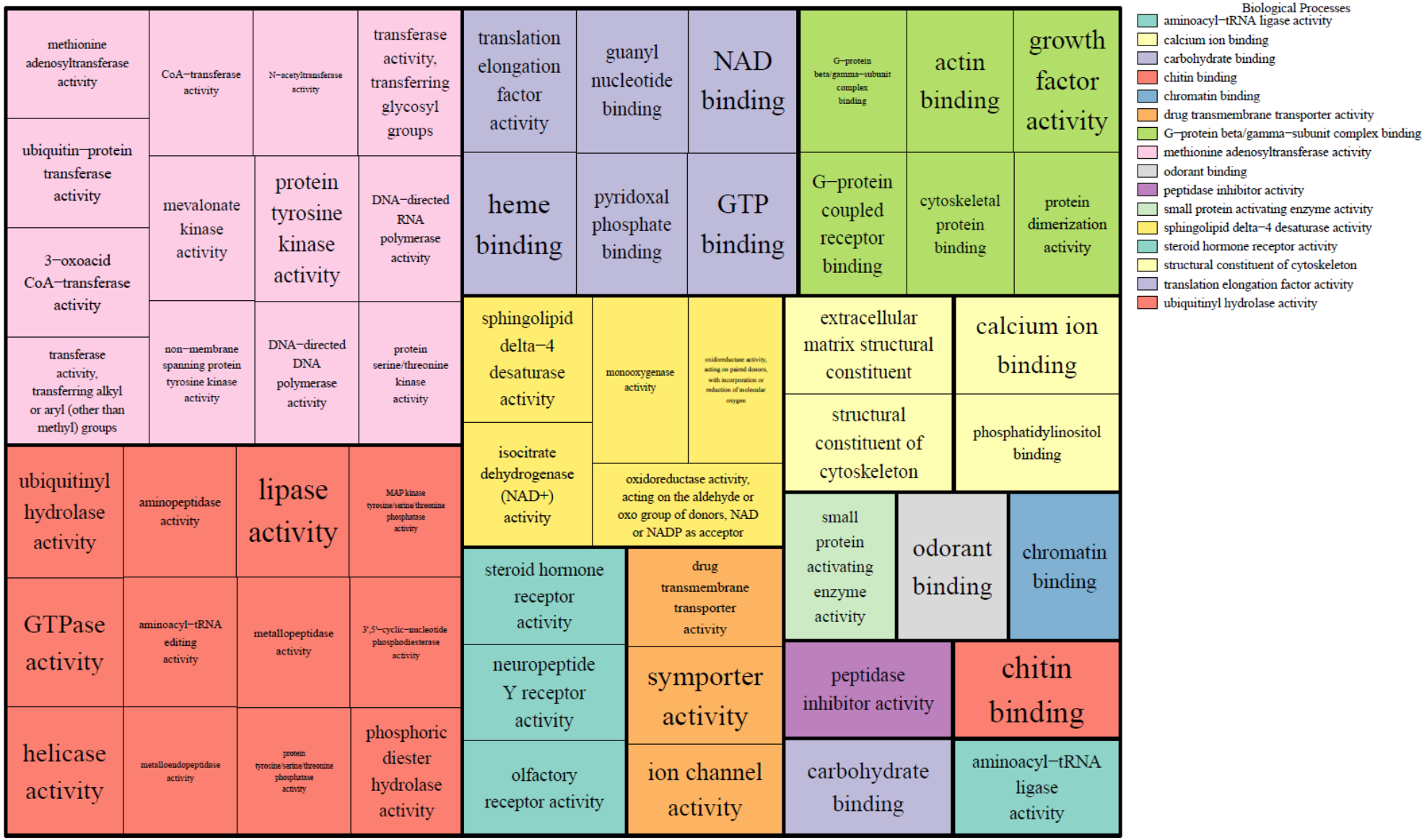


Fig 10: Treemap containing clustered GO terms for biological processes. The treemap was sized by the uniqueness score calculated in Blast2GO (Conesa et al., 2005).

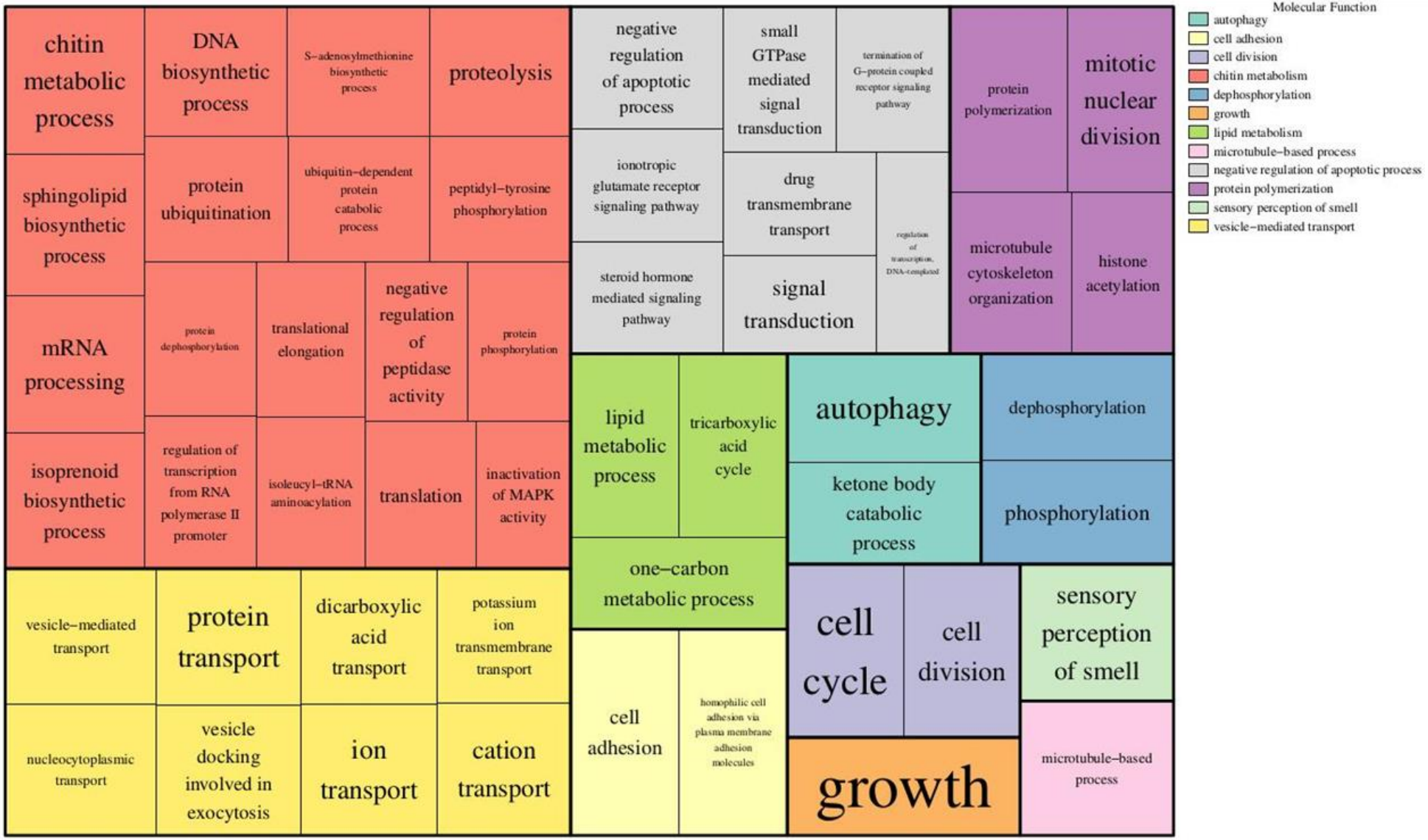


Fig 11: Treemap containing clustered GO terms for molecular functions. The treemap was sized by the uniqueness score calculated in Blast2GO (Conesa et al., 2005).

6.4 Discussion

6.4.1 Summary of key findings

I found evidence to suggest that variation in social organisation between populations of *L. acervorum* is at least partially underpinned by a genomic architecture with similarities to that found in *F. selysi* (Purcell et al., 2014) and *S. invicta* (Wang et al., 2013). Of the 121 markers that passed criteria to make them social markers (0.53% of all called SNPs), 35 markers (28.9%) were successfully aligned to at least one of four myrmicine ant genomes, with 85.7% of these aligned to at least two species. Of these 35 markers, 12 were found to have a significant SoS associated with differences in the social phenotype. The 12 social markers were also associated (either by direct alignment to *S. invicta* or indirect alignment to another myrmicine scaffold which then aligned to *S. invicta*) with three *S. invicta* social reference scaffolds. Importantly, these three social reference scaffolds were found to be contiguous on *S. invicta* LG2 when cross-referenced with the linkage map, and so likely form what I term a 'social region' in *L. acervorum.* Furthermore, GO analysis of genes within the main part of the social region revealed genes with functions relevant to known differences between the social phenotypes, for example: odorant binding, signal transduction and genes known to affect aggression in social insects. These findings do not support the distributed genetic architecture that would likely underpin a pleiotropic gene regulatory network.

