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Polymorphic Social Organisation in a Eusocial Insect: The Ultimate and Proximate Causes

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ABSTRACT

A fundamental variable in cooperative breeding animal species is the degree to which reproduction is partitioned among group members - termed reproductive skew. Understanding the causes for variation in skew contributes to our understanding of social evolution because skew directly impacts on the inclusive fitness gained through cooperation. In this thesis I present a novel model system for investigating skew, by providing detailed sociogenetic data to show a polymorphism in colony social organisation in a species of ant, *Leptothorax acervorum*. In multiple queen colonies queens reproduce relatively evenly in most populations (polygyny), but I show that skew is particularly high in a Spanish and Japanese population where just one queen out of many monopolises all reproduction (functional monogyny). I further investigated how high skew among queens was maintained in the functionally monogynous Spanish population by undertaking behavioural observations and experiments. In contrast to what is assumed by the majority of skew theory - that control lies with individuals in direct competition over reproduction (queens) - I show that a third party (the workers) plays a principle role in determining which queen reproduces in the colony. Genetic analyses also revealed that workers favour the queen who meets their fitness interest, showing that workers posses both the information and power for their interests to prevail. Furthermore, such worker influence is not observed in polygynous colonies and tellingly multiple queens reproduce.

Functional monogyny maintains high relatedness and therefore high indirect fitness benefits among colony members, yet polygyny reduces such benefits because of multiple genetic lineages within the colony. Polygyny is therefore seemingly paradoxical

when only considering relatedness, so presumably other parameters are important. I compared life-history traits and ecological factors associated with each social organisation and discuss the potential importance of habitat patchiness, limited dispersal and queen turnover in shaping the marked contrast in skew between populations. Furthermore, I detected high levels of triploid females in the functionally monogynous populations supporting a high frequency of matched matings between sexuals at the complementary sex determination locus. Importantly, there have been no reports of triploidy in polygynous populations showing that variation in social organisation, along with associated life-history traits and ecological factors, can determine the frequency of matched matings and increase the risk of genetic load.

The research presented in this thesis overall highlights two important issues: first, the basic assumptions of skew theory must be tested if skew models are to be applicable, and the gap which has developed between skew theory and associated empirical testing needs to be bridged. Second, we cannot focus on relatedness alone to explain skew or test kin selection theory, because factors within an ecological parameter are also fundamental.

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Social evolution: importance of kin selection

Cooperation is a fundamental property of some of the major transitions in the evolution of life, from gene assemblages that replicate as one linked complex (chromosomes) to the cooperation of cells to form multicellular life (Maynard Smith & Szathmary 1995). A pinnacle of cooperation is found in cooperative breeding animals where non-clonal individuals forego their own reproduction to help rear the offspring of others. Generally this means that reproduction is partitioned unevenly among group members so reproduction is skewed between individuals (known as reproductive skew). Explaining skew in cooperative breeding groups is important because we need to understand why individuals are willing to give up their reproductive potential to help others at a direct cost to themselves.

Take the extreme behaviour of honey bee workers who generally forego reproduction to help rear offspring of the colony queen and are even willing to give up their life to protect the colony. This paradoxical behaviour appeared damning to Darwin's theory of selection on the individual, a behaviour which was "insuperable" to Darwin himself (1859). How can such altruistic behaviour be selected under Darwinian natural selection? For over a century there have been many attempts to address this question (e.g. A. R. Wallace, T. H. Huxley, P. Kropotkin, W. C. Allee, (see Dugatkin 2006)), some of which were on the verge of achieving the answer. However, it was the pioneering work of Hamilton (1964) who conceived and formulated the solution which subsequently revolutionised our understanding of the process of natural selection (see Dawkins 1976) and united the studies of evolutionary and behavioural ecology.

Hamilton proposed that an altruistic trait can be selected under Darwinian evolution (natural selection) if the actor gains an indirect fitness benefit through helping an individual who is genetically related. Consequently, by helping related individuals it ensures that shared genes, and importantly the gene for the altruistic trait, is propagated to the next generation. Hamilton showed that the coefficient of relatedness between individuals mediates the costs and benefits of cooperation; shown by Hamilton's rule:

$r \times b > c$ (r = relatedness between helper and recipient; b = benefit of cooperation to the recipient; c = cost to the cooperator)

Altruistic behaviour may be selected if the indirect benefit of helping another individual (b) outweighs the direct cost of the act (c) when accounting for the degree of relatedness between the helper and recipient *(r)*. In other words, helping individuals that are related, relative to the average genetic similarity among individuals in the population, provides an indirect fitness benefit. This rule was aptly named by Hamilton his 'inclusive fitness theory', which was later named 'kin selection theory' (Maynard Smith 1964).

To understand reproductive skew within a kin selective framework, we must understand exactly why the direct cost of cooperation (c) does not always outweigh the indirect benefit (rb), given that breeding solitarily provides a greater fitness payoff. Emlen (1982) showed that if ecological constraint limiting successful dispersal is high then remaining in the natal area (natal philopatry) and helping may be selected. Natal philopatry means group members are on average related (incl. sibs) therefore cooperation is then reinforced through high kin selected benefits. In support, over 30% of bird species

in the harsh arid zones of Australia and Africa cooperatively breed compared to only 5% in the northern temperate areas of Europe (Koenig & Dickinson 2004). The union of Hamilton's kin selection theory (1964) and Emlen's ecological constraint models (1982) has provided a theoretical framework that allows investigation into the genetic, ecological and social basis for the evolution of social organisation and behaviour.

The kin selective benefits promoting cooperation among group members is also a cause for conflict. Individuals increase their direct fitness by gaining a disproportionate amount of reproduction within the group, but this also affects the indirect fitness benefits gained by other cooperative individuals. Therefore there is not only conflict between individuals competing directly for reproduction, but also among other non-reproductive group members over which individual(s) should succeed. This is further complicated because reproductive skew is known to vary across cooperative breeding species. Subsequently, understanding the evolution of stable reproductive skew, whose interests prevail, and how conflict is resolved, is fundamentally important in testing kin selection theory and ultimately understanding social evolution (Vehrencamp 1983a; Keller & Reeve 1994, 1999).

CHAPTER 1

Evolutionary explanation for variation in reproductive skew

Animal societies vary in organisation and behaviour, and explaining the factors responsible for such variation are important goals of evolutionary biology and behavioural ecology. A fundamental aspect of animal social groups is how reproduction is shared among group members, termed reproductive skew. Understanding the factors underlying variation in skew is considered to be an important step towards a 'unifying theory' of social evolution (Keller & Reeve 1994; Sherman *et al.* 1995; Reeve *et al.* 1998; Crespi & Ragsdale 2000; Johnstone 2000; Reeve *et al.* 2000; Buston *et al.* 2007). In addition, investigating the conflict over skew and whose interests prevail allows tests of kin selection theory (Hamilton 1964) and a further understanding of the levels of selection on social traits (Keller 1999).

The spectrum for skew (Figure 1A) spans from one extreme where reproduction can be shared evenly among group members (low skew), as found in the African lion *Panthera leo* (Heinsohn & Packer 1995), groove-billed Anis *Crotophaga sulcirostris* (Vehrencamp 1977), acorn woodpecker *Melanerpes formicivorus* (Haydock & Koenig 2002), and banded mongoose *Mungos mungo* (De Luca & Ginsberg 2001), to the opposite extreme where reproduction can be monopolised by one or a minority of group member(s) (high skew), as found in the meerkat *Surricatta surricatta* (Clutton-Brock *et al.* 2001b), naked mole-rat *Heterocephalus glaber* (Sherman *et al.* 1991), dwarf mongoose *Helogale parvula* (Creel & Waser 1991) and superb fairy-wren *Malurus cyaneus* (see Cockburn 1998).

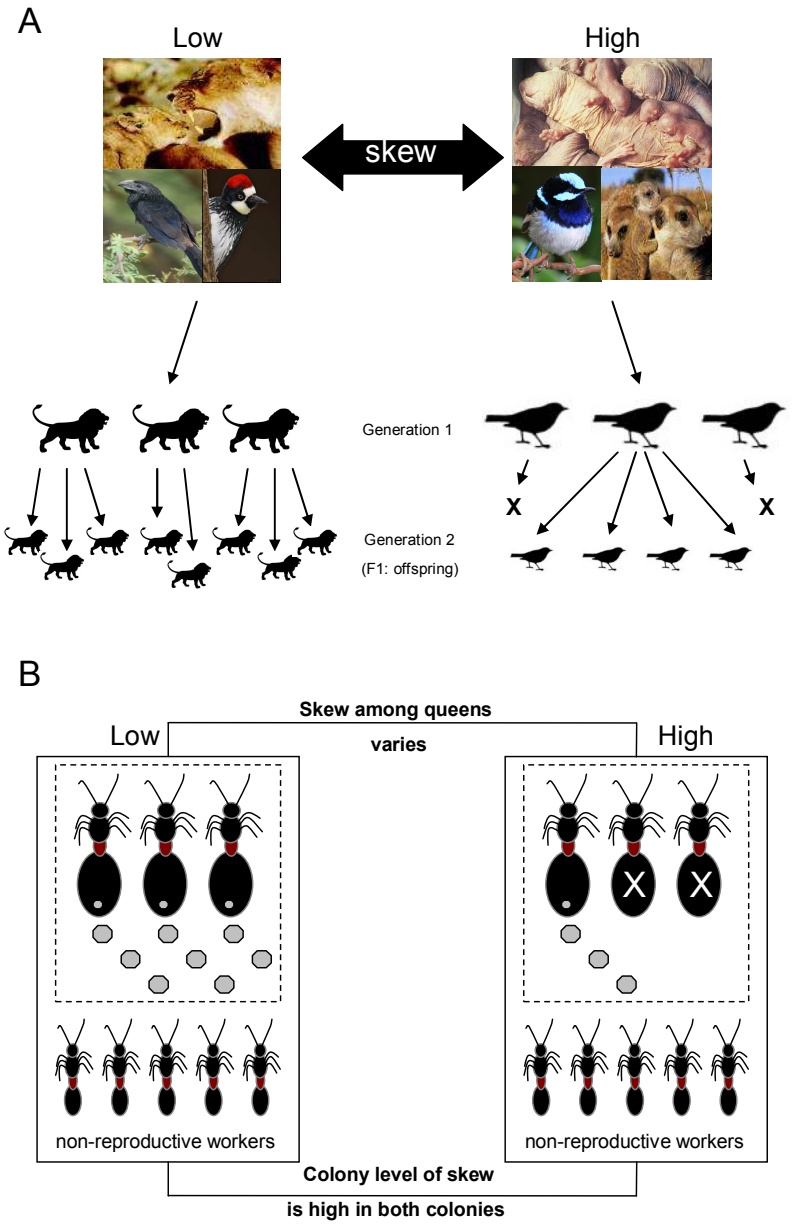

Figure 1. A) Variation in reproductive skew among group members where all members have the potential to breed (e.g. cooperative breeding bird and mammal species). **B)** Variation in skew in the eusocial Hymenoptera, where skew is high at the colony level as queens reproduce but workers generally do not, and variation in skew at the level among queens where reproduction can range from being monopolised by one queen or shared evenly among all queens.

In the social birds and mammals, all individuals have the capability to reproduce, but in the eusocial Hymenoptera (ants, bees & wasps) skew can be found at two primary levels. A distinguishing feature of the eusocial Hymenoptera is the presence of a reproductive caste where individuals are morphologically divided into reproductive queens and non-reproductive workers that rear offspring and protect the colony resulting in high skew at the colony level. A secondary level of skew can also be found in species with multiple queen colonies (Figure 1B), because skew can vary among queens (see Holldobler & Wilson 1990; Bourke & Franks 1995), and it is this secondary level of skew in the eusocial Hymenoptera which is to be the focus of the later chapters of this thesis.

In this introductory chapter I briefly discuss an evolutionary explanation for variation in skew including the development of a conceptual framework, and the subsequent expansion of skew modelling and associated empirical testing. Studies addressing skew have provided an important step in understanding the evolution of social strategies/traits, but in this chapter I highlight some of the fundamental problems with current skew theory, the potential for confusion, and how such problems can be addressed in future studies.

1.1 Skew theory

Skew theory attempts to explain the evolutionary stable sharing of reproduction in a social group, and is rooted within the pioneering works of Hamilton's inclusive fitness (kin selection) theory (1964) and Emlen's ecological constraints models (1982). Hamilton's rule states that the coefficient of relatedness between group members determines the indirect fitness benefits individuals can gain through cooperation (Hamilton 1964).

Therefore, in the context of skew, relatedness should play an important role in determining how reproduction is shared among individuals, because this impacts on the indirect fitness of other group members. Ecological constraints directly affect an individual's success in dispersal and solitarily breeding (Emlen 1982), which consequently impacts on the direct costs and benefits incorporated within Hamilton's rule. Furthermore, high constraint promotes natal philopatry (remaining in the natal group/area) maintaining high relatedness among group members (kin selected benefits), and determines the level of potential conflict over skew.

The 'optimal skew model' developed by Vehrencamp (1983a), was originally designed to explain despotic (high skew) and egalitarian (low skew) cooperative bird systems. Relatedness and ecological constraints were the basic parameters incorporated within the framework, with group productivity as an additional parameter. Group productivity is considered to be an important factor, and describes the total productivity of the group in terms of offspring reared. For example, the reproductive success of any one individual may be lower when breeding solitarily than when in a group because of other benefits associated with group living. Group productivity may thus partially counteract a decrease in relatedness benefit when reproduction is shared among group members because rearing numerous less related individuals may be a better option than rearing few highly related individuals. The optimal skew model makes key assumptions over the control of skew, stating that in every cooperative breeding group a single dominant individual has control over skew and group membership, with other potential reproductive individuals, known as 'subordinates', having little or no control over group reproduction but in someway benefiting the group. Importantly, if subordinate inclusive fitness is higher when

leaving the group and breeding solitarily than that gained from the amount of reproduction achieved by remaining in the group, the subordinate will either depart the group or fight the dominant for a higher reproductive portion or the dominant position.

The development of the optimal skew model fuelled high interest in skew theory and subsequently led to a number of model extensions (see Keller & Reeve 1994; Reeve *et al.* 1998; Johnstone 2000; Buston *et al.* 2007). Below I discuss the assumptions and predictions of two primary models most often considered (transactional and tug-of-war), along with empirical support. I further go on to discuss another model, 'the majority rules model', which proposes a shift in perspective over control of skew in social groups. The majority rules model has received relatively little attention and empirical support, but in the later chapters of this thesis I show that the assumption of this model over the control of skew should not be overlooked.

1.1.1 Transactional models

Transactional models (concession and restraint models) assume that a dominant individual has control over group membership and that there is a form of 'social contract' between the dominant and subordinate over the partitioning of reproduction between themselves (see Reeve & Nonacs 1992; Reeve 2000).

Concession model

The premise of this model is that the dominant concedes a proportion of reproduction to the subordinate as an incentive to retain the subordinate in the group (Vehrencamp 1983a, b; Reeve 1991; Reeve *et al.* 1998; Johnstone *et al.* 1999), because the

subordinate is predicted to leave the group if breeding solitarily is more beneficial. The incentive is termed the 'staying incentive', and can also be considered as a bribe to stay in the group (Reeve & Keller 1997). Reeve & Ratnieks (1993) showed that fighting ability can also be important in influencing skew when considering concessions, for example, it may benefit the dominant to provide a 'peace incentive' in order to prevent any costly fighting. Therefore, variation in relative fighting ability will have an influence on this peace incentive and/or the decision of the dominant/subordinate to become involved in a fight. This may be particularly important in species that form dominance hierarchies through aggressive disputes, as described in species of birds (e.g. Koenig 1981b, a), mammals (e.g. Cowlishaw & Dunbar 1991; Ellis 1995; Drews 1996), and social insects (e.g. Heinze & Smith 1990; Heinze *et al.* 1997; Monnin & Peeters 1999). The dominant, however, faces a trade-off over the amount it is willing to allocate or 'give-up' in relation to the benefit it will receive from the subordinate staying. Skew is predicted to positively correlate with the degree of ecological constraints, productivity, and relatedness between the dominant and subordinate (see Table 1). This is because the dominant can provide a lower incentive to retain the subordinate in the group when there is high constraint (low success) on breeding solitarily, and the subordinate gains inclusive fitness benefits through high productivity and high relatedness with the dominant.

Restraint model

Like the concessions model, the restraint model assumes that the dominant controls membership of the group, but in contrast, subordinates are assumed to be able to control their own allocation of reproduction (Johnstone & Cant 1999). The subordinate must not

Table 1. Assumptions and predictions of three primary reproductive skew models (this table is an edited version of that previously published by Hammond *et al.* 2006).

	Transactional Models		Tug-of-war model	Majority rules model
	Concession model	**Restraint model**		
Main assumptions:	1. Dominant controls group membership	1. Dominant controls group membership	1. Limited control between dominant & subordinate	1. Colony members collectively control group membership
	2. Dominant controls skew	2. Subordinate controls skew	2. Gaining share of reproduction is costly	2. Colony members collectively determine skew
Predictions:				
1. Skew vs relatedness	Positive or negative	Negative	Negative or no correlation	Positive
2. Skew vs degree of ecological constraints	Positive	Negative	No correlation	Positive
3. Skew vs per capita productivity	Positive	Negative	No correlation	Positive
4. Relatedness vs group productivity	No correlation	No correlation	Positive	No correlation

attain more reproduction than the dominant is willing to tolerate, otherwise the dominant is predicted to evict the subordinate from the group. The subordinate must therefore strike a fine balance between selfishly increasing its own direct reproductive share and 'restraining' itself from taking too much, otherwise it risks being evicted from the group. The change in the assumption over dominant control of skew consequently leads to a contrast in predictions to those made by the concession model. Skew is predicted to be high under low ecological constraint, low group productivity, and low relatedness (see Table 1). This is because, eviction from the group is the only form of control a dominant has. Therefore, a subordinate is able to take a higher proportion of reproduction (low skew)

when the dominant gets a fitness return through being highly related to the subordinate and productivity is high, and under these circumstances the threat of eviction would be low when ecological constraint is high.

1.1.2 Support for transactional skew models

Evidence for the concession and restraint models is largely based on studies that have found support for some of the model(s) predictions rather than specifically identifying the presence of a transaction (social contract). For instance, support for the concessions model has been primarily based on the relationship with high group relatedness and high skew, such as found in the African lion (Packer *et al.* 1991), banded mongoose (De Luca & Ginsberg 2001), white-winged chough *Corcorax melanorhampos* (Heinsohn *et al.* 1999), Arabian babblers *Turdoides squamiceps* (Lundy *et al.* 1998) and social paper wasps *Polistes* (Reeve *et al.* 2000). Some studies have also found evidence to support the presence of both high relatedness and high ecological constraints in favouring high skew, for example in the pukekko (Jamieson 1997), white-fronted bee-eater *Merops bullockoides* (Emlen & Wrege 1988, 1991) and species of the ant genus Leptothorax (Bourke & Heinze 1994).

A few studies have provided evidence to suggest the presence of reproductive concessions by the dominant. In the social paper wasp *Polistes fuscatus*, skew among queens increased as the colony became more established (Reeve *et al.* 2000). This is thought to be because at the foundress stage rapid establishment of the colony is important for survival and therefore it pays for reproduction to be shared evenly (low skew), and raise group productivity. However, once the production of colony members becomes less

important the dominant is less inclined to concede reproduction and skew becomes high (see Reeve & Nonacs 1992; Gamboa & Stump 1996). This example shows that concessions provided by a dominant should correlate with the relative value of a subordinate. For example, in the dwarf mongoose older individuals are known to have a greater ability to disperse and successfully obtain reproductive status in a neighbouring group. As predicted by the concessions model, dominant individuals appeared to provide higher reproductive incentives to older members of the group than to younger ones (Creel & Creel 1991).

Support for the restraint model is relatively limited, but this is possibly due to problems with identifying control over restraint. However, a recent study showed that in the American crow *Corvus brachyrhynchos* the dominant breeders of the group did not appear to have any control over skew but did have the power to forcibly evict subordinate individuals (Townsend *et al.* 2009). The authors of this study, however, propose that their study provides greater support for a synthetic approach of explaining skew incorporating both a restraint and tug-of-war model (Johnstone 2000), because competition over reproduction often occurred before individuals were potentially evicted (i.e. within a window of selfishness (Reeve 2000)). Investigation into the foundress stage of the social paper wasp *Polistes dominulus*, found that dominant individuals appear to regulate their aggression towards subordinates depending on the amount of resource (food sharing) taken by the subordinate (Tibbetts & Reeve 2000). This suggests that the dominant is able to punish the subordinate if it takes too much, but the dominant does not necessarily have direct control. This supports the assumption of the restraint model for within group conflict, but does not directly show conflict over reproduction. There are a number of

other studies supporting the action of subordinate self restraint (e.g. Wasser & Barash 1983; Snowdon 1996; Clarke *et al.* 2001; Arruda *et al.* 2005; Saltzman *et al.* 2009), but the underlying reason for such self restraint is often unclear.

1.1.3 Tug-of-war model

This model (Reeve *et al.* 1998) does not assume that there is a reproductive transaction between the dominant and subordinate. The tug-of-war model states that there is a power struggle over reproduction, referred to as 'incomplete control'. The degree of reproduction the dominant and subordinate gain is dependent on the ability of each individual to outcompete each other, with the dominant being able to incur the least cost and hence gain the highest reproductive proportion. However, the tug-of-war over reproduction is detrimental to group productivity as competition uses resources which could be allocated to reproduction, and could potentially destabilise the group. The tug-of-war model makes few directional predictions because the basic skew parameters are not considered to directly determine the outcome of the tug-of-war between individuals, although high relatedness between individuals should prevent escalation of too costly competition and may promote lower skew (see Table 1).

1.1.4 Support for the tug-of-war model

The evidence to support the tug-of-war model is often based on the inability to find direct dominant control, rather than support for a power struggle *per se*. For example, studies of the meerkat *Suricatta suricatta* (Clutton-Brock *et al.* 2001b) showed that subordinates often breed because dominants appear unable to enforce full control (as also

suggested in the mountain gorilla (Bradley *et al.* 2005)). Dominant female meerkats are known to be aggressive towards subordinates and even temporarily evict them in order to prevent subordinate reproduction (Clutton-Brock *et al.* 1998; Young *et al.* 2006). However, dominants are known to be at their most vulnerable in the three-months after usurping the previous dominant. Accordingly, subordinates were found to increase their reproduction during this time, suggesting that dominants have incomplete control (Young *et al.* 2006). In the cooperative breeding cichlid *Neolamprologus pulcher* (see Taborsky & Limberger 1981; Balshine *et al.* 2001), dominant individuals are known to suppress subordinate helper reproduction, but when breeding resources are sparse and size differences between dominants and subordinates is small, dominants are unable to fully control subordinate reproduction which suggests that a form of tug-of-war is present (Heg & Hamilton 2008).

The tug-of-war model makes few directional predictions between the basic parameters and skew, therefore, studies finding little relationship with skew have been considered to show support. A study of the social wasp *Polistes carolina* (Seppa *et al.* 2002), found skew did not appear to be particularly high or low, nor was there any correlation between relatedness or group productivity and skew, a result that has been similarly found in other Polistes species (e.g. Peters *et al.* 1995; Field *et al.* 1998a; Queller *et al.* 2000). A study on the social allodapine bee *Exoneura nigrescens* (Langer *et al.* 2004), was the first to directly manipulate all three of the main parameters that are thought to affect reproductive skew. Specific rearing experiments allowed control of relatedness between queens, the manipulation of availability of nest sites determined ecological constraints, and the availability of resources determined colony productivity. The study showed relatedness was negatively correlated with skew, but positively correlated with

group productivity, and there was no association with skew and ecological constraint or group productivity, all of which conforms to the general predictions of the tug-of-war model.

1.1.5 Majority rules model

The majority rules model (Reeve & Jeanne 2003) does not assume that skew is controlled by specific individuals, in fact, no single individual has the ability to determine its own reproductive share in the group. Instead, skew is determined by all group members as a 'collective genetic interest', where individuals gain a reproductive share dependent on their potential to maximise the fitness optima of all group members. For example, this can result in reproduction being disproportionately skewed towards a single individual perceived as the 'virtual dominant'. The majority rules model assumes that the virtual dominant is the individual that group members have the maximum average genetic relatedness to. The predictions of the majority rules mode are the same as the concessions models of skew because there is a 'virtual' dominant individual in control of skew, but each model makes contrasting assumptions over who is in control of skew in 'reality' (i.e. specific individuals (concessions) vs the collective (majority-rules)).

1.1.6 Support for majority rules model

The majority rules model has received relatively little attention. The predictions of the model are the same as found in the concession model, therefore, testing of the predictions alone cannot distinguish between models (discussed later). Thus, it is the assumption of either individual or collective control that should be investigated. Reeve &

Jeanne (2003), highlight that the collective interest of group members is particularly important in cooperative species with large groups, and provide two primary examples from the literature as support. In macaque species living in small groups reproduction is dominated by a single individual through physical aggression, but in macaque species living in large groups it appears that the dominant is the individual that shows an optimum relatedness to all group members (Dunbar 1988). The dominant queen in social paper wasps, which have relatively small colonies, does not always meet the interests of all colony members (Queller *et al.* 1997), but in the neotropical swarm founding wasp which have larger colonies, the dominant does appear to meet the interests of the majority of colony members (Jeanne 1991; Hastings *et al.* 1998).

The majority-rules model may be particularly applicable in species of the eusocial Hymenoptera because of generally large colony sizes and the presence of a reproductive caste system where non-reproductive workers may play a collective role. In small colonies queens may be able to play an important role in determining skew as there are possibly only few individuals (workers and other queens) who have a conflict of interest, whereas in large colonies the number of opposing individuals may be too many for a queen's selfish interests to prevail.

1.2 Limitations of current reproductive skew models

Contrary to the supporting evidence for skew models described above, there have been many empirical tests showing little or somewhat mixed support, raising questions over the validity of such models (e.g. Creel & Waser 1991; reviewed in Clutton-Brock 1998; Field *et al.* 1998a; Crespi & Ragsdale 2000; Magrath & Heinsohn 2000; Clutton-Brock *et al.* 2001b; Fournier & Keller 2001; Haydock & Koenig 2002; Seppa *et al.* 2002; Hannonen & Sundstrom 2003a; Fournier *et al.* 2004; Fanelli *et al.* 2005; Liebert *et al.* 2005b; Hammond *et al.* 2006; Heg *et al.* 2006; Liebert & Starks 2006; Nonacs *et al.* 2006; Buston & Zink 2009). This appears to be particularly evident in studies looking at the eusocial Hymenoptera, for example, Sumner *et al*. (2002) found that despite uniformly high skew throughout colonies of the tropical hover wasp *Liostenogaster flavolineata,* the parameters incorporated in skew models were highly variable. Similarly, Nonacs *et al.* (2004) found that aggression over skew was not correlated with relatedness, colony size, or group productivity in the social wasp *Polistes fuscatus*, as was also found in the Stenogastrine wasp *Parischnogaster mellyi* (Fanelli *et al.* 2008).

One problem is that empirical studies claiming support for skew models are often based on only one or some of the predictions being met, leading to speculative and suggestive conclusions rather than definitive ones. Furthermore, such studies often do not explicitly test how skew is controlled (see next section), for example, similar predictions can be made from different assumptions (discussed later). There is little contention that each parameter incorporated within the framework of skew is important in shaping social organisation: relatedness (e.g. Metcalf & Whitt 1977a, b; Strassmann 1989; Creel & Waser 1991; Packer *et al.* 1991; Bourke *et al.* 1997; Jamieson 1997; Reeve & Nonacs 1997; Field

et al. 1998a; Burda *et al.* 2000), ecological constraint (e.g. Koenig & Pitelka 1981; Rowley 1981; Komdeur 1992; Bourke & Heinze 1994; Peters *et al.* 1995; Jamieson 1997; Field *et al.* 1998b), and group productivity (Metcalf & Whitt 1977a, b; Vehrencamp 1983a, b; Reeve & Nonacs 1997). However, the problem with skew theory is that each model's fundamental assumptions, for example who controls skew or group membership, are often not fully understood. This is not helped by the mind-boggling array of models all of which have little data to support the assumptions made and make highly overlapping predictions (examples: value aggression (Reeve & Nonacs 1997), costly young (Cant & Johnstone 1999), synthetic models (e.g. Johnstone 2000; Buston & Zink 2009), subordinate manipulation (Crespi & Ragsdale 2000), N-person staying incentive (Reeve & Emlen 2000), bordered tug-of-war (Reeve & Shen 2006), clutch size adjustment (Hamilton & Heg 2007)). To progress further in our aim of a unifying theory of social evolution, the assumptions of skew models must be investigated.

1.2.1 Applicability of model assumptions

Dominant control and the interpretation of control

The most fundamental assumption of skew theory is that a dominant individual has the ability to control group membership and/or skew among group members. Although there is a relatively large amount of evidence to support control over group membership (e.g. birds: Reyer *et al.* 1986; Holekamp & Smale 1993; Mulder 1995; Clutton-Brock *et al.* 1998; Cant 2000; Cooney & Bennett 2000; mammals: Young *et al.* 2006), there is comparatively little evidence for control over skew (e.g. Abbott *et al.* 1997; Creel 2001; Faulkes & Bennett 2001; Young *et al.* 2006). Indeed, Clutton-Brock (1998) highlights that

the apparent provision of an incentive may actually be the inability or failure of a dominant to prevent/control a subordinate's reproduction ('limited control'). The implication that a dominant individual can issue concessions to others appears to have no definitive supporting evidence (e.g. Jeanne 1991; Queller *et al.* 1997; Hastings *et al.* 1998; Johnstone & Cant 1999; Crespi & Ragsdale 2000; Haydock & Koenig 2002; Nonacs *et al.* 2004). In fact, studies have shown that a lack of direct control over skew may be a common scenario, such as in the acorn woodpecker (Mumme *et al.* 1983; Koenig *et al.* 1995) and African painted hunting dogs *Lycaon pictus* (Malcolm & Marten 1982; Creel *et al.* 1997). In the meerkat, female subordinates have even been known to kill the dominant pairs pups (Young & Clutton-Brock 2006). Moreover, there appear to be few studies showing control over skew and group membership in the social insects (see Beekman & Ratnieks 2003; Ratnieks *et al.* 2006). Hannonen & Sundstrom (2003a) suggested that the assumptions of current reproductive skew models do not apply to multiple queen ant societies, due to a low threat of eviction and lack of a dominance hierarchy between queens/subordinates.

Skew models also rarely account for the influence of non-reproductive group members, or 'third party' (Reeve & Jeanne 2003), which is likely to be important in both the cooperative vertebrates and invertebrates. For instance, in social vertebrates many helpers-at-the-nest do not reproduce in any one season, but could potentially discriminate between reproductive individuals by biasing their help towards favoured individuals which in turn is likely to affect skew. Workers in eusocial insects generally do not reproduce, and can even be sterile, yet they are known to play a major role in a number of colony conflicts, such as sex ratio.

When testing skew theory, if assuming that specific individuals do have control over skew, we must determine correctly which individual this is, and their social status, as this underpins a major assumption of all skew models (see Kokko 2003). Dominant control may be hard to distinguish from subordinate self restraint. Are we able to identify whether subordinate reproduction is determined by a dominant providing a reproductive incentive, as the concession model states, or that the subordinate has directly taken a reproductive share against the dominants intention, as stated by the restraint and tug-of-war models? Unless this is known it is almost impossible to distinguish between such models and their subsequent predictions. In the meerkat (Clutton-Brock *et al.* 1999b; Clutton-Brock *et al.* 1999a; Clutton-Brock *et al.* 2001b) and banded mongoose (Creel & Waser 1991) subordinate reproduction correlates with age, but so does their ability to disperse and gain dominant status in another group. Therefore, does the dominant concede more reproduction to retain the subordinate because of her increased ability to successfully disperse, or could it be that subordinates have a greater ability to attain more reproduction directly through behaviours such as fighting or evading punishment? In addition, in social mammals dominant and subordinate positions are often dependent on age, yet skew models often do not account for dynamics in age structure. Furthermore, there is contention over whether forcible eviction or voluntary departure is a more evolutionary stable strategy because of the potential costs of competition involved (e.g. Reeve & Ratnieks 1993; Johnstone & Cant 1999; Buston *et al.* 2007; Buston & Zink 2009).

Interestingly, it seems that many skew models assume that dominant control is cost free, but this seems unlikely as competition for reproductive opportunities is predicted to use resources which could be invested in reproduction. Importantly, there is a lack of data

to show a quantitative measure of the direct costs to individuals when competing over skew (see Rubenstein & Shen 2009). The 'majority-rules' model of skew (Reeve & Jeanne 2003), explains that no single individual has the direct ability to control reproductive allocation but that group members as a collective determine skew. Therefore, this assumption may be highly relevant considering that the cost incurred by a single individual attempting to control skew should be significantly higher than that incurred per individual when acting as a collective control over skew (Beekman *et al.* 2003).