6.4.2 Verification of experimental rationale

My approach depended on fulfilling a number of criteria which I discuss in the following sections.

6.4.2.1 Confirmation of a P social phenotype in colonies collected from the PF population

A pairwise comparison between populations with the same social phenotype was essential to control for neutral population structure as FM and P colonies have never been found in the same population and therefore cannot be compared within populations (see Fig 1). Genetic data indeed confirmed the PF population was of a P social phenotype because siblingship and colony relatedness analysis revealed all colonies contained workers and/or larvae from multiple families and colony relatedness was low. These data are clear confirmation of a P social phenotype and similar values have been reported in other P populations from the UK, including the SD population that was included in my RAD experiment (Hammond et al., 2006, Hammond et al., 2002, Bourke et al., 1997), and in another Pyrenean population (Heinze et al., 1995a).

6.4.2.2 Population structure is low

As expected (Foitzik et al., 2009, Gill et al., 2009), pairwise F_{ST} was generally low for the majority of SNPs, with population averages ranging between 0.020±0.049 and 0.056±0.131 (Median ± IQR). The comparison between the two Spanish FM populations, OT vs V, showed the lowest median F_{ST} (0.02±0.049) and tellingly, the second lowest median F_{ST} was found between the P populations, PF vs SD (0.032±0.081). Therefore, population divergence (F_{ST}) was generally low between populations regardless of social phenotype or geographical population origin. Furthermore, median F_{ST} between a UK P and a Spanish P population (PF-SD, 1100Km) was lower than the median F_{ST} between FM and P Spanish populations (PF-OT, PF-V; 323Km, 286Km), despite a greater than three times geographic distance, suggesting that population divergence may be more closely associated with social phenotypic differences, rather than neutral population structure. Finally, the paucity of population specific RAD tags, with 98% being shared by all four populations and the SD population possessing no unique RAD tags whatsoever, bolsters my conclusion.

Low genetic differentiation between geographically separate populations of *L. acervorum* (see 2.2 and Fig 2) support previous findings of a recent shared history between Spanish and UK populations (Gill et al., 2009, Heinze et al., 1995a). Most importantly, however, it justified my rationale that an extreme outlier approach to detecting SNP markers associated with the social phenotype could be confidently distinguished from neutral population variation, so limiting the known confounding effects of population structure on outlier analysis (Westram et al., 2014).

6.4.2.3 Social markers were successfully detected

It was important to test whether the outlier filtering criteria (see 2.1 and 2.7.2) produced a robust list of social markers that were associated with the differences in social phenotype and not F_{ST} outliers that were associated with random neutral variation (Via, 2012, 2014O'Brien et al., 2014, Oleksyk et al., 2010, Beaumont and Balding, 2004, Nielsen, 2005). This was especially important since my analysis was based on between population comparisons, rather than within population comparisons which inherently control for population structure (Renaut et al., 2012, Westram et al., 2014). However, the social marker filtering criteria was very effective at detecting SNP outliers associated with a difference in social phenotype since all tests for low F_{ST} values between social phenotypes yielded zero in all comparisons bar one which yielded 1 marker (see Table 4). This strongly suggests that the 121 social markers detected by my criteria were associated with the differences in social phenotype and were not false positives (see 2.1).

6.4.3 Similarities and differences between the *L. acervorum* social region and previously reported ant social chromosomes

6.4.3.1 Similarities between the social region and the ant social chromosomes

Within species differences in social phenotype have been attributed to genetic polymorphisms located in a single genomic region in the ant species *F. selysi* and *S. invicta*,

which have been termed social chromosomes (Purcell et al., 2014, Wang et al., 2013). In *L. acervorum*, my analysis likewise showed a large contiguous region with significantly elevated F_{ST} which locates to a single chromosomal location: LG2 on the *S. invicta* linkage map. This pattern is similar to the social chromosome in *F.selysi*, where a single contiguous chromosomal region shows high population divergence (F_{ST}) between the social phenotypes (Purcell et al., 2014), and *S. invicta*, where all polymorphic regions associated with differences between the social phenotypes are located on the same chromosome (Wang et al., 2013).

In addition, the social region is of a similar scale (~54% of the recombination distance of LG2) to that observed in *S. invicta* (~55% of the recombination distance of LGSB Wang et al., 2013) and *F. selysi* (~80% of LG3 Purcell et al., 2014). Similarly to *F. selysi*, median F_{ST} was elevated along the whole length of the social region in all between social phenotype population comparisons (see Fig 6 and Tables 7-9). However, not all markers within the region displayed elevated F_{ST}, which again is similar to the results found within the *F. selysi* social chromosome (see Fig 1B in Purcell et al., 2014) and the supergene underpinning Batesian mimicry in *Heliconius numata* butterflies (see Fig 2 in Joron et al., 2011).

Although the evidence for a single social region is strong, I cannot rule out the possibility that there are additional regions associated with social phenotype. I was only able to align ~30% of the social markers to any of the four myrmicine genomes, which leaves ~70% with no positional information. Therefore, it is possible that other social regions exist at different chromosomal positions associated with social markers that I was unable to align. Whether these un-aligned markers would form another contiguous region or align to multiple places across the genome is unknown and would require a genome and linkage map for *L. acervorum* to investigate further. However, the lack of information about these un-aligned markers does not in any way undermine my conclusion that there is at least one social region associated with social phenotype.

6.4.3.2 Differences between the *L. acervorum* social region and the ant social chromosomes

In both *F. selysi* and *S. invicta*, the social chromosomes are characterised by large regions of suppressed recombination as revealed by linkage analysis (Purcell et al., 2014, Wang et al., 2013). In *S. invicta*, two large chromosomal inversions are responsible for suppressed recombination (Wang et al., 2013), whereas, in *F. selysi,* although chromosomal rearrangements have been suggested (Purcell et al., 2014), there is, as yet, no definitive evidence for their presence. In this RADseq study, I used a pooling approach, which made it impossible to investigate patterns of linkage disequilibrium in *L. acervorum*. However, large regions of repressed recombination in the social region are unlikely for the following reasons.

First, filtering for high F_{ST} SNP outliers associated with a difference in social phenotype produced a small number of social SNP markers (0.53%; 121 of 22,693 of SNPs called). In contrast, 3.5% of SNPs (643) were associated with the social phenotype in *F. selysi* (Purcell et al., 2014) and between 10.2% - 13.2% of the markers detected in *S. invicta* were associated with the non-recombining regions of the social chromosome (Wang et al., 2013). High levels of linkage disequilibrium caused by chromosomal rearrangements in *S. invicta,* and likely in *F. selysi,* enables the detection of high proportions of high F_{ST} SNP outliers (social markers in my terminology) (Thompson and Jiggins, 2014, Schwander et al., 2014). Therefore, the comparatively low numbers of social markers detected in *L. acervorum* suggests that a large social chromosome characterised by a large chromosomal rearrangement is unlikely.

Second, population divergence within the social region between populations with alternate social organisations, although significantly higher than the genome average, was still low. Despite containing 12 highly divergent social markers, the median F_{ST} for all between social phenotype population comparisons within the social region ranged between 0.1-0.2,

which is much lower than the highly divergent F_{ST} present in the ant social chromosomes and butterfly supergene (Purcell et al., 2014, Wang et al., 2013, Joron et al., 2011).

However, in the butterfly *Heliconius numata*, multiple re-arrangements underpin recombination suppression in a 400kb region controlling wing colouration (Joron et al., 2011). The social region in *L. acervorum* is an order of magnitude larger (6.2Mb) than the butterfly in comparison, suggesting the potential for small chromosomal rearrangements within the social region in *L. acervorum*.

It is therefore unlikely that the social region contains large chromosomal rearrangements, which are typical of supergenes (Thompson and Jiggins, 2014, Schwander et al., 2014). However this doesn't discount the possibility that a supergene is only recently forming in *L. acervorum*, and has not yet evolved substantial linkage disequilibrium via chromosomal rearrangements. Indeed, the low levels of population divergence reported in this study (see 6.3.4) and by previous authors (Gill et al., 2009, Foitzik et al., 2009) suggests a recent shared history between FM and P populations. This might support the hypothesis that that divergence in social organisation is recent and not yet underpinned by a substantial social chromosome characterised by chromosome rearrangements.

One further key difference is that the social region in *L. acervorum* aligned to LG2 on the *S. invicta* linkage map, which is a separate chromosomal location to the *S. invicta* social chromosome (LGSB) (Wang et al., 2013). Furthermore, the social chromosome in *F. selysi* was found to share homology with multiple *S. invicta* LGs, but tellingly, did not share homology with LG2 (location of the social region) (see Fig 1A in Purcell et al., 2014). This not only demonstrates a key difference in the homology of the social region in comparison to the social chromosomes but also provides an additional example of convergent origin of genome architecture associated with divergent social phenotypes.

6.4.4 Explaining the evolutionary differences between the social region in *L. acervorum* and the ant social chromosomes

The differences between the social region in *L. acervorum* and the social chromosomes of *F. selysi* and *S. invicta* might be explained by differences in the selective environment linked to differences in ecology. First I address what these differences are and then interpret how they may influence the selective environment.

6.4.4.1 Differences in social phenotypes between *L. acervorum* and *F. selysi* / *S. invicta*.

L. acervorum social phenotypes differ in the number of reproducing queens in multiple queen colonies (Keller, 1995, Trettin et al., 2011, Gill and Hammond, 2011b, Gill et al., 2009, Hammond et al., 2006, Ito, 2005, Felke and Buschinger, 1999, Bourke, 1994). This contrasts with both *F. selysi* and *S. invicta*, where social organisation is either monogynous (M) or polygynous (P) and multiple mated queens are never tolerated in monogynous colonies (Purcell et al., 2015, Ross and Keller, 1995, Chapuisat et al., 2004). This difference extends further than just the number of queens and the number of reproducing queens as in *F. selysi* and *S. invicta* the social polymorphism is integrally linked to colony foundation (Ross, 1993, Ross and Keller, 1995), with M founding colonies independently and P founding colonies dependently, and this is linked to a suite of co-adapted morphological and behavioural traits (Rosset and Chapuisat, 2007, Schwander et al., 2005, Purcell and Chapuisat, 2012, Lawson et al., 2012, Krieger and Ross, 2002, Keller and Ross, 1999, DeHeer et al., 1999, Keller and Ross, 1995, Keller and Ross, 1993). In contrast, both FM and P colonies in *L. acervorum* contain multiple mated queens and likely found colonies dependently. Although there are well documented behavioural differences between P and FM colonies (Trettin et al., 2011, Gill and Hammond, 2011a, Gill and Hammond, 2011b, Ito, 2005), there is no evidence, to date, of morphological traits co-varying with social organisation. For example, colony size and

composition does not seem to vary significantly between P and FM colonies (see Chapter 5) and queens from alternate social organisations do not differ in size (pers. obs.).

6.4.4.2 Differences in selective environment between *L. acervorum* and *F. selysi* / *S.invicta*.

L. acervorum populations appear to be fixed for alternative social phenotypes (Trettin et al., 2011, Gill and Hammond, 2011b, Gill et al., 2009, Hammond et al., 2006, Ito, 2005, Bourke et al., 1997), whereas in contrast, *F. selysi* and *S. invicta* colonies possess alternate social phenotypes and are found together in the same habitats and so experience the same environmental pressures (Purcell and Chapuisat, 2013, Chapuisat et al., 2004, Ross and Keller, 1995, Purcell et al., 2015). The presence of divergent social phenotypes in the same populations in *F. selysi* and *S. invicta* is therefore problematic as the persistence of alternative phenotypes requires them to have equal fitness otherwise one phenotype will quickly reach fixation (Thompson and Jiggins, 2014, Schwander et al., 2014). In *S. invicta,* negative frequency-dependent selection that arises because of homozygous lethality (DeHeer et al., 1999, Hallar et al., 2007, Keller and Ross, 1999, Ross, 1997) and differential selection in a heterogeneous environment in *F. selysi* (Richman, 2000, Purcell et al., 2015) likely prevent the fixation of one social phenotype. This is obviously a fundamental difference in *L. acervorum*, where social phenotypes are distributed in allopatry and the two social phenotypes appear to be fixed in different populations and can therefore be thought of as adaptations to local environments (Trettin et al., 2011, Gill and Hammond, 2011b, Gill et al., 2009, Hammond et al., 2006, Ito, 2005, Bourke et al., 1997).

The sympatric nature of the social polymorphisms in *F. selysi* and *S. invicta* may be important in driving the evolution of the social chromosomes. This is because where there is free gene-flow; recombination can lead to maladapted combinations of genes and undermine the fitness of the social forms. This is antagonistic selection, with genes beneficial in one

social form being maladaptive in the other social form (Wang et al., 2013), a situation analogous to the evolution of sex chromosomes (Kirkpatrick and Guerrero, 2014, Bachtrog et al., 2011). This establishes strong selection for recombination suppression between genes involved in the social phenotype (Wang et al., 2013).

In contrast, this mechanism is unlikely to be operating in *L. acervorum* because of the allopatric distribution of social phenotypes. This raises the question as to how the contiguous social region that underlies the divergent social phenotypes has evolved in *L. acervorum*. One possible explanation might be divergence hitchhiking (DH) which is 'the coordinated evolution of multiple genes in a genomic region extending over several megabases due to partial protection from between population recombination and gene exchange' (Via, 2012). The patterns of F_{ST} shown within the social region are compatible with those predicted to be produced by DH (Via, 2012). Specifically, the presence of spatially clustered outlier F_{ST} markers which are interspersed with non-outlier F_{ST} markers, which is the signature of DH regions. Furthermore, between population recombination and gene exchange are likely to be low between populations of *L. acervorum.* Importantly, this mechanism does not require structural rearrangements to reduce recombination, although there is currently no evidence to suggest either an absence or a presence of structural rearrangements in the *L. acervorum* social region.

6.4.5 Gene content within the social region

A further similarity between the social region detected in *L. acervorum* and the social chromosome of *S. invicta* was the presence of genes associated with chemical communication and odour perception. The example SoS (see 6.3.7) was found to possess two different odorant binding receptors as well as other genes involved in steroid hormone signalling and G-protein signal transduction (see Figs 10-11). Historically, polymorphic social organisation in *S. invicta* was thought to be under the control of a single gene, GP-9 (Keller, 1993), which coded

for an odorant binding protein (Keller and Ross, 1998, Krieger and Ross, 2005). Queens that expressed the GP-9_b type allele within a monogynous population were quickly detected and aggressed by workers (Ross and Keller, 1998, Keller and Ross, 1998). Interestingly, worker aggression in FM colonies of *L. acervorum* may be directed by chemical cues administered by queen sting smearing events (Chapter 2, Gill and Hammond, 2011a). Therefore, it seems possible that regulation of aggression in FM colonies is determined by genes involved in chemical recognition. Furthermore, a loss of sensitivity to chemical cues in P workers may also explain the lack of worker aggression observed in P colonies (Chapters 2, 4 and 5, Bourke, 1991).

Searching for genes within close proximity to the social SNP markers revealed an especially interesting candidate gene involved in regulating aggression. A gene encoding for choline o-acetyltransferase was located within 3,860bps of the social SNP marker consensus_1896. It is an enzyme responsible for the synthesis of the neurotransmitter acetylcholine, which is highly abundant in the insect brain (Gauthier, 2010). Acetylcholine targets nAChR receptors, which are abundant throughout the CNS in all insects and therefore make excellent targets for pesticides (Pisa et al., 2015, Matsuda et al., 2001). As such, neonicitinoid pesticides targeting nAChR receptors have impacted upon the behaviour of some hymenopteran insects including; chemical communication, olfactory learning and memory behaviour in bees (Gauthier, 2010, Pisa et al., 2015, Rabhi et al., 2014) and foraging and aggression behaviour in ants (Barbieri et al., 2013, Galvanho et al., 2013). A recent study investigating the impact of neonicitinoid pesticides on ant behaviour demonstrated that exposure significantly altered aggressive behaviour in two different species (Barbieri et al., 2013). The colonies of the Argentine ant, *Linepithema humile*, showed increased aggression when exposed to neonicitinoids, whereas the myrmicine Southern ant, *Monomorium antarcticum*, showed decreased levels of aggression. These findings suggest that acetylcholine and nAChR receptor activity can play important roles in regulating behavioural aggression in

ants. Importantly, there is potential for variation in the Choline o-acetyltransferase gene to be involved in regulating the aggression that ultimately determines the colony social organisation in *L. acervorum* (Trettin et al., 2011, Gill and Hammond, 2011a, Gill and Hammond, 2011b, Bourke, 1991). It is important to emphasise, that while interesting and of relevant function, there is no direct evidence linking these genes to functional differences between the two social phenotypes.