Benefits of subordinates

In the social birds and mammals, groups are relatively small and all members are potential breeders, therefore, we can presume that subordinate individuals are likely to be beneficial to the group (e.g. for territorial defence: Clutton-Brock *et al.* 1999b; Bradley *et al.* 2005; or rearing offspring: Komdeur 1996; Clutton-Brock *et al.* 2000; Clutton-Brock *et al.* 2001a). However, it is less clear regarding the actual benefits provided by subordinate individuals in species with large groups, as the per capita benefit provided by each subordinate should decrease as group size increases. In eusocial insect societies with multiple queen colonies, the benefit of a subordinate queen to the group may be small as the majority of work is undertaken by non-reproductive workers (but see Heinze & Oberstadt 2003), and in most species queens have been shown to do little work (see Holldobler & Wilson 1990). Therefore, in social insects as the number of possible reproductives (i.e. queens) increases, the benefit each queen (which would include subordinates) provides will decrease. Hence, the assumption that a subordinate benefits the group becomes less applicable in large groups. In addition, the value of subordinate

individuals may also be dependent not only on the number of potential queen reproductives but also the size of the workforce. For example, in species where colonies are founded via pleometrosis (multiple queens) each queen may be highly valued when there are no workers present (e.g. Reeve *et al.* 2000). In addition, little is known regarding the variation in benefit provided by different subordinates both in social vertebrates and invertebrates (see Heg *et al.* 2006).

Individual's ability to assess parameters

The most fundamental assumption of skew models is that individuals can assess the amount that reproduction is skewed (Vehrencamp 1983; Reeve & Ratnieks; but see Kokko 2003). Supportive evidence to show individuals have this ability is lacking, and it is possible that in many species the extent of this knowledge may be that individuals only have the ability to assess whether themselves and others are simply reproductive rather than a quantifiable assessment of skew. An understanding of the ability of individuals to assess ecological constraints and group productivity is also limited. Ecological constraint encompasses numerous variables which are arguably difficult to quantify especially in a dynamic environment (see Strassmann 1993; Magrath & Heinsohn 2000). We must be clear on the extent to which individuals can assess the constraint of the environment, in particular the ability to quantify specific variables and/or whether individuals act upon thresholds and the time span that such a decision is made over (Nonacs 2007). This is also applicable to us as researchers in regards to our own ability to truly assess the constraints of the environment (discussed later) as studies often identify only a subset of variables which are sometimes not even known to directly affect the study system in question (see

Magrath & Heinsohn 2000). Group productivity may be an easier parameter to assess, although this may become harder in large groups and those with multiple breeders. Similarly, we must understand more about how individuals react to the rate of productivity in conjunction with other variables and whether certain thresholds exist.

Do individuals have the ability to assess and quantify the coefficient relatedness between themselves and another individual? Can they distinguish between siblings and cousins, for example? Kin selection theory potentially favours a kin recognition mechanism, allowing kin discrimination among group members and hence nepotistic behaviour. In many bird and mammal societies individuals are known to discriminate in favour of kin and against non-kin which is considered to be a learnt trait. For example, long-tailed tits *Aegithalos caudatus* who fail during the breeding season are known to redirect their behaviour and help rear the offspring of neighbouring kin, by distinguishing between related and non related pairs based on individual's calls (Sharp *et al.* 2005). Kin discrimination also appears to be exhibited in the termite *Cryptotemes secundus* where limited food conditions favour interactions between related individuals (Korb 2006). There is also some evidence supporting a genetic basis for individual chemical profiles in social insects (Carlin & Holldobler 1986; Dronnet *et al.* 2006) which may be important for within colony kin recognition to occur. In other colonial organisms such as bacterial colonies (e.g. Griffin *et al.* 2004; Diggle *et al.* 2007) and slime moulds (Gadagkar & Bonner 1994; Kaushik *et al.* 2006) 'individuals' are known to aggregate preferentially with those descended from the same lineage.

However, many studies have shown a lack of evidence for intra-group kin discrimination, particularly in the social insects (Queller *et al.* 1990; Frumhoff 1991; Breed

et al. 1994; Balas & Adams 1996; Bernasconi & Keller 1996; DeHeer & Ross 1997; Strassmann *et al.* 1997; Solis *et al.* 1998; Tarpy *et al.* 2004; Holzer *et al.* 2006). This may be because the cost of making discrimination errors is too high for a kin discrimination mechanism to be selected. Therefore, individuals may show 'indiscriminate altruism' (Keller 1997), because the close integration of individuals and the occurrence of natal philopatry increases the likelihood of living, and hence cooperating with, closely related group members and so within colony kin discrimination may not be necessary (Ratnieks 2006).

In summary, a greater understanding of the ability of individuals to make a quantifiable estimate of skew and assess the general parameters incorporated in skew models is required. We must gain a better understanding of the potential response thresholds involved, and investigations into the potential behavioural 'rules of thumb' (Strassmann 1993; Reeve *et al.* 1998; Beekman *et al.* 2003; Kokko 2003; Hart & Ratnieks 2005).

1.2.2 Behavioural vs evolutionary time

Skew models rely on the ability of an individual to assess both the basic parameters and the strategic behaviour of other individuals over skew (mentioned above). A major concern of this assumption is that skew models are unclear on whether individual strategic responses, such as a subordinate staying or leaving the group, is in 'behavioural time' where individuals have 'perfect knowledge' (can directly assess parameters and respond immediately), or in 'evolutionary time' where individuals have 'imperfect knowledge' (cannot directly assess changes in the parameters) (Kokko 2003). In other words, do

individuals 'know' only the level of skew and parameters at the population level, and cannot respond in behavioural time if a change occurs at the group level? For instance, using an example proposed by Kokko (2003), if individuals have imperfect knowledge then the social contract or competitive response between the dominant and subordinate will be based on the average population level of skew. However, this is then susceptible to the invasion of a mutant strategy where a dominant concedes less, because the subordinate 'knows' only the population level of skew even though there is a change in skew at the group level. This can also be applicable to the average value of the three basic parameters even though there may be variation within the population. Consequently, selection should always favour a decrease in concessions over time (i.e. a selfish strategy), which would eventually lead to low dominant concession at the population level and therefore over time selection will favour departure of subordinates. If this is the case group cohesion is then jeopardised because stable groups cannot form under social transactions (Kokko 2003).

This leads me to conclude two possibilities, either: 1) individuals have imperfect knowledge of skew and the basic parameters in behavioural time, therefore reproductive transactions cannot exist; or 2) individuals have perfect knowledge and are able to directly assess and respond in behavioural time meaning that transactions are possible. Intriguingly, many population studies have found little variation in skew to explain (e.g. social insects: (Field *et al.* 1998a; Reeve *et al.* 2000; Fournier & Keller 2001; Seppa *et al.* 2002; Sumner *et al.* 2002; Hannonen & Sundstrom 2003a; Nonacs *et al.* 2004; Liebert & Starks 2006), and studies investigating changes in skew when experimentally manipulating skew model parameters have found little response (Langer *et al.* 2004; Heg *et al.* 2006), supporting hypothesis 1. In contrast, some studies on birds and mammals

have found that variation in relatedness between group members directly affects skew among dominant and subordinate individuals (e.g. Packer *et al.* 1991; Piper & Slater 1993; Keane *et al.* 1994; Whittingham *et al.* 1997). This may suggest that organisms such as birds and mammals are more able to directly assess parameters, in contrast to organisms such as social insects. However, we must further investigate the possibility of perfect knowledge in behavioural time if our understanding of stable skew is to progress.

1.2.3 Empirically assessing/quantifying parameters

A parameter such as ecological constraint may contain a multitude of variables each of which can be difficult to quantify when empirically testing skew models. In addition, different species experience a range of environmental variables all differing in influence and association to a particular species life-history (e.g. Arnold & Owens 1998; Hatchwell & Komdeur 2000; Pen & Weissing 2000; Kokko & Lundberg 2001; Kokko & Ekman 2002). Environmental variables may also not only differ between populations, but vary locally within populations perhaps even between neighbouring groups. This makes it increasingly difficult to accurately assess ecological constraint as a parameter (see Magrath & Heinsohn 2000). In addition, more knowledge is needed to assess how the fundamental variables that constitute the parameter ecological constraint, directly affect the non-genetic costs and benefits incorporated within Hamilton's rule (1964). Tests of reproductive skew models have often focused upon a parameter that is comparable over all species and is easily quantifiable; relatedness (e.g. Field *et al.* 1998a; Reeve *et al.* 2000; Langer *et al.* 2004; Langer *et al.* 2006; Nonacs *et al.* 2006). Relatedness, however, has been shown to be a non-independent parameter (Emlen 1996b; Magrath 1999; see Magrath & Heinsohn

2000). For example, high ecological constraints may promote natal philopatry, and consequently, maintain high group relatedness (Emlen 1982, 1995, 1996b; but see Kokko & Lundberg 2001). Furthermore, high skew in itself would maintain high relatedness and therefore is relatedness a cause or simply a consequence of skew, and importantly is there any way of distinguishing between the two? Both the competitive ability of a subordinate and the fertility (productivity) of an individual might co-vary with relatedness (Emlen 1996 a,b), and any apparent avoidance of inbreeding can also confound the assumed relationship of relatedness and skew (Emlen 1995; Koenig *et al.* 1998). Many variables considered independent in reproductive skew models may in fact be non-independent (Magrath & Heinsohn 2000).

1.2.4 Social queuing

The majority of skew models do not account for the potential of a future breeding component. If a subordinate has the opportunity to inherit or supersede the dominant in the future (e.g. Emlen 1991; Lucas *et al.* 1997), the benefits of remaining in the group and receiving no incentive may still outweigh those from leaving (Kokko & Johnstone 1999; Ragsdale 1999; Kokko & Lundberg 2001). This is an important consideration as any studies finding skew which appears to be higher than expected based on model predictions, might be because subordinates have a future breeding opportunity (Queller *et al.* 2000). For example, Sumner *et al.* (2002) proposed that their finding of high skew in colonies of the tropical hover wasp could not be explained by the concessions model unless it incorporated a future breeding component. In addition, social queuing for dominance rank is found in the mountain gorilla and is dependent on age (Bradley *et al.* 2005), therefore,

age may also be an important factor in determining skew among individuals and may need to be considered in skew models.

1.2.5 Two-individual scenario

The majority of skew models are based on a two-individual scenario; a dominant and a subordinate. However, in most cooperative societies there are often numerous individuals who have the potential to reproduce or establish a dominant position. Therefore, how relevant are skew models when considering multiple breeding individuals? This specific criticism of skew models is perhaps harsh as modelling using more than two individuals may be mathematically complex, but it may be of fundamental importance. Johnstone *et al.* (1999) showed that accounting for more than two individuals within a skew model can alter model predictions, because the relatedness among subordinates must be considered, and there is also the potential for other factors to vary among subordinates, such as their contribution to productivity, and fighting ability (Reeve & Emlen 2000; Haydock & Koenig 2003; Reeve & Jeanne 2003; Rubenstein & Shen 2009).

Questions remain: is dominant control over numerous subordinates a plausible scenario? Can individuals assess skew across so many individuals as many skew models assume? In addition, should models assume a two-individual scenario or potentially a two-party scenario (i.e. the principle reproductives and the helpers-at-the-nest / the queens and the workers)? This latter question returns to the perspective of minority vs majority, as mentioned previously in respect to the individual vs the collective.

1.2.6 Measure of reproductive skew and fertility of individuals

Testing of skew models requires empirically measuring skew among group members, but it is important that we first define how skew is measured, or more specifically what is measured. This can be illustrated by considering the reproductive system of the eusocial Hymenoptera and the relative value of offspring. The production of sexual offspring (queens and males) is likely to be more valuable in terms of individual lifetime fitness than the production of non-sexual offspring (workers). For example, in the fire ant *Solenopsis invicta* multiple queens that appeared to be equivalent in reproduction, in fact differed significantly in the number of sexuals each queen produced (Ross 1988). Skew among queens was therefore high when considering sexual production and low when considering reproduction as a whole. Similarly, in polygynous populations of the ant *Leptothorax acervorum*, queens were found to vary in the proportions of males produced (Hammond *et al.* 2006). Interestingly, the extreme division of labour exhibited in the eusocial Hymenoptera means that workers may have a direct influence over the caste fate of reared offspring, for instance workers can determine the fate of females developing into either queens or workers (Bourke & Ratnieks 1999; Hammond *et al.* 2002). A study on the ant *Formica fusca* conducted by Hannonen & Sundstrom (2003b), suggested selective manipulation of queen offspring by workers, although this may have been a consequence of egg viability (Holzer *et al.* 2006). Although social insects have been discussed, how skew is measured also has implications for the social birds and mammals. For example, although group members may seemingly produce equal numbers of offspring, the resources invested in each offspring may be unequally distributed, and those offspring receiving a higher investment may be more likely to both survive and gain a higher hierarchal position, and therefore gain a greater fitness advantage.

When investigating skew among potential reproductive colony members, we must know the fertility of each individual otherwise a measure of skew can be erroneous if all individuals are assumed to be potentially functional when they are not. In the cooperative birds and mammals, sexual maturity is likely to vary between species and may vary between cooperative groups and/or individuals. Skew may therefore be highly influenced by the fertility of individuals. A more extreme example of the effect of reproductive fertility on skew can be found in the eusocial Hymenoptera. In many species, the production (see chapter 5 for detail of the mechanisms and processes involved) of infertile diploid males and triploid females has been reported (see Cook 1993; Cook & Crozier 1995; Krieger *et al.* 1999; Liebert *et al.* 2004). Therefore, when considering skew among individuals we must consider both male and female fertility (Liebert *et al.* 2005a), particularly when significantly high frequencies of triploid females have been found (Krieger *et al.* 1999). An example would be that in a two queen scenario, if one of the queens is diploid (fertile) and one of the queens is triploid (infertile) then only one queen has the potential to be reproductive, but if this is not known, then observed high skew is an artefact.

1.2.7 Multiple models with overlapping predictions

The predictions of reproductive skew models should allow empirical tests to distinguish between multiple models, but this is difficult when they often make the same predictions (Magrath & Heinsohn 2000). The ease with which relatedness can be quantified suggests it is a good parameter to investigate and test skew models. However, relatedness is predicted to negatively correlate with skew by both transactional and tug-of-

war models. Consequently, empirical tests often do not support any single model creating potential confusion and a lack of explanatory power. To more accurately test skew models, assessment of further parameters may be required, but this brings us back to the problem previously discussed (section 1.2.1: assessment of parameters). Therefore, we again come back to the importance of testing the fundamental assumptions of skew models: who is in control?

1.3 Conclusion: future directions

The advances in skew theory are an important progression towards a fuller understanding of social evolution, but such theoretical studies should not lose sight of empirical testing. The aim of this thesis is not to strictly test skew models, but to address some of the key issues highlighted in this introductory chapter. I conclude by suggesting two primary directions I think research should progress in order to close the gap between theoretical and empirical studies and subsequently further our understanding surrounding the evolution of variation in skew.

First, we must critically evaluate the fundamental assumptions of skew models requiring knowledge at a proximate level. Indeed, the ultimate cause of variation in skew has received significantly higher attention than the proximate mechanisms regulating it, and a more proximate approach should be at the forefront in our attempts to understand variation in skew, but importantly should not be considered as a "competing approach" (West *et al*. 2007). In other words, understanding the proximate basis of skew variation will subsequently lead to an understanding of the ultimate basis and the two should be appropriately integrated (Saltzman *et al.* 2009). Fortunately, awareness of this gap has

recently increased (e.g. Magrath & Heinsohn 2000; Hannonen & Sundstrom 2002; Owens 2006), but we are far from understanding whether the implicit assumptions of skew models are truly valid. For instance, we still have a relatively limited understanding concerning control over skew and whether transactions among individuals even exist. To gain a significant insight into this, we must first empirically identify the 'potential' and 'actual' conflict over skew among group members, and importantly how such conflicts are resolved and whose interests prevail (see Ratnieks *et al.* 2006). The importance of such studies cannot be underestimated as they allow testing of a fundamental assumption of skew theory – who is in control of skew?

Example of conflict and its resolution over reproductive skew

Cooperative breeding systems harbour many conflicts, particularly over individual reproductive strategies. This is because in groups consisting of non-clonal members the optimal reproductive strategy for one individual may not meet the optimal fitness interest of another (Hamilton 1964). Indeed, in both social vertebrates and invertebrates there are often aggressive disputes over how reproduction should be skewed among group members (see Keller & Reeve 1994). The resolution of such conflict is consequently important as escalation can be costly because it impacts detrimentally on the reproductive output of the group which can begin to counteract the benefits of breeding cooperatively in the first place (i.e. the direct and indirect fitness benefits gained from cooperative breeding) (see Beekman *et al.* 2003).

The behavioural mechanisms by which conflicts are resolved are perhaps most widely documented in the eusocial Hymenoptera (see Beekman & Ratnieks 2003; Ratnieks *et al.* 2006). Such reproductive conflicts predominantly stem from both the close integration of non-clonal individuals and the haplodiploid reproductive system. Haplodiploidy means males are derived from unfertilised haploid eggs and females from fertilised diploid eggs. The importance of this can be illustrated when considering how the sex of offspring is skewed in favour of either sexual females or males (sex ratio). A queen has the ability to determine the sex of her eggs and so can manipulate the sex ratio to meet her own interest, whereas workers can attempt to manipulate sex ratio post queen reproduction leading to a balance of power between the two parties. For example, in species where queens are singly mated, queens are equally related to either offspring sex (0.5), yet her daughter workers are related to each other (0.75) by three times as much as their brothers (0.25). Therefore, there is conflict over the rearing of daughter queens and males, because the queen optimum female:male sex ratio is 1:1, which conflicts with the worker optimum which is 3:1. Trivers and Hare (1976) were the first to raise this point, suggesting that workers should bias the sex ratio towards females (also see Boomsma 1989; Boomsma & Grafen 1990). Indeed, empirical evidence has shown that workers are able to manipulate sex ratios by killing male eggs (e.g. Sundstrom *et al.* 1996; Mehdiabadi *et al.* 2003), or biasing the caste fate of female (diploid) eggs into queens (Hammond *et al.* 2002). Moreover, evidence has also shown that queens still hold significant power as queens are known to adjust the sex ratio of

eggs in an attempt to negate worker coercion (Passera *et al.* 2001; Rosset & Chapuisat 2006).

In many species of the eusocial Hymenoptera workers have lost the ability to mate but retained the ability to lay unfertilised haploid male eggs. Subsequently, workers are more related to their own sons (0.5) than they are to their brothers (0.25), therefore, workers can gain a fitness advantage by producing males and replacing queen sons. However, this does not meet the interest of the queen as she is more related to her own sons (0.5) than to worker sons (0.25). Queens are thus predicted to police worker reproduction ('queen policing'; Ratnieks 1988), of which has empirical support (see Ratnieks *et al.* 2006). An additional complexity may be found in species where the queen is multiply mated. In such colonies, cohorts of workers (those sharing the same patriline) are more related to queen's sons (0.25) than they are to the sons of other worker cohorts (av. <0.25). Therefore, workers are predicted to police other selfish worker reproduction ('worker policing'; Ratnieks 1988). Evidence for this has been found in a multitude of eusocial Hymenopteran species (see Ratnieks *et al.* 2006). Furthermore, reproductive workers are known to carry out little work (Wenseleers *et al.* 2004a), therefore if worker reproduction persists it may negatively impact on group productivity causing a "tragedy of the commons" scenario and it is known that in species that have effective policing behaviour workers should show a form of self restraint (Wenseleers *et al.* 2004b).

Conflict over skew in the eusocial Hymenoptera can also be similar to that found in social vertebrates; queens can compete directly among each other over reproductive shares. As previously discussed, skew theory suggests that such conflict is resolved through reproductive transactions (social contracts), or a compromise (tug of war), yet there is little evidence to support this. We therefore return to the problem that evidence to support a fundamental assumption of skew theory – that control over skew is among only the individuals that are involved in direct reproduction - is lacking. Non-reproductive parties (i.e. workers in eusocial insects, or helpers-at-the-nest in social vertebrates) are not considered even though there is ample evidence (for example that described above) that non-reproductive parties (i.e. workers) can be influential.

It is also important that we understand what information a 'decision' or response is based upon (see Nonacs 2007). We also know little regarding the direct costs of control over skew, in addition to the direct and indirect benefits subordinates provide, and this should be investigated. Furthermore, skew theory predictions are often based upon a two-individual scenario, but this is likely to be rare in nature. In addition, the influence of a non-reproductive third party is often neglected, something that is particularly relevant when considering the worker caste in social insects. A third party may play a primary role because their interests must be met if the group is to remain stable. Studies are also needed to identify whether members of a social group have the ability to assess both skew

and the basic parameters incorporated within the skew framework, and their response over behavioural time (perfect knowledge hypothesis, Kokko 2003).

Secondly, a deeper understanding of the ecological variables that constitute constraint on dispersal and solitary breeding is required, in addition to investigating the importance of group productivity on skew. We must avoid focusing too heavily on relatedness alone in explaining stable skew as it is not the sole component of Hamilton's rule (1964), the factors affecting the direct costs and benefits of cooperation must also be accounted for (Herbers 2009). We must also identify suitable study systems to investigate the underlying factors and mechanisms determining variation in skew. Unlike many studies that have either found little variation within a population to explain (e.g. Field *et al.* 1998a; Reeve *et al.* 2000; Fournier & Keller 2001; Seppa *et al.* 2002; Hannonen & Sundstrom 2003a; Nonacs *et al.* 2004; Liebert & Starks 2006), or have carried out cross-species comparisons (e.g. Bourke & Heinze 1994; Kutsukake & Nunn 2006), a system is needed which exhibits both high variation in skew, and that does not suffer from large inter-species differences in life-history and ecology, and where the confounding effects of phylogeny can be controlled (Magrath & Heinsohn 2000).

Image references *(taken from websites).*

Acorn woodpecker: www.ejphoto.com
African lion: www.saviodsilva.net
Groove-billed anis: www.gilquintanilla.net
Meerkats: sciencetrio.wordpress.com
Naked mole rats: www.popsci.com (popular science website)
Superb fairy wren: u1.ipernity.com

CHAPTER 2

Polymorphic social organisation in the ant

Leptothorax acervorum

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2.1 INTRODUCTION

A common misconception of social organisation in the eusocial Hymenoptera (the ants, bees and wasps) is that all species have colonies headed by a single queen. There are in fact many species with colonies containing multiple queens which each have the potential to reproduce (see Holldobler & Wilson 1990). Multiple queen colonies have become popular models to test skew theory, because skew can be highly variable among queens (e.g. Keller & Reeve 1994; Reeve *et al.* 1998; Bourke 2001; Reeve & Keller 2001; Hammond *et al.* 2006). Skew among queens can be shared relatively evenly (low skew), a situation known as polygyny - a particularly common social organisation among ant species (Bourke & Franks 1995). In contrast, one queen out of many can monopolise all reproduction (high skew), a situation known as functional monogyny (Buschinger 1968) - a rare social organisation reported in just a handful of ant species: *Formicoxenus hirticornis* (Buschinger 1979); *F. nitidulus* (Buschinger & Winter 1976); *F. provancheri* (Buschinger 1980; Heinze *et al.* 1993); *Leptothorax gredleri* (see Heinze *et al.* 1992;

Lipski *et al.* 1992); *L. species* A (see Heinze & Buschinger 1989; Heinze & Smith 1990) and *L. sphaginocolus* (see Buschinger & Francoeur 1991).

Functional monogyny has also been reported in multiple queen colonies of the ant *Leptothorax acervorum* (Ito 1990; Seppa *et al.* 1995; Felke & Buschinger 1999). This is intriguing as studies of UK and central European populations show multiple queen colonies to be polygynous based on strong and comprehensive evidence, including data on egg maternity (Hammond *et al.* 2006), low nestmate relatedness (Douwes *et al.* 1987; Stille *et al.* 1991; Chan & Bourke 1994; Heinze *et al.* 1995a; Heinze *et al.* 1995b; Bourke *et al.* 1997; Hammond *et al.* 2001), queen ovary development, and behaviour (Buschinger 1968; Bourke 1991, 1993; Heinze *et al.* 1995b). By contrast the evidence for functional monogyny is much weaker. Ovary dissections and observations of colonies suggest that a single queen is reproductive in multiple queen colonies from a population in Spain (Felke & Buschinger 1999), and that reproduction is biased towards one queen in multiple queen colonies from Japan (Ito 1990). In addition, two genetic studies using allozyme data revealed high relatedness among workers in populations from Finland (Seppa *et al.* 1995) and Spain (Heinze *et al.* 1995a), but crucially queen number and mating status in the studied colonies was unknown. Furthermore, according to Buschinger, functional monogyny is the persistent coexistence of mated queens with only a single queen actively reproducing, which may be confused with monogyny in which multiple queens are present only short-term due possibly to overwintering (hibernation), or pleometrosis (mated queens founding a new colony) (Heinze & Buschinger 1988). There is a lack of data to show that functional monogyny is a stable social organisation in

L. acervorum, and there is a further question over whether these populations are a different species (Felke & Buschinger 1999).

Altogether the above studies tentatively suggest that *L. acervorum* may exhibit a marked polymorphism in social organisation, with low skew in some populations because queens share reproduction equally (polygyny), and high skew in others because reproduction is monopolized by a single queen (functional monogyny). The identification of such a polymorphism would be very interesting because it would have important implications for our understanding of the evolution of social organisation and the genetic underpinning of such variation, but more solid data supporting functional monogyny are needed. Furthermore, a polymorphism in such an important aspect of social organisation as reproductive skew has not been described in any other animal species.

This chapter presents data on the social organisation of the potentially functionally monogynous population from Spain (Felke & Buschinger 1999). A detailed genetic analysis of colony kin structure was undertaken to gain information on the number of reproductive queens, queen turnover, queen re-adoption, and temporal stability of colony social organisation. In addition, colonies from a UK polygynous population and colonies from the Spanish population were kept under identical laboratory conditions to investigate the environmental and genetic basis for the difference found in social organisation. Finally, the genetic relationships between populations described as polygynous and putatively functionally monogynous was investigated by comparing mitochondrial and nuclear DNA sequence data.

2.2 METHODS

2.2.1 Colony collection and maintenance

In the Spanish population of the ant *L. acervorum,* both single queen (SQ) and multiple queen (MQ) colonies can be found. Colonies were collected from a population (~1700m altitude) in Orihuela del Tremendal, Sierra de Albarracin, Spain, by Rob Hammond (RH) in 2004 (OT04), and by myself and RH in 2006 (OT06). In 2004 colonies were collected in June, before eclosion of sexual offspring, and in 2006 in October, after eclosion of sexual offspring and mating. Colonies were also collected from a known polygynous population in Sherwood Forest, UK in March and October 2007. To increase the geographical spread of populations sampled for investigation of the genetic relationship among populations, colonies were collected from an additional five populations (Table 1, Figure 1): Valdelinares, Spain (V); Solvorn, Norway (SO); Umea, Sweden (UM); Tvarminne, Finland (TV); and Vaasa, Finland (VN). Workers were also used from colonies from a previous collection in Santon Downham, UK (SD) (Hammond *et al.* 2006).

Table 1. Total number of SQ, MQ and queenless (XQ) colonies collected from seven European *L. acervorum* populations in 2006 and 2007.

Collection	Date	No. colonies	SQ	MQ	XQ
OT04, Spain	Jun'04	**74**	31	40	3
OT06, Spain	17-18th Oct'06	**89**	28	60	1
V, Spain	19th Oct'06	**11**	2	9	0
SF, UK	20th Mar'07	**16**	7	7	2
SO, Norway	30th May'07	**8**	3	3	2
UM, Sweden	2nd Jun'07	**13**	8	2	2
VN, Finland	4th Jun'07	**18**	9	8	1
TV, Finland	5-6th Jun'07	**15**	5	4	6

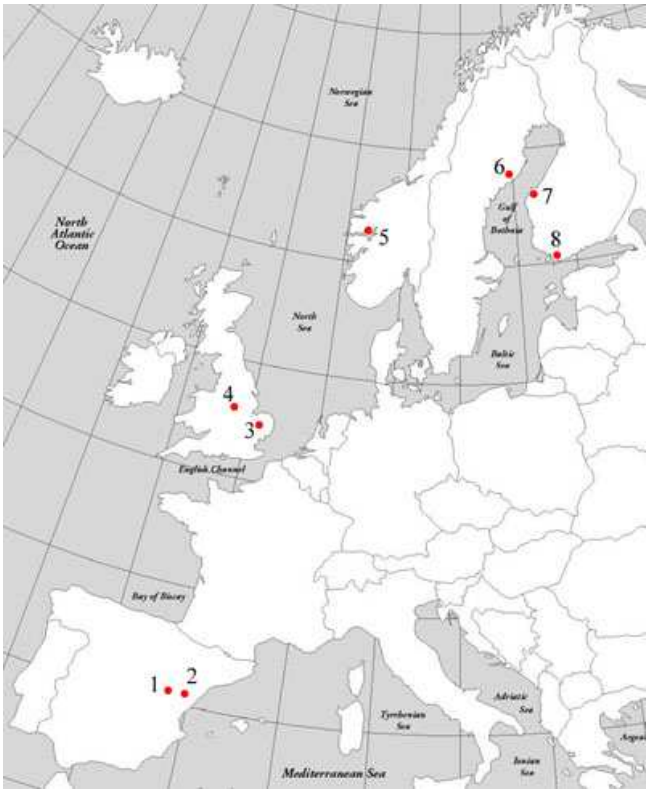


Figure 1. Map showing the locations of eight European *L. acervorum* populations where colonies were collected. **1.** Orihuela del Tremendal, Sierra de Albarracin, Spain (OT); **2.** Valdelinares, Sierra de Gudar, Spain (V); **3.** Santon Downham, Thetford Forest, Norfolk, UK (SD); **4.** Sherwood Forest, Nottinghamshire, UK (SF); **5.** Solvorn, Norway (SO); **6.** Umea, Sweden (UM); **7.** Vaasa, Finland (VN); **8.** Tvarminne; Finland (TV).

Colonies were found in cavities within partially decayed twigs on the ground of coniferous forests (Figure 2), and removed from twigs within five days. As whole twigs were collected, it was likely that all queens and the vast majority of workers were collected. Scandinavian (SO, UM, TV, VN) colonies were stored in 75% ethanol for later genetic analysis. Spanish (OT) and UK (SF) colonies were transferred to laboratory nests, censused (Table 2), kept in identical conditions in environmental chambers (Sanyo

MLR-351H) and fed chopped meal worms and dilute honey solution two to three times per week. OT and SF colonies collected in October were kept in autumn conditions (light/dark:14h/10h, temp.: 20/10ºC, humidity: 80/70%) for eight weeks, winter conditions (light/dark:13h/11h, temp.:10/0ºC, humidity: 60%) for six weeks, then transferred to spring conditions (light/dark:14h/10h, temp.:20/10ºC, humidity: 80/70%) for 8 weeks (A. Buschinger pers. comm.). SF colonies collected in March had subsequently overwintered in the field. During spring, colonies (OT: n=44, SF (October): n=5, SF (March): n=9) were monitored to determine the number of queens showing reproductive activity once egg-laying began. Reproductive queens were classified as having an enlarged (physogastric) abdomen (Figure 3) and occupying a central position among nestmates.

Figure 2. *L. acervorum* nests are found in cavities in partially decayed twigs (arrow points to colony individuals within the nest).

Figure 3. *L. acervorum* queen with enlarged (physogastric) abdomen [the scale bar represents 1mm].

Table 2. Queen and worker number in SQ and MQ colonies collected from the OT population.

	No. colonies collected	Total queens	Mean ± s.e. per colony	Total workers	Mean ± s.e. per colony	No. colonies genotyped
OT04 collection						
SQ	31	31	1.0	592	19.1 ± 2.98	6
MQ	40	245	6.1 ± 0.84	1675	41.9 ± 4.31	13
Total:	**71**	**276**	-	**2267**	-	**19**
OT06 collection						
SQ	28	28	1.0	1991	71.1 ± 9.37	21
MQ	60	551	9.2 ± 0.99	6335	105.6 ± 8.42	53
Total:	**88**	**579**	-	**8326**	-	**74**

2.2.2 Colony sampling

From the OT04 collection, four workers from each of 19 colonies (13 MQ, six SQ) were removed and frozen (-20°C). In the OT samples, MQ colonies were classed as those with multiple dealate (wingless) queens (from hereon: 'queens'). From the OT06 collection, 15 colonies (11 MQ, four SQ) were randomly selected and frozen immediately after removal from the twig to provide a snap-shot of colony social structure upon collection (referred to as 'snap-shot' colonies). From the remaining OT06 colonies a sample of workers (range=4-12 per colony) and larvae (range=3-8 per colony) were removed and frozen (-20ºC) from 60 colonies (42 MQ, 17 SQ, one queenless) for genetic analysis of colony social structure. Larvae were categorised as being small (1st instar to half grown larvae) or large (fully grown larvae to pre-pupae). To investigate genetic relationships among populations using mtDNA and nDNA one worker per colony was sampled from eight populations (colonies sampled per population, mtDNA: OT=7, V=6, SF=8, SD=7, SO=5, UM=5, VN=6, TV=3; nDNA: OT=7, V=3, SF=6, VN=3, TV=2).

2.2.3 Dissection

In the 11 snap-shot MQ colonies, the ovaries of all dealate queens were dissected (n=81 queens, range=2-16 per colony). Mated queens had an opaque spermatheca (sperm filled) whereas unmated queens had a transparent spermatheca. I classified ovarian development into: A = elongated ovarioles each with large yolk-filled eggs and large numbers of corpora lutea; B = shorter ovarioles with <5 yolk-filled eggs and some corpora lutea; C = short ovarioles, small eggs and no corpora lutea; and D = very short

ovarioles with no yolky eggs and no corpora lutea. The length of ovaries was scored relative to the size of the spermatheca (Figure 4).