6.4.6 Conclusions and future directions

I was able to detect a large region of the genome (the social region) that was associated with polymorphic social phenotype in *L. acervorum*. This social region possessed a number of similarities to the social chromosomes present in *F. selysi* and *S. invicta*, including; a single chromosome locale, high F_{ST} between alternate social phenotypes and genes involved in odour detection and signal transduction. Furthermore, this region contained genes with functions relevant to the behavioural differences between social phenotypes in *L. acervorum.* However, it was not possible to determine whether chromosomal rearrangements play a role within the social region. Additionally, there was no support for a pleiotropic gene regulatory network underpinning variation in social organisation.

Future directions should focus on establishing a full *L. acervorum* genome and linkage map to allow more appropriate mapping of RAD tags and social SNP markers. Furthermore, fine scale linkage mapping of the social region has the potential to resolve the genetic architecture of the social region, including the presence or absence of chromosomal rearrangements.

Chapter 7

Main thesis conclusions

7.1 Introduction

My main objectives were to investigate the roles of behavioural and genetic mechanisms in diversifying complex social phenotypes and to explore how variation within these mechanisms can facilitate the evolution and elaboration of eusocial life (Bourke, 2011).

Using the socially polymorphic ant species *L. acervorum*, as a model:

- I explored the role of aggressive interactions between queens and workers as a mechanism that potentially enforces reproductive altruism, and investigated how variation in aggressive behaviour directly influences reproductive skew (Chapters 2 and 4). I also tested the worker control hypothesis, set out by Gill and Hammond (2011b), by investigating skew in functionally monogynous (FM) queens when worker aggression was manipulated to be absent (Chapter 2).
- I investigated the potential for social plasticity in social organisation by testing the effects of queen:worker ratio (Q:W) on reproductive skew in polygynous colonies from the UK (Chapter 5).
- I tested for evidence of selection across the genome that is associated with the alternate social phenotypes using restriction associated DNA sequencing (RADseq). Furthermore, I tested whether variation in social phenotype was associated with a social chromosome architecture similar to that found in *S. invicta* and *F. selysi* (Purcell et al., 2014, Wang et al., 2013).

In this chapter I review my main results, discuss their implications in light of previous work, highlight emerging research questions and propose suggestions for future work.

7.2 The role of aggression in enforcing queen reproductive altruism and testing the worker control hypothesis

7.2.1 Investigating skew in the absence of worker enforcement

In Chapter 2, I explored the possibility that worker aggression directed at potentially reproductive queens in FM colonies acts as an enforcement mechanisms for coercing queen altruism (worker enforcement) and maintaining high reproductive skew (worker control hypothesis). These hypotheses were based on previous findings that the rate of worker aggression negatively correlated with queen reproduction, which ultimately determined colony skew (Gill and Hammond, 2011a, Gill and Hammond, 2011b). If worker aggression is an important enforcement mechanism, I expected queens to reproduce equally in the absence of aggression, which happens in polygynous (P) colonies (Hammond et al., 2006, Bourke et al., 1997) where worker aggression is absent (see Chapter 5, Bourke, 1991). However, I observed high rates of queen-queen (Q-Q) aggression when worker enforcement was removed and the total per capita rates of aggression observed in colonies without worker enforcement were significantly higher than the total rates of aggression observed in un-manipulated FM colonies.

Importantly, the removal of worker enforcement did not lower skew amongst the FM queens as colonies only ever contained a single reproductive queen and there were no significant differences in egg production or the beginning of egg laying when compared with un-manipulated FM colonies. Rates of aggression did not predict the reproductive fate of queens, but this was likely due to low statistical power.

Furthermore, I observed frequent sting smearing behaviour amongst queens in colonies without worker enforcement, which highlights a possible mechanism for directing worker enforcement and is common for establishing dominance hierarchies in many ant species including leptothoracines (Smith et al., 2012, Monnin and Ratnieks, 2001, Heinze et al., 1994, Heinze et al., 1992, Heinze et al., 1998). However, further studies are required to fully confirm sting smearing and the role of Dufour's gland secretions as an appropriate mechanism for directing worker enforcement as a previous study found sting smearing to provoke indiscriminate aggression from workers towards all queens involved in the behaviour (Gill and Hammond, 2011a).

However, despite strong evidence indicating queen control over skew through Q-Q aggression, worker enforcement is likely still important for maintaining high skew in FM colonies as evidenced by significantly higher rates of worker-queen (W-Q) aggression in un-manipulated FM colonies. Therefore, I found new evidence suggesting that ultimate control over skew resides with the queens and that dominance is established through aggressive Q-Q behaviour, which supports the queen control hypothesis in *L.acervorum* (Trettin et al., 2011). Furthermore, worker enforcement is clearly important to maintaining an FM social organisation and high skew, which suggests it does act as an enforcement mechanism. Finally, my observations in Chapter 2 indicate that regulating high skew in FM colonies involves complex Q-Q and W-Q aggression.

7.2.2 Investigating skew in the presence of worker enforcement

In Chapter 4, I tested whether reproductive altruism can be coerced in P queens through the application of aggressive enforcement to dissect further the role of worker enforcement behaviour in regulating reproductive skew. I tested this hypothesis by regularly exposing P queens from a UK population to aggressive worker enforcement and, assuming that aggressive worker enforcement behaviour is universally capable of coercing queen

reproductive altruism, I expected queens aggressed by workers to forgo reproduction. I found no evidence to indicate that worker enforcement affected skew as ovary dissections revealed no significant differences between enforced and control queens, egg productivity and the onset of laying were not affected and there was no significant difference in *vg* expression between treatment and control queens (a gene associated with reproduction, Chapter 3). The observations that skew was not affected in P colonies treated with worker enforcement contrast with the findings of Chapter 2 where aggressive enforcement behaviour was important in maintaining queen altruism and high skew in FM colonies.

Possible explanations for the discrepancy in effects of worker enforcement between P and FM queens on reproductive altruism and skew are as follows. First, the worker enforcement treatment used in Chapter 4 may not have been sufficient to cause an effect. Second, P colony queens may not be sensitive to worker enforcement behaviour, perhaps due to genetic differences (Gill et al., 2009), and are therefore not affected by worker enforcement (discussed in 7.3).

In summary, aggressive enforcement behaviours are clearly important in maintaining high skew in FM colonies by coercing queen altruism and FM queens engaged in frequent aggressive interactions, which supports the queen control hypothesis over skew (Chapter 2). However, queen responses to aggressive enforcement behaviour are likely to have a genetic basis, as demonstrated by the lack of effect worker enforcement had on skew in P colonies (Chapter 4). This suggests that genetic variation in aggressive enforcement behaviour and/or queen sensitivity to enforcement underpins phenotypic differences in social organisation, which I discuss further in this Chapter.

7.2.3 Future directions concerning the relationship between queen control and worker enforcement over colony skew

The role of Dufour's glad secretions in directing worker enforcement in FM *L. acervorum* colonies needs further clarification as queen signalling behaviour may directly impact colony skew. One possibility is that P queens have been selected to down regulate the production of Dufour's gland chemicals, since worker aggression is largely absent in P colonies (see Chapter 5, Bourke, 1991). Secretions from the Dufour's glad have been shown to be important directing worker aggression in many ant species (Smith et al., 2012, Monnin and Ratnieks, 2001, Heinze et al., 1994, Heinze et al., 1992, Heinze et al., 1998). Therefore, dissecting Dufour's glands from reproductive P and FM queens and comparing the differences in size would provide insights into the level of chemical production. For example, one might expect the Dufour's glands in FM queens to be significantly larger in comparison to P queens if they produce more chemical signals for directing worker enforcement.

Furthermore, nothing is currently known about the specificity of queen signals in *L. acervorum*. There is wide variation in specificity of signalling chemicals amongst dominance hierarchy forming ant species, from general specificity where workers can respond to the queen secretions of different colonies (Monnin et al., 2002), to highly specific where workers are only able to respond to colony specific queen secretions (Heinze et al., 1998). Evidence from Chapter 2 demonstrates that queen signals are population specific, as cross-fostered workers from a UK population never acted upon queen secretions. However, it cannot be determined how fine scale the specificity truly is. To investigate the specificity of queen signals, workers from P and FM colonies could be cross-fostered with FM queens. If specificity rests at the population level, you would expect workers from different populations to be unresponsive to queen secretions. Alternatively, if the social phenotype is important, then you would only expect FM workers, regardless of colony or population origin, to act upon queen

signals. Finally, in the unlikely scenario that specificity operates at the colony level, only un-manipulated colony workers would be expected to react.