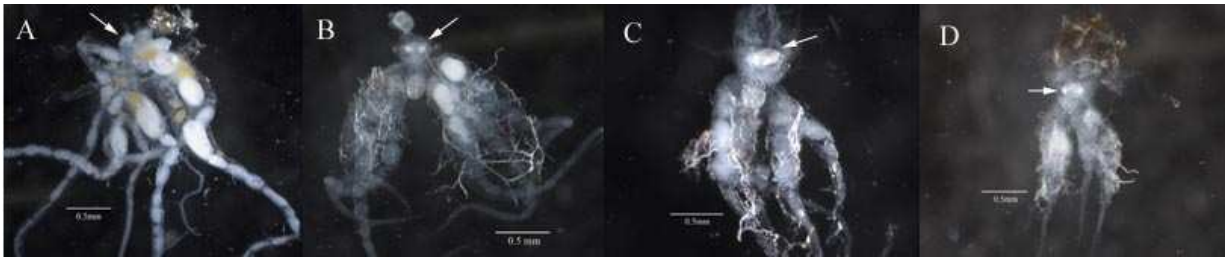


Figure 4. Examples of queen ovarian development classes. The type A, C and D panels show ovary development of queens from a MQ colony from the Spanish OT population. The type B panel shows ovarian development of a queen from a MQ colony from the Finnish TV population. White arrows point to the spermatheca, and scale bars show 0.5mm.

2.2.4 Genetic Analysis

DNA was extracted by grinding each individual in 200µl (queens, workers, and large larvae) or 50µl (small larvae) of 10% Chelex solution (10mM Tris-HCl, pH 7.5) followed by heating for 10mins at 100ºC.

Microsatellite genotyping

Individuals were genotyped at three (OT04, not LXAGA2) or four (OT06) polymorphic microsatellite loci: LXAGT1, LXAGA1, LXAGA2 (Bourke *et al.* 1997), and L18 (Foitzik *et al.* 1997) with allele sizes determined by reference to an internal standard (GenomeLab standard-400) using a Beckman Coulter CEQ 8000. PCR

conditions for each loci were as follows: for all loci there was an initial denaturation step of 95ºC for 5mins. This was followed by: L18 – 30 cycles of 94 for 20secs; 50 for 30secs; 72 for 1min. LXAGA1 - 30 cycles of 94 for 20secs; 58 for 30secs; 72 for 30 secs. LXAGA2 - 30 cycles of 94 for 20secs; 52 for 20secs; 72 for 1min; ending with a final extension step of 72ºC for 45mins. LXAGT1 - 27 cycles of 94 for 20secs; 52 for 20secs; 72 for 1min.

Only individuals genotyped at two or more (OT04 cols) and three or more (OT06 cols) loci were analysed (OT04/OT06: 100/86% of individuals; mean number of loci per individual=2.65/3.85). From OT04, four workers per colony from 19 colonies were genotyped. From OT06, individuals from 75 colonies (53 MQ, 21 SQ, one queenless) were genotyped, with an average of 7.3 workers per colony (n=70 colonies; range=3-11) and 5.0 larvae per colony (n=55 colonies, range=1-12). In the majority of colonies (50/75) both workers and larvae were genotyped. Larval sex was determined by ploidy, with individuals having one allele at all genotyped loci classified as male. The likelihood of misclassifying diploids as haploids was low as only 1.4% of diploids (workers: n=511) were homozygous at three loci, and none were homozygous at four loci. Seventy-two percent of larvae were diploid (n=54 colonies; diploids: mean=3.6 per colony; range=0–11; haploids: mean=1.4 per colony; range=0-6). From the 11 snap-shot MQ colonies, all queens were genotyped (n=81; mean=7.4 queens per colony; range=2–16).

Sibship, relatedness analysis, and queen turnover

The sibship of all workers and larvae genotyped from OT06 colonies (n=75) was investigated using the program COLONY (Wang 2004) which grouped individuals into

fullsib families assuming that queens mate singly (Hammond *et al.* 2001). The level of allelic dropout and genotyping errors was set to 0.05 for each locus. The predicted maternal genotypes (PMG) generated by COLONY were checked to see if they matched observed queen genotypes for each fullsib family in the 11 snap-shot MQ colonies.

Regression relatedness (Queller & Goodnight 1989) between various parties from OT colonies was calculated using the program Relatedness 5.08 (available from: http://www.gsoftnet.us/GSoft.html). Population allele frequencies were estimated with individuals weighted equally and allele frequency bias corrected by colony, and standard errors were estimated by jackknifing over colonies. Relatedness estimates were not always normally distributed (Kolmogorov-Smirnov test), therefore, estimates were analysed using Mann-Whitney U tests, and the statistical significance between relatedness estimates and expected point values was tested by seeing if expected point values fell outside 95% confidence limits.

From OT06 colonies, queen turnover was estimated by comparing relatedness within and between small diploid larvae and adult workers using equation four in Pedersen & Boomsma (1999).

$$QT = 1 - \frac{r_{W \leftrightarrow L}}{(r_W + r_L) - r_{W \leftrightarrow L}}$$

QT	Queen turnover
r	Relatedness coefficient
W	Worker (old cohort)
L	Larvae (new cohort)
W ↔ L	Relatedness between workers and larvae

Only colonies (21 MQ, eight SQ) with multiple small diploid larvae (MQ: mean=3.5 per colony; range=2-7; SQ: mean=4.0 per colony; range=2-6) and multiple workers (MQ: mean: 7.5 per colony; range=4-10; SQ: mean=7.5 per colony; range=6-8) were used to estimate queen turnover.

Genetic relationship among populations

PCR amplification of a region of the mitochondrial cytochrome *b* gene (cyt*b*) was undertaken using primers CB1 and tRs (Simon *et al.* 1994). PCR conditions were an initial denaturation step of 95ºC for 5mins, followed by 10 'touchdown' cycles of: 94ºC for 20secs; 60-50ºC for 20secs (-1ºC per cycle); 72ºC for 90secs, followed by a further 25 cycles of: 94ºC for 20secs; 50ºC for 20secs; 72ºC for 90secs, ending with a final extension step of 72ºC for 10mins. In addition, PCR amplification of a region of the nuclear encoded cGMP-activated protein kinase gene (*foraging*) was undertaken using primers designed (courtesy of Thomas Mathers in 2008) from published sequences from the ant *Pogonomyrmex barbatus* (Ingram *et al.* 2005), Genbank: AY800387). POGO_for_RG2_F: 5'-TCC AAA AGT AAA TTT TCC GGT TTA-3', and, POGO_for_RG2_R: 5'-CAC TGA TAC CGC CTC TTT GA-3'. PCR conditions were an initial denaturation step of 95ºC for 3mins, followed by: 95ºC for 30secs, 62ºC for 15secs, 72ºC for 30secs, and a final step of 72ºC for 7mins (optimised by Thomas Mathers in 2008). Both mtDNA and nDNA PCR products were cleaned and sequenced in both directions using PCR amplification primers by a commercial company (Macrogen Inc., Korea, or SymBio Inc., USA).

Foraging sequence trace files were inspected for heterozygotes and sorted into alleles. As the majority of individuals in all populations were homozygous for *foraging* allele H1 (see results) other alleles (H2-H5) were inferred by subtracting the H1 allele. Sequences were aligned using ClustalW in MEGA 4.0 (Tamura *et al.* 2007). For mtDNA data, a Neighbour-Joining tree was constructed using MEGA 4.0 and the robustness of tree topology investigated using 1000 bootstrap re-samples of the data.

2.3 RESULTS

2.3.1 Dissection

Ninety six percent of queens in the 11 snap-shot MQ colonies were mated (70/73, eight undetermined because of dissection errors) with an average of 6.4 mated queens per colony (range=2-14). In all colonies, only one queen per colony had type A ovarian development and all such queens were mated. All remaining queens had either type C or D ovaries (none possessed type B ovaries).

2.3.2 Genetic analysis

Sibship

In all 75 colonies the majority of workers and larvae (range=50-100%) were fullsisters as they were assigned to the same fullsib family ('the majority fullsib family' from hereon). An average of 90% of workers and larvae per colony grouped into the majority fullsib family (Figure 5) with a mean of 1.5 fullsib families per colony (range=1-5). Importantly, there was no significant difference between MQ and SQ colonies in the proportion of workers and larvae assigned to the majority fullsib family (Figure 5: Fishers Exact Test: df=4; p=0.90) showing that MQ and SQ colonies have the same colony sib structure.

Table 3. Sibship analysis of the 11 snap-shot MQ colonies, showing the number of queens (Q), workers (W) and larvae (L) grouped into fullsib families. In 'fullsib family membership', numbers in brackets are the number of each type (e.g. L(4) = 4 larvae) and letter superscripts for queens (e.g. Q^A) show ovarian class (see text). 'Q^A genotype match' shows which family's predicted maternal genotype matches the observed Q^A genotype.

Colony	Number genotyped:			Fullsib family membership:					Q^A genotype match:
	Q	W	L	Majority fullsib family	2	3	4	5	
A08_1710	7	-	6	Q^C(2), Q^D(4), L(4)	**Q^A**	L(2)			Majority fullsib family
A09_1910	8	8	5	Q^C(2), Q^D(3), W(8), L(4)	**Q^A**, Q^C, Q^D	L			Family 3
A10_1910	2	8	2	Q^C, W(7), L(2)	**Q^A**	W			Majority fullsib family
A14_1910	4	8	5	Q^C, Q^D(2), W(8), L(5)	**Q^A**				Majority fullsib family
B02_1710	9	7	6	Q^C(4), Q^D(4), W(7), L(6)	**Q^A**				Majority fullsib family
B04_1910	9	8	5	Q^C(3), Q^D(4), W(8), L(5)	**Q^A**	Q^C			Majority fullsib family
B11_1810	11	7	6	Q^C(10), W(6), L(6)	**Q^A**	W			Majority fullsib family
B13_1710	7	-	6	Q^C, L(4)	**Q^A**, Q^C(2), Q^D, L	Q^D	Q^C	L	None
B17_1810	16	8	11	Q^C(2), Q^D(12), W(4), L(11)	**Q^A**, Q^D, W(4)				Majority fullsib family
B18_1710	5	6	3	Q^C(2), Q^D(2), W(4), L(2)	**Q^A**	W(2), L			Majority fullsib family & family 3
B19_1810	3	8	6	W(6), L(6)	**Q^A**, Q^C, Q^D, W	W			Majority fullsib family

In nine of the 11 snap-shot MQ colonies the observed genotype of the type A queen matched the PMG generated by COLONY for the majority fullsib family (Table 3). In colony A09_1910 the type A queen's genotype matched the PMG of a single larva, whereas in colony B13_1710 the type A queen's genotype matched no genotyped colony member. All queens with type C or D ovaries did not match the PMG of any worker or larvae, in fact, 86% of these queens were assigned to the majority fullsib family. In colonies A09_1910, B13_1710, B17_1810, and B19_1810, a number of type C or D queens (2, 3, 1 & 2 queens per colony) were fullsisters of the type A queen (Table 3). The sibship data showed that never more than one resident queen per colony was the mother of group members (Figure 6).

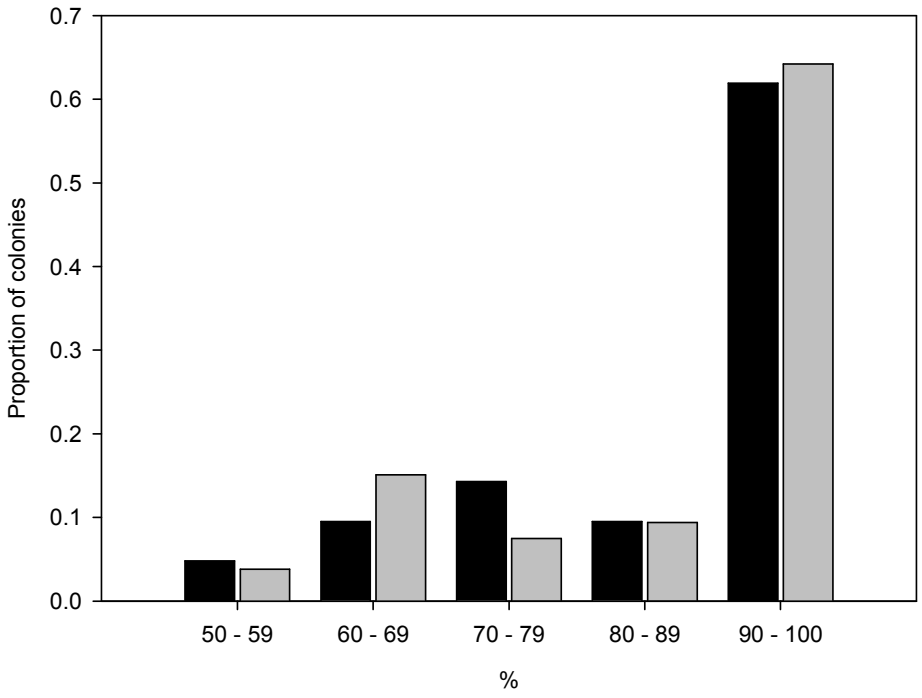


Figure 5. Proportion of MQ (black bars; n=53) and SQ (grey bars; n=21) colonies with a particular percentage of workers and larvae assigned to the majority fullsib family.

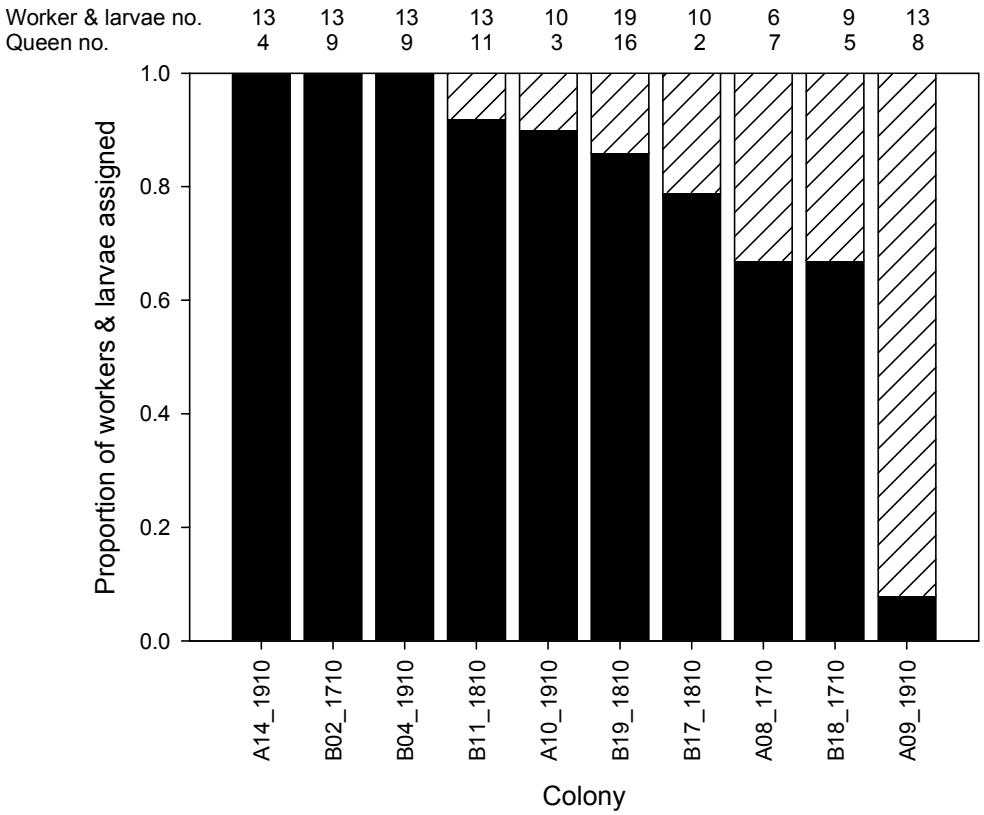


Figure 6. The proportion of workers and larvae (black bars) per colony whose PMG matched the genotype of the type A queen. The PMG of all remaining colony individuals (striped bar) did not match the genotype of any resident queen. Colony B13_1710 was not included as no resident queen's genotyped matched the PMG of any colony individuals. The number of workers, larvae and queens that were analysed is shown by the numbers at the top of the table.

Relatedness

Within colony relatedness was high in OT samples (Table 4). In OT04 MQ colonies, the average relatedness among workers (0.83±0.05, n=13 cols) was not significantly different from 0.75, nor different to worker relatedness in SQ colonies (0.83 vs 0.76; U=30, n_1=14, n_2=6, p=0.46). In OT06 MQ colonies, the average relatedness among workers (0.64±0.02, n=48 cols/349 ind.) was significantly lower than 0.75, whereas relatedness among larvae (0.70±0.02, n=38 cols/151 ind.) was not significantly

different from 0.75, but there was no significant difference between worker and larvae relatedness (0.64 vs 0.70; U=747, n_1=48, n_2=38, p=0.15). Like OT04, importantly there was no difference in worker relatedness in OT06 MQ and SQ colonies (0.67 vs 0.64; U=404, n_1=48, n_2=21, p=0.19), or larvae (0.66 vs 0.70; U=217, n_1=38, n_2=12, p=0.81). In OT06 MQ colonies, the average relatedness of workers to larvae (0.65±0.02, n=37 cols/274W-132L) was significantly lower than 0.75, a difference most likely explained by the few workers and larvae that did not belong to the majority fullsib family.

In the snap-shot MQ colonies, the average within colony relatedness among mated queens was 0.59±0.05 (n=11 cols/70 ind.). Sibship analysis indicated that most type C or D queens were fullsisters, and daughters of the type A queen (Table 3). Accordingly, one would expect the average relatedness among nestmate type C or D queens to approach that between fullsisters (r=0.75), and relatedness between type C or D queens and type A queens to approach that expected for mother-offspring (r=0.5). Observed values agreed with these predictions as relatedness between type C or D queens (0.64±0.06; n=9 cols/57 ind.) did not differ significantly from 0.75 and the relatedness of type C or D queens to type A queens (0.41±0.04; n=11 cols/59Q^{CD}- 11Q^{A}) did not differ significantly from 0.5, but the two values differed significantly from each other (0.64 vs 0.41; U=3, n_1=8, n_2=10, p=0.019). The average relatedness of workers to type A queens (0.39±0.08; n=9 cols, 68W-9Q^{A}) and larvae to type A queens (0.41±0.06; n=11 cols, 51L-11Q^{A}) was not significantly different from that expected between daughters and mothers (r=0.5). The average relatedness of workers to type C or D queens (0.66±0.03; n=9 cols, 68W-48Q^{CD}), and larvae to type C or D queens (0.65±0.05; n=11 cols, 51L-59Q^{CD}), were significantly higher than 0.5 but lower than 0.75. The lower than expected

value is most likely explained because of the few workers and larvae that did not belong to the majority fullsib family (Table 3).

Table 4. Summary of relatedness values for SQ and MQ colonies from the OT population.

	r-value ± s.e.m.	n=colonies (individuals)
OT04		
MQ colonies		
Workers	0.83 ± 0.05	13 (52)
SQ colonies		
Workers	0.76 ± 0.09	6 (24)
OT06 (all)		
MQ colonies		
Workers	0.64 ± 0.02	48 (349)
Larvae	0.70 ± 0.02	38 (151)
Small larvae	0.71 ± 0.04	22 (76)
Large larvae	0.73 ± 0.04	17 (46)
Workers & Larvae	0.66 ± 0.02	53 (500)
Workers → Larvae	0.65 ± 0.02	37 (274/132)
SQ colonies		
Workers	0.67 ± 0.04	21 (154)
Larvae	0.66 ± 0.05	12 (44)
Workers & Larvae	0.67 ± 0.03	21 (198)
Snap-shot MQ colonies		
Mated Queens	0.59 ± 0.05	11 (70)
$Queens^{CD}$	0.64 ± 0.06	9 (57)
Workers → $Queen^{A}$	0.39 ± 0.08	9 (68/9)
Larvae → $Queen^{A}$	0.41 ± 0.06	11 (51/11)
$Queens^{CD}$ → $Queen^{A}$	0.41 ± 0.04	11 (59/11)
Workers → $Queens^{CD}$	0.66 ± 0.03	9 (68/48)
Larvae → $Queens^{CD}$	0.65 ± 0.05	11 (51/59)

Queen turnover

Queen turnover was 19.7% in MQ colonies (n=21), 11.3% in SQ colonies (n=8), and 17.3% for MQ and SQ colonies combined. Given that small larvae most likely arise from eggs laid in the year of collection, these queen turnover estimations are directly comparable to those reported in Hammond *et al.* (2006). The estimation of queen turnover is considerably less than that reported by Hammond *et al.* (2006) and by Bourke *et al.* (1997) in a low skew UK population of *L. acervorum*.

Genetic relationship among populations

Alignment of 685bp of cyt*b* from 47 workers revealed 17 unique haplotypes (Genbank accession numbers: HQ259995 - HQ2560011). Tree building showed that the majority of haplotypes (76%) grouped into a single clade with high bootstrap support (Figure 7). Within this clade, branch lengths were short with an overall uncorrected distance of just 0.5%, and importantly the haplotypes from the OT population were scattered throughout this clade with no evidence that low skew populations were genetically distinct. In fact, one haplotype was found both in known low skew populations (SD and SF) and in the high skew population (OT). Furthermore, the largest distance between a high skew OT haplotype and a haplotype from a well studied low skew population, SD (Hammond *et al.* 2006), was the same as that between the two most divergent haplotypes within the low skew population, SD (both 0.6%).

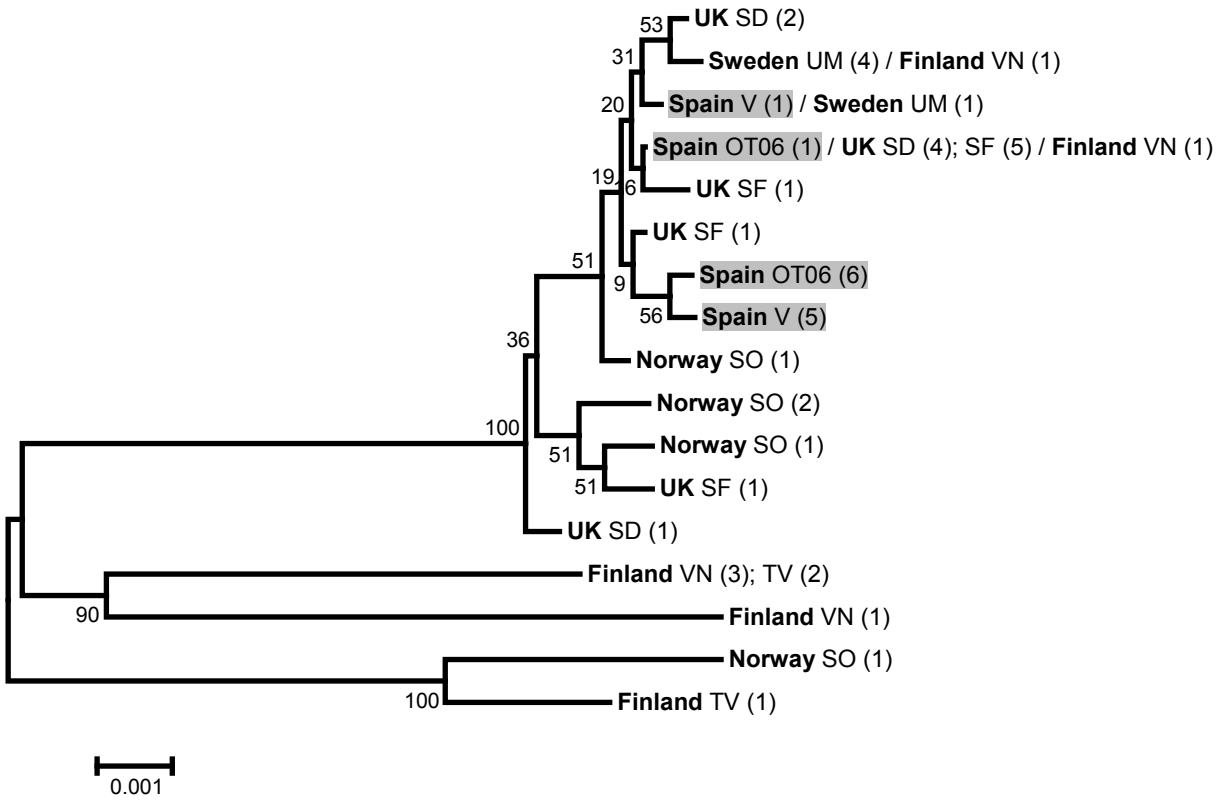


Figure 7. Neighbour-joining tree of the 17 haplotypes recovered from 685bp of cytochrome *b* with bootstrap support shown. Populations are: OT=Orihuela del Tremendal, Spain; V=Valdelinares, Spain; SD=Santon Downham, UK; SF=Sherwood Forest, UK; SO=Solvorn, Norway; UM=Umea, Sweden; TV=Tvarminne, Finland; VN=Vaasa, Finland. For each haplotype the population(s) is shown and in brackets the number of individuals in which it was found. The Spanish high skew populations are highlighted in grey and the scale bar shows 0.1% sequence divergence.

Alignment of a 287bp fragment of the *foraging* gene from 21 workers (five populations) revealed five alleles which differed by a maximum of four substitutions (Genbank accession numbers: HQ2560012 - HQ2560016). In all populations the same allele (H1) was at high frequency (range 0.667-0.917) with every individual having at least one copy of this allele (Table 5). In each geographical area (Spain, UK and Finland)

we found area specific alleles, but these differed by only one to three substitutions from H1. The sharing of the same allele in all populations, and the minimal sequence differences among all alleles therefore provides additional evidence for a close genetic relationship between populations.

Table 5. Variation in a 287bp fragment of the *foraging* gene. The sample size is the number of diploid workers with each sampled from a different colony.

Alleles	Variable sites	Populations			
		UK (SF)	Spain (OT)	Spain (V)	Finland (TV/VN)
H1	CGGCGT	0.917	0.857	0.667	0.900
H2	.A..AA		0.071	0.167	
H3	G.....		0.071	0.167	
H4	...T..	0.083			
H5	..A...				0.100
Sample size		6	7	3	5

2.3.3 Colony observations

Out of 44 OT MQ colonies overwintered in the laboratory (mean no. queens per colony=10.7±1.6; range=2-30), 37 colonies had just a single queen that showed signs of reproductive activity in the eight weeks of observation. In the remaining seven colonies no queens showed evidence of reproductive activity and laying was not observed. In contrast, in all 14 SF MQ colonies (mean no. queens per colony=6.0±1.0; range=2-13) more than one queen per colony showed signs of reproductive activity during the eight weeks of observation (average percentage of queens showing reproductive activity=80%; range=24-100%).

2.4 DISCUSSION

This study shows that the Spanish population of *Leptothorax acervorum* studied is functionally monogynous. Dissections showed that in all MQ colonies, every queen (with the exception of two queens in two colonies) was mated. However, only one queen per colony had developed ovaries and showed signs of recent egg-laying (type A queens). In support of this, workers and larvae within MQ colonies were highly related, and sibship analysis showed that the majority of colony members (including type C and D queens) grouped into a single 'majority fullsib family'. Furthermore, the type A queen was, in most cases, genetically compatible with being the mother of the 'majority fullsib family' (exceptions discussed below), and no type C or D queen was compatible with being the mother of any other colony member. These data provide firm evidence to confirm a previous report of functional monogyny (Felke & Buschinger 1999).

The data also reveal that functional monogyny is temporally stable, and not solely the consequence of daughter queens overwintering before dispersal (Felke & Buschinger 1999). First, both worker relatedness and dealate queen number were high in samples collected in both early summer (OT04) and late autumn (OT06). Second, in two colonies (B17_1810 and B19_1810) the type A queen was a member of a fullsib family (Table 1) that included other queens and workers. Interestingly, in both colonies the type A queen was also the mother of the majority fullsib family, which included, at least in one case (B17_1810), mated daughter queens. Given that it takes two years for queens to develop from egg to adulthood (see Heinze *et al.* 1995b), this means that mated queens can remain non-reproductive within their natal colonies for at least two years. Third, queen turnover (19.7%) was lower than the rate estimated in polygynous *L. acervorum*

populations (Bourke *et al.* 1997; Hammond *et al.* 2006), and other ant species (e.g. Pedersen & Boomsma 1999; Bargum *et al.* 2007). Finally, the sibship analyses showed that in the majority of snap-shot MQ colonies (7/11) the type A queen was assigned to a fullsib family containing no other individuals and was the mother of the majority fullsib family which also contained most type C and D queens. In addition, high relatedness among type C and D queens, a low level of queen turnover, and a reproductive tenure of multiple years, further suggests daughter queen re-adoption is frequent.

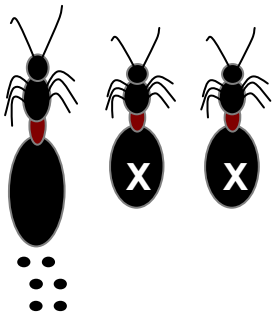
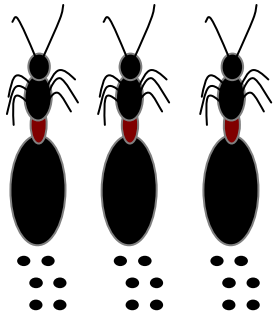
The genetic data also showed that a very small number (13/210, 6%) of workers, larvae, and non-reproductive (type C and D) queens did not belong to the type A queen fullsib family and were not the offspring of any queen within the colony. Adult members of these families might be the offspring of queens lost because of queen turnover events (e.g. death or colony budding), or perhaps have drifted into non-natal nests, a credible explanation given the high population densities (up to 4 nests per m^2). It is less obvious, however, why a small number of larvae could not be attributed to either fullsib family (mismatches at multiple loci discount genotyping errors and mutations). One possibility is that these are brood left behind during colony emigrations and later collected by workers of another colony (Hare 1996).

2.4.1 Social polymorphism in L. acervorum

The social organisation of the studied population strongly contrasts (Table 6) with that reported from other polygynous *L. acervorum* populations (Stille *et al.* 1991; Chan & Bourke 1994; Heinze *et al.* 1995a; Heinze *et al.* 1995b; Bourke *et al.* 1997; Hammond *et al.* 2001; Hammond *et al.* 2006). For example, in a UK population Hammond *et al.*

(2006) showed that in the majority of nests (70% of cols, n=17) skew was not significantly different from that expected if all queens reproduced equally. Moreover, our estimates of worker relatedness (0.64 and 0.83), which agreed with a previous estimate based on allozyme data from a Spanish population (r=0.72 Heinze *et al.* 1995a), were much higher than the values calculated with microsatellites reported from MQ colonies in polygynous populations (e.g. UK: r=0.26 Bourke *et al.* 1997; r=0.28 Hammond *et al.* 2006; Germany: r=0.49 Heinze *et al.* 2001).

Table 6. Comparison of the fundamental differences between the functionally monogynous Spanish population (current study) and the well-studied polygynous UK populations.

Population	**Present study:** Spain	UK
Social organisation	Functional monogyny	Polygyny
Skew	High (complete skew)	Low
3-Queen scenario		
Description	Single queen monopolises all reproduction in a multiple queen colony	More than one queen shares reproduction in a multiple queen colony
Worker relatedness	0.83 & 0.64	$0.28^{(1)}$; $0.26^{(2)}$; $0.28^{(3)}$; $0.44^{(4)}$
Queen relatedness	0.59	$0.26^{(1)}$; $0.48^{(1)}$; $0.48^{(2)}$; $0.26^{(3)}$; $0.17^{(4)}$; $0.28^{(5)}$
Queen turnover	19.7%	43 - 67.2%$^{(1)}$

* (1) Hammond *et al.* 2001; (2) Bourke *et al.* 1997; (3) Heinze *et al.* 1995a; (4) Chan & Bourke 1994; (5) Hammond *et al.* 2006

There was limited genetic differentiation between the Spanish and UK populations at both mitochondrial and nuclear markers suggesting that the two populations share a common history in the recent past. For the *foraging* gene (nDNA) an allele was found to be frequent in all populations, and in cyt*b* (mtDNA) there was no evidence of haplotypes sorting into groups concordant with social organisation or geography. Lack of mtDNA differentiation is particularly telling as mtDNA is sensitive to differentiation by drift because effective population size (Ne) is low on account of uniparental inheritance and haploidy. Furthermore, in ants with queen re-adoption, female dispersal is limited and so gene flow will likely have only a weak homogenizing effect on mtDNA haplotype frequencies, supporting a recently shared history as the most likely explanation of limited differentiation between populations.

Two lines of evidence suggest that this social polymorphism has a genetic basis, rather than being a plastic social phenotype in response to environmental factors. First, populations appear to show exclusivity in social phenotype, as in polygynous populations multiple queens always reproduce in nests containing several mated queens (Hammond *et al.* 2006), whereas only a single queen was ever found to reproduce in nests containing several mated queens in the Spanish population. In addition, as the local environment is likely to be somewhat variable within the Spanish population, my finding of just functional monogyny further supports a fixed rather than plastic response. Second, in the common garden experiments both OT (functionally monogynous) and SF (polygynous) colonies were kept in a common laboratory environment during and after overwintering but this did not lead to a convergence in social organisation. In none of the OT colonies did more than one queen reproduce after overwintering, by contrast, in all SF colonies

multiple queens showed signs of reproductive activity. Such stability does not support the hypothesis that social organisation tracks current environmental cues but points to a fixed genetic difference, but this is not to say that environmental factors are not important in shaping the evolution of the polymorphism in social organisation.

There are few cases of genetically based differences in social organisation, for example in the fire ant *Solenopsis invicta* a single genomic element, marked by the odorant-binding protein gene Gp-9, is responsible for the existence of two distinct social forms (Keller & Ross 1998; Krieger & Ross 2002). This shows that a complex social phenotype can have a simple genetic basis, so variation at a single genetic region or a quantitative genetic effect, are both possible explanations for the contrasting social organisation in *L. acervorum*. That said, complex explanations such as maternal effects, or social organisation being environmentally influenced early in colony development in a similar fashion to that seen in the process of caste determination cannot be ruled out. Breeding studies are needed to show conclusively that polygyny and functional monogyny are heritable.

So far an important limitation of studies on reproductive skew has been the relatively low variance in reproductive skew within and between populations which reduces the power to identify social or ecological factors that affect skew (e.g. Field *et al.* 1998a; Magrath & Heinsohn 2000; Sumner *et al.* 2002; Nonacs *et al.* 2004; Hammond *et al.* 2006; Liebert & Starks 2006). Ecological constraint on dispersal (Emlen 1982) have been considered important both in the evolution of polygyny, *per se* (Keller 1993), and in determining the level of skew among queens within colonies (Bourke & Heinze 1994; Keller & Reeve 1994; Reeve & Keller 2001). In this study, functionally monogynous

colonies were restricted to sites above 1500m in altitude, and nest density appeared patchy (RJG and RLH pers. obs.). This seems to suggest that constraints on dispersal are indeed high and so at least partly explain functional monogyny (Bourke & Heinze 1994). However, high ecological constraint should also select for the re-adoption of all daughter queens due to high costs associated with solitary nest founding. We would therefore expect a higher proportion of MQ colonies in functionally monogynous populations (high skew) than in polygynous (low skew) populations. However, the proportion of MQ colonies found (61%) is within the range found in low skew UK populations (21-69%, Chan & Bourke 1994). From colony collections (RJG & RLH) it seems that colonies in polygynous populations are, like in the functionally monogynous population, also distributed relatively patchily.

Concession models predict that skew should positively correlate with relatedness between potential reproductives (Vehrencamp 1983a; Reeve & Ratnieks 1993; Reeve & Keller 1997). In line with this prediction queen relatedness is higher in the functionally monogynous population than in polygynous populations (Chan & Bourke 1994; Heinze 1995; Hammond *et al.* 2001; Hammond *et al.* 2006). The data also fits Reeve and Keller's (1995) prediction that skew should be higher in societies comprising the mother and her offspring than in colonies comprising sisters as non-reproductive queens are generally the daughters of the reproductive queen. However, queens in polygynous colonies are also related because of daughter queen re-adoption (Hammond *et al.* 2001), yet in these colonies re-adopted queens reproduce (Hammond *et al.* 2006). It therefore remains to be investigated whether the relationship of skew and relatedness is a consequence of skew rather than a cause. The contrast in skew between populations may

be explained if a future breeding component is incorporated into skew models (Kokko & Johnstone 1999; Ragsdale 1999; see Sumner *et al.* 2002), and such models predict queuing for a reproductive position when individual survivorship is high (Kokko & Johnstone 1999). In line with this, daughter queens do supersede their mother in functionally monogynous colonies (Table 2) and queen turnover is lower than in polygynous colonies, suggesting that differences in survivorship may underlie differences in skew.

More fundamentally, transactional skew models, which include concession models, assume that there is a social contract between group members. Thus when model parameters such as ecological constraints on solitary breeding vary, the behaviour of group members is predicted to change. For instance, in concession models if constraints on solitary breeding reduce, dominants should concede more reproduction to subordinates (Reeve & Ratnieks 1993). However, the lack of variation in skew in the functionally monogynous Spanish population, despite almost certain variation in constraints on solitary breeding within populations, and that skew was not obviously changed when both functionally monogynous and polygynous colonies were kept in a common and importantly novel lab environment, suggests that behavioural adjustments are not made. Furthermore, the likely genetic polymorphism suggests that the level of skew is an evolved response rather than a behavioural one, an important issue that has previously been highlighted (Kokko 2003).

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RJG and RLH wrote the paper.

AA helped with microsatellite genotyping

LK provided comments and corrections with the manuscript and provided facilities required for RLH's 2004 collection.

RJG carried out all remaining field and laboratory work.

CHAPTER 3: Part 1

Worker policing of royal reproduction

The work presented here (part 1 of this chapter) is a manuscript in preparation for submission: Gill, R. J. and Hammond, R. L.

3(1).1 INTRODUCTION

In social living animals the benefits of cooperation are potentially undermined by selfish individual behaviour (Frank 1995), and revealing such conflict and its resolution is important in understanding sociality, a major transition in evolution (Trivers 1974; Maynard Smith & Szathmary 1995). Social conflicts are well studied in the eusocial Hymenoptera where the close integration of related, but non-clonal, individuals leads to potential conflicts over many aspects of reproduction (e.g. Ratnieks 1988). Parties often use coercive behaviour in order for their interests to prevail (Ratnieks *et al.* 2006; Ratnieks & Wenseleers 2008), for example, research has shown that both workers and queens are known to police worker male production (Ratnieks & Visscher 1989; Foster & Ratnieks 2000; Halling *et al.* 2001; Oldroyd *et al.* 2001; Bonckaert *et al.* 2008), workers are able to manipulate sex ratios (Sundstrom *et al.* 1996; Mehdiabadi *et al.* 2003) and influence caste development (Bourke & Ratnieks 1999; Hammond *et al.* 2002), and also queens can adjust the sex ratio of eggs in an attempt to negate worker coercion (Passera *et al.* 2001; Rosset & Chapuisat 2006).

In species with multiple queen colonies (a common social organisation), reproductive skew among queens is another primary basis for conflict, because skew among queens affects colony kin structure and can thus impact on worker indirect fitness. It is therefore surprising, given that workers are influential over many aspects of colony reproduction, that there is a lack of evidence to support worker influence over skew among queens. Workers can potentially increase their indirect fitness by maintaining high skew so rearing close kin (i.e. fullsibs), however, conflict may arise because this may not meet the direct fitness interests of all queens in the colony. Despite this, almost all theoretical treatments of reproductive skew assume control rests with the reproductive individuals (queens) (e.g. Vehrencamp 1983a; Vehrencamp 1983b; Reeve & Ratnieks 1993; Emlen 1995; Emlen *et al.* 1998; see Johnstone 2000) (chapter 1), but queen control can potentially allow queens to exploit the altruistic behaviour of workers against their interests. This raises an important question: who is in control of reproductive skew among queens in multiple queen colonies and whose interests prevail?

I investigated the potential behavioural mechanism/s maintaining high skew in the functionally monogynous Spanish population of *L. acervorum,* by studying the specific roles of queens and workers over queen reproduction. Reproductive monopolization may be because of 'queen control', with the reproductive queen aggressively dominating other potentially reproductive queens and forming dominance hierarchies (e.g. Heinze & Lipski 1990; Heinze & Smith 1990; Heinze *et al.* 1997), or 'worker control', with workers influencing which queen reproduces. I tested the queen control and worker control hypotheses by looking to see if aggressive and non-aggressive behaviour and which party (queens and/or workers) determines which queen becomes reproductive in multiple queen

colonies. I further investigated whether relatedness between specific queens and other colony members determined which queen became the dominant reproductive.

3(1).2 METHODS

3(1).2.1 Colony collection, maintenance and composition

L. acervorum colonies were collected from two Spanish populations, Orihuela de Tremendal, Sierra de Albarracin (OT) and Valdelinares, Sierra de Gudar (V) in October 2006 (chapter 2; Gill *et al.* 2009) and 2007. All colonies were found in decaying twigs, and colonies were removed from their twigs 2-6 days after collection, censused for number of workers and queens, and provided with an artificial nest in the laboratory. Artificial nests (Figure 1) were modified from that used by Bourke (1991), made from two transparent glass slides (52x75mm) separated by 1mm thick cardboard. The cardboard had a 27x24mm (648mm^2) area cut-out to provide a nesting area, and a nest entrance 10mm in length and 3mm in width. The thickness of the cardboard encouraged a single layer of individuals within the nesting area allowing efficient observations of colony behaviour. Each nest was placed in a foraging arena (transparent container: 77x121mm) with the vertical sides (18mm) coated in Fluon® to prevent individuals escaping. Damp cotton wool and a diet of honey solution and chopped-up meal worm were provided once a week in winter, and 2-3 times a week in spring/autumn and summer.

Lab colonies were kept in a versatile environmental chamber (*Sanyo* MLR-351H) and temperature, light, and humidity controlled. Colonies experienced the following conditions: autumn (6-8 weeks), winter (6 weeks), spring (8 weeks) and summer (6 weeks) (Table 1). The composition of the 22 multiple queen colonies observed (OT=19; V=3) was as found in the field, with 2-7 queens per colony at the start of winter. After

overwintering in the lab, colonies had an average of 3.1 queens (range=2-6), and 80 workers (range=36-164).

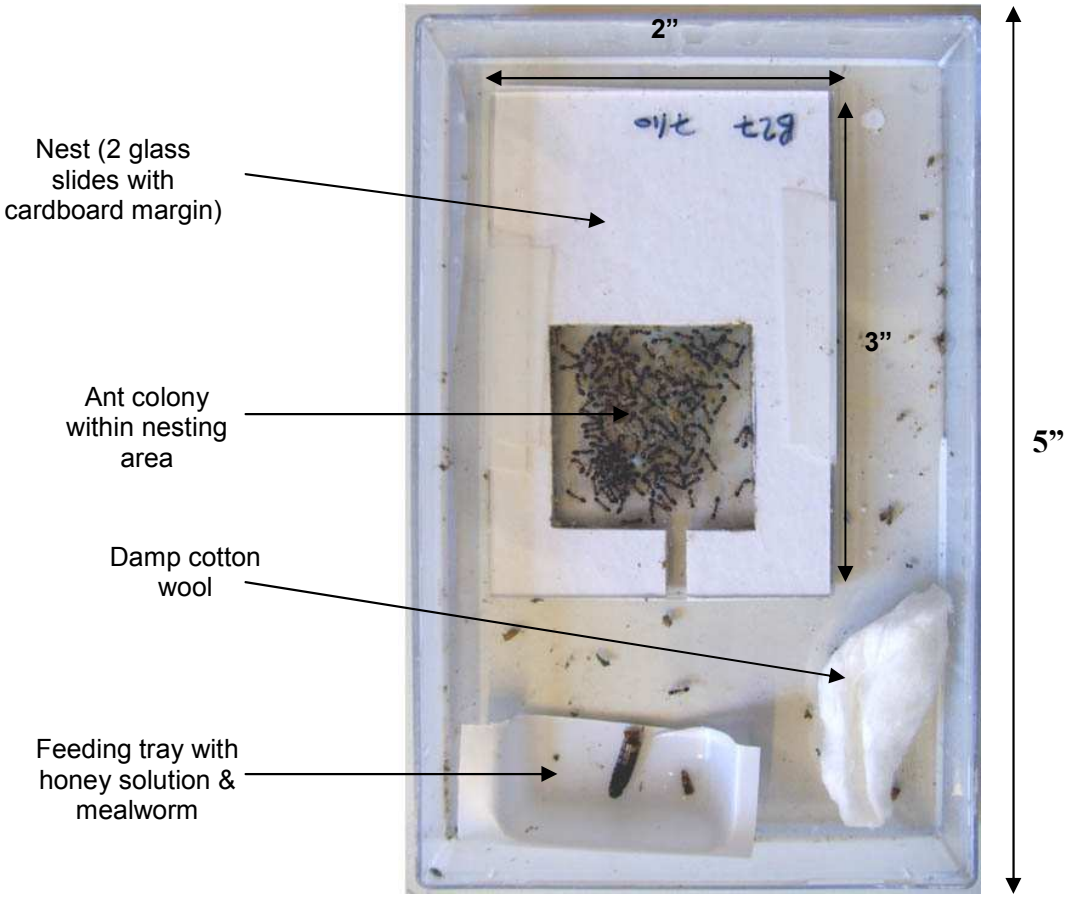


Figure 1. Photograph showing the artificial nest and foraging arena provided.

Table 1. Seasonal conditions.

Season	No. weeks	Hourly rhythm	Temp./°C	Photoperiod (N-D-D-D) (relative light intensity)	Humidity/%
Winter	6	12-1-10-1	0-5-10-5	0-1-2-1	60-60-60-60
Spring/Autumn	8/6-8	11-1-11-1	10-15-20-15	0-2-3-2	70-70-80-70
Summer	6	9-1-13-1	15-20-25-20	0-2-3-2	70-70-80-70

*Conditions were based on a personal communication with A. Buschinger in 2003.

3(1).2.2 Behavioural observations and analysis

After overwintering in the laboratory, I observed aggressive and non-aggressive behaviour received by queens (individually marked using Humbrol model paint) in all 22 MQ colonies over the 'spring' period when queen ovary development occurs. Colony behaviour was sampled by taking short videos from the onset of spring until eggs were laid (Figure 1). Behaviour was sampled between 07.00-19.00 and recording bouts were made over this period (see supplementary material, S3), with each bout for each colony made on separate days and the mean duration per bout being 0.68hrs (range=0.26-1.5hrs). Behavioural recording was stopped once egg laying began (Figure 1), however, in three colonies (OT4.09, OT4.15 & OT4.35) recording was stopped early as all but a single queen (the future reproductive) had either been evicted or had left the nest permanently.

Colony behaviour was recorded using four Panasonic WV-CL270/6 colour CCTV cameras with 10x zoom lenses, connected to a digital video recorder (model: DVR24). Behavioural interactions were analysed by re-watching recordings and behaviour scored using the programme VAR (designed by D. S. Gill & R. J. Gill and available from RJG) which helps measure the length of each type of interaction and the individuals involved. All behaviour was sampled using a focal queen approach with the type and duration of all behaviour a queen received or performed recorded. Rates of behaviour were calculated as the duration of behaviour (seconds) / the total length of time a focal queen had been observed (hours). Per capita rates were calculated as the rate of behaviour / number of workers or queens (which ever was applicable) in the colony. During recording there were occasions where queens strayed outside the nest or out of camera view, this time

was deducted from the focal queen observation time, so explaining variation among queens from the same nest in the time they were observed (see supplementary data S4).

Table 1.1. Colony composition and information on colony recordings. For each colony the number of recording bouts (separate days), the period during which recording bouts took place within, and when the first egg was recorded (* the exact 'lay date' was unknown for three colonies) is shown. The number of days shown in 'recording period' and 'lay date' corresponds to the number of days after the first day of spring.

Colony	W no.	Q no.	No. recording bouts	Recording period (days)	Lay date (days)
A01_1810	54	3	6	7-21	24
A10_1810	53	2	5	8-17	17
A11_1910	29	2	7	12-24	41
B07_1810	84	4	10	12-40	41
B13_1910	70	5	10	6-32	34
B14_1810	47	2	10	11-29	32
OT3.07	46	5	6	8-21	24
OT3.13	84	2	14	4-49	66
OT3.27	122	6	6	9-30	32
OT3.32	62	5	11	7-38	42
OT4.03	107	3	9	4-21	23
OT4.09	103	2	2	7-8	*
OT4.13	38	4	11	4-36	38
OT4.15	67	2	9	7-28	*
OT4.19	102	3	4	11-30	36
OT4.35	47	2	5	4-10	*
OT5.02	105	3	16	4-38	42
OT5.03	100	3	13	4-38	38
OT6.01	164	3	16	4-53	56
V.01	43	3	17	4-52	57
V_06	137	3	5	12-19	20
V.22	36	2	12	4-28	30
Average	77.3	3.1	9.3	-	36.5

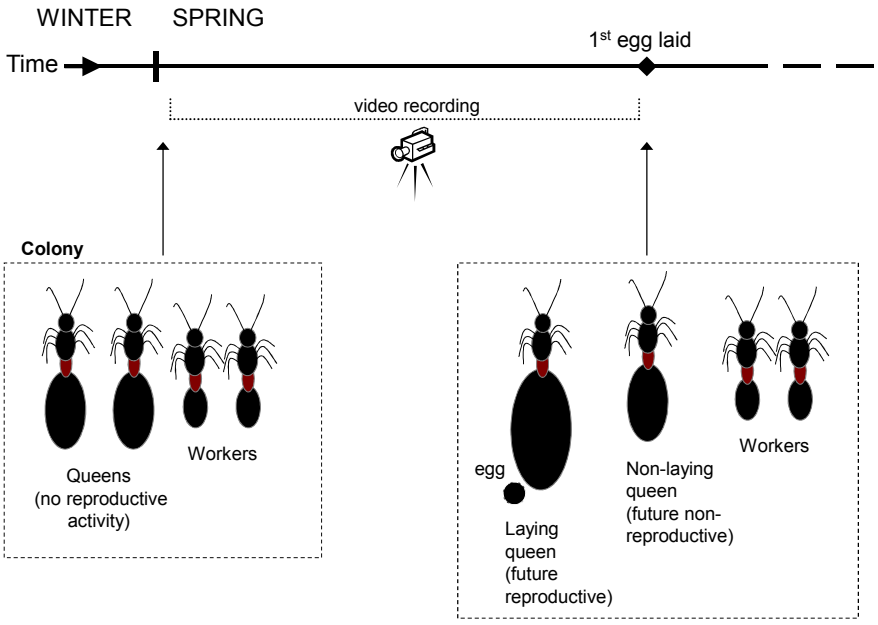
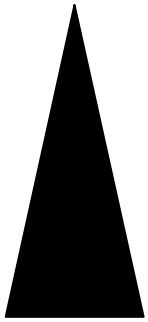


Figure 2. Experimental setup for behavioural observations. Multiple queen colonies (n=22) were recorded from the onset of artificial spring until the first egg was laid and the reproductive queen identified. A total of 354.6 hours of individual queen behaviour was observed for a total of 69 queens.

Four types of aggressive interactions were scored: 1) a single bite; 2) biting; 3) dragging; and 4) spreading, each increasing in their degree of aggressiveness (Table 2). As the duration of a single bite was difficult to measure, a standard time of one second per bite was used to calculate a rate. Grooming behaviour involved individuals cleaning others using their mouthparts, but I also categorised trophallaxis (an individual feeding another) as grooming, because it was often difficult to distinguish between these behaviours. In addition, it has been reported that queens may feed from larval secretions (Bourke 1991), however, this was also difficult to distinguish from grooming of larvae

and therefore was also included under grooming. During recording there were occasions where queens sometimes strayed outside of the nest or out of camera view, this was noted, and the time was deducted from the focal queen observation time. This explains within nest variation in the time queens were observed (see Table 3).

Table 2. Classification and description for each type of aggressive interaction.

Type of aggression	Degree of aggressiveness	Definition
Single Bite	Low	A single individual bites another for ≤1 second.
Biting		A single individual bites another individual for an extended period of time (>1 second), which often immobilises the attacked individual.
Dragging		A single individual bites another individual usually on an appendage (i.e. legs, antennae, neck and petiole) and drags the attacked individual.
Spreading	High	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.

3(1).2.3 Queen size

At the end of the experiment queen thorax width was measured (Figure 3) as an estimate of body size (Bourke 1991). Measurements were made using the Leica Application Suite V.2.7.1 from digital images taken with a Leica S8APO binocular microscope equipped with a DFC290 digital camera.

Figure 3. Thorax width of queens was measured; the white line represents where the measurement was taken which was at the widest part of the thorax.

3(1).2.4 Queen reproductive and mated state

Reproductive queens could be identified once egg-laying had begun by an enlarged (physogastric) abdomen and a central position among nestmates (Gill *et al.* 2009). Ovary dissection was undertaken to investigate the mated status of queens.

3(1).2.5 Genetic analysis

After behavioural observations samples of workers and larvae, and all queens (n=58) still present in 21 of the 22 colonies observed (9 queens from 8 colonies were evicted or left the nest and escaped during observations) were genotyped at four polymorphic microsatellite loci (loci and PCR conditions described in chapter 2). Worker and larval DNA was extracted using a 10% Chelex solution as previously described

(chapter 2; Gill *et al.* 2009), and queen DNA was extracted using a Pure Gene extraction kit.

Sibship patterns using the program COLONY (Wang 2004) were investigated to group individuals into fullsib families based on queens being singly mated (Hammond *et al.* 2001; Gill *et al.* 2009). Maternal queens were identified by checking all mated queen genotypes against the predicted maternal genotype (PMG), as predicted by COLONY, for each fullsib family per colony. Colonies with a resident queen matching the PMG for the majority fullsib family (containing the majority of workers) were classed as maternal colonies, whereas colonies with no resident queen matching the PMG of any fullsib family containing workers were classed as non-maternal colonies. In addition, regression relatedness (Queller & Goodnight 1989) was calculated using the program Relatedness 5.08 for only diploid (female) individuals.

3(1).2.6 Statistical analyses

Investigation into whether specific behavioural interactions predicted the future reproductive status of queens (reproductive queens coded 1, non-reproductive queens coded 0) was carried out using logistic regression. Prior to analyses the assumption that independent variables had a linear relationship with the log of the dependent variable was tested, as described in Field [p.296] (2009). In all cases there was no significant interaction term between the independent variable and its natural logarithm so this assumption was met. Behavioural variables were all found to be non-normally distributed (Kolmogorov-Smirnov tests) and so non-parametric tests were used (incl.

Mann-Whitney U test, Spearman's rank correlation, Wilcoxon signed rank test). All statistical analyses were performed with either Minitab version 13.1 or SPSS version 17.

3(1).3 RESULTS

3(1).3.1 Behavioural observations

In total 354.6 hours of individual queen behaviour was observed for a total of 69 queens (mean per queen=5.14 hours, range=0.11-12.09 hours).

Aggression

In all colonies, and as expected (chapter 2; Gill *et al.* 2009), only a single queen per colony became reproductive. Behavioural observations showed that aggression received by queens was strikingly biased, with more than 99% carried out by workers towards queens (W→Q, mean per queen±s.e.m.: 292.4±67.4 sec/hr; Figure 4 & 5A), and less than 1% carried out by queens (Q→Q, mean per queen: 0.6±0.4 sec/hr; Wilcoxon signed-rank test: $Z=-6.51$, $n=69$, $p<0.001$). The numerical superiority of workers did not explain this difference as the per capita rate of W→Q aggression was still significantly higher than the per capita rate of Q→Q aggression (3.26±0.69 sec/hr vs 0.17±0.14 sec/hr; Wilcoxon signed-rank test: $Z=-5.11$, $n=69$, $p<0.001$).

Further analysis showed that the future reproductive state of queens ('reproductive', n=22; 'non-reproductive', n=47) was predicted by the amount of worker aggression received (logistic regression: ß(s.e.)=-0.117(0.047), Wald=6.32, df=1, p=0.01, odds ratio (95% CI)=0.89(0.812-0.975)). On average, non-reproductive queens received over 100 times more W→Q aggression than reproductive queens (427.8±92.8 vs 3.1±1.2 sec/hr; Figure 5B & 6, Table 3; Mann-Whitney: $U=91.0$, $n_1=22$, $n_2=47$, $p<0.001$), and the level of aggression was often extreme with seven queens killed by workers (10% of queens, five colonies) and five permanently evicted (7% of queens, five colonies). In

contrast, in ten colonies the reproductive queen received no worker aggression and in over 80% of colonies (18/22) she received the least worker aggression among all colony queens (Table 3). In the four colonies where a non-reproductive queen received less W→Q aggression than the reproductive queen, in two cases the non-reproductive queen was permanently evicted from the nest before laying began, whereas in the other two colonies the difference in aggression rate was minimal (4.4 vs 0 and 10.0 vs 8.4 sec/hr). In contrast to the high W→Q aggression, 91% of queens received no aggression from other queens, and there was no difference in the level of Q→Q aggression received between reproductive and non-reproductive queens (reproductive vs non-reproductive: zero vs 0.81±0.62; Mann-Whitney: $U=472$, $n_1=22$, $n_2=47$, $p=0.56$).

Figure 4. Worker aggression towards queens. Two examples of workers spreading a queen that later did not reproduce (the queen is in the centre of both images).

For W→Q aggression received by non reproductive queens (n=47), single bites contributed the least (0.95±0.20 sec/hr, or 3.7 bites/queen, range=0-15) then spreading (89.5±25.5 sec/hr), dragging (130.1±27.4 sec/hr) and biting (207.3±56.0 sec/hr) (Figure 6). In contrast, queens that later became reproductive (n=22) were never subjected to spreading by workers and the average rates were low for bites (0.12±0.06 sec/hr, or 0.5 bites/queen, range=0-3), biting (0.17±0.128 sec/hr) and dragging (2.8±1.19 sec/hr).

Comparisons between reproductive and non-reproductive queens for each behaviour separately (Figure 6) were all significantly different (Mann-Whitney: all U<242, all n_1=22, all n_2=47, all P<0.001). I observed 38% (18/47) of non-reproductive queens (n=15 colonies) outside the nest for an average of 0.24±0.07 hours (range=0.02-0.65), whereas, reproductive queens were never seen outside the nest.

Table 3. Mated state, the rate of worker aggression received, and the total focal queen time observed for each queen in 22 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 mated status of queens which permanently left, or were evicted from the nest, and escaped (X) could not be determined. The rate (sec/hr) of W→Q aggression is in **bold** and the total focal queen observation time (hrs) is in brackets. All queens were genotyped except those highlighted.

Colony	Queen reproductive status					
	RQ	NRQ_1	NRQ_2	NRQ_3	NRQ_4	NRQ_5
A01_1810	M **0.16** (6.37)	M **1.26** (6.37)	M **0 (**1.37)			
A10_1810	M **0** (5.92)	M **41.59** (5.92)				
A11_1910	M **22.27** (9.16)	X **297.24** (7.36)				
B07_1810	M **13.67** (9.07)	M **844.92** (8.92)	M **9.0** (0.11)	M **2484.6** (0.6)		
B13_1910	M **9.29** (5.6)	U **454.33** (5.66)	? **12.6** (5.32)	X **348.89** (5.43)	X **156.23** (5.59)	
B14_1810	M **0.81** (9.91)	? **46.76** (9.77)				
OT3.07	M **4.15** (2.65)	M **181.05** (2.47)	M **445.49** (1.78)	U **680.76** (2.21)	? **42.42** (2.62)	
OT3.13	M **1.22** (9.82)	? **26.06** (9.63)				
OT3.27	M **0** (3.26)	M **878.13** (2.56)	M **2601.55** (1.01)	M **842.76** (0.82)	? **126.33** (2.94)	? **951.09** (2.74)
OT3.32	M **0** (7.21)	M **35.29** (7.2)	M **211.4** (6.67)	? **332.03** (1.64)	X **0** (0.48)	
OT4.03	M **0** (4.85)	M **239.86** (5.27)	? **793.7** (5.02)			
OT4.09	M **0.53** (1.96)	? **1133.46** (1.45)				
OT4.13	M **0** (5.91)	M **7.3** (5.75)	M **11.09** (5.95)	M **9.62** (5.72)		
OT4.15	M **0** (5.37)	X **30.04** (5.36)				
OT4.19	M **0** (2.74)	M **1768.75** (0.96)	? **1407.27** (2.57)			
OT4.35	M **4.42** (2.94)	X **0** (2.58)				
OT5.02	M **9.96** (10.15)	M **270.21** (4.54)	? **8.43** (10.2)			
OT5.03	M **0.46** (6.48)	M **188.12** (6.16)	X **65.97** (2.83)			
OT6.01	M **1.1** (7.28)	M **233.72** (7.22)	M **200.87** (7.39)			
V.01	M **0** (12.09)	M **35.88** (11.48)	X **2.93** (6.48)			
V_06	M **0** (4.78)	M **1596.46** (3.8)	X **0.58** (1.71)			
V.22	M **0** (5.89)	M **50.06** (5.57)				

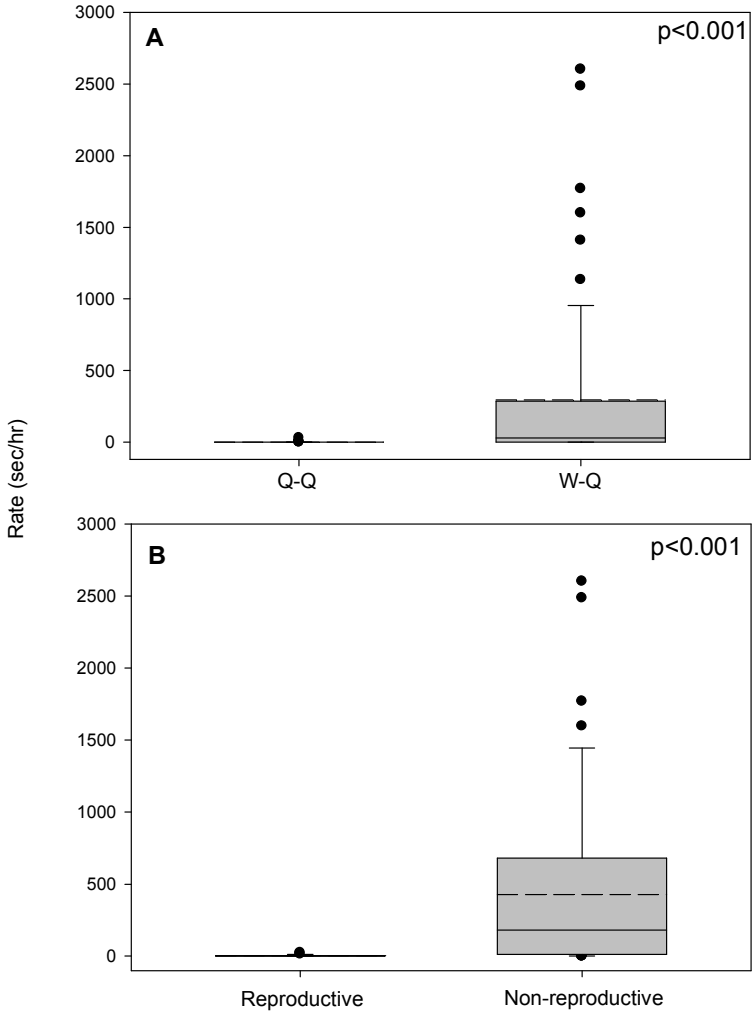


Figure 5. Observed aggressive behaviour. **A**) The rate (sec/hr) of aggression received by queens (n=69) from other queens (Q-Q) and from workers (W-Q). The p-value was calculated using a Wilcoxon signed-rank test. **B**) The rate of W→Q aggression received by queens that became either reproductive (n=22) or non-reproductive (n=47). The p-value was calculated using a Mann-Whitney U test. Box-plots show the median (line), mean (dashed line), quartiles (box limits) and 10^{th} and 90^{th} percentiles (error bars).

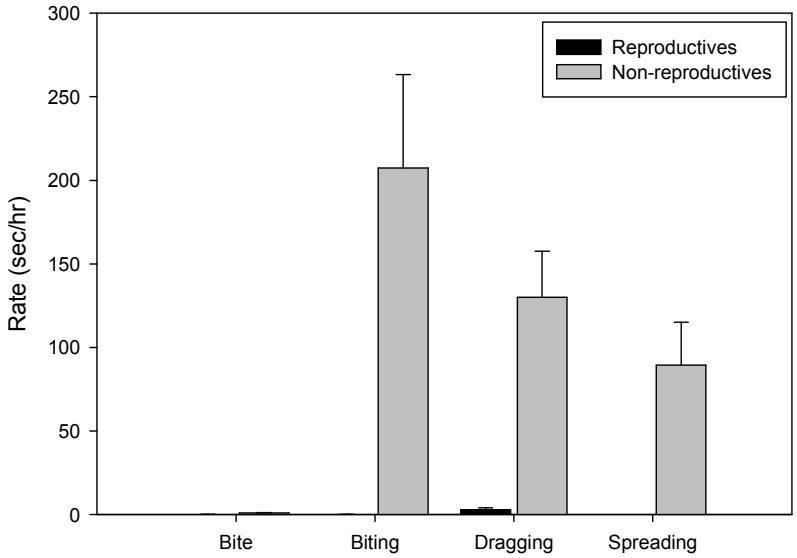


Figure 6. Comparison of the mean (±s.e.m.) rate of each type of W→Q aggressive behaviour received by queens that became reproductive (n=22; black bars) and remained non-reproductive (n=47, grey bars).

Grooming

The rate of non-aggressive W→Q 'grooming' behaviour (mean=495.5±46.6 sec/hr) was significantly higher than the rate of Q→Q grooming (mean=12.5±4.2 sec/hr; Wilcoxon sign rank test: Z=7.17, n=69, p<0.001). W→Q grooming was also biased but in the opposite direction to that found for aggression, with future reproductive queens receiving a significantly higher rate of worker grooming than non-reproductive queens (667.7±107.0 sec/hr vs 415±42.9 sec./hr; Mann-Whitney: U=335, n_1=22, n_2=47, p=0.019; see Appendix 1). Indeed, W→Q grooming predicted the future reproductive status of queens (logistic regression: ß(s.e.)=-0.002(0.001), Wald=5.11, df=1, p=0.024, odds ratio (95% CI)=0.998(0.997-1.0)). In contrast, there was no significant difference in the amount of Q→Q grooming received (Mann-Whitney: U=478, n_1=22, n_2=47, P=0.62).

3(1).3.2 Genetic analyses

Sibship

Parentage analysis revealed that in 14/21 colonies a single resident queen was the mother of all genotyped workers and larvae ('maternal' colonies) and so had been the reproductive queen the previous year(s) (Table 4). In all 14 maternal colonies it was this mother queen who became the reproductive during our observations. Workers thus discriminated in favour of their less related mother (r=0.5) if she was resident in the colony in spite of there being in eight colonies other potentially functional queens that were fullsisters (r=0.75) to the workers (i.e. the offspring of the maternal queen). One possible explanation for maternal queens always becoming reproductive might be that they develop their ovaries faster than previously non-reproductive queens and so have a reproductive head start. There was no support for this, however, as there was no difference in the time that eggs appeared in the colony between maternal colonies (40.3±3.8 days) and colonies where no mother queen was present (non-maternal, 33.6±4.2 days; t-test=1.03, df=15 p=0.32). Crucially, in non-maternal colonies W→Q aggression was still found to be significantly biased towards the queen that became reproductive (reproductive=5.1±3.5 sec/hr vs non-reproductive=219.2±65.8 sec/hr; Mann-Whitney: U=12, n_1=6, n_2=15, p=0.008; Figure 7A), and as in maternal colonies, in 5/6 colonies it was the queen who received the lowest level of aggression and remained in the nest that became the reproductive. There was also no significant difference in the average rate of W→Q aggression between the 14 maternal colonies and the six non-maternal colonies (Mann-Whitney: U=395, n_1=43, n_2=21, p=0.42; Figure 7B).

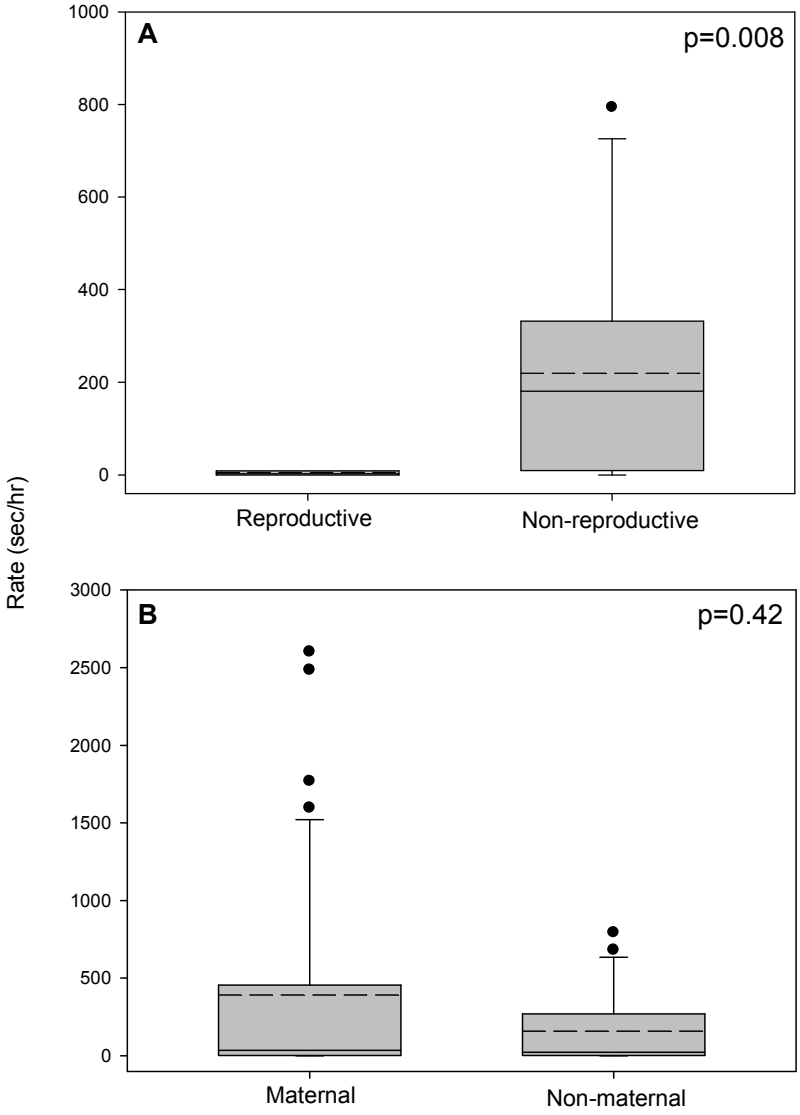


Figure 7. Observed W→Q aggression in non-maternal and maternal colonies. **A**) In the six non-maternal colonies alone, the rate of W→Q aggression received by queens that became reproductive (n=6) and non-reproductive (n=15). The p-value was calculated using a Mann-Whitney U statistical test. **B**) The rate of W→Q aggression received by all queens in maternal (n=43 queens in 14 colonies) and non-maternal colonies (n=21 queens in 6 colonies). The p-value was calculated using a Mann-Whitney U statistical test. Box-plots show the median (line), mean (dashed line), quartiles (box limits) and 10^{th} and 90^{th} percentiles (error bars).

Table 4. Sibship analysis of 21 of the observed MQ colonies. Fullsib family membership shows the number (shown in brackets) of queens (Q), workers (W) and larvae (L) assigned to the same fullsib family. Queens are classed as either the future reproductive queen (RQ) or non-reproductive queens (NRQ). 'RQ genotype match' shows which fullsib family's predicted maternal genotype matches the RQ genotype. Colonies were classified as maternal if the mother of the majority fullsib family was resident in the nest.

Colony	No. genotyped			Fullsib family membership			RQ genotype	Maternal or
	Q	W	L	Majority fullsib family	2	3	match	non-maternal
A01_1810	3	8	2	NRQ(2); W(8)	**RQ**	L(2)	Family 3	-
A11_1910	1	7	-	**RQ**; W(7)			None	NM
B07_1810	4	8	-	NRQ(2); W(8)	**RQ**; NRQ		Majority	M
B13_1910	3	9	4	NRQ(2); W(9); L(4)	**RQ**		Majority	M
B14_1810	2	8	-	NRQ; W(8)	**RQ**		Majority	M
OT3.07	5	8	5	**RQ**; NRQ(4); W(8); L(5)			None	NM
OT3.13	2	8	-	W(8)	**RQ**	NRQ	Majority	M
OT3.27	6	8	3	NRQ(5); W(8); L(3)	**RQ**		Majority	M
OT3.32	4	8	-	**RQ**; NRQ(3); W(8)			None	NM
OT4.03	3	8	5	**RQ**; NRQ(2); W(8); L(5)			None	NM
OT4.09	2	8	6	W(8); L(6)	**RQ**	NRQ	Majority	M
OT4.13	4	8	-	**RQ**; NRQ(3); W(8)			None	NM
OT4.15	1	8	6	W(8); L(6)	**RQ**		Majority	M
OT4.19	3	8	6	NRQ(2); W(8); L(6)	**RQ**		Majority	M
OT4.35	1	8	6	**RQ**; W(5); L(6)	W(3)		None	NM
OT5.02	3	8	6	NRQ(2); W(8); L(6)	**RQ**		Majority	M
OT5.03	2	8	6	NRQ; W(8); L(6)	**RQ**		Majority	M
OT6.01	3	8	6	NRQ; W(8); L(6)	**RQ**; NRQ		Majority	M
V.01	2	7	6	W(7); L(6)	**RQ**	NRQ	Majority	M
V_06	2	8	5	W(8); L(5)	**RQ**	NRQ	Majority	M
V.22	2	8	3	W(8); L(3)	**RQ**; NRQ		Majority	M

* Colony A10_1810 was not included in the sibship analysis because resident queens were not genotyped.

Relatedness

Average colony relatedness over all 22 colonies was high (0.68±0.02; n=293 individuals). Relatedness among colony workers (0.74±0.03; n=172W) and larvae (0.71±0.04; n=63L over 16 colonies) supports the sibship analysis that the majority of individuals within colonies belong to a single fullsib family (i.e. are fullsisters). The average relatedness among colony queens was also high (0.57±0.07; n=55RQ over 18 colonies), and relatedness among just future non-reproductive queens (0.74±0.05; n=30NRQ over 11 colonies) supports that they are mostly fullsisters.

3(1).3.3 Queen size

There was no significant difference in queen size between future reproductive and non-reproductive queens (reproductive: 0.52±0.01, range=0.44-0.59 vs non-reproductive: 0.51±0.01, range=0.43-0.56; Mann-Whitney: U=304, n_1=21, n_2=36 P=0.22). There was also no correlation between queen size and the rate of W→Q aggression received (Spearman's rank correlation: r=-0.24, P=0.074), Q→Q aggression carried-out (r=-0.03, P=0.85), or Q→Q received (r=0.115, P=0.40).

3(1).3.4 Queen mated state

The mated status of 49 queens was confirmed, showing that 96% of these queens were mated (see Table 3). The mated status of 20 queens (from 16 colonies) could not be determined but based on the proportion of mated queens in this and a previous study (also 96% of queens mated; see chapter 2; Gill *et al.* 2009) these queens were likely mated. In two colonies a single queen was unmated, and these queens were included in the

behavioural analysis for two reasons: 1) excluding the data made no significant effect on the outcome of the analyses (i.e. W→Q aggression still predicted future queen reproduction; logistic regression: Wald=6.31, p=0.012), and 2) it is not known whether workers in this species can discriminate between mated and unmated queens.

3(1).4 DISCUSSION

This study provides evidence contradicting a fundamental assumption of almost all skew models, namely that skew is controlled by those individuals that compete directly over reproduction (e.g. Vehrencamp 1983a, b; Reeve & Ratnieks 1993; Johnstone 2000). In the eusocial Hymenoptera this assumes queen control, but my findings support the worker control hypothesis, providing evidence to support worker aggression as the causative factor influencing queen reproduction. High worker aggression towards queens predicted which queen per colony became the reproductive, a contrast to the particularly low among queen aggression that did not predict which queen became reproductive. Queens that became reproductive overall received a low amount of W→Q aggression (in many cases no aggression) compared with those that remained non-reproductive. A similar trend was also found in W→Q grooming of queens where the queen that became reproductive received a higher amount of grooming, further supporting that workers favour one queen over all other resident queens.

The worker regulation we have demonstrated is, in many senses, a type of worker 'policing' behaviour akin to that found between queens and workers and among workers over male parentage (see Ratnieks *et al.* 2006). Furthermore, worker regulation of queen reproduction extends the repertoire of reproductive conflicts over which workers are influential. In other social insects excess queens are reared but then culled (e.g. Wenseleers *et al.* 2004), and although worker aggression in *L. acervorum* did sometimes result in queen death, the majority of *L. acervorum* queens were not killed and remained in the nest. This largely non-lethal aggression can therefore be considered a conflict resolving mechanism (see Ratnieks *et al.* 2006) which allows non-reproductive queens to

remain in the nest as insurance against death of the reproductive queen (Gill *et al.* 2009), or perhaps for future budding (Stille & Stille 1993; Heinze *et al.* 1995) or solitary dispersal (Bourke & Heinze 1994; Field *et al.* 1998; Sumner *et al.* 2002; 2010). Social queuing - where queens wait for a reproductive opportunity (Kokko & Johnstone 1999; Ragsdale 1999) - could thus be potentially important in functionally monogynous *L. acervorum* colonies.

Based on the evidence, I conclude that workers effectively police queen reproduction through aggression, which can be as extreme as killing and evicting queens to maintain high skew in the colony. This mechanism is similar to aggression observed in some queenless ants where lower ranking workers are aggressive towards beta females in order to maintain the reproductive monopoly of alpha females (Monnin & Ratnieks 2001; Monnin *et al.* 2002). But this is the first study to show worker policing of queens in a species with morphologically distinct castes and extends the repertoire of reproductive conflicts over which workers have the power and information to influence (Beekman *et al.* 2003; Beekman & Ratnieks 2003).

Worker policing of queens cannot be motivated by the selfish desire of workers to reproduce directly, because workers cannot replace queen-derived diploid offspring (workers and new queens), they can only produce haploid offspring (males). Therefore, this behaviour should be motivated by favouring the queen that meets the overall interests of the workers as a collective. The majority rules model of skew (Reeve & Jeanne 2003), predicts that the 'virtual dominant' (reproductive queen in this case) should be the individual that workers as a collective have the maximum average relatedness to. However, in this study, parentage analysis showed that when a mother queen is resident

in the colony, workers always favour their less related mother (r=0.5) over fullsister queens (r=0.75). Although at first this seems counterintuitive, such worker policing of queens in favour of their mother does allow worker interests to prevail. This is because workers are more related to their mother's offspring (average r=0.5) than to their fullsisters' offspring (average r=0.375) based on queens being mated to a single unrelated male (Heinze *et al.* 1995b; Hammond *et al.* 2001; Gill *et al.* 2009) (chapter 2). Therefore, this suggests that a possible modification of the majority rules model is that individuals do not necessarily base their behaviour on their direct relatedness to an individual(s) *per se* but is based on the indirect fitness benefit they receive from their relatedness to future generations.

The finding of workers favouring their mother supports the prediction of Reeve & Keller (1995) who proposed that skew should be high in groups constituting a mother-daughter association, and this further supports the importance of relatedness in selecting for policing behaviour, as found in the majority of examples of worker policing of worker reproduction (Ratnieks & Wenseleers 2005; Ratnieks *et al.* 2006; Wenseleers & Ratnieks 2006). However, in non-maternal colonies all genotyped queens were fullsisters to the workers, so workers were equally related to all queens. The prediction of Reeve & Keller (1995) and the majority rules model (Reeve & Jeanne 2003) is that skew should be low in daughter-daughter associations. In spite of this, in non-maternal colonies W→Q aggression was still biased, and only one queen became reproductive. This suggests that worker policing of queens may be an evolved rule of thumb that maintains high colony relatedness, rather than a facultative response to changes in worker-queen relatedness asymmetry (see also discussion in part 2 of this chapter).

A corollary of the worker control hypothesis is that an absence of worker aggression towards queens should lead to all queens reproducing. In full support, in closely related polygynous populations of the same species (chapter 2; Gill *et al.* 2009) queens receive little or no aggression from workers (Bourke 1991; Heinze *et al.* 1997) and tellingly all queens within multiple queen colonies reproduce (low skew polygynous populations) (Heinze *et al.* 1995a; Heinze *et al.* 1995b; Bourke *et al.* 1997; Hammond *et al.* 2006). This contrast in behaviour and associated low skew social organisation further supports my conclusion that the selective aggression of workers towards queens regulates queen reproduction and is a form of worker policing. Importantly, the contrast between worker aggression leading to high skew and worker passivity leading to low skew, also shows that policing behaviour can vary even within a single species; something that has rarely been investigated (Foster & Ratnieks 2000). Furthermore, multiple queens reproducing in low skew populations of *L. acervorum* cannot be because there is no mechanism of control available to workers as this study clearly shows a workable mechanism by which workers can successfully maintain high skew. Therefore there must be a benefit in multiple queens reproducing that outweighs the dilution of relatedness in these polygynous populations. This highlights that although relatedness is a vital component of Hamilton's inclusive fitness model (Hamilton 1964), the benefits and costs of cooperation (*b* & *c* components of Hamilton's rule) are important components and must also be considered for a fuller understanding of social behaviour (Hammond & Keller 2004; West *et al.* 2007; Herbers 2009).

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RJG and RLH wrote the manuscript.

RJG carried out field and laboratory work.

Additional data

See Appendix 1.

CHAPTER 3: Part 2

Further evidence for worker policing of queens

3(2).1 INTRODUCTION

Understanding conflict and its resolution in social animal groups means we must identify where the balance of power lies among group members and the behavioural mechanisms that allow control (Alexander 1974; Trivers 1974; Trivers & Hare 1976; Beekman *et al.* 2003; Beekman & Ratnieks 2003). Hamilton's inclusive fitness theory (1964) proposes that conflicts of interest are inevitable in any social group consisting of non-clonal individuals ('potential conflict'). But ultimately, which individual(s) or party holds power is what determines the outcome of such conflict ('actual conflict') (Ratnieks *et al.* 2006). This can be illustrated when considering the potential conflict over offspring sex ratio in monogamous species of the eusocial Hymenoptera. The haplodiploid reproductive system means that workers are more related to fullsisters (0.75) than to their brothers (0.25) whereas the queen is equally related to both (0.5). Therefore, the optimal ratio for workers is more female biased (3:1) than it is for the queen (1:1) (Trivers & Hare 1976; also see Boomsma 1989; Boomsma & Grafen 1990). Workers can attempt to control sex ratio by preferentially rearing females over males, but queens are known to attempt to negate such coercion by biasing the sex of their eggs (male biased). Therefore there is a power struggle over sex ratio among workers and queens with both parties having some form of control (e.g. Chapuisat *et al.* 1997; Chapuisat & Keller 1999; Helms *et al.* 2000; Passera *et al.* 2001; de Menten *et al.* 2005; Rosset & Chapuisat 2006).

Unlike sex ratio theory, however, theoretical models developed to explain stable skew often overlook the influence of a third party (workers) and assume that control is among the primary reproductive individuals (see Johnstone 2000). Indeed, a number of empirical studies have indicated that among queen interactions determine queen reproductive dominance in social insects (e.g. Roseler & Roseler 1989; Heinze & Lipski 1990; Heinze & Smith 1990; Ortius & Heinze 1995; Premnath *et al.* 1996; Bernasconi & Strassmann 1999; Cuvillier-Hot *et al.* 2002). However, the interests of non-reproductive workers cannot be ignored, because if worker interests are neglected then the stability of the group may be jeopardised. If skew among queens is determined by queens themselves then the outcome may not necessarily meet the interests of the workers. For instance, workers may favour one queen over another based on higher relatedness to the queen or offspring they rear (see part 1 of this chapter). Furthermore, having multiple reproductive queens can dilute colony relatedness and thus lowers worker indirect fitness because workers end up rearing less related brood. Therefore, it is in the interests of the workers to play a primary role in determining skew among queens, and the general numerical superiority of workers in most species means as a collective force they potentially hold significant power (Trivers & Hare 1976; Beekman & Ratnieks 2003).

The 'majority rules model' of skew proposes that the 'decision' over which queen(s) become reproductively dominant is determined by colony members as a collective (Reeve & Jeanne 2003). In the previous study (part 1 of this chapter), I provided supportive evidence for the majority rules model; that collectively workers play an important role in determining skew among queens through aggressive behaviour in a functionally monogynous population of the ant *Leptothorax acervorum*. This 'worker

policing of queens' provides support for the hypothesis of worker control over skew, as opposed to the generally assumed queen control. However, the previous study could not entirely rule-out the queen control hypothesis because workers may base their aggressive behaviour on a predetermined establishment of dominance between queens (e.g. Monnin & Ratnieks 2001; Monnin *et al.* 2002). This may be relevant considering that in the majority of cases the reproductively dominant queen was also the reproductive queen in the previous year. Although the outcome of the worker and queen control hypotheses is the same (one queen becomes reproductively dominant), knowing who is in control and which individuals/party possess the power to directly ensure their interests prevail, has important implications on our understanding of within group conflicts and the assumptions of skew theory.

I present here a further test of the worker and queen control hypotheses by investigating the response of workers and queens to the loss of the established reproductive queen during the laying period. The objective of this experiment was to identify the influence of queens and workers in establishing a new reproductive queen within a reproductive season, by investigating the behavioural mechanism(s) that determine which queen inherits the new reproductively dominant position. Other studies have shown that removal of a dominant reproductive queen or change in colony composition can have effects on the reproduction of other queens and queen hierarchies (e.g. Monnin & Peeters 1999; Ortius & Heinze 1999; Kummerli *et al.* 2005), and aggression among workers and queens (e.g. Liebig *et al.* 1999; Vander Meer & Alonso 2002). Given these responses to such experimental manipulation, I can expect to observe a direct response by colony members to the loss of the reproductive queen during my

experiment. Queen control suggests a centralised role of control by specific individual/s, and predicts there should be aggression among queens when the reproductive position becomes vacant (for example an assumption made by the tug-of-war model of skew; Reeve *et al.* 1998). Worker control predicts that worker aggression will predict which queen fills the newly vacant reproductive dominant position (as implied by the majority rules model; Reeve & Jeanne 2003).

In this study I experimentally split multiple queen colonies in half to produce two colony fractions, one that always contained the reproductive queen with the other fraction thus only containing non-reproductive queens, and crucially previously non-reproductive queens. This allowed observation of the responses of both queens and workers to the loss of the reproductive dominant queen in one group, with the other group containing the original reproductive dominant queen acting as a type of control. If queen control was important we would expect to see form of aggression among queens competeing for the dominant position, whereas, if worker control was important we might expect that worker aggression predicts which queen becomes reproductive as previously shown in pre-split experiments (see Chapter 3(1)).

3(2).2 METHODS

3(2).2.1 Colony collection and maintenance

Colonies used in this experiment were from populations (OT & V) from the same collections (2006 & 2007) as reported in chapters 2 (Gill *et al.* 2009) and 3. Once brought into the lab, colonies were kept in artificial nests through artificially induced autumn, winter, spring, summer (as described in chapter 2 (Gill *et al.* 2009) and 3), and finally back into autumn conditions (up to 8 weeks).

3(2).2.2 Experimental Procedures

Thirty-three multiple queen (MQ) colonies (mean=4.8 queens per colony, range=2-13) were monitored from the onset of spring to establish when egg laying began and which queen showed reproductive activity (Figure 1). The reproductive queen was determined by enlargement of the abdomen (physogastric) and occupation of a central position among nestmates (see chapter 2; Gill et al. 2009). For identification purposes all queens per colony were painted using Humbrol enamel paint of a different colour on the thorax. In the majority of colonies (n=20) queen marking was undertaken a few days before the transition from winter to spring, and in the remaining colonies (n=13) this was done when colonies were split (see below).

Split experiment

Each MQ colony was split when at least two eggs had been laid and the reproductive queen identified (Figure 1). Splitting a colony involved dividing exactly half the number of workers and queens (at this point each colony possessed an average of 3.5

queens; range=2-10; see Table 1), and approximately half the number of larvae, into two groups ('group 1' & 'group 2' colonies). This was done by placing the artificial nest on ice and chilling individuals to restrict their movement. Each group was then provided with a new nest within a separate foraging arena (i.e. separate plastic containers). Group 1 colonies were always assigned the fraction of queens containing the reproductive queen ('original-reproductive queen' from hereon), and group 2 colonies were thus always assigned the fraction of queens containing all previously, and currently, non-reproductive queens. Each half of the workers and larvae were then assigned randomly to each group (Figure 1, Table 1). The formation of the group 1 colonies provided a control for the splitting process and confirmed that the original reproductive queen had been identified correctly. The formation of the group 2 colonies allowed observations of colony behaviour in response to the loss of the original reproductive queen. Colonies with an odd number of queens resulted in the split of queens being unequal, for example, in a three-queen split colony the original-reproductive queen would be assigned to the group 1 colony, and both non-reproductive queens would be assigned to the group 2 colony. During the split experiment, all eggs that had been laid in the colony were removed (stored in 75% ethanol and frozen at -20ºC) to control for any possible cues eggs may have on queen reproductive status or colony behaviour (e.g. Vander Meer & Alonso 2002).

All 33 group 1 colonies were monitored to establish when egg laying by the original-reproductive queen resumed and whether queens remained in the nest (Figure 1). Of the 33 group 1 colonies, there were 11 colonies with other non-reproductive queens assigned (referred to as 'group 1 MQ colonies'). The 33 group 2 colonies were also

monitored to see whether any non-reproductive queens became reproductive and when the date of egg laying began. In 12 of the group 2 colonies there was only a single non-reproductive queen assigned (referred to as 'group 2 SQ colonies'), and in the remaining 21 group 2 colonies, there were multiple non-reproductive queens assigned (referred to as 'group 2 MQ colonies') (see Figure 2).

i) Behavioural recordings

In 17 of the group 2 MQ colonies, colony behaviour was recorded using CCTV cameras (description in part 1 of this chapter). Behaviour was sampled using a focal queen approach, recording the type and duration of all behaviour a queen received or performed. Aggressive and non-aggressive behavioural interactions involving individually marked queens were scored using previously described methods (part 1 of this chapter). Observations were undertaken haphazardly, starting an average of four days after colonies had been split (range=1-11 days). Group 2 MQ colony recordings ended when either: i) the first egg had been laid in the colony, ii) if all but one queen had permanently evicted/left the nest and/or was killed/died, or iii) if no queen had become reproductive by four weeks into the autumn period (which is 18 weeks since the onset of spring). After the experiment and following observations all colonies were frozen (-20ºC) for dissection analysis (see below).

3(2).2.3 Dissection

At the end of the study, queens were dissected to determine their mated state (see chapter 2 for methods) as it was important to ascertain whether queens had the potential to be reproductive.

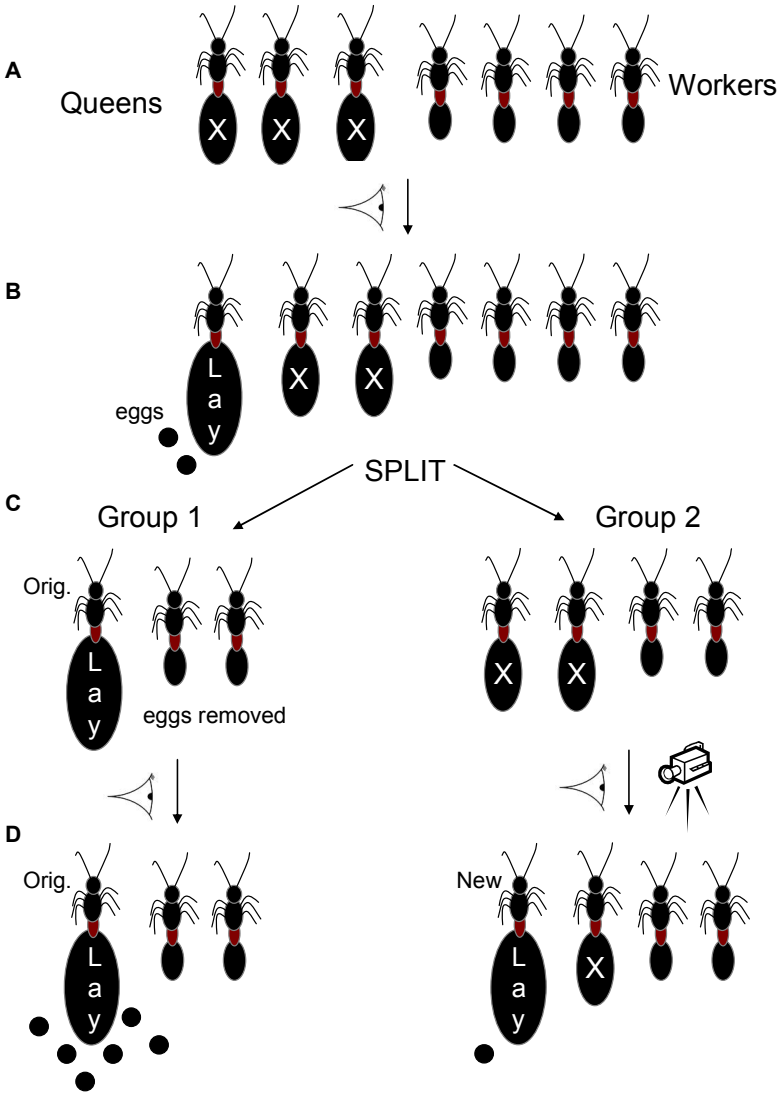


Figure 1. Procedure for the splitting experiment. **A**) Represents a three-queen colony at the start of spring when there is no reproductive queen and ovaries are undeveloped. **B**) Colonies are monitored until one queen becomes reproductive and at least two eggs have been laid. **C**) Colonies are split into two groups (1 & 2), group 1 is assigned the original-reproductive queen (Orig.) and half the workers, and group 2 is assigned both non-reproductive queens and the other half of the workers. All eggs that had been laid are removed. **D**) Group 1 colonies are monitored and the original-reproductive queen resumes egg-laying. The group 2 colonies are monitored, but colony behaviour is also recorded in the group 2 MQ colonies. Recordings stop when one of the queens becomes the new reproductive (New) and the first egg is laid.

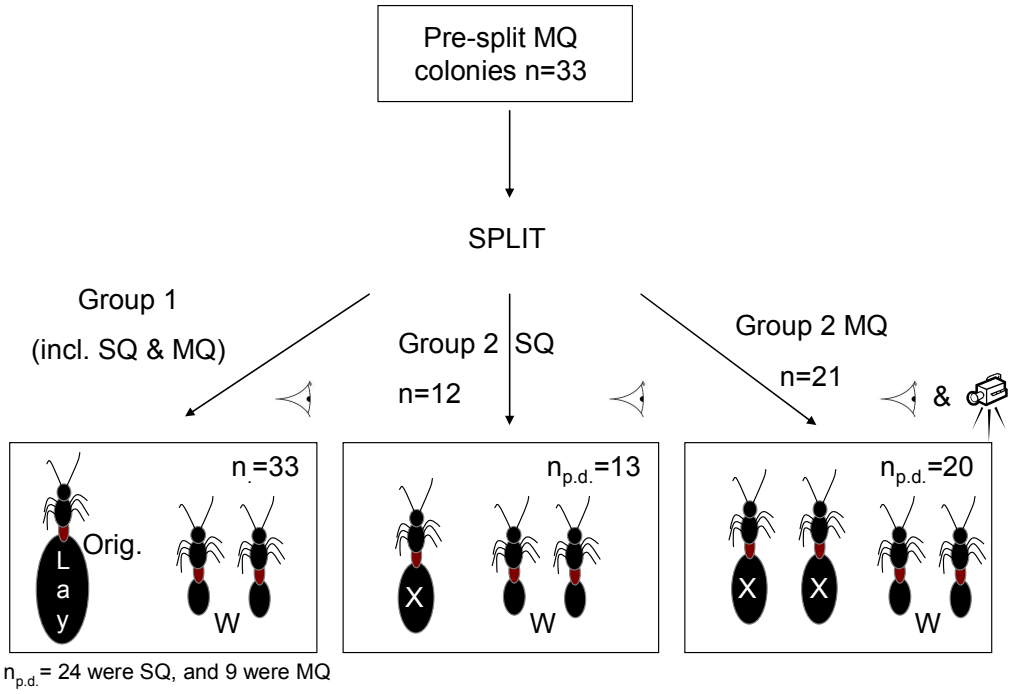


Figure 2. Thirty three MQ colonies were split in half to produce 33 group 1 colonies all of which were assigned the original reproductive queen (Orig.), and group 2 colonies which were assigned non-reproductive queens. Of the 33 group 2 colonies, 12 had a single queen and 21 had multiple queens, although dissections showed that one of the group 2 MQ colonies had only one mated queen. Therefore, the number of group 2 colonies after dissection ($n_{p.d.}$) that were SQ was 13 and MQ was 20. All group 1 and 2 colonies were monitored to look at queen eviction and queen reproduction. Colony behaviour of the group 2 MQ colonies was recorded.

Table 1. Census of the 33 MQ colonies after being split into group 1 and 2 colonies, showing the number of queens (Q) and workers (W) assigned to each group. Classification of a single queen (SQ) or multiple queen (MQ) group is determined by whether one or more queens are functional (mated) based on queen dissection data.

Colony	Group 1			Group 2		
	Q	W	SQ/MQ	Q	W	SQ/MQ
A1_1810	1	25	SQ	1	25	SQ
A10_1810	1	26	SQ	1	25	SQ
B07_1810	1	31	SQ	1	30	SQ
B13_1910	2	16	SQ	2	16	MQ
B14_1810	1	20	SQ	1	20	SQ
OT3.07	2	22	MQ	2	21	SQ
OT3.10	3	31	MQ	3	31	MQ
OT3.11	6	16	MQ	4	15	MQ
OT3.13	1	31	SQ	1	31	SQ
OT3.22	1	84	SQ	2	85	MQ
OT3.24	2	109	MQ	2	109	MQ
OT3.26	1	46	SQ	2	45	MQ
OT3.27	1	60	SQ	2	59	MQ
OT3.29	1	81	SQ	2	81	MQ
OT3.32	1	30	SQ	1	30	SQ
OT4.03	1	53	SQ	2	52	MQ
OT4.12	3	26	MQ	3	26	MQ
OT4.13	2	17	MQ	2	18	MQ
OT4.16	1	42	SQ	2	42	MQ
OT4.19	1	40	SQ	1	40	SQ
OT4.24	3	15	MQ	2	16	MQ
OT4.31	1	30	SQ	2	29	MQ
OT4.33	3	9	MQ	2	8	MQ
OT4.36	2	23	SQ	2	23	MQ
OT4.38	4	57	MQ	4	57	MQ
OT5.02	1	38	SQ	1	38	SQ
OT5.03	1	38	SQ	1	38	SQ
OT5.05	1	35	SQ	2	35	MQ
OT6.01	1	55	SQ	2	55	MQ
V.01	1	15	SQ	1	15	SQ
V.18	1	37	SQ	1	37	SQ
V.22	1	16	SQ	1	17	SQ
V_06	1	65	SQ	2	65	MQ

3(2).3 RESULTS

3(2).3.1 Dissection

From the 33 split MQ colonies (n=115 queens), 106 queens were dissected (nine queens were lost and therefore could not be dissected), with the mated state of 90/106 queens being successfully determined (Tables 1 & 2). Ninety-one percent (82/90) of these queens were mated, and eight queens were found to be unmated (over six colonies). From hereon the unmated queens were not considered in the rest of the analyses. Importantly, the dissections showed that of the 11 group 1 MQ colonies, there were two colonies (B13_1910 & OT4.36) that had only one mated queen and therefore were considered as group 1 SQ colonies from hereon. In addition, of the 17 recorded group 2 MQ colonies, there was one colony (OT3.07) that had only one mated queen, and therefore, this colony was not included in the behavioural analysis and was considered as a group 2 SQ colony from hereon. All remaining group 1 MQ colonies and group 2 MQ colonies had more than one mated queen.

In summary (see Figure 2 & Table 2), the dissection data showed that of the 33 group 1 colonies; 24 were SQ colonies and nine were MQ colonies. Of the 33 group 2 colonies; 13 were SQ colonies, and 20 were MQ colonies of which 16 were recorded for direct behavioural observations.