7.2.4 Future directions concerning the insensitivity of P queens to worker enforcement

In Chapter 4, I make the argument that the worker enforcement treatment may not have been sufficient to have an effect on queen reproduction. Therefore, the extent to which P queens are sensitive to worker enforcement behaviour may not have been sufficiently tested. It may be possible to develop a more appropriate methodology for treating P queens with aggressive worker enforcement. One simple solution might be to immobilise queens, simulating the immobilisation behaviour witnessed in FM colonies, in a block of flexible foam. This would allow queens to be immobilised for much longer periods of time without the danger of serious damage and allow treatment schedules to more closely match the rates of aggression observed in natural FM colonies. However, this is obviously more artificial than the natural behaviour and works on the assumption that queen immobilisation is the key enforcement mechanism (Monnin and Ratnieks, 2001, Gobin et al., 1999).

An alternative approach might be to cross-foster P queens with FM workers using the method outlines in Chapter 2. FM workers may direct aggression towards queens in response to chemical signalling from queen Dufour's gland secretions (discussed in 7.2.1). Therefore, FM queen Dufour's glad secretions could be extracted and applied to P queens to direct FM worker aggression in cross-fostered colonies (Smith et al., 2012). This method assumes that Dufour's gland signals from non-native queens can be detected by FM workers and used to direct aggression and so the stability is dependent on the results of future investigations into Dufour's glad signalling specificity (see7.2.3). Providing that workers behaved aggressively towards smeared queens, P queens would receive a more natural worker enforcement

treatment, which would facilitate a more robust test of the hypothesis that worker enforcement is a universal mechanism for coercing queen altruism.

7.3 Evidence supporting a genetic basis for polymorphic social organisation

I uncovered evidence to support a genetic basis for regulating variation in social organisation, which supports the conclusions of previous studies (Gill and Hammond, 2011b, Gill et al., 2009). First, P queens from a UK population showed no variation in social organisation when colony queen:worker (Q:W) ratios were manipulated, which suggests that P colonies are not socially plastic to environmental constraints as suggested by Trettin et al. (2014). Second, as already discussed (7.1), P queen reproduction was not affected by aggressive worker enforcement. Assuming that FM is the ancestral social phenotype (see Discussion chapter in Gill, 2010), P queens may have been selected to become insensitive to aggressive worker enforcement as a result of selection for a P social phenotype. Third, P workers cross-fostered with FM queens did not respond aggressively to queens that had been the victim of a sting smearing event, suggesting that P workers have lost their sensitivity to queen Dufour's glad secretions. Again this might be explained by selection for the P social phenotype, which should promote a decrease in aggressive response to queen secreted signals. However, the lack of response to queen signals may also be explained by differences in signal specificity as explained (see 7.1.2).

Finally, a genome-wide scan for population differentiation between P and FM populations revealed a large contiguous social region (see Chapter 6) with similarities to the social chromosomes/supergene architecture of *S. invicta* and *F. selysi* (Purcell et al., 2014, Wang et al., 2013). First, only one social region was detected, which mapped to a 6.2Mb chromosomal region (LG2) on the *S. invicta* linkage map. Second, SNP markers mapping to the

region presented high F_{ST} in all comparisons between alternate social phenotypes, indicating potential linkage disequilibrium. Finally, genes involved in odour detection and signal transduction, which have been implicated with differences in the social phenotypes of *S. invicta* (Krieger and Ross, 2005, Keller and Ross, 1998) and variation in ant aggression (Barbieri et al., 2013), were present within the region under selection. However, it is important to iterate that only ~30% of detected social markers mapped to any of the ant genomes, leaving ~70% of the social markers with no positional information. Therefore, I cannot discount the possibility that other social regions are present in *L. acervorum*.

The social region contrasted with the social chromosomes of *S. invicta* and *F. selysi* in a two main characteristics. First, a relatively low proportion (0.5%) of social markers were detected and F_{ST} within the social region between social phenotypes was comparatively low. The low proportion of detected social markers might suggest that supergene architecture is in the early stages of development and has not yet achieved high linkage disequilibrium through substantial chromosomal rearrangements. This would also explain why the population divergence along the selected region was not as constantly high as that present in the *F. selysi* social chromosome (Purcell et al., 2014). To support this idea, it is likely that P and FM populations have recently diverged (Gill et al., 2009, Foitzik et al., 2009) and the patterns of F_{ST} I observed in social region match the predictions of evolution by divergence hitchhiking (DH), which can reduce recombination without chromosomal rearrangements between allopatrically separated populations (Via, 2012). Furthermore, the average genome-wide population divergence between FM and P populations was very low, which similarly supports a recent shared history (see Chapter 6).

The selection regimes acting upon *S. invicta*, *F. selysi* and *L. acervorum* to diversify alternate social phenotypes are likely to be different in each case, which might explain the architectural differences. The social chromosome in *S. invicta* is likely to have evolved under a

regime of antagonistic selection, similar to sex chromosomes (Wang et al., 2013), where alleles have different fitness values depending on the social phenotype. This type of selection is appropriate for *S. invicta* because populations of each social phenotype occupy the same habitat types and are likely to have evolved the divergent social phenotypes in a shared environment (Ross and Keller, 1995). Conversely, the social chromosome in *F. selysi* may have evolved through local adaptation to spatial heterogeneity in habitat composition, since populations have mixed social phenotypes but certain environmental constraints favour one social phenotype or the other (Purcell et al., 2015). It is difficult to fully understand the selection regime acting to diversify the social phenotypes in *L. acervorum*, because the distribution of alternate social phenotypes is allopatric, therefore the barriers to sympatric social phenotype divergence experienced by *S. invicta* and *F. selysi* do not apply. One explanation is that strong local adaptation in a small number of traits may have driven the evolution of a the social region through DH (Via, 2012), which over evolutionary time may develop into a full social chromosome similar to *S. invicta* and *F. selysi*. Hypothetically, traits under selection might include queen sensitivity to worker enforcement (Chapter 4) and worker sensitivity to queen produced aggression signals (Chapter 2), which would facilitate the evolution of the P social phenotype. Furthermore, the number of variable traits that co-vary with social organisation is much lower than *F. selysi* and *S. invicta*, supporting the idea that the social chromosome is only recently evolving and that variation in social phenotype need only be underpinned by a relatively small number of genes.

7.3.1 Future directions concerning the social region

The discovery of a large social region associated with differences in the social phenotype, represents an excellent opportunity to explore the role of genome architecture in diversifying complex social phenotypes further. Despite the discovery, very little is known about the specific architecture of the social region or how variation within the region

translates phenotypically into alternate social phenotypes. My study was limited by the unavailability of a *L. acervorum* genome and relied heavily on the genomes of the most closely related ant species available *S. invicta* is the closest ancestor to *L. acervorum*, however the two species diverged roughly 75mya (Moreau, 2006), which is likely to explain why the majority (~70%) of the social markers did not map with high confidence to the *S. invicta* genome. Therefore it is essential that the genome of *L. acervorum* is sequenced to facilitate future exploration of the social region. Furthermore, a linkage map would allow more accurate mapping of social markers and would provide definitive evidence for at least one social region in *L. acervorum*. Furthermore, linkage mapping would provide insights into whether chromosomal rearrangements are present within the social region, which have been shown to be important in social chromosomes (Wang et al., 2013) and supergenes (Thompson and Jiggins, 2014, Schwander et al., 2014, Joron et al., 2011).

7.4 Final conclusions

I have demonstrated that behavioural and genetic mechanisms are important in diversifying and maintaining complex social traits. I have provided evidence that polymorphic social organisation in *L. acervorum* is underpinned by variation in a social region with similarities to the social chromosomes present in *S. invicta* and *F. selysi*. Furthermore, I have investigated the role of aggressive worker enforcement behaviour and shown how variation within this mechanism can impact upon a fundamental aspect of eusocial living, reproductive skew. Finally, my work reveals new areas for investigation and highlights new and exciting opportunities to investigate the convergent genomic architecture underpinning divergent social phenotypes in *L. acervorum*.

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Appendices

Appendix 1: relates to Chapter 3

Table 1: Queen and worker qPCR data for housekeeper gene stability analysis. Efficiency relates to the average PCR amplification of target gene during each cycle. Avg C(t) relates to the average C(t) for each of the three sample technical replicates. C(t) is the cycle time where exponential amplification of the gene product is achieved.

Colony	Queen ID	Housekeeper	Efficiency	Avg C(t)	Colony	Worker ID	Housekee	Efficiency	Avg C(t)
VA.13.15	queen1	*elf*	1.54	21.42	HH.13.03	worker1	*elf*	1.44	22.25
SD.13.15	queen1	*elf*	1.61	23.33	DA.13.16	worker1	*elf*	1.5	22.13
VA.13.26	queen2	*elf*	1.41	21.78	DA.13.13	worker2	*elf*	1.41	21.54
SD.13.19	queen2	*elf*	1.53	21.22	DA.13.08	worker2	*elf*	1.5	23.46
PF.13.14	queen3	*elf*	1.4	22.82	DA.13.03	worker3	*elf*	1.47	23.36
OTD.13.10	queen3	*elf*	1.55	23.46	DA.13.11	worker3	*elf*	1.43	24.42
PF.13.40	queen4	*elf*	1.34	21.91	DA.13.12	worker4	*elf*	1.46	21.5
OTD.13.17	queen4	*elf*	1.51	22.39	HH.13.06	worker4	*elf*	1.52	22.36
VA.13.15	queen1	*rps*	1.45	22.25	HH.13.03	worker1	*rps*	1.36	22.59
SD.13.15	queen1	*rps*	1.53	24.09	DA.13.16	worker1	*rps*	1.46	23.52
VA.13.26	queen2	*rps*	1.52	22.99	DA.13.13	worker2	*rps*	1.49	22.99
SD.13.19	queen2	*rps*	1.44	22.7	DA.13.08	worker2	*rps*	1.55	24.15
PF.13.14	queen3	*rps*	1.55	25.11	DA.13.03	worker3	*rps*	1.34	23.52
OTD.13.10	queen3	*rps*	1.51	24.48	DA.13.11	worker3	*rps*	1.42	24.81
PF.13.40	queen4	*rps*	1.41	23.77	DA.13.12	worker4	*rps*	1.42	22.33
OTD.13.17	queen4	*rps*	1.46	23.99	HH.13.06	worker4	*rps*	1.54	24.14
VA.13.15	queen1	*ubiq*	1.48	23.32	HH.13.03	worker1	*ubiq*	1.41	24.33
SD.13.15	queen1	*ubiq*	1.4	23.94	DA.13.16	worker1	*ubiq*	1.5	22.38
VA.13.26	queen2	*ubiq*	1.33	23.79	DA.13.13	worker2	*ubiq*	1.43	23.29
SD.13.19	queen2	*ubiq*	1.51	22.5	DA.13.08	worker2	*ubiq*	1.49	23.52
PF.13.14	queen3	*ubiq*	1.39	24.49	DA.13.03	worker3	*ubiq*	1.43	25.22
OTD.13.10	queen3	*ubiq*	1.5	24.31	DA.13.11	worker3	*ubiq*	1.53	24.45
PF.13.40	queen4	*ubiq*	1.38	22.82	DA.13.12	worker4	*ubiq*	1.36	23.59
OTD.13.17	queen4	*ubiq*	1.43	22.94	HH.13.06	worker4	*ubiq*	1.46	22.58

Appendix 2: relates to Chapter 6

Tables:

Table 1: genotype data for PF workers. Four polymorphic microsatellites (L18, LXAGA1, LXAGA2 and LXAGT1) were used to genotype workers. Colony codes are translated as so: population ID-year-colony number-individual number. So PF11-3-W1= Pla de la Font, year 11, colony number 3, worker number 1. Genotype data for each of the 4 microsatellite loci are given. -1 and -2 microsatellite suffixes relate to allele 1 and allele 2 respectively. Samples highlighted in grey were omitted from siblingship analysis.

Colony	L18-1	L18-2	LXAGA1-1	LXAGA1-2	LXAGA2-1	LXAGA2-2	LXAGT1-1	LXAGT1-2
PF11-3-W1	131	143	164	164	138	155	265	265
PF11-3-W2	134	136	166	172	157	166	273	282
PF11-3-W3	134	134	166	170	144	166	282	320
PF11-3-W4	0	0	166	172	0	0	0	0
PF11-3-W5	130	143	164	170	138	167	265	265
PF11-3-W6	134	151	166	170	166	180	0	0
PF11-3-W7	134	143	164	166	129	155	225	331
PF11-3-W8	134	136	166	170	138	167	272	326
PF11-4-W1	134	143	158	164	127	180	271	276
PF11-4-W2	134	134	168	170	161	161	280	280
PF11-4-W3	134	134	168	170	161	161	276	278
PF11-4-W4	134	143	158	164	127	146	280	280
PF11-4-W5	131	143	158	164	127	180	271	276
PF11-4-W6	134	134	168	170	161	161	277	280
PF11-4-W7	134	134	168	170	0	0	277	280
PF11-4-W8	134	170	168	170	161	161	277	280
PF11-15-W1	134	142	166	170	0	0	224	305
PF11-15-W2	134	134	166	172	146	146	237	269
PF11-15-W3	143	143	166	170	138	144	224	269
PF11-15-W4	134	143	166	170	0	0	237	328
PF11-15-W5	143	143	164	176	140	157	224	328
PF11-15-W6	143	143	166	170	144	144	224	307
PF11-15-W7	143	143	166	170	144	144	224	267
PF11-15-W8	134	143	166	170	146	146	237	268
PF11-21-W1	139	143	164	168	0	0	237	315
PF11-21-W2	134	143	164	170	151	161	231	239
PF11-21-W3	138	143	168	168	148	155	237	315
PF11-21-W4	143	143	166	170	138	144	271	280
PF11-21-W5	143	143	164	170	138	148	269	278
PF11-21-W6	139	143	168	168	148	155	237	315
PF11-21-W7	138	143	158	170	127	154	246	248
PF11-21-W8	143	166	166	170	138	144	271	280

PF11-22-W1	142	142	157	170	0	0	272	287
PF11-22-W2	142	142	157	164	129	138	270	287
PF11-22-W3	143	143	157	170	129	148	280	287
PF11-22-W4	142	142	157	170	129	167	272	287
PF11-22-W5	142	142	157	170	129	148	280	287
PF11-22-W6	142	142	164	170	0	0	278	283
PF11-22-W7	142	142	157	170	129	148	272	287
PF11-22-W8	142	142	164	170	138	167	272	287
PF11-24-W1	135	143	166	170	0	0	276	293
PF11-24-W2	139	139	151	170	126	140	215	241
PF11-24-W3	135	139	0	0	142	142	257	271
PF11-24-W4	143	143	0	0	0	0	276	317
PF11-24-W5	131	143	166	170	163	169	273	284
PF11-24-W6	143	143	170	170	161	161	278	319
PF11-24-W7	134	143	153	170	129	169	269	280
PF11-24-W8	134	143	170	170	161	161	278	319
PF11-25-W1	138	143	166	166	0	0	272	282
PF11-25-W2	131	138	166	172	130	155	271	315
PF11-25-W3	131	138	166	166	129	130	270	319
PF11-25-W4	131	138	168	176	0	0	282	282
PF11-25-W5	131	138	166	172	130	155	271	319
PF11-25-W6	131	134	168	170	129	184	272	325
PF11-25-W7	131	138	166	172	130	155	271	319
PF11-25-W8	131	138	166	166	130	155	271	319
PF11-29-W1	143	143	166	166	0	0	275	280
PF11-29-W2	143	151	0	0	144	151	271	280
PF11-29-W3	131	143	164	166	128	161	275	275
PF11-29-W4	143	143	164	164	144	146	285	300
PF11-29-W5	139	143	166	168	161	161	275	307
PF11-29-W6	135	135	166	170	153	153	243	243
PF11-29-W7	143	0	164	164	144	146	285	300
PF11-29-W8	143	164	164	166	130	138	224	224
PF11-30-W1	131	143	166	172	0	0	278	283
PF11-30-W2	315	315	172	172	166	184	317	317
PF11-30-W3	0	0	166	172	144	148	278	283
PF11-30-W4	134	134	166	170	148	161	261	269
PF11-30-W5	143	151	166	166	153	153	237	245
PF11-30-W6	131	131	164	172	144	148	281	281
PF11-30-W7	131	143	166	170	161	161	237	237
PF11-30-W8	0	0	168	170	129	129	233	352

Table 2: genotype data for PF larvae. Four polymorphic microsatellites (L18, LXAGA1, LXAGA2 and LXAGT1) were used to genotype larvae. Colony codes are translated as so: population ID-year-colony number-individual number. So PF11-3-W1= Pla de la Font, year 11, colony number 3, worker number 1. Genotype data for each of the 4 microsatellite loci are given. -1 and -2 microsatellite suffixes relate to allele 1 and allele 2 respectively. Samples highlighted in grey were omitted from siblingship analysis.