3(2).3.2 Pre-split observations

In all 33 colonies before the split, and as expected (chapter 2; Gill *et al.* 2009), just a single queen became reproductive with the first egg being laid an average of 39.0 days after the start of spring (range=17-66 days). Colonies were split an average of 7.2 days after the first egg lay date (range=0-22), with an average of seven eggs being present at the time of the split (range=2-49) (see Figure 3). Expectedly, the number of days after the first egg was laid until colonies were split correlated with the number of eggs present at the time of splitting (Spearman's Rank correlation=0.504, n=33, p=0.003). This variation means that across colonies the dominant reproductive queen will have been established for a longer period of time, which could result in variation in observed colony behaviour. However, the high end of this variation was primarily caused by five colonies (OT3.10, OT3.22, OT3.29, OT4.33, V.18) as these colonies were split more than 2 weeks after the first egg lay date (mean=19 days; range=17-22; mean=22 eggs, range=4-49) (see Figure 3). Of these five colonies, two (OT3.10 & OT4.33) were included in the 16 sampled group 2 MQ colonies that were recorded. If all five colonies were excluded from the analysis (therefore: n=28 colonies), the average number of days colonies were split after the first egg lay date was 5.1 (range=0-13), with an average of 4.4 eggs present at the time of the split (range=2-12), showing that among the vast majority of colonies there is little variation and that colonies were split relatively soon after the first egg was laid (Figure 3).

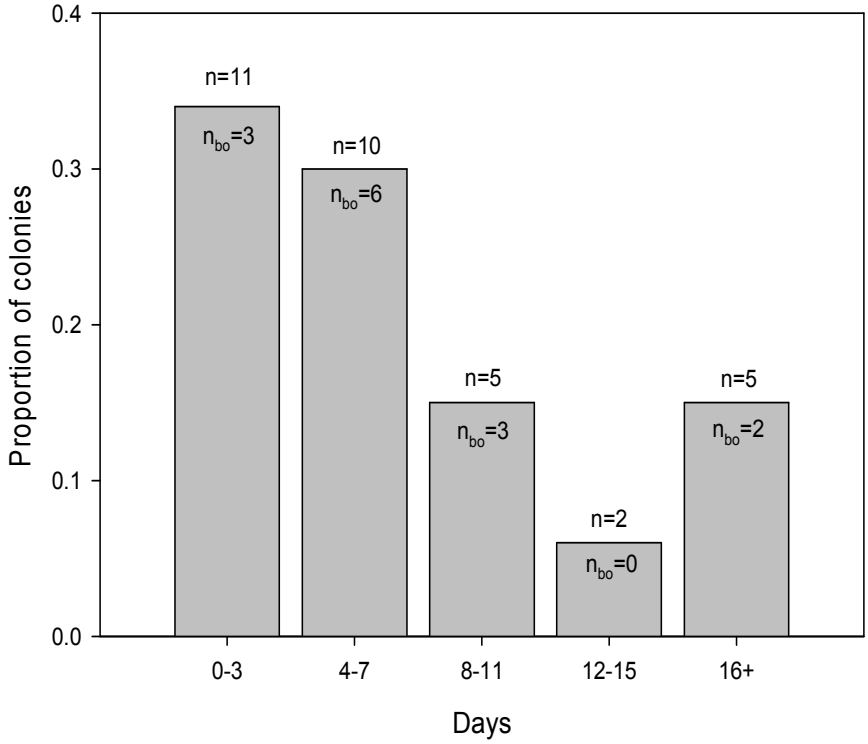


Figure 3. Number of days colonies were split after the first egg was laid in the original colonies. Majority of colonies were split within two weeks of the first egg being laid. The number of colonies is shown at the top of each bar, and the number of group 2 MQ colonies considered in the behavioural analysis (n_{bo}), is shown inside the bar.

3(2).3.3 Split experiment

Group 1 colonies

In all 33 group 1 colonies (n=48 queens), the original-reproductive queen remained in the nest and reproductive activity (egg-laying) resumed an average of 3.0 days (range=1-9) after the split (n=23 colonies; for the remaining 10 colonies the exact date was not recorded) (Figure 4), and showed reproductive activity for the full monitoring period. During the monitoring period in only two colonies did I find non-reproductive queens evicted or killed - one queen in colony OT3.24 was killed by

workers (1/48 queens (2.1%)), and two queens in colony OT4.33 permanently left the nest for unknown reasons.

Group 2 colonies

Over all 33 group 2 colonies (n=58 queens), in 24 colonies a single queen became reproductive ('new-reproductive queen' from hereon), and in nine colonies no queen became reproductive (see Table 2). In the 24 colonies where a new-reproductive queen was established, the first egg was laid an average of 15.8 days after each colony was split (range=5-34; n=23 colonies; in one colony the exact date was not recorded), which was significantly longer than that found in group 1 colonies (Mann-Whitney: $U=10$, $n_1=23$, $n_2=23$, $p<0.001$) (Figure 4). A pair-wise test also confirmed this finding, as in 17 colonies the exact date the first egg was laid after the split was known for each corresponding group 1 and group 2 colonies (Wilcoxon test: $Z=3.61$, $n=17$, $p<0.001$).

Specifically, of the 13 group 2 SQ colonies, in eight colonies the single queen became established as the new-reproductive queen, in four colonies the queen was permanently evicted and/or killed by workers leaving the colony queenless, and in colony OT5.03 the queen remained in the nest but did not become reproductive during our observations. Of the 20 group 2 MQ colonies, in 16 colonies a single queen became established as the new-reproductive queen. In the remaining four colonies no queen became reproductive: in two colonies all queens were permanently evicted and/or killed by workers leaving the colony queenless, and in the other two colonies just one queen was permanently evicted and/or killed by workers but the remaining queen did not become reproductive. There was no significant difference between group 2 SQ and group

2 MQ colonies in the number of days until the first egg was laid after colonies had been split (means=14.6±2.4 vs 16.4±2.2; Mann Whitney: U=56, n_1=8, n_2=15, p=0.82).

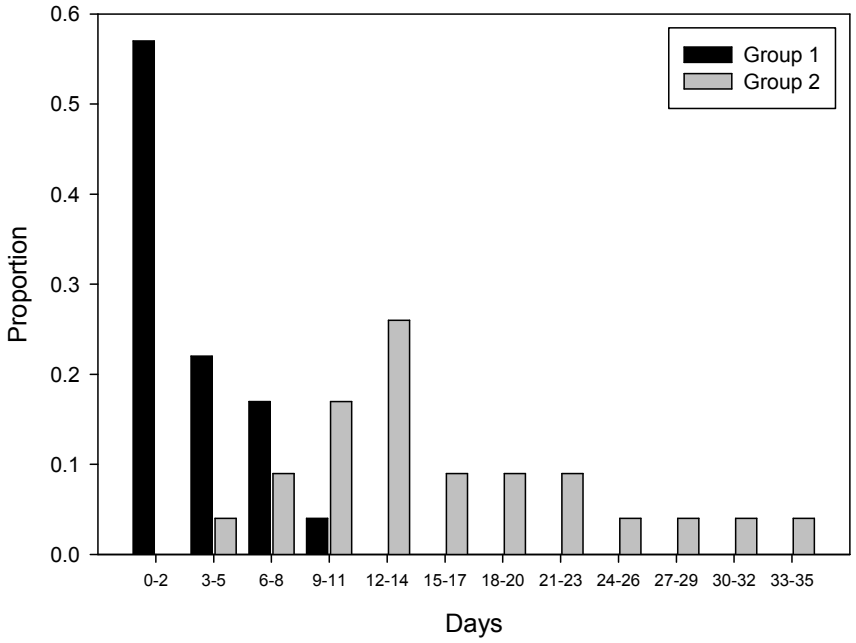


Figure 4. Frequency distribution graph showing the specific number of days until the first egg was laid after the split in group 1 (black bars) and group 2 (grey bars) colonies. Eggs appeared in group 1 colonies significantly earlier than in group 2 colonies (Mann Whitney U: p<0.001).This confirms that the original reproductive queen was determined correctly, otherwise there would be no lag in group 2 colonies.

Importantly, in all group 2 colonies there was no situation where there were multiple reproductive queens. In total, over all 33 group 2 colonies there were 20 queens that were permanently evicted and/or killed by workers (20/58 queens (34.5%)), and one queen that left the nest permanently for an unknown reason. Overall, 50% of group 2 MQ colonies (n=10) ended up either single queened or queenless colonies because workers

permanently evicted and/or killed queens (Table 2), which was a significantly higher proportion than that found in group 1 colonies (Fishers Exact Test: df=1, $p<0.001$).

Behavioural observations of the recorded group 2 MQ colonies

Aggression

Of the 16 recorded and observed group 2 MQ colonies, the 14 colonies where a previously non-reproductive queen became established as a new-reproductive were considered in the behavioural analysis. In total 76.4 hours of individual queen behaviour was observed for a total of 32 queens (range=2-4 queens per colony; mean per queen=2.39 hrs, range=0.08-3.92). In all 14 colonies aggressive behaviour towards queens was observed (Figure 5), with the vast majority of aggression received from workers (W→Q; 95.4%), with an average rate per queen of 280±123 sec/hr. In comparison, the average rate of queen aggression received by other queens (Q→Q) was considerably lower (4.7±3.0 sec/hr; 1.3%). The rate of W→Q aggression was significantly higher than the rate of Q→Q aggression (Wilcoxon signed rank: $Z=4.45$, $n=32$, $p<0.001$), which was still found when accounting for the numerical superiority of workers (mean no. workers=31 per colony, range=8-65; Wilcoxon signed rank test: $Z=2.99$, $n=32$, $p=0.001$). In addition, there was a positive trend in the number of workers per colony and the average rate of W→Q aggression across all queens, but this was not significant (Spearman's rank correlation: 0.49, df=13, $p=0.08$) (see Appendix 2 for Q→W interactions).

Importantly, there was variation among queens in the rate of W→Q aggression received (Figure 5A). The rate of W→Q aggression received by queens that became the

new-reproductive as significantly lower than that received by queens that remained non-reproductive (means=34.3±17.2 vs 470±209 sec/hr; Mann-Whitney: U=40, n_1=14, n_2=18, p=0.011; Figure 6A). Logistic regression showed that W→Q aggression was very close to significantly predicting future queen reproductive status (logistic regression: ß(s.e.)=-0.10(0.005), Wald=3.5, df=1, p=0.06, odds ratio (95% CI)=1.01(1.0-1.02)). Specifically, in 11/14 colonies the new-reproductive queen received the least amount of W→Q aggression among all colony queen/s, although unlike that found in part 1 of this chapter there was only one colony where the future new-reproductive queen received no W→Q aggression. These results, as in the previous chapter, show a bias in W→Q aggression towards all but one queen which subsequently becomes the new reproductive.

There was no significant difference in the rate of Q→Q aggression carried out by new-reproductive queens and non-reproductive queens (means±s.e.m.=0.18±0.13 vs 8.22±5.31; Mann-Whitney: U=111, n_1=14, n_2=18, p=0.63; Figures 6B), and it did not predict queen future reproductive status (logistic regression: ß(s.e.)=-0.151(0.212), Wald=0.511, df=1, p=0.48, odds ratio (95% CI)=1.163(0.768-1.761)) (see Appendix 2 for Q→W interactions). However, there was a specific act of aggression between queens that has not previously been observed (part 1 of this chapter) or to my knowledge in this species. Observations showed that in some colonies on rare occasions a queen tried to sting another queen. This behaviour involved a queen holding on to another queen whilst bending her gaster so as to touch the attacked queen, a behaviour similar to that reported during a 'sting-smearing' event in a study on the queenless ant *Dinoponera quadriceps* (Monnin *et al.* 2002).

Overall, I observed 11 sting smearing (S.S.) acts over three colonies (range=2-7 acts per colony; Table 3). After S.S. act workers reacted overall with increased W→Q aggression (Figure 7). Analysis of the rate of W→Q aggression received by each queen involved (both the actor and recipient queen of the SS act) in the 10 minute period leading up to a S.S. event and the 10 minute period after the event, showed a significantly higher W→Q aggression after a S.S. event (pre: 0.63±0.63 vs post: 79.1±54.4 sec/hr; Wilcoxon signed rank test: $Z=3.67$, $n=8$, $p=0.014$). Only eight pair-wise values were used in the analysis (each event involved two queens, and there were four events analysed (two in colony B13_1910, and one in both OT3.10 and OT4.12)). This is because in three cases (one case in each of the three colonies) there was more than one S.S. event within the same video recording. Therefore, it was possible that the reaction to the first SS event was still present during the 10 minutes prior to the second SS event, so I took the rate of W→Q aggression before the first sting smear and after the last sting smear. For one case (B13_1910) I could not analyse the rate because the event occurred right at the end of the recording so I was unable to measure the aggressive rates after the event. In all observed cases (which included eight queen observations) the rate of W→Q aggression received after a S.S. event was higher for all queens. Furthermore, there was no major difference in the number of S.S. acts carried-out by the new-reproductive queen (n=six acts by two queens) and non-reproductive queens (n=five acts by three queens).

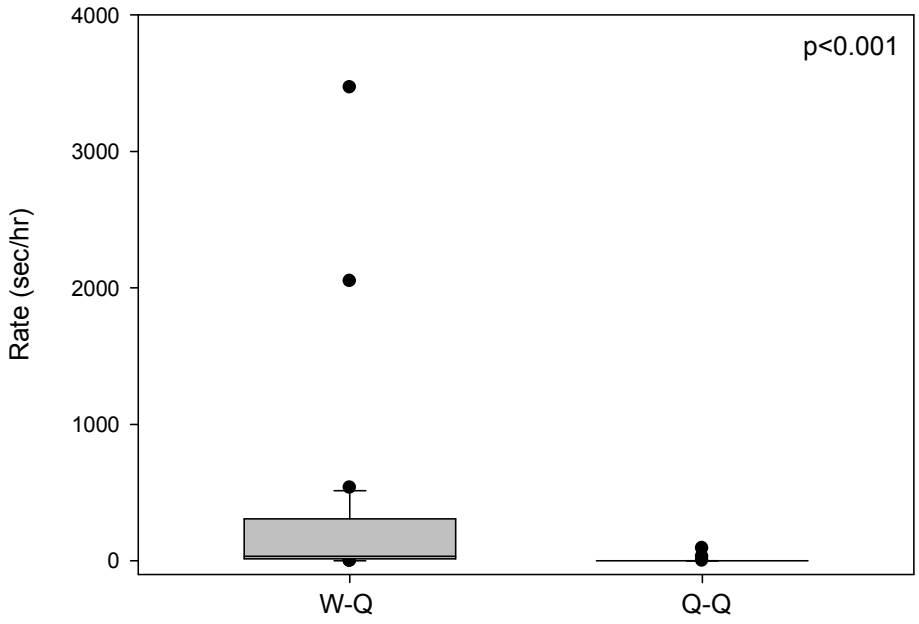


Figure 5. Rate (sec/hr) of W→Q and Q→Q aggression observed over all queens (n=32) in 14 group 2 MQ colonies. The p-value was calculated using a Wilcoxon signed rank test. Box-plots show the median (line), mean (dashed line), quartiles (box limits) and 10th and 90th percentiles (error bars).

Table 2. Mated state of queens across all 33 split MQ colonies, with queens classified as either mated (M, yellow), unmated (U, green) or undetermined (?, purple), and queens which were not dissected are shown as X (grey). Columns show whether each queen was the original reproductive queen (orig. RQ) in group 1, the new reproductive queen (new RQ) in group 2, or non-reproductive queens in both groups (NRQ). Group 2 colonies possessed either a single queen (SQ), or multiple queens (MQ), and the 16 group 2 MQ colonies are symbolised by an asterix (*) if they were used in the behavioural analysis and a hash (#) if they were excluded from the analysis because no queen became reproductive. Queens that were permanently evicted or killed by workers have a diagonal lined pattern, and queens which were permanently evicted or died from unknown causes are boxed.

Colony	Behav.	Group 1						SQ/MQ	Group 2				
	Obs.	Orig._RQ	NRQ1	NRQ2	NRQ3	NRQ4	NRQ5		New_RQ	NRQ1	NRQ2	NRQ3	NRQ4
B13_1910	*	M	U					MQ	?	X			
OT3.10	*	M	M	U				MQ	?	M	M		
OT3.11	*	M	M	U	U	?	?	MQ	M	M	M	X	
OT3.22		M						MQ	-	M	M		
OT3.24		M	?					MQ	M	M			
OT3.26		M						MQ	-	M	?		
OT3.27	*	M						MQ	?	?			
OT3.29		M						MQ	M	M			
OT4.03	*	M						MQ	M	?			
OT4.12	*	M	M	M				MQ	M	M	?		
OT4.13	*	M	M					MQ	M	M			
OT4.16	*	M						MQ	M	M			
OT4.24	*	M	M	M				MQ	M	M			
OT4.31	*	M						MQ	M	X			
OT4.33	*	M	?	X				MQ	M	M			
OT4.36	*	M	U					MQ	M	M			
OT4.38	#	M	M	U	X			MQ	-	M	M	U	X
OT5.05	*	M						MQ	M	M			
OT6.01	#	M						MQ	-	M	M		
V_06	*	M						MQ	X	M			
A01_1810		M						SQ	M				
A10_1810		M						SQ	M				
B07_1810		M						SQ	-	M			
B14_1810		M						SQ	?				
OT3.07		M	?					SQ	M	U			
OT3.13		M						SQ	-	?			
OT3.32		X						SQ	-	M			
OT4.19		M						SQ	-	?			
OT5.02		M						SQ	?				
OT5.03		M						SQ	-	M			
V.07.01		M						SQ	M				
V.07.18		M						SQ	X				
V.07.22		M						SQ	M				

Grooming

Overall, the majority of grooming carried out was W→Q grooming (54.8%), with the average rate received per queen being 179.5±21.4 sec/hr, which was significantly higher than Q→Q grooming received by queens (mean=21.2±10.9; 6.2%; Wilcoxon signed rank test: Z=4.9, n=32, p<0.001). However, when accounting for the numerical

superiority of workers, the per capita rate of W→Q grooming was not significantly different than Q→Q grooming (Wilcoxon signed rank test: Z=-1.05, n=32, p=0.86). There was no significant difference in the rate of W→Q grooming between new-reproductive and non-reproductive queens (means=172.3±33.5 vs 185.2±28.5; Mann-Whitney: U=118, p=0.78) nor was there in the rate of Q→Q grooming (means=4.73±1.88 vs 33.9±18.9; Mann-Whitney: U=94, n1=14, n2=18, p=0.89) (see Appendix 2 for Q→W interactions).

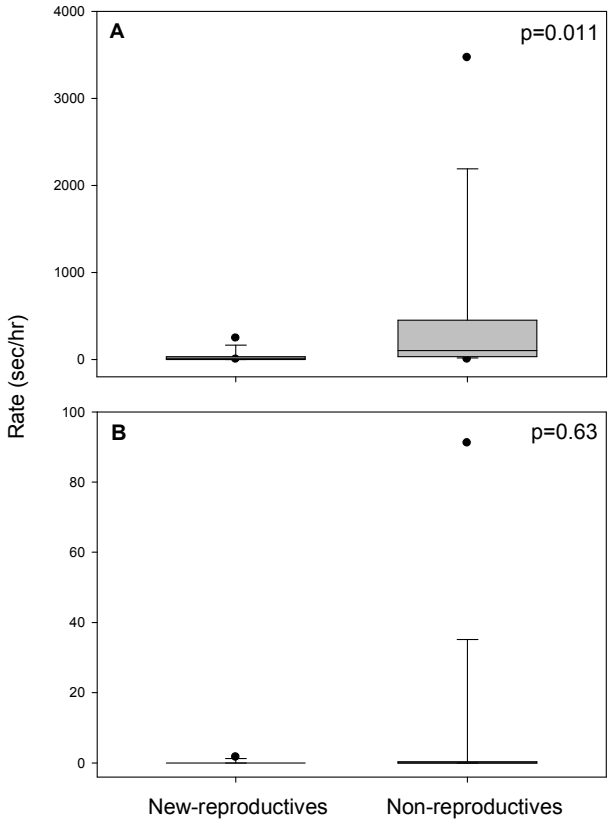


Figure 6. Rate of aggression observed in 14 group 2 MQ colonies received by queens that became the new-reproductive (n=14) and queens that remained non-reproductive (n=18). **A**) W→Q aggression; **B**) Q→Q aggression. The p-values were calculated using a Mann-Whitney U statistical test.

Table 3. Summary of the 11 observed S.S. acts among queens. The direction and description of each act between queens is shown and whether queens are new-reproductives (NewRQ) or non-reproductives (NRQ). The actor is the individual carrying out the sting smear, and the recipient the individual receiving it. If acts occur simultaneously among queens (that each queen tries to sting each other at the same time) then both acts are classed under the same event, and it is this event that is considered in the analysis looking at pre and post S.S. aggression rates.

Colony	Actor	NewRQ/ NRQ	Recipient	NewRQ/ NRQ	Act	Response	Event
B13_1910	GQ	NewRQ	PQ	NRQ	Act 1	Act 1 & 2 happened simultaneously; GQ and PQ tried to sting	Event 1*
	PQ	NRQ	GQ	NewRQ	Act 2	each other, resulting in workers attacking both queens	Event 1*
	GQ	NewRQ	PQ	NRQ	Act 3	Workers attacked PQ	Event 2
	GQ	NewRQ	PQ	NRQ	Act 4	GQ tried to sting PQ whilst workers were attacking PQ	Event 3^
	PQ	NRQ	GQ	NewRQ	Act 5	Workers attacked GQ	Event 4
	PQ	NRQ	GQ	NewRQ	Act 6	Act 6 & 7 happened simultaneously; GQ and PQ tried to sting	Event 5*
	GQ	NewRQ	PQ	NRQ	Act 7	each other, resulting in workers attacking both queens	Event 5*
OT3.10	GQ	NewRQ	OQ	NRQ	Act 8	Workers attacked GQ	Event 6
	GQ	NewRQ	OQ	NRQ	Act 9	Workers attempted to bite the abdomen of the GQ where sting is.	Event 7
OT4.12	OQ	NRQ	BQ	NRQ	Act 10	Act 10 & 11 happened simultaneously; BQ and OQ tried to sting	Event 8*
	BQ	NRQ	OQ	NRQ	Act 11	each other, resulting in workers attacking both queens	Event 8*

*events 1 & 2, 4 & 5, and 6 & 7 occurred in the same video recording, therefore the rate of pre W-Q aggression was analysed before the first event (i.e. event 1), and the rate of post W-Q aggression was analysed after the second event (i.e. event 2).

^event 3 occurred at the end of a recording so could not be analysed.

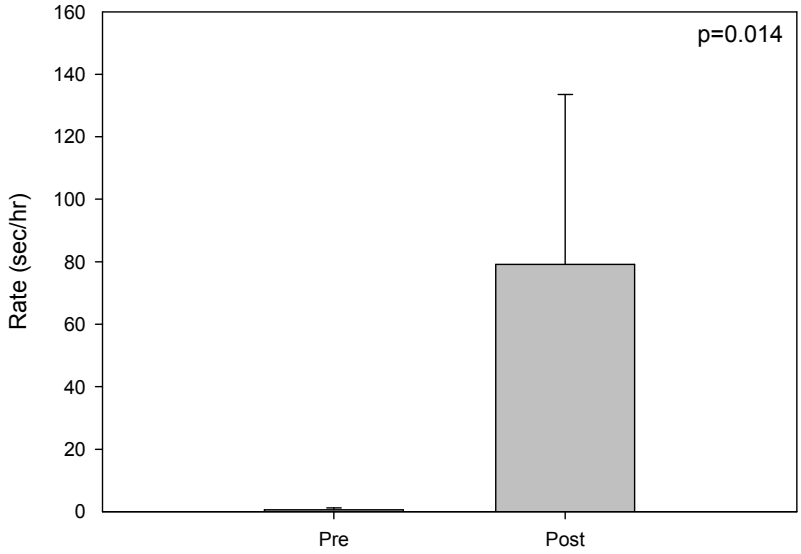


Figure 7. Average rate (±s.e.m.) of W→Q aggression received per queen during the 10-minute period leading up to a S.S. event (Pre) and the rate received in the 10-minute period immediately after a S.S. event (Post). This result is based on four S.S. events (over three colonies) including data from both queens involved in each event. The p-value was calculated using a Wilcoxon signed rank test).

Comparison with previous study (part 1)

To be clear, this section compares the behavioural observations of colonies that were not manipulated ('Part 1' of this chapter) with the behavioural observations found in the current study where colonies had been manipulated ('Part 2'). In otherwords, I am comparing the pre-laying observations between unmanipulated and manipulated colonies to see whether there is an effect of the dominant queen being removed.

A comparison of the behaviour observed in Part 1 showed that the average rate of W→Q aggression was very similar (Part 1: 292, vs, Part 2: 280, sec/hr) and is not significantly different (Mann-Whitney: $U=990$, $n_1=69$, $n_2=32$, $p=0.41$; Figure 8).

Furthermore, there is no statistical difference between the average rate of Q→Q aggression (Part 1: 0.6, vs, Part 2: 4.7, sec/hr; Mann-Whitney: U=986, n_1=69, n_2=32, p=0.39; Figure 6). This shows that W→Q aggression remains high when the reproductively dominant queen is removed, and Q→Q aggression remains low. This, therefore, does meet the prediction of the queen control hypothesis, as we would expect an increase in Q→Q aggression when the dominant position becomes vacant.

The rate of W→Q aggression received by queens who became the reproductive in Part 1 was significantly lower than the rate received in Part 2 (Part 1: 3.1, vs, Part 2: 34.3, sec/hr; Mann-Whitney: U=61, n_1=22, n_2=14, p<0.01; Figure 9A). But, there was no significant difference in the rate received by queens that remained non-reproductive in both studies (Part 1: 428, vs, Part 2: 470, sec/hr; Mann-Whitney: U=405, n^1=47, n^2=18, p=0.79; Figure 9B). Importantly, the rate of Q→Q aggression received by queens who became the reproductive in Part 1 was not significantly different than the rate received in Part 2 (Part 1: zero, vs, Part 2: 0.18, sec/hr; Mann-Whitney: U=138, n_1=22, n_2=14, p=0.62), nor was it by queens that were non-reproductive (Part 1: 0.81 , vs, Part 2: 8.2 , sec/hr; Mann-Whitney: U=375, n_1=47, n_2=18, p=0.49), which again does not support the queen control hypothesis.

Interestingly, the rate of W→Q grooming during Part 1 was significantly higher than in Part 2 (Part 1: 496, vs, Part 2: 180, sec/hr; Mann-Whitney: U=410, n_1=69, n_2=32, p<0.001). In contrast, there was no difference in the rate of Q→Q grooming (Part 1: 12.5, vs, Part 2: 21.2, sec/hr; Mann-Whitney: U=1021, n_1=69, n_2=32, p=0.55). Furthermore, W→Q grooming towards the queen that became reproductive in Part 1 was significantly

higher than received in Part 2 (Part 1: 668, vs, Part 2: 172, sec/hr; Mann-Whitney: U=31, n_1=22, n_2=14, p<0.001).

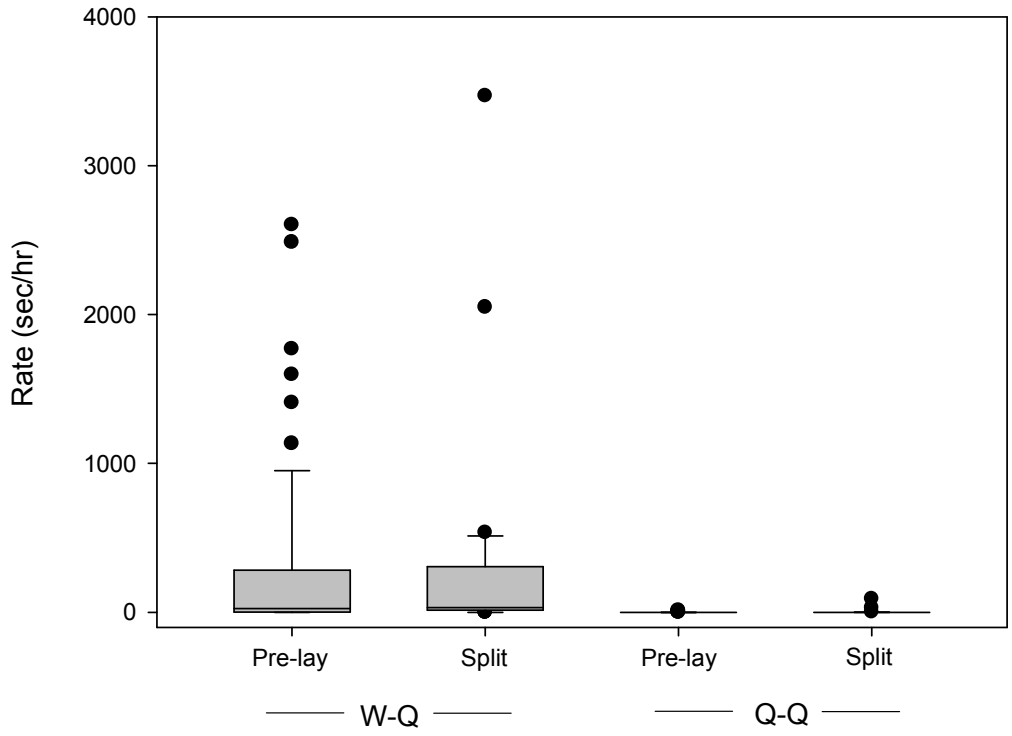


Figure 8. Comparison of the rates of W→Q and Q→Q aggression observed during Part 1 ('pre-lay') and that observed in the current study – Part 2 ('split').

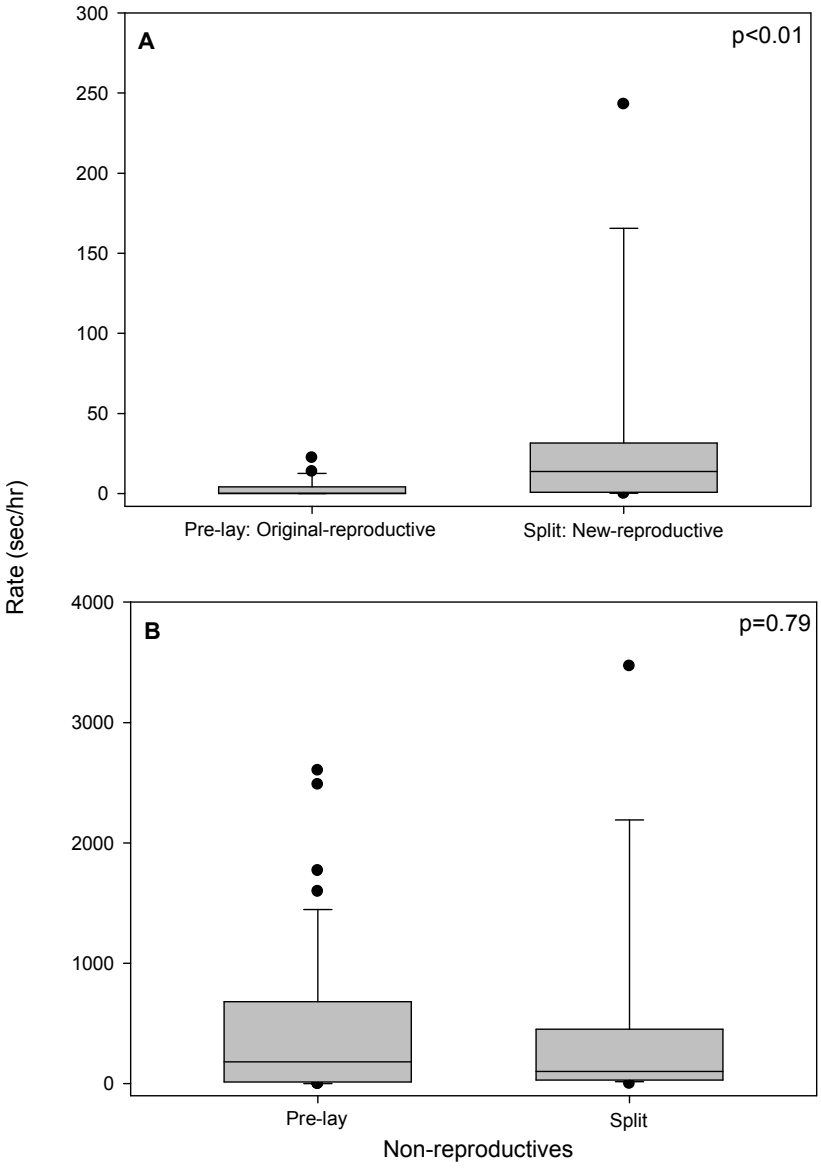


Figure 9. A) Comparison of the rate of W→Q aggression received by the queen who became reproductive in Part 1 ('pre-lay: original-reproductive') and that in Part 2 ('split: new-reproductive'). **B)** The rate of W→Q aggression received by non-reproductive queens in Part 1 ('pre-lay') and in Part 2 ('split'). The p-values were calculated using a Mann-Whitney U statistical test.

3(2).4 DISCUSSION

The experimental study described here provides further support for the fundamental role of worker policing of queens in maintaining complete skew among queens in a functionally monogynous population of *L. acervorum*. In all group 2 MQ colonies never more than a single queen became reproductive, hence monopolisation of reproduction was maintained even after the loss of the original reproductively dominant queen. Crucially, behavioural observations showed that the queen that became the new-reproductive received a significantly lower rate of W→Q aggression compared with the queens that remained non-reproductive. In contrast, there was no correlation with the rate of Q→Q aggression and which queen future reproductive status, providing little support for the queen control hypothesis.