Colony	L18-1	L18-2	LXAGA1-1	LXAGA1-2	LXAGA2-1	LXAGA2-2	LXAGT1-1	LXAGT1-2
PF11-3-L1	0	0	0	0	0	0	0	0
PF11-3-L2	131	143	163	172	138	138	224	265
PF11-3-L3	131	143	163	172	138	155	224	265
PF11-3-L4	131	143	164	172	138	182	265	323
PF11-3-L5	134	143	164	164	138	155	224	265
PF11-3-L6	131	143	164	172	138	182	224	265
PF11-3-L7	134	143	164	172	138	138	0	0
PF11-3-L8	134	151	166	172	157	166	273	282
PF11-4-L1	131	143	157	170	126	146	270	276
PF11-4-L2	134	143	157	164	126	180	270	276
PF11-4-L3	134	143	157	170	126	146	271	321
PF11-4-L4	134	143	157	164	126	180	271	321
PF11-4-L5	134	134	170	170	146	146	276	276
PF11-4-L6	131	143	157	170	126	146	270	276
PF11-4-L7	134	134	170	170	146	146	276	276
PF11-4-L8	134	143	157	170	126	146	271	276
PF11-15-L1	134	143	166	170	138	144	224	305
PF11-15-L2	143	143	166	170	144	144	224	267
PF11-15-L3	134	143	164	176	139	157	224	224
PF11-15-L4	143	143	166	170	138	144	224	305
PF11-15-L5	135	135	166	170	142	146	237	328
PF11-15-L6	135	135	168	172	146	155	237	276
PF11-15-L7	143	143	166	166	144	157	224	224
PF11-15-L8	134	143	166	170	144	144	224	267
PF11-21-L1	143	143	157	170	127	138	270	278
PF11-21-L2	134	138	157	170	126	154	245	270
PF11-21-L3	138	143	164	168	148	155	237	315
PF11-21-L4	138	143	157	157	146	146	0	0
PF11-21-L5	134	143	164	170	138	148	269	278
PF11-21-L6	139	143	168	168	148	155	237	315
PF11-21-L7	134	138	157	170	126	154	245	271
PF11-21-L8	134	143	157	170	127	138	248	271
PF11-22-L1	143	143	166	166	0	0	280	280
PF11-22-L2	143	143	170	170	148	148	284	284
PF11-22-L3	143	143	170	170	148	148	271	271
PF11-22-L4	143	143	166	166	148	148	271	271

PF11-22-L5	143	143	166	166	166	166	280	280
PF11-22-L6	143	143	170	170	148	148	284	284
PF11-22-L7	143	143	157	170	129	148	280	286
PF11-22-L8	143	143	166	166	166	166	286	286
PF11-24-L1	134	143	170	170	129	129	276	319
PF11-24-L2	143	143	170	170	162	162	272	280
PF11-24-L3	143	143	170	170	162	162	245	272
PF11-24-L4	131	143	0	0	140	140	0	0
PF11-24-L5	131	135	166	170	169	180	272	272
PF11-24-L6	131	143	166	170	140	169	280	280
PF11-24-L7	135	143	166	166	140	140	272	278
PF11-24-L8	135	143	166	166	0	0	246	272
PF11-25-L1	0	0	0	0	0	0	0	0
PF11-25-L2	0	0	38	38	0	0	0	0
PF11-25-L3	131	143	166	166	146	151	271	271
PF11-25-L4	131	134	166	166	167	184	325	332
PF11-25-L5	131	143	151	164	144	151	241	282
PF11-25-L6	131	134	166	170	0	0	272	325
PF11-25-L7	131	134	166	170	0	0	272	325
PF11-25-L8	134	139	166	170	0	0	325	332
PF11-29-L1	139	143	0	0	144	144	285	320
PF11-29-L2	139	143	166	170	144	144	285	285
PF11-29-L3	139	143	166	170	127	144	255	320
PF11-29-L4	137	137	168	170	0	0	321	321
PF11-29-L5	139	143	166	170	144	144	285	285
PF11-29-L6	131	134	164	166	182	182	307	319
PF11-29-L7	143	143	166	168	161	161	271	276
PF11-29-L8	143	143	158	164	129	129	276	276
PF11-30-L1	131	143	166	172	144	148	224	277
PF11-30-L2	143	143	164	170	140	153	277	277
PF11-30-L3	135	143	166	170	129	161	279	311
PF11-30-L4	0	0	166	170	129	161	265	265
PF11-30-L5	131	143	166	172	144	144	278	278
PF11-30-L6	134	151	164	166	144	159	243	278
PF11-30-L7	131	143	166	172	144	153	246	280
PF11-30-L8	134	143	170	170	0	0	0	0

Table 3: PF colony siblinship analysis data for workers and larvae. *N*=total number of workers or larvae genotyped at ≥3 microsatellite loci. Fullsib families = the number of predicted fullsib families based on worker genotypes.

Colony	Worker *N*	Fullsib families	Colony	Larvae *N*	Fullsib families
PF11.03	7	6	PF11.03	7	3
PF11.04	8	4	PF11.04	7	3
PF11.15	8	6	PF11.15	8	5
PF11.21	8	4	PF11.21	8	6
PF11.22	8	4	PF11.22	1	1
PF11.24	8	7	PF11.24	8	4
PF11.25	8	5	PF11.25	6	3
PF11.29	8	7	PF11.29	8	5
PF11.30	8	6	PF11.30	8	6

Table 4: PF colony relatedness data for workers and larvae. *N*=total number of workers or larvae genotyped at ≥3 microsatellite loci. SD = standard deviation.

Colony	Workers	Mean relatedness	SD	Colony	Larvae	Mean relatedness	SD
PF11.03	7	0.086590476	0.23972464	PF11.03	7	0.235757143	0.335422015
PF11.04	8	0.288796429	0.382813474	PF11.04	7	0.428292857	0.263397119
PF11.15	8	0.199346429	0.290484838	PF11.15	8	0.202	0.396199085
PF11.21	8	0.104775	0.278129053	PF11.21	8	0.08535	0.266250365
PF11.22	8	0.489357143	0.26047495	PF11.22	1	0.324971429	0.269947215
PF11.24	8	0.057860714	0.220568513	PF11.24	8	0.15207619	0.249646258
PF11.25	8	0.319557143	0.368728612	PF11.25	6	0.21354	0.384206371
PF11.29	8	0.076489286	0.225417656	PF11.29	8	0.052714286	0.303828384
PF11.30	8	0.002814286	0.142937909	PF11.30	8	0.036142857	0.221945736

Table 5: Barcode sequences used for RADseq.

Library ID	Barcode
PF.pooled.07.2013	TCTCTCGA
NF.pooled.07.2013	ACGTAGCA
SD.pooled.07.2013	CATGATCA
OT.pooled.07.2013	GATCGTGA
NF.pooled.07.2013	TACGATAT
SD.pooled.07.2013	AGCTGTGA
OT.pooled.07.2013	CGCGCATA
V.pooled.07.2013	GCATGTGC
PF.pooled.07.2013	GACTGCAG
V.pooled.07.2013	CACACAGT
SD.pooled.07.2013	GCTACAGC
NF.pooled.07.2013	CTAGTGTC
V.pooled.07.2013	TCAGCATC
PF.pooled.07.2013	AGTCACGA
OT.pooled.07.2013	ACACGACA

R script: social marker filtering

```
# Script for social marker filtering

#Read the 1snp_nopair.fst.csv data table. Fst file from popoolation2 filtered for read1 seqs with 1 SNP

data<-read.table("1snp_nopair.fst.csv", header=T, sep=",",as.is=T)
dim(data) #22693    11

#check the data
data[1:10,]
data[338166:338176,]

#Calculate the quantiles. Quantiles used to set the thresholds

quantile(data$ot.v,seq(0,1,0.05))
quantile(data$ot.pf,seq(0,1,0.05))
quantile(data$ot.sd,seq(0,1,0.05))
quantile(data$v.pf,seq(0,1,0.05))
quantile(data$v.sd,seq(0,1,0.05))
quantile(data$pf.sd,seq(0,1,0.05))

#Prints the names and the summary data for each pairwise fst

for(i in 6:11){
  print(names(data[i]))
  print(summary(data[,i]))
}

# Filter for bottom 60% and top 95% quantile. FM vs FM and P vs P threshold= <60% and all FM vs P threshold= >95%

quantile95<-
which(data$ot.v<0.030101822&data$pf.sd<0.048189602&data$ot.sd>0.320815362&
data$ot.pf>0.390785330&data$v.pf>0.378682068&data$v.sd>0.308920774)
length(quantile95) #121
data[quantile95,]

#Write the white list of social marker fst

write.table(data[quantile95,], "quantile_95.txt", quote=F,row.names=F,col.names=T,
sep="\t")
```