In all group 1 colonies the original-reproductive queen maintained her reproductive position. Furthermore, monitoring of colonies suggested that within colony aggression was low, as over all colonies only one non-reproductive queen was known to be killed by workers (2.1% of queens). In contrast, over all group 2 colonies 20 queens were known to be permanently evicted or killed by workers (34.5% of queens). This significant difference between group 1 and group 2 colonies suggests that the elevated aggression found in group 2 colonies is not simply because of the physical disruption of the splitting process, otherwise we should expect little difference between the two groups, but is a consequence of losing the original-reproductive queen. The high frequency of queens being killed or evicted by workers shows that workers respond aggressively to the loss of the reproductive queen, and that workers ultimately hold the power to determine the fate of queens.

Behavioural observations of group 2 MQ colonies did not support the queen control hypothesis as there was very little Q→Q aggression observed and it was not significantly different from that found during the pre-laying period (part 1). This is particularly telling, as none of these queens will have been previously reproductive so we would expect aggression among queens when competing for the vacant reproductive position, but only 25% of queens carried-out Q→Q aggression. Instead, a high rate of W→Q aggression was overtly biased towards all but a single queen who subsequently became the new-reproductive, suggesting that workers are the controlling party over who becomes the new dominant reproductive queen. This pattern of aggressive behaviour appears to be in response to the loss of the original-reproductive queen because, as shown in part 1 of this chapter, the new-reproductive queen will have received W→Q aggression when the original-reproductive was previously present. Hence, workers can be dynamic in their aggressive response towards specific queens dependent on the composition of queens in the colony. Furthermore, an important point is that workers still have the ability to evict and kill queens, therefore even if queens have the potential to compete over skew amongst themselves ('potential conflict') workers hold the power and information to determine queen reproductive fate ('actual conflict' Beekman *et al.* 2003; Beekman & Ratnieks 2003; Ratnieks *et al.* 2006).

Although the rate of Q→Q aggression observed was relatively low it could be that such interactions are important events, such as the acts of 'sting-smearing' (S.S.) observed between specific queens (n=three colonies). It seems that S.S. might only occur when queens experience the loss of the reproductive queen, as in the previous study (part 1) S.S. was not observed. A S.S. act among queens was immediately followed by high

W→Q aggression, therefore, is S.S. a cue used by workers to attack particular queens? A previous study looking at *Dinoponera quadriceps,* an ant species that has lost the morphological caste system, showed that S.S. can act as a signal to low ranking females to attack beta and gamma females who try to compete for the alpha position (Monnin & Ratnieks 2001; Monnin *et al.* 2002). In the current study, if S.S. was used as a similar cue for worker aggression towards smeared queens, we would expect that the queen carrying out the S.S. is more likely to become the new-reproductive queen. However, there appeared to be no clear pattern in the future reproductive status of queens and the number of sting smearing acts carried-out. S.S. was carried out by non-reproductive queens nearly as many times as carried out by new-reproductive queens (five vs six acts respectively).

A potential problem regarding the proposal that the act of S.S. is a possible cue for W→Q aggression is that a queen who does not meet the optimum interests of the workers (i.e. an unrelated queen) might be able to S.S. a queen that is closely related to the workers. This is credible considering that sibship analysis of MQ colonies from the same population (see chapter 2; Gill *et al*. 2009; chapter 3 part 1) shows that resident queens may not always be fullsisters or mother to the workers. This highlights a potential problem with the concept of queen control, in that queens could exploit the altruistic behaviour of workers against their best interests. Perhaps therefore, S.S. is accompanied by an honest signal which advertises the queen relationship to the workers (see Keller & Nonacs 1993), similar to that found in honest signalling of queen fecundity (e.g. Seeley 1985; Monnin & Peeters 1999; Cuvillier-Hot *et al.* 2002; Hannonen *et al.* 2002; Cuvillier-Hot *et al.* 2004; Hartmann *et al.* 2005; Smith *et al.* 2009). However, when S.S. was observed there were multiple occasions where both the queen carrying out the S.S.

and the recipient received aggression from workers (Table 3 & Figure 6). This makes it unclear why queens would S.S. when there is a risk of getting attacked themselves. Also, if S.S. is not an important cue in determining skew among queens, why do workers respond with high activation and aggression? It is possible that workers respond aggressively to any form of stinging action, as for example occurs in response to attack from predators, or in attacking prey (see Holldobler & Wilson 1990).

Surprisingly, in four group 2 SQ and two group 2 MQ colonies, workers killed or permanently evicted all queens resulting in a queenless colony. The consequence of queenless colonies seems paradoxical considering this could potentially lead to the death of the colony. A possibility is that there were queen larvae in the colony and it may have been a better option to rear potential fullsister queens than to allow the resident queen(s) in the colony to become the new-reproductive if (as previously discussed) there are resident queens which are not fullsisters or mother to the workers.

The evidence to support the worker control hypothesis presented in both parts of this chapter has important implications surrounding the primary assumption that many skew models make; that the primary reproductive party is in control. Power over skew can be dependent on the cost each individual incurs when manipulating skew to optimise their own reproductive interest. The cost incurred by a single individual when trying to influence skew is substantially higher than that incurred per individual when individuals act as a collective. Incorporating the interests and power of a third party, in this case the workers, within the framework of skew theory is likely to advance our understanding of conflicts over skew and the evolution of social organisation. The majority rules model is one such skew model which considers this. However, a primary prediction of the majority

rules model is not supported by this study. Based on the proposal by Reeve & Keller (1995), in parent-daughter associations skew is predicted to be high (as found in the functionally monogynous colonies), but if the parent is lost, skew is predicted to decrease among members, as the collective still gains high inclusive fitness even when skew is low. In the functional monogynous colonies the original reproductive queen is usually the mother of the majority of workers and queens (see chapter 2; Gill *et al.* 2009; chapter 3 part 1), but my experiment clearly shows that when the original reproductive queen is lost, (leaving multiple fullsister queens which are also fullsisters to the workers) complete skew among queens is maintained. Keeping skew high may therefore be a 'rule-of-thumb' perhaps to prevent dilution of relatedness within colonies in future generations. But this raises the question: why does a polygynous social organisation persist in other *L. acervorum* populations? It is this question that will be addressed in the next chapter.

Additional Data

See Appendix 2.

CHAPTER 4

High reproductive skew in a Japanese population of *Leptothorax acervorum*

4.1 INTRODUCTION

Animal social organisation not only varies between species, but also between different populations and even cooperative groups of the same species, and investigating such variation contributes to our understanding of the processes and levels of selection on social traits (Bourke & Franks 1995; Keller 1999). Reproductive skew is a primary aspect of social organisation that can be studied to uncover the ecological, genetic and social factors involved. Furthermore, studying variation in skew allows investigation of the principle components (*r*, *b* & *c*) that constitute Hamilton's inclusive fitness rule (kin selection theory, Hamilton 1964). In eusocial Hymenopteran species with multiple queen colonies, providing an evolutionary explanation for the occurrence of functionally monogynous (high skew) and polygynous (low skew) social organisations is a direct test of kin selection theory. Functional monogyny is associated with high colony relatedness and consequently high indirect fitness benefits to colony members. Polygyny, however, is seemingly paradoxical because it is associated with low colony relatedness, hence lowered indirect fitness benefits to colony members (Keller 1993, 1995) and competition is predicted among queens (Holldobler & Wilson 1977; Rosengren & Pamilo 1983). If only considering relatedness, then functional monogyny should always be the most stable

strategy, yet in ant species for example, functional monogyny is only found in a handful of species (see Gill *et al.* 2009) whereas polygyny is common (see Holldobler & Wilson 1990; Bourke & Franks 1995).

The common occurrence of polygyny presumably means that the dilution in relatedness benefit must be outweighed by other factors influencing the benefit (*b*) - cost (*c*) tradeoff incorporated in Hamilton's rule (Hamilton 1964; Holldobler & Wilson 1977; Herbers 1986; Keller 1993; West *et al.* 2007; Herbers 2009). These factors are likely to lie within an ecological parameter (see Herbers 2009), particularly the constraint on dispersal and success of solitary breeding (Emlen 1982). Investigating the influence of such factors in shaping social organisation is key to furthering our understanding of the evolution of polygyny (Keller 1993; Bourke & Heinze 1994; Keller 1995), but they are often overlooked (see West *et al.* 2007; Herbers 2009).

The polymorphic social organisation described in *Leptothorax acervorum* (chapter 2; Gill *et al.* 2009) is a particularly good system to identify the underlying genetic and ecological factors that shape social organisation. Furthermore, a comparison between closely related populations of the same species can control for differences confounded by phylogeny (Bourke & Heinze 1994; Magrath & Heinsohn 2000; Gill *et al.* 2009). There is a relatively large amount of data surrounding multiple *L. acervorum* polygynous populations, with data on colony kin structure (Douwes *et al.* 1987; Bourke 1991; Stille & Stille 1992, 1993; Heinze 1995; Heinze *et al.* 1995a; Heinze *et al.* 1995b; Heinze *et al.* 1997; Hammond *et al.* 2001; Hammond *et al.* 2006), aspects of colony life history (Bourke 1991; Stille *et al.* 1991; Heinze 1993a), and ecology (Franks *et al.* 1991; Bourke & Heinze 1994; Heinze *et al.* 2003). However, similar data on functional

monogyny in *L. acervorum* is mostly restricted to a single population in Spain (Felke & Buschinger 1999; Gill *et al.* 2009), which means there is limited power in identifying the critical variables responsible for the polymorphism. Therefore, confirming functional monogyny in another *L. acervorum* population is important, and by obtaining sociogenetic and ecological data it allows identification of the trends in parameters/factors that are indicative of each social phenotype.

L. acervorum can be found in Japan (Figure 1), and reports suggest high skew among queens in multiple queen colonies based on dissection data and behavioural observations (Ito 1990, 2005). Queen dissections support that there is a reproductively dominant queen, but also suggest that there are additional 'supplementary' laying queens present in the colony (Ito 1990). A behavioural study also reported reproductive dominance by a single queen over other queens but based on only four colonies (Ito 2005). This evidence has led to the conclusion that Japanese populations are functionally monogynous, but the presence of possible supplementary laying queens queries whether functional monogyny is truly exhibited, or whether multiple queens reproduce but skew among queens is high. Moreover, there is no sociogenetic data to support functional monogyny in the Japanese populations. There is also limited genetic data surrounding the taxonomic relationship between the described Japanese and European *L. acervorum* populations (Baur *et al.* 1995), which is important if any comparative analysis is to control for phylogeny.

In this study I carried out a detailed genetic analysis of colony kin structure in single and multiple queen colonies from a Japanese *L. acervorum* population to confirm or reject functional monogyny. I investigated the rate of queen turnover and frequency of

queen re-adoption, and also carried out queen dissections which provided additional data on the number of reproductive queens. The genetic relationship between the Japanese and European *L. acervorum* populations was also deduced using mtDNA and nDNA sequence data.

4.2 METHODS

4.2.1 Colony collection and maintenance

Previous reports on colony social organisation of *L. acervorum* in Japan (Ito 1990, 2005) were based on a population from the large northern island of Hokkaido. In the current study, however, colonies were collected from the Japanese mainland where *L. acervorum* is widely distributed and its range extends to the north of the mainland close to Hokkaido (see Figure 1).

Colonies were collected on the south facing slope of Mount Fuji, Japan on the 7th and 10th September 2008, in two areas where colonies were found in high density. The first area (MF1) was at an altitude of ~2200m in an open area near the edge of the tree line. The second area (MF2) was found at an altitude of ~2100m where there were sparsely distributed small coniferous trees. Between MF1 and MF2 was a road dividing the two areas (~200m apart). Nests were found under pieces of volcanic rock on the ground (Figure 2), and were collected by placing the rock into a plastic bag and using an aspirator to collect any remaining individuals on the ground. The type and position of nests made it difficult to collect fully intact colonies, unlike collections from twigs in other populations (see Gill *et al.* 2009, chapter 2). Once the rock was over-turned individuals (workers and queens) on the ground scattered, so some individuals may not have been collected, including resident queens. Thus, it is possible that colonies with only a single resident queen (when censused in the laboratory) may have had multiple queens in the field (addressed in the discussion). In addition, at the time of collection colonies possessed eggs, larvae, developing pupae, and in some colonies alate (winged) queens. The presence of developing pupae (including sexuals) and alate queens suggests that

colonies were collected before the mating period. Individuals from the MF population were darker around the thoracic part of the body in comparison to individuals from European populations, but they still possessed the characteristic thoracic spines and 11 segmented antennae of *L. acervorum.*

In the Japanese populations of *L. acervorum*, as found in Europe, colonies may either contain a single queen or multiple queens. In total 48 colonies were collected, of which 23 had more than one queen, 18 with only a single queen, and seven with no queen. At the time of collection eggs and alate queen were present in the nest. Six to seven days after collection, colonies were censused and provided with an artificial nest. Any known alate queens were removed and frozen. Four of the 23 MQ colonies were then frozen (referred to as 'snap-shot' MQ colonies) to gain information on the reproductive state of queens at the point of collection. All remaining colonies were kept in autumn conditions for two weeks, followed by winter (5-weeks), spring (7-weeks), and summer (12-weeks), under the same seasonal conditions as previously described (Gill *et al.* 2009; chapters 2 & 3). Any individuals found dead between collection and the end of summer were frozen (-20ºC) for possible later genetic analysis, and dissection in the case of queens.

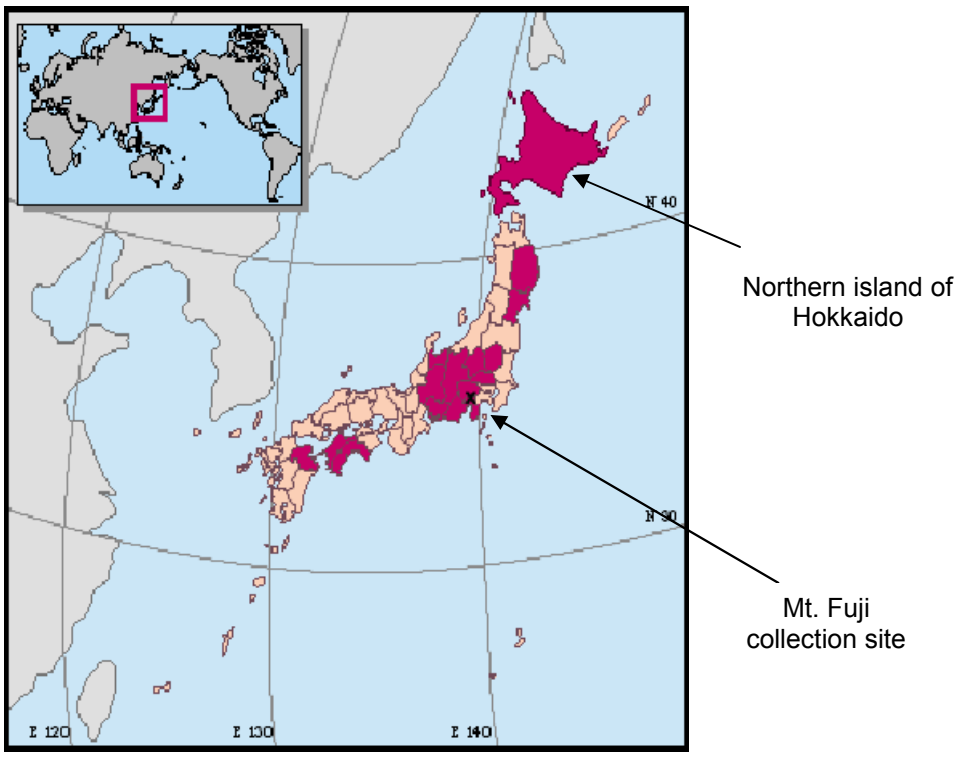


Figure 1. Geographical distribution of *L. acervorum* in Japan (image taken from the Japanese Ant Database Group website©2003). **X** marks the location of Mt Fuji which is the collection site for the current study.

Figure 2. A typical nest site of *L. acervorum* in the MF population in Japan. Colonies were found within crevices of small volcanic rocks on the ground (the arrow points to larvae found in the nest).

4.2.2 Colony sampling and queen dissection

Of the 48 colonies collected, 30 colonies (incl. the four snapshot MQ colonies) had all queens, eight workers, and up to six large larvae if present, were sampled for genetic analysis (22 colonies with more than one queen (n=96 queens; range=2-10), and 8 with a single queen; Tables 1 & 2). In the four snap-shot MQ colonies, queens were dissected to determine both queen mated status and ovary development using an ovary classification previously described (Gill *et al.* 2009; chapter 2). In the 26 remaining colonies individuals were sampled at the end of the summer season, and queens were dissected to determine their mated status. Ovary development could not be determined because colonies had been kept and manipulated for behavioural observations (not reported here), therefore, ovary development may not reflect true reproductive status in an unmanipulated colony state. At the time of sampling the majority of larvae were in their final instar (pre-pupal stage, referred to as 'large' larvae).

Table 1. Colony composition of the 30 studied colonies (queen number excludes any known alate queens at the time of the census).

Studied colonies	Total Qs	No. queens ± s.e.m.	Range	Total Ws	No. workers ± s.e.m.	Range
1 Queen (8 cols)	8	1.0	-	253	31.6 ± 6.0	15 - 67
2+ Queens (22 cols)	96	4.4 ± 0.6	2-10	870	39.6 ± 5.8	5 - 119
Total	104			1123		

Table 2. Census of the 30 studied colonies taken 6-7 days after collection before queen dissection analysis (queen number excludes any known alate queens at the time of the census).

Colony	Q no.	W no.
MF1.08.02	10	53
MF1.08.03	4	50
MF1.08.04	2	7
MF1.08.05	1	67
MF1.08.06	4	56
MF1.08.12	4	24
MF1.08.13	3	9
MF1.08.14	1	26
MF1.08.15	1	17
MF1.08.16	3	58
MF1.08.17	1	22
MF1.08.18	7	40
MF1.08.19/20	10	119
MF2.08.24	2	26
MF2.08.25	3	12
MF2.08.27	1	15
MF2.08.28	6	5
MF2.08.29	2	54
MF2.08.30	1	43
MF2.08.31	3	41
MF2.08.34	2	39
MF2.08.36	7	87
MF2.08.37	1	28
MF2.08.38	2	50
MF2.08.40	3	41
MF2.08.41	2	13
MF2.08.42	4	44
MF2.08.44	10	12
MF2.08.45	1	35
MF2.08.46	2	30

4.2.3 Genetic Analysis

Worker and larval DNA was extracted using a 10% Chelex solution as previously described (Gill *et al.* 2009; chapter 2), and queen DNA was extracted using a PureGene extraction kit. To investigate relatedness and sibship within colonies, individuals were genotyped at four polymorphic microsatellite loci as previously described (Gill *et al.* 2009; chapter 2). Only individuals which amplified successfully at three or four loci were used in the analysis.

Sibship, relatedness analysis, and queen turnover

Sibship analysis was performed using the program COLONY (Wang 2004) assuming that queens mate singly (Hammond *et al.* 2001; Gill *et al.* 2009; chapter 2), and in this analysis female (diploid) and male (haploid) larvae were included. Regression relatedness was calculated using the program Relatedness 5.08, and only female (diploid) individuals were used (larvae which had only a single allele at all loci (males) were excluded from the analysis; 16%, 21/130). Relatedness estimates were not always normally distributed (Kolmogorov-Smirnov test), therefore, estimates were analysed using Mann-Whitney U tests, and the statistical significance between relatedness estimates and expected point values was tested by seeing if expected point values fell outside 95% confidence limits. Queen turnover was estimated using equation four in Pedersen & Boomsma (1999), by comparing relatedness within and between worker and larval cohorts. Only colonies with both multiple workers and multiple diploid (female) larvae were used in the analysis.

Genetic relationship between Japanese and European populations

To determine the genetic relationship between the Japanese *L. acervorum* population and other European *L. acervorum* populations, a region of the mitochondrial cytochrome *b* gene and a region of the nuclear *foraging* gene were amplified, as previously described (chapter 2; Gill *et al.* 2009). From the Japanese MF population, one worker individual from each of 14 colonies was sampled. The sequence data was compared with the sequence alignments obtained from the European populations (both UK and European polygynous and Spanish functionally monogynous populations) analysed in chapter 2 (Gill *et al.* 2009).

4.3 RESULTS

4.3.1 Dissection

Eighty-nine per cent of queens over all 30 colonies were dissected (93/104; Table 3). The mated state of queens was successfully determined in 88% of these queens (82/93), in which 76% were mated (the 8 colonies with a single queen = six mated & two undetermined; the 22 colonies with more than one queen = 56 mated, 20 unmated, nine undetermined).

Queen dissections showed, importantly, that not all colonies with more than one dealate queen possessed more than one mated (potentially functional) queen (Table 3). Of the 22 colonies with more than one queen, 17 definitively had more than one mated queen. In two other colonies (MF1.16 & MF2.29), only one of the queens was dissected (each was mated), but I classed these two colonies as having more than one mated queen because the probability of the other non-dissected queens being mated was higher than being unmated. In the remaining three colonies (MF2.25, MF2.28 & MF2.41) only a single queen was mated and all other queens were unmated. From hereon, colonies with multiple, potentially functional, queens were classed as 'multiple queen (MQ) colonies', whereas all colonies with only a single potentially functional queen were classed as 'single queen (SQ) colonies'. Therefore, the dissection results show there to be 19 MQ colonies and 11 SQ colonies in which all further analyses are to be based on.

The proportion of unmated queens (24%) is comparatively higher than that found in the Spanish population (4%) (chapter 2; Gill *et al.* 2009). This supports the idea that colonies were collected before newly developed alate queens had left the nest to mate, especially as alate queens and developing sexual pupae were found at the time of

collection. These unmated queens did not possess wings when censused suggesting that either these queen failed to mate in the previous year and are still resident (virgin queens), or during the period between collection and colonies being censused, alate queens somehow lost their wings (e.g. workers bit them off).

Table 3. Mated state of queens across all 30 studied colonies, with queens classified as either mated (M), unmated (U) or undetermined (?). Queens not dissected are shown as X. Colonies are classed as those with either one mated queen (SQ), or more than one mated queen (MQ).

Colony	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10	SQ/MQ
MF1.02	M	M	M	M	U	U	U	U	U	U	MQ
MF1.03	M	M	X	X							MQ
MF1.04	M	M	U								MQ
MF1.05	M										SQ
MF1.06	M	M	M	?							MQ
MF1.12	M	M	?	X							MQ
MF1.13	M	M	M								MQ
MF1.14	M										SQ
MF1.15	M										SQ
MF1.16	M	X	X								MQ
MF1.17	M										SQ
MF1.18	M	M	M	M	M	M	X				MQ
MF1.19/20	M	M	M	M	M	U	U	?	X	X	MQ
MF2.24	M	M									MQ
MF2.25	M	U	U								SQ
MF2.27	M										SQ
MF2.28	M	U	U	U	U	U					SQ
MF2.29	M	X									MQ
MF2.30	M										SQ
MF2.31	M	M	X								MQ
MF2.34	M	M									MQ
MF2.36	M	M	M	M	M	U	X				MQ
MF2.37	?										SQ
MF2.38	M	M									MQ
MF2.40	M	M	M								MQ
MF2.41	M	U									SQ
MF2.42	M	M	U	U							MQ
MF2.44	M	M	M	M	?	?	?	?	?	?	MQ
MF2.45	?										SQ
MF2.46	M	M									MQ

Dissection of queens from the four snap-shot MQ colonies revealed that all colonies had more than one mated queen but only a single queen per colony showed signs of recent reproductive activity (Table 4). In each of the four colonies only a single queen possessed either type A or B ovary development, with all remaining queens possessing type D. The presence of type B ovary development might be that ovaries had started to regress suggesting that I collected near the end of the laying period.

Table 4. Dissection of the four snapshot MQ colonies showing both the mated status and ovary development type for each queen.

Colony	Queen	Mated status	Type
MF2.31	Q1	M	**B**
	Q2	M	D
	Q3	X	-
MF2.34	Q1	M	**A**
	Q2	M	D
MF2.44	Q1	M	**B**
	Q2	M	D
	Q3	M	D
	Q4	M	D
	Q5	?	D
	Q6	?	D
	Q7	?	D
	Q8	?	D
	Q9	?	D
	Q10	?	D
MF2.46	Q1	M	**B**
	Q2	M	D

* see chapter 2 for classification of ovary development type.

4.3.2 Genetic analysis

In total, 436 individuals were genotyped, with each individual genotyped at an average of 3.81 loci. From the 11 SQ colonies, this constituted 72 workers (mean=6.5 per colony, range=4-8), 19 queens (eight colonies had one queen, and three colonies had more than one queen but only one was mated), and 54 larvae (45 females & nine males; mean=4.9 per colony, range=1-6). From the 18 MQ colonies, 142 workers (mean=7.5 per colony, range=4-16) and 73 queens (mean=3.8 per colony, range=1-10) were genotyped (one unmated queen from colony MF2.42 was not successfully genotyped), and from 15 colonies 76 larvae (64 females & 12 males; mean=4.0 per colony, range=3-6) were genotyped.

Sibship

i) SQ colonies

In each of the 11 SQ colonies the majority (mean=83%) of workers and larvae as a collective were assigned to a single fullsib family (referred to as the 'majority fullsib family'; range=63-100%), with an average of 2.4 fullsib families per colony (Table 5A). On average 78% of workers and 100% of larvae per colony were assigned to the majority fullsib family. This showed that workers did not always belong to the same fullsib family perhaps due to variation in age, but in every colony larvae, which are the same age cohort, belonged to the same fullsib family.

In five colonies the mated queen genotype matched the predicted maternal genotype (PMG) provided by COLONY for the majority fullsib family. In one of these four colonies (MF2.30), the queen was also assigned to the majority fullsib family, this

was because the predicted maternal and paternal genotype shared alleles at each locus making it impossible to determine whether the queen in question is a fullsister or the mother of the majority fullsib family. In six colonies, the mated queen genotype did not match the PMG for any fullsib family.

ii) MQ colonies

In the 19 MQ colonies, on average 81% of workers and larvae as a collective were assigned to the majority fullsib family (range=40-100%), with an average of 2.9 fullsib families per colony (Tables 5A). An average of 76% of workers per colony and 89% of larvae per colony (n=15 colonies) were assigned to the majority fullsib family, showing that workers and/or larvae were not always assigned to the same fullsib family. The average proportion of queens assigned to the fullsib family was 56% showing that the majority of queens are fullsisters, are also fullsisters to the workers and larvae, and in some cases daughters of another resident queen. Importantly, when considering only mated queens in MQ colonies (Table 5B), I found that 77% of mated queens were assigned to a fullsib family containing other adult individuals (workers and other queens). Moreover, 58% were assigned to the majority fullsib family, supporting daughter queen re-adoption after mating.

There was no evidence to show that there were multiple reproductive queens, as there were no instances where group members were the offspring of more than one queen still resident in the colony. In 10 colonies the PMG of the majority fullsib family matched the genotype of just a single queen, and the PMG of all other fullsib families did not match any other queen genotype in the colony. In two colonies (MF1.18 & MF2.34) the

genotype of a single queen who was assigned to the majority fullsib family matched the PMG of another fullsib family (family 2). In the remaining seven colonies no queen's genotyped matched the PMG for any fullsib family.

In three colonies (MF1.12, MF1.18 & MF2.34), the genotype of more than one queen matched the PMG of a single fullsib family. The maternal queen could not be distinguished because in each case queens possessed identical genotypes. However, it is unlikely that these queens could all be the mother of the single fullsib family, unless queens either mated with the same male, or mated with different males sharing the same alleles, but the probability of this occurring would be particularly low if mating is random (Stille & Stille 1992; Hammond *et al.* 2001).

Table 5A. Sibship analysis of the 30 studied colonies, including all queens regardless of mated status. The 'number of individuals genotyped', shows the total number of individuals per colony (Col), and the number of queens (Q), workers (W), female larvae (LF) and male larvae (LM) per colony. Genotypes of queens, workers, and larvae are grouped into fullsib families. In 'fullsib family membership', numbers in brackets are the number of each type (e.g. L(4) = 4 larvae), and the asterix (i.e. Q*) shows that the PMG for the fullsib family stated under 'Q* genotype match' is the fullsib family that matches the genotype of that queen(s).

See table 5A on next page.

Table 5A.

Colony	No. ind. genotyped					Fullsib family membership						Q* genotype
	Col	Q	W	LF	LM	Maj. fullsib family	2	3	4	5	6	match
SQ colonies												
MF1.05	15	1	8	2	4	Q, W(8), L(6)						None
MF1.14	15	1	8	6	-	W(8), L(6)	**Q***					Majority
MF1.15	13	1	7	5	-	W(6), L(5)	**Q***	W				Majority
MF1.17	14	1	7	6	-	Q, W(6), L(6)	W					None
MF2.25	15	3	6	6	-	Q(2), W(3), L(6)	**Q***	W(2)	W			Majority
MF2.27	15	1	8	5	1	W(4), L(6)	Q, W(3)	W				None
MF2.28	17	6	5	6	-	**Q(2)***, Q(4), W(4)	L(6)	W				Family 2
MF2.30	11	1	7	3	-	**Q***, W(7), L(3)						Majority: mother or fullsister
MF2.37	8	1	6	1	-	Q, W(5), L	W					None
MF2.41	10	2	4	-	4	W, L(4)	Q, W	Q	W	W		None
MF2.45	12	1	6	5	-	Q, W(6), L(5)						None
Total	**145**	**19**	**72**	**45**	**9**							
MQ colonies												
MF1.02	22	10	8	3	1	Q(9), W(8), L(4)	**Q***					Majority
MF1.03	15	2	8	3	2	Q, W(6), L(4)	Q,L	W	W			None
MF1.04	9	3	6	-	-	Q(3), W(6)						None
MF1.06	16	4	8	4	-	Q, W(8), L(2)	**Q***	Q, L(2)	Q			Majority
MF1.12	17	3	8	6	-	Q, W(8), L(6)	**Q(2)***					Majority
MF1.13	13	3	5	4	1	W, L(5)	**Q***, Q	Q, W(3)	W			Majority
MF1.16	12	1	6	5	-	Q, W(3), L(4)	W(2)	W	L			None
MF1.18	19	6	7	6	-	**Q(3)***, Q(3), W(6), L(3)	W, L(3)					Family 2
MF1.19/20	27	8	16	2	1	Q(7), W(15), (L3)	**Q***	W				Majority
MF2.24	12	2	6	3	1	Q, W, L(3)	Q, W	W(2)	W	W	L	None
MF2.29	7	1	6	-	-	W(6)	**Q***					Majority
MF2.31	16	2	8	6	-	W(7), L(6)	Q, W	Q				None
MF2.34	16	2	8	5	1	**Q(2)***, W(7), L	W, L(5)					Family 2
MF2.36	16	6	4	5	1	Q(2), W, L(6)	**Q***, Q(2), W(3)	Q				Majority
MF2.38	10	2	8	-	-	Q, W(8)	**Q***					Majority
MF2.40	10	3	7	-	-	Q(2), W(7)	**Q***					Majority
MF2.42	16	3	8	2	3	Q(3), W(7), L(5)	W					None
MF2.44	23	10	7	6	-	Q(4), W(2), L(6)	Q(3), W(2)	**Q***, Q(2), W	W	W		Majority
MF2.46	15	2	8	4	1	Q, W(6), L(4)	Q, W(2)	L				None
Total	**291**	**73**	**142**	**64**	**12**							
Totals	**436**	**92**	**214**	**109**	**21**							

Table 5B. Sibship analysis of the 30 studied colonies but only including known mated queens.

Colony	No. ind. genotyped					Fullsib family membership						Q* genotype
	Col.	Q	W	L_F	L_M	Majority fullsib family	2	3	4	5	6	match
SQ colonies												
MF1.05	15	1	8	2	4	Q, W(8), L(6)						None
MF1.14	15	1	8	6	-	W(8), L(6)	**Q***					Majority
MF1.15	13	1	7	5	-	W(6), L(5)	**Q***	W				Majority
MF1.17	14	1	7	6	-	Q, W(6), L(6)	W					None
MF2.25	15	1	6	6	-	W(3), L(6)	**Q***	W(2)	W			Majority
MF2.27	15	1	8	5	1	W(4), L(6)	Q, W(3)	W				None
MF2.28	17	1	5	6	-	**Q***, W(4)	L(6)	W				Family 2
MF2.30	11	1	7	3	-	**Q***, W(7), L(3)						Majority: mother or fullsister
MF2.37	8	-	6	1	-	W(5), L	W					None
MF2.41	10	1	4	-	4	W, L(4)	Q, W	W	W			None
MF2.45	12	-	6	5	-	W(6), L(5)						None
Total	**145**	**9**	**72**	**45**	**9**							
MQ colonies												
MF1.02	22	4	8	3	1	Q(3), W(8), L(4)	**Q***					Majority
MF1.03	15	2	8	3	2	Q, W(6), L(4)	Q,L	W	W			None
MF1.04	9	2	6	-	-	Q(2), W(6)						None
MF1.06	16	3	8	4	-	Q, W(8), L(2)	**Q***	Q, L(2)				Majority
MF1.12	17	2	8	6	-	Q, W(8), L(6)	**Q***					Majority
MF1.13	13	3	5	4	1	W, L(5)	**Q***, Q	Q, W(3)	W			Majority
MF1.16	12	1	6	5	-	Q, W(3), L(4)	W(2)	W	L			None
MF1.18	19	6	7	6	-	**Q(3)***, Q(3), W(6), L(3)	W, L(3)					Family 2
MF1.19/20	27	5	16	2	1	Q(4), W(15), (L3)	**Q***	W				Majority
MF2.24	12	2	6	3	1	Q, W, L(3)	Q, W	W(2)	W	W	L	None
MF2.29	7	1	6	-	-	W(6)	**Q***					Majority
MF2.31	16	2	8	6	-	W(7), L(6)	Q, W	Q				None
MF2.34	16	2	8	5	1	**Q(2)***, W(7), L	W, L(5)					Family 2
MF2.36	16	5	4	5	1	Q(2), W, L(6)	**Q***, Q(2), W(3)					Majority
MF2.38	10	2	8	-	-	Q, W(8)	**Q***					Majority
MF2.40	10	3	7	-	-	Q(2), W(7)	**Q***					Majority
MF2.42	16	2	8	2	3	Q(2), W(7), L(5)	W					None
MF2.44	23	4	7	6	-	Q(2), W(2), L(6)	**Q***, Q, W	W(2)	W	W		Majority
MF2.46	15	2	8	4	1	Q, W(6), L(4)	Q, W(2)	L				None
Total	**291**	**53**	**142**	**64**	**12**							
Totals	**436**	**62**	**214**	**109**	**21**							

Relatedness

Relatedness (see Table 6) among all colony members was high in both SQ (0.64±0.04, n=136 indivduals/11 colonies) and MQ colonies (0.59±0.03, n=279/19), and were not significantly different from each other (Mann-Whitney: U=98, p=0.80). Within SQ colonies, both the average relatedness among workers (0.61±0.07, n=72/11) and larvae (0.78±0.03, n=44/9) were not significantly different from 0.75, nor were they significantly different from each other (Mann-Whitney: U=26, p=0.81).

Within MQ colonies, the average worker relatedness (0.63±0.05, n=142/19) was not significantly different to that found in SQ colonies (Mann-Whitney: U=96, p=0.73), but was significantly lower than 0.75. The average relatedness among larvae (0.66±0.04, n=64/15), however, was significantly lower than that found in SQ colonies (Mann-Whitney: U=34, p=0.05), and was not significantly different from 0.75. There was also no significant difference between worker and larvae relatedness in MQ colonies (Mann-Whitney: U=139, p=0.92). The average relatedness among all queens (0.53±0.06, n=71/17), and mated queens (0.54±0.07, n=51/17) was not significantly different (Mann-Whitney: U=139, p=0.85), and both were significantly lower than 0.75, but not significantly higher than 0.5.

Queen Turnover

Queen turnover was calculated to be 13.3% in SQ colonies (n=9), and 17.4% in MQ colonies (n=15), similar to that estimated in a known functionally monogynous Spanish *L. acervorum* population (11.3% and 19.7%; chapter 2; Gill *et al.* 2009). The study on the Spanish population considered a larval cohort composed of small larvae

sampled at the time of collection. In the current study large larvae were used, but because larvae were sampled after colonies had been overwintered and kept in spring and summer conditions, these larvae will have been at an earlier stage in development (i.e. small larvae) at the time of field collection, and so the estimate was comparable.

Table 6. Summary of relatedness values for the 30 studied colonies, and the number of colonies (n) and individuals per colony ((ind)) used for the analysis.

	r-value ± s.e.	**n (ind)**
SQ colonies		
Colony	0.64 ± 0.04	11 (136)
Workers	0.61 ± 0.07	11 (72)
Larvae	0.78 ± 0.03	9 (44)
Workers→Larvae	0.67 ± 0.06	10 (68/45)
Workers & Larvae	0.65 ± 0.06	10 (113)
SQ colonies (values for queen turnover)		
Workers	0.62 ± 0.08	9 (62)
Larvae	0.78 ± 0.03	9 (44)
Workers & Larvae	0.65 ± 0.06	9 (106)
MQ colonies		
Colony	0.59 ± 0.03	19 (279)
Workers	0.63 ± 0.05	19 (142)
Larvae	0.66 ± 0.04	15 (64)
Workers→Larvae	0.59 ± 0.04	15 (115/64)
Workers & Larvae	0.57 ± 0.04	15 (179)
Queens (all)	0.53 ± 0.06	17 (71)
Mated queens	0.54 ± 0.07	17 (51)
Workers→Queens (all)	0.56 ± 0.05	19 (142/73)
Workers→Queens (mated)	0.57 ± 0.05	19 (142/53)
Larvae→Queens (all)	0.53 ± 0.03	15 (64/64)
Larvae→Queens (mated)	0.55 ± 0.03	15 (64/45)
MQ colonies (values for queen turnover)		
Workers	0.60 ± 0.06	15 (115)
Larvae	0.66 ± 0.04	15 (64)
Workers & Larvae	0.57 ± 0.04	15 (179)

Genetic relationship between Japanese and European populations.

A 685bp fragment of cyt*b* (mtDNA) was compared with that previously analysed in European populations of *L. acervorum* (Figure 3). In the Japanese population the sequence data revealed four unique haplotypes that are not shared in other European populations. However, all four haplotypes nested within the majority clade containing haplotypes from all other European populations. Variation among the Japanese haplotypes was as high as variation between Japanese and other European haplotypes (i.e. SF and SO populations), and low bootstrap support showed a lack of genetic distinction at cyt*b* locus between the Japanese and European populations.

A 264bp fragment of the *foraging* gene from seven workers from the Japanese (MF) population was compared with that previously found in the Spanish functionally monogynous populations (OT & V) and polygynous UK (SF) and Finnish populations (TV/VN). There were two alleles found in the Japanese population, one allele (H6) that was found in two heterozygous individuals and was unique to the Japanese population which differed by only one base from the common allele (H1). The common allele was found in all individuals (five homozygous individuals), and this common allele is found in every other population.

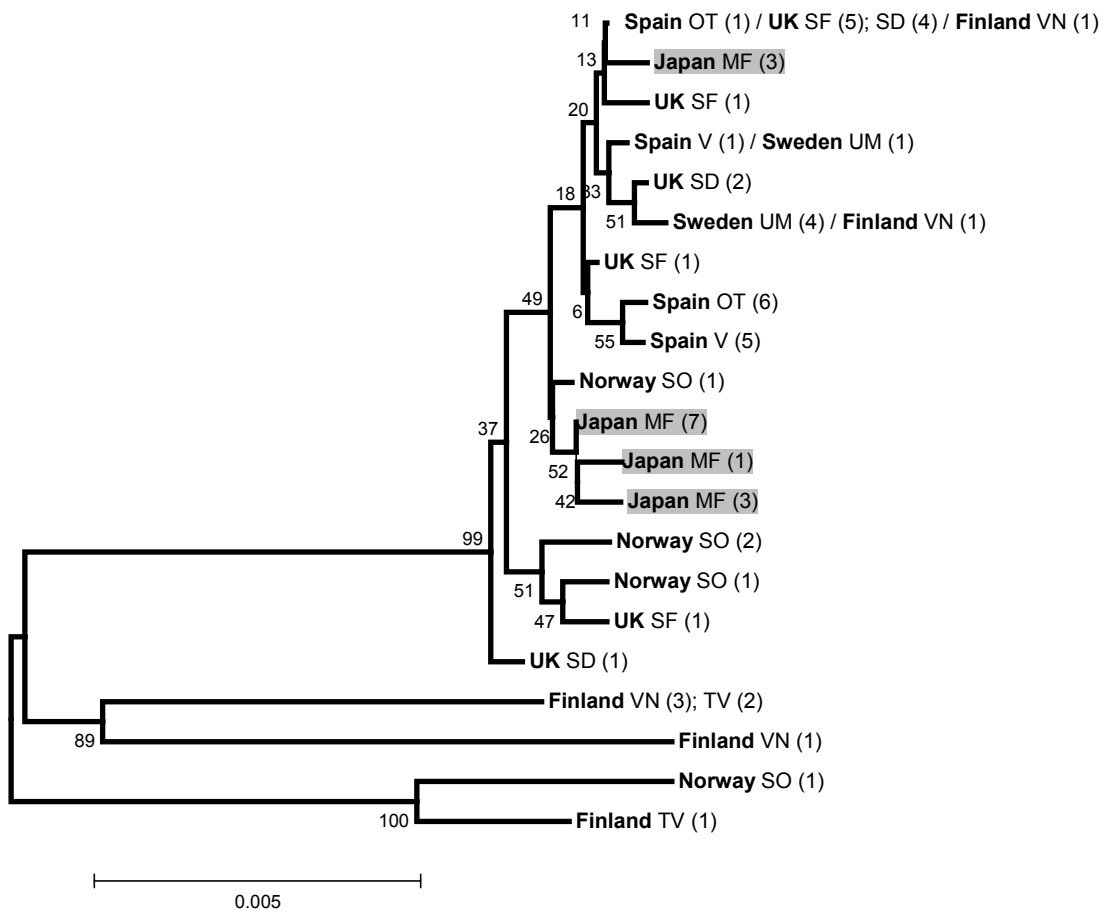


Figure 3. Neighbour-joining tree of the 21 haplotypes recovered from 685bp of cyt*b* with bootstrap support shown. Populations are: MF=Mt Fuji, Japan; OT=Orihuela del Tremendal, Spain; V=Valdelinares, Spain; SD=Santon Downham, UK; SF=Sherwood Forest, UK; SO=Solvorn, Norway; UM=Umea, Sweden; TV=Tvarminne, Finland; VN=Vaasa, Finland. For each haplotype the population(s) is shown and in brackets the number of individuals in which it was found. The Japanese population is highlighted in grey and the scale bar shows 0.5% sequence divergence.

Table 5. Variation in a 264bp fragment of the *foraging* gene. The sample size is the number of diploid workers with each sampled from a different colony.

Alleles	Variable sites	Populations				
		UK (SF)	Spain (OT)	Spain (V)	Finland (TV/VN)	Japan (MF)
H1	CGGCGT	0.917	0.857	0.667	0.900	0.857
H2	.A..AA		0.071	0.167		
H3	G.....		0.071	0.167		
H4	...T..	0.083				
H5	..A...				0.100	
H6	.A....					0.143
Sample size		6	7	3	5	7

4.4 DISCUSSION

Dissection data and genetic analyses support that skew is particularly high among queens in multiple queen *L. acervorum* colonies from a Japanese population, confirming previous reports of high skew (Ito 1990, 2005). Queen ovary dissections from the snap-shot MQ colonies showed that only a single queen possessed developed ovaries. Moreover, at the time of collection (i.e. seven days prior to when the snap-shot colonies were frozen) eggs were present in the colonies which further supports that only one queen was responsible for egg production and not that all but one queen had regressed their ovaries. Sibship analysis supported that in all MQ colonies there were no cases where colony members were the offspring of more than one resident queen. In addition, the majority of individuals (i.e. 81% of workers and larvae) belonged to a single matriline, which was supported by high relatedness among workers and larvae. Altogether there is a lack of evidence to support the presence of supplementary laying queens as has been previously suggested (Ito 1990), and supports a functionally monogynous social organisation.

Sibship analysis of MQ colonies showed that 55% of mated queens were grouped into the majority fullsib family, supported by high relatedness among queens. This shows that in many colonies queens are the daughters of a resident queen confirming daughter queen re-adoption after mating. Furthermore, on average 75% of all mated queens were grouped into a fullsib family with other adult individuals (workers and other queens), in fact, in five MQ colonies there were queens assigned to the same fullsib family as the maternal (reproductive) queen. This shows that queens must have been resident in the colony for at least the time required to rear workers and/or larvae (approx. one year), and

in two cases (MF2.36 & MF2.44) daughter queens (approx. two years). The additional finding of low queen turnover, shows that high skew in the Japanese population is a temporally stable social organisation, as also found in the Spanish *L. acervorum* population (chapter 2; Gill *et al.* 2009).

There was little difference in colony kin structure between SQ and MQ colonies supporting the assertion that the same number of queens are reproductive. However, in many SQ and MQ colonies a minority of workers were not assigned to the same fullsib family. If considering functional monogyny is exhibited, one explanation could be that there is more than one age cohort among workers within the colony. Potentially this could be due to multiple queen turnover events occurring within the lifetime of the workers, but this is not supported by the finding of low turnover. Another explanation could be that workers have been accepted into the nest from neighbouring colonies – a type of drifting behaviour – where workers move between neighbouring colonies (see Hare 1996; Sumner *et al.* 2007; Bourke pers. comm.). Indeed in the field, colonies are often found in dense patches. That said, it seems less clear how in not all (av. 80%) larvae in MQ colonies were assigned to the same fullsib family. This means that MQ colonies often possessed larvae that did not share the same mother even though they were from the same age cohort (all large in size). Does this suggest supplementary queen layers? It seems unlikely considering that over 18 colonies and 218 individuals (workers and larvae) in no case did I find more than one resident queen whose genotype matched the PMG of different fullsib families. Possible explanations for the unrelated larvae may be as follows:

i) One possibility could be that a queen turnover event had occurred during the egg laying period in the previous season resulting in larvae differing only slightly in age (have different mothers) even though all were large in size. However, we would then expect (given that turnover is relatively low) that such larvae would be grouped into the same fullsib family as other workers, yet this was not often the case.

ii) The evidence surrounding the mating system of *L. acervorum* supports that queens are monogamous (singly mated) and has been shown in a UK population (Hammond *et al.* 2001) and supported in a German population (Heinze *et al.* 1995b). However, it may be possible that in the Japanese population queens are not always monogamous, and may mate with multiple males (a low instance of polygamy). If this were true, however, we might expect to see a similar situation in SQ colonies, but in every colony analysed larvae were assigned to the same fullsib family and relatedness was high. In addition, in the high skew (functionally monogynous) Spanish population the proportion of colony members of fullsib families was similarly very high, and in SQ colonies relatedness was also not significantly different from 0.75 (Gill et al. 2009). Yet, I cannot rule out a low instance of polygamy; queens could mate with never more than two males and one male may be significantly less successful. For example, sperm stored in the queen's spermatheca may be heavily biased by one male perhaps due to sperm competition or female discrimination among sperm (see Birkhead & Moller 1988; Parker 2006), or perhaps even low physical copulation success.

iii) Eggs could have been laid by a supplementary laying queen who, once detected as reproductive, was evicted from the colony. It is known that workers can evict queens from the colony (chapter 3), and this may be because the queen in question may had developed her ovaries against the interests of other colony members. Furthermore, if eggs were laid by a supplementary queen, evidence suggests that workers would be unable to discriminate among eggs laid by different queens (Bourke 1991) and therefore such eggs would be reared and not cannibalised.

iv) Another possibility could be the occurrence of intra-species social parasitism, where offspring of a non-resident queen are reared (e.g. Foitzik & Heinze 2000; Lopez-Vaamonde *et al.* 2004). This could occur if queens from neighbouring colonies enter a nest and lay eggs, or if brood is transferred by colony members and then reared by conspecific workers. This would require investigation into the local nest distribution and looking at genetic relationships between and within colonies.

v) Brood may be dropped during colony emigrations and workers from neighbouring colonies pick up and transfer the brood into their own nest (see Hare 1996).

There were a number of SQ and MQ colonies where no resident queen matched the PMG of any fullsib family. Explanations for this could be: i) that not all resident queens were collected in the field because of the difficult position of nests, meaning that some

SQ colonies may actually have been MQ colonies in the field. Despite this my analysis is still relatively conservative because queen number can only be underestimated and in all colonies there was a low number of matrilines. ii) That eggs were not genotyped and perhaps not enough individuals were genotyped in the respective colonies. If a resident queen had only recently become the reproductive after a queen turnover event, then she will have only been able to produce eggs and perhaps be the mother of a small number of larvae. The average number of larvae genotyped per colony (n=4) may not have been enough to detect the small proportion that could be attributable to the resident queens. In addition, this may also be a problem when detecting the presence of supplementary laying queens. iii) These colonies could have been the result of a recent budding event, where a fraction of a colony has split off (a 'bud') and founded a new colony (Stille & Stille 1992, 1993). If this had happened near to the time of collection then larvae and workers will be the offspring of a queen who is resident in a previous (possibly neighbouring) colony and not any resident queen. However, support for budding in *L. acervorum* is not conclusive.

Population differences in social organisation in L. acervorum

The data on sociogenetic structure of MQ colonies in the Japanese population and the previously studied Spanish population show a number of similarities (see Table 8), supporting that both populations exhibit the same social structure – functional monogyny. Skew is particularly high in both populations as is intra-colony relatedness and daughter queen re-adoption. In addition, each population shows the same average number of matrilines per colony and queen turnover is low.

The similarity in social organisation also highlights the marked contrast organisation to that found in other polygynous populations of *L. acervorum* found in the UK and central Europe (Figures 4 & 5). Furthermore, mtDNA and nDNA sequence data importantly reveals little differentiation between the Japanese, Spanish, and other European populations, supporting that they are the same species. This also suggests that the evolutionary time since the divergence between the polygynous and functionally monogynous populations is relatively recent. There was little evidence of any geographical structure based on the sequence data (both mitochondrial and nuclear), which was surprising given the large geographical distance between populations, especially between the European populations and the Japanese population. Indeed, similarly in previous studies no geographic structure has been found between populations throughout Europe in *L. acervorum* (Brandt *et al.* 2007; Foitzik *et al.* 2009). However, a distinct difference was reported between a Spanish and German population, and the European populations and a Japanese population but based on a single individual per population (Baur *et al.* 1995). Genetic distances (e.g. FST, GST, D_{EST}) using microsatellite data could provide a deeper insight into phylogenetic structure of these populations. However, in this study both the low number of loci and extent of polymorphism at some loci (highly mutable) would have little power and potentially suffer from problems such as homoplasy (see Estoup *et al.* 2002; reviewed in Selkoe & Toonen 2006). Therefore, an important evolutionary question remains: what is the ancestral social organisation of *L. acervorum* is and/or whether functional monogyny has arisen independently multiple times (see overall discussion)? In addition, a future avenue

of worker should investigate what ecological factors have driven such a divergence in social organisation (see overall discussion).

Table 8. Comparison of the data and information from MQ colonies from the high skew Spanish and Japanese (current study) populations.

Population	Spain*	Japan
Skew	High (complete skew)	High (complete skew)
Worker relatedness	0.83 & 0.64	0.63
Queen relatedness	0.59	0.57
Mean number of matrilines in colony	2.9	2.9
Queen turnover	19.7%	17.4%
Queen re-adoption	Yes (high)	Yes (high)
Worker policing of queens	Yes	?

* data from chapter 2 (Gill *et al.* 2009)

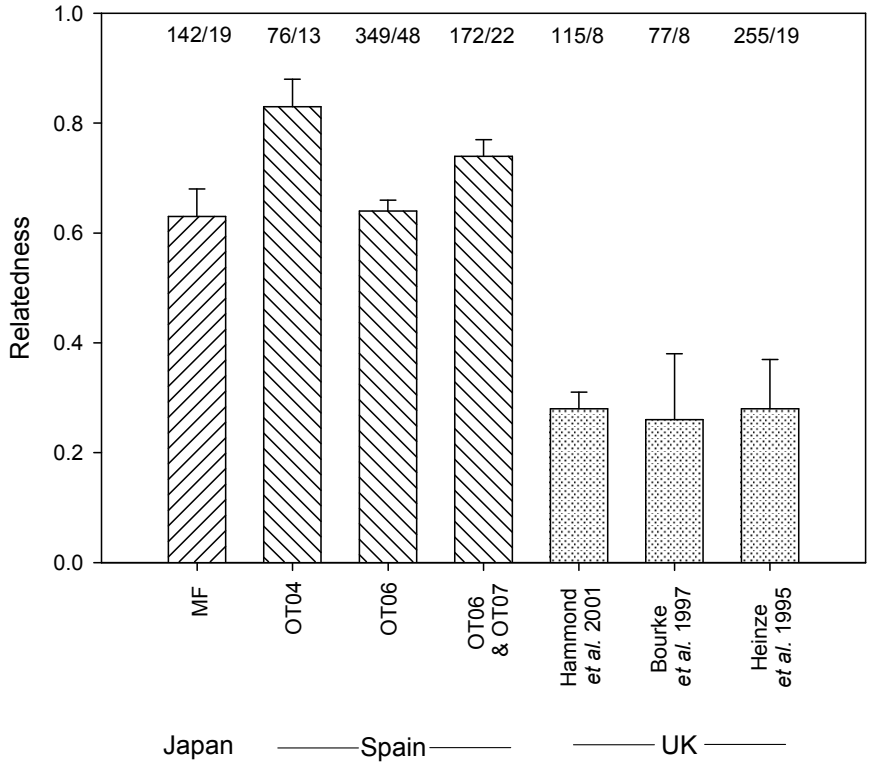


Figure 4. Comparison of worker relatedness values found in the high skew Japanese (MF) population (current study), and the Spanish (OT) population from a 2004 (OT04) collection, 2006 (OT06) collection (chapter 2, Gill *et al.* 2009), and another study (chapter 3) which includes colonies from the 2006 and a 2007 (OT06 & OT07) collection. These are compared with worker relatedness values reported from studies of a known low skew UK population (Santon Downham (SD): Hammond *et al.* 2001; Bourke *et al.* 1997; Heinze *et al.* 1995a). The bars represent the mean (±s.e.m.) relatedness coefficient values, and the numbers at the top of the bars represent the number of individuals/the number of colonies that were analysed.

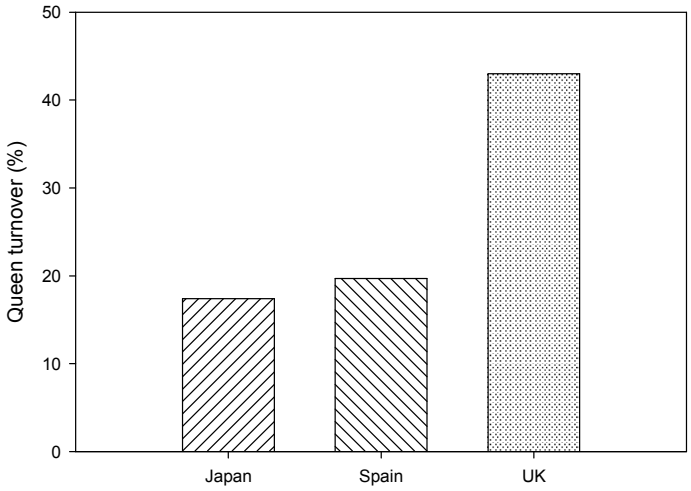


Figure 5. Comparison of queen turnover estimates in the high skew Japanese (MF) population (current study), high skew Spanish (OT) population (chapter 2; Gill *et al.* 2009), and low skew UK (SD) population (Hammond *et al.* 2006). Queen turnover was calculated in all cases using equation 4 in Pedersen & Boomsma (1999), considering within and between relatedness among a young cohort (larvae that developed from eggs laid at the start of the reproductive season in the same year of collection) and old cohort (workers).

Additional Data

See Appendix 3.

CHAPTER 5

Triploidy in functionally monogynous populations of the ant *Leptothorax acervorum*

INTRODUCTION

A common mode of sex determination found across approximately 20% of animal species (including the whole Hymenopteran order), is arrhenotokous parthenogenesis also known as haplodiploidy. In a haplodiploid reproductive system, fertilised eggs (diploid) develop into female individuals, whereas non-fertilised eggs (haploid) develop into males. In many species with haplodiploid reproduction (including most Hymenopteran species), the genetic basis is a single-locus complementary sex determination system (slCSD), where allele heterozygosity at this locus produces females, and hemizygosity (a single allele) produces males (see Whiting 1943; Beye *et al.* 2003). In the Hymenoptera, allele diversity at the CSD locus is known to be high in the majority of species and can range between approximately nine and 85 alleles across species (e.g. Ross *et al.* 1993; Antolin *et al.* 2003; Hasselmann & Beye 2004). The high allelic diversity means that mating individuals generally do not share identical CSD alleles, thus diploid offspring are usually heterozygous and subsequently develop as females. However, in the event that mating individuals do share alleles, known as a 'matched mating', diploid offspring which are homozygous at the CSD locus are produced resulting in diploid males (Adams *et al.* 1977) (Figure 1).

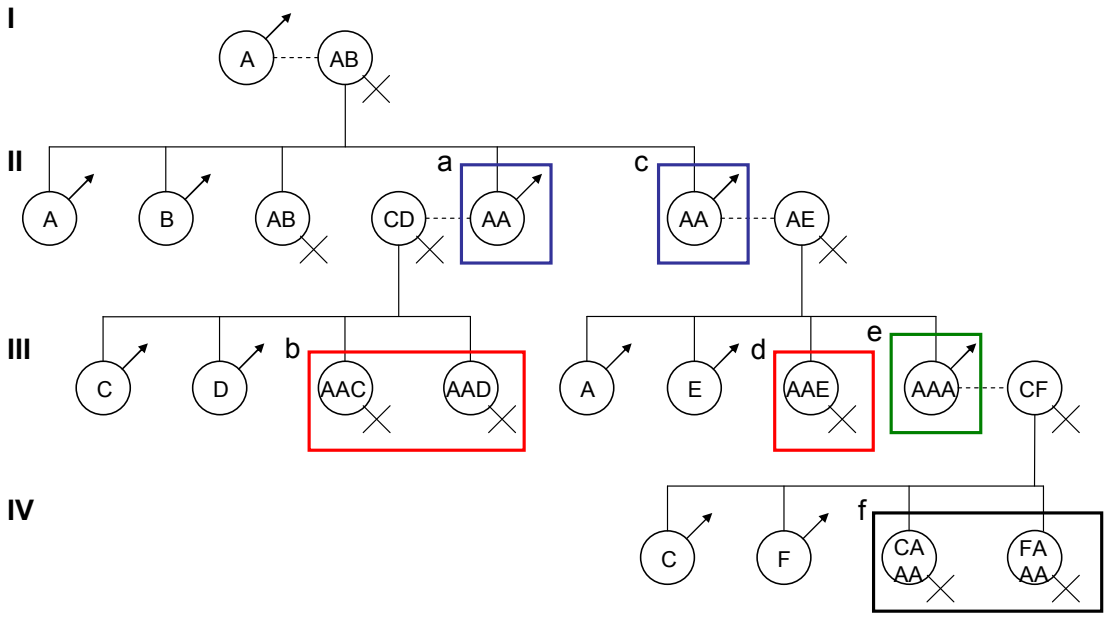


Figure 1. An example pedigree showing individual ploidy and the possible (hypothetical) genotypes at a single locus CSD site assuming that diploid males are fertile and triploid females are infertile (this does not show the correct sex ratio or allele frequencies). Solid lines represent the production of offspring from the female (queen) and dashed lines show female-male mating. Each capital letter within an individual (i.e. the circle) represents a single allele at the CSD locus, with the same letter depicting a shared allele, and the number of letters representing ploidy (n). Blue boxes = diploid males; red boxes = triploid females (workers and queens); green box = triploid male; black box = tetraploid females. **a)** Diploid male mates with a female not sharing any allele at the CSD locus - progeny is therefore either haploid male or triploid female; **b)** female offspring of a diploid male are triploid; **c)** diploid male mates with a female that shares an allele at the CSD locus - progeny is therefore either haploid male, triploid female, or triploid male; **d)** female offspring of a diploid male are triploid; **e)** triploid male mates with a female that shares an allele at the CSD locus - progeny is therefore either haploid male or tetraploid female; **f)** female offspring of a triploid male are tetraploid.

Diploid males have been found in over 60 species of the Hymenoptera (see van Wilgenburg *et al.* 2006), and it has been acknowledged that diploid male production may be a more widespread phenomenon than previously thought (see Bourke & Franks 1995; Cook & Crozier 1995; Krieger *et al.* 1999). In many cases, diploid males have been shown to be sterile (e.g. Petters & Mettus 1980; Cook 1993; Cook & Crozier 1995; Duchateau & Marien 1995) and can have physiological problems with mating (Smith & Wallace 1971; Elagoze *et al.* 1994). However, some studies have reported the presence of fertile diploid males who are able to mate resulting in the production of triploid female offspring (Figure 1, for refs see Table 1).

Diploid male production means that half of all fertilised eggs develop as males which significantly decreases the number of intended females reared. This not only impacts on colony sex ratio and queen production, but also greatly reduces worker production which may be of fundamental importance for the efficient functioning, survival, and founding/establishment of colonies (Ross & Fletcher 1986; Cook & Crozier 1995). For instance, studies have shown that colonies possessing diploid males have a lowered fitness and have an increased population extinction risk (Plowright & Pallett 1979; Ross & Fletcher 1986; Zayed 2004; Zayed & Packer 2005; Whitehorn *et al.* 2009). In addition, triploid female offspring are considered sterile in most species (refs in Table 1) resulting in a detrimental impact on the direct fitness of the parents who are investing in a dead end progeny, and also the indirect fitness benefits to other group members, and could potentially lead to the collapse of the colony (but see Garofalo & Kerr 1975; e.g. Naito & Suzuki 1991; Yamauchi *et al.* 2001; Ayabe *et al.* 2004; Cowan & Stahlhut 2004; Beukeboom & Kamping 2006). The production of diploid males and triploid females is in

effect a wasted investment and hence is considered a 'genetic load' in the eusocial Hymenoptera. The genetic load imposed as a result of diploid male and triploid female production, and the possible widespread occurrence across many species, means understanding the underlying causes and implications is important to evolutionary and conservation biology. In addition, skew theory assumes that all individuals have the potential to be reproductive, but the occurrence of infertile individuals has a fundamental impact on this assumption.

The probability of matched matings, and consequently diploid male and triploid female production, is increased under two primary scenarios: 1) low population genetic diversity; and 2) high inbreeding. Low allele diversity at the CSD locus at the population level leads to an increased probability that individuals will mate with another that shares a CSD allele assuming mating is random. This can be caused by having small populations where the effects of genetic drift are high (Zayed *et al.* 2004; Takahashi *et al.* 2008), which is enhanced in localised and patchy populations where there may be little gene flow (Henshaw *et al.* 2002). In addition, low allele diversity can occur in populations that have experienced relatively recent bottlenecking (e.g. Ross *et al.* 1993; Packer & Owen 2001). Inbreeding will also raise the probability of matched matings, as related individuals are more likely to share CSD alleles. The proximity at which mating occurs from the natal area can be a primary determinant of the level of inbreeding; the greater the distance from the natal area the lower the probability of mating with other individuals sharing CSD alleles.

Aspects of social organisation play a fundamental role in determining genetic diversity within the group/colony and at the local population scale. Reproductive skew

among queens determines the number of matrilines within a colony and therefore the genetic diversity. Low skew generally causes higher diversity which has been shown to increase disease resistance (Reber *et al.* 2008) and group productivity (Mattila & Seeley 2007). However, direct investigations into the impact of skew among queens on genetic diversity in connection to diploid male and triploid female production is limited (but see Krieger *et al.* 1999; Cournault & Aron 2009). Furthermore, high and low skew colonies are often associated with specific life history traits such as mating and dispersal strategies (Holldobler & Wilson 1990; Herbers 1993; Bourke & Franks 1995), which are fundamental factors affecting gene flow between populations and the probability of inbreeding.

The polymorphic social organisation found in *L. acervorum* is a good model to investigate how social organisation (comparing low and high skew populations) determines the potential risk of diploid male and triploid female production. In the low skew polygynous populations (UK & c. Europe) genetic diversity is considered to be relatively high as colonies consist of multiple genetic lineages and relatedness is low (Douwes *et al.* 1987; Bourke 1991; Bourke & Heinze 1994; Heinze *et al.* 1995a; Heinze *et al.* 1995b; Bourke *et al.* 1997; Chan & Bourke 1994; Hammond *et al.* 2001; Hammond *et al.* 2006). Inbreeding may also be relatively low due to a mating strategy that includes nuptial mating swarms away from the nest (Franks *et al.* 1991), and the habitat is considered more extensive and uniform suggesting ecological constraint on dispersal ability is moderate and populations may be larger and more connected (see Bourke & Heinze 1994; chapter 4). In contrast, in high skew functionally monogynous populations (Japan and Spain), genetic diversity is lower as colonies consist of a low number of

genetic lineages and relatedness is high. Furthermore, populations appear altitudinally restricted and relatively isolated, hence dispersal between neighbouring local populations may be severely constrained (Bourke & Heinze 1994; Felke & Buschinger 1999; Gill *et al.* 2009; chapter 2). In addition, mating is thought to occur close to the natal area/nest through 'female calling' (Bourke & Heinze 1994; Felke & Buschinger 1999); R. Hammond pers. comm.; A. Buschinger pers. comm.), which could increase the risk of inbreeding. Therefore, I raise the prediction that there should be an increased probability of matched matings in the functionally monogynous populations compared to that found in polygynous populations.

In this study I provide support for matched matings in two functionally monogynous populations of *L. acervorum* by detecting the presence of triploid female individuals. This was done by analysing a previously established genetic data set (microsatellite genotypes) from a Spanish (OT & V; chapters 2 & 3), and Japanese (MF; chapter 4) population. Using the microsatellite data I can determine not only ploidy of individuals, but also carry out paternity analysis which allows investigation into whether the production of triploid females is a consequence of fertile diploid males. In addition, for both the Spanish and Japanese populations I provide an estimate of the average inbreeding coefficient (F_{IS}). Ultimately, I compare the data from the current study with that previously published on polygynous *L. acervorum* populations, and discuss the link between social organisation and the frequency of matched matings and its implications.

Table 1. Reports of triploid females in the Hymenoptera, showing whether triploidy was detected in natural (wild) populations and the level of triploidy found, or detected in laboratory bred lines (for some of the studies I was unable to obtain these facts (?)).

Species	Laboratory or wild pop.	Level of triploidy in wild pop.	References
Ants			
Crematogaster sp 2.	Wild	?	Imai *et al.* 1977
Lasius sakagamii	Wild	?	Yamauchi & Hashikura (reported in Yamauchi *et al.* 2001)
Solenopsis invicta	? / Wild	? / 7.7-9.8%	Hung *et al.* 1974 / Krieger *et al.* 1999
Tapinoma erraticum	Wild	2.6%	Cournault & Aron 2009
Bees			
Apis cerana japonica	?	?	Hoshiba *et al.* 1981
Apis mellifera	Lab