Figures:

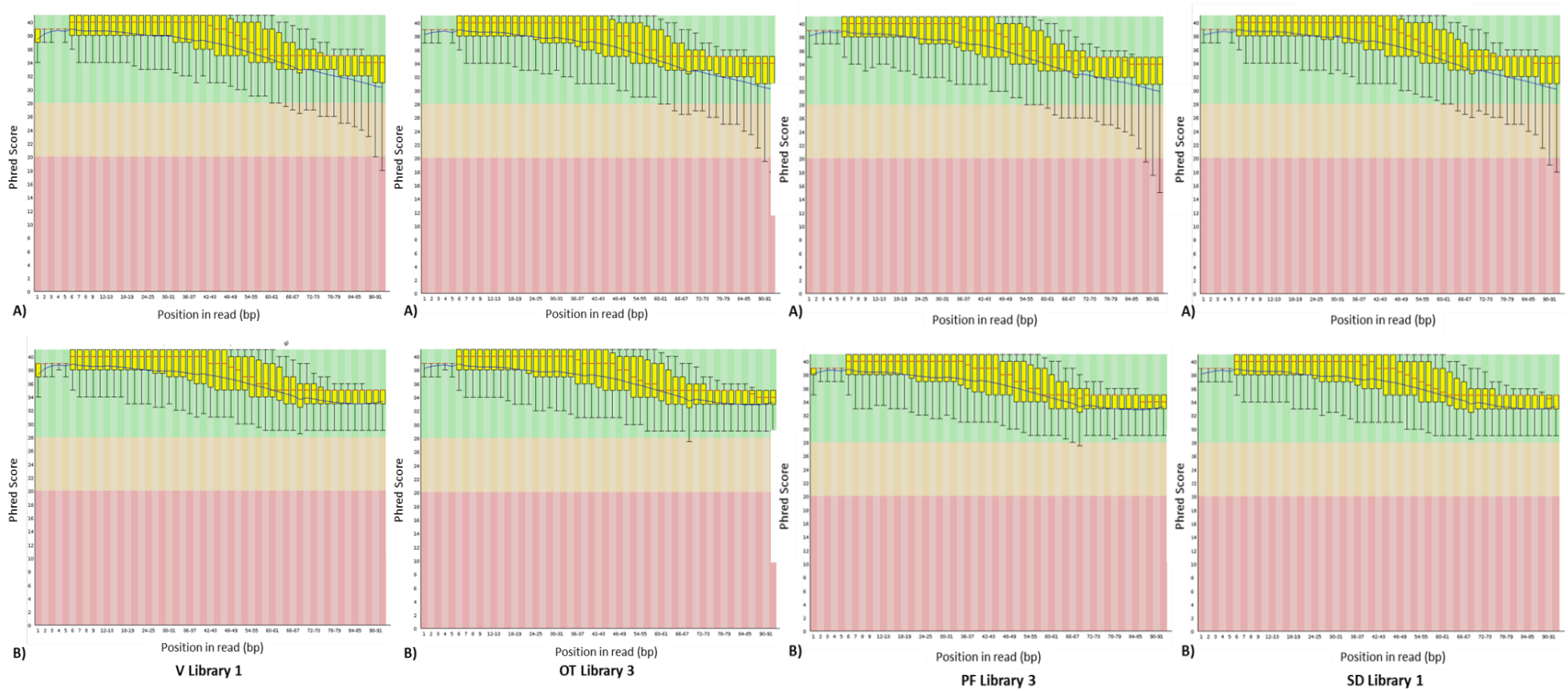

Fig 1: Average bp Phred score per sequence for each selected population library (Table 2). **A)** QC after de-multiplexing and PCR duplicate removal using STACKS. **B)** QC after low quality base trimming using FastX. Box plots show the median (red line) and 1st and 3rd quartiles (yellow box). The whiskers show the 10-90% quantile range. Green bands represent phred score 28-40. Orange bands represent phred score 20-28.

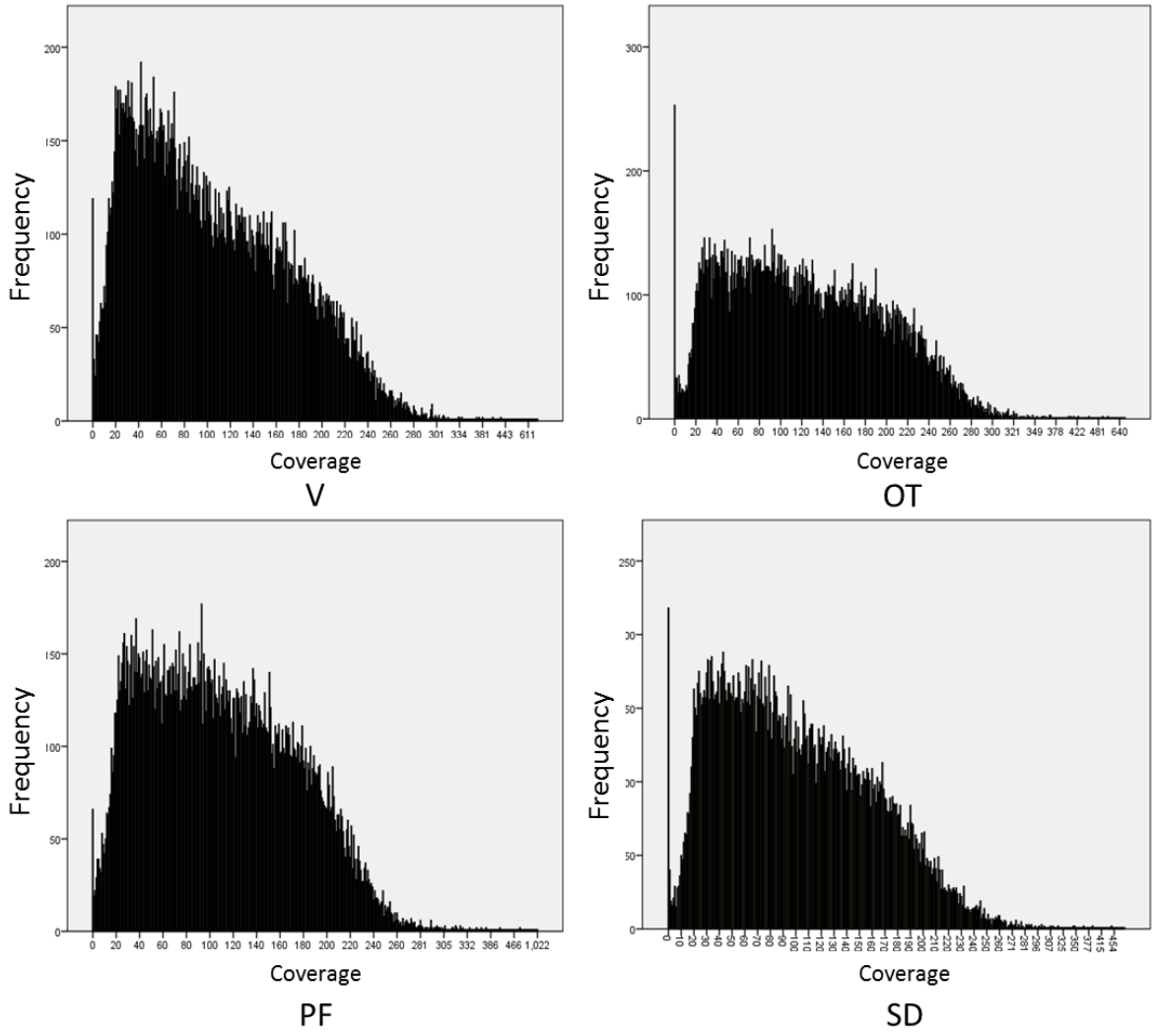

Fig 2: Frequency plots of RADtag coverage for each population. Each bar represents the number of RADtags (y axis) with x coverage (x axis). The majority of RAD tags had a coverage between 20x and 200x. 20x coverage was set to the minim threshold and 200x was set to the maximum threshold for SNP calling in Popoolation2 analysis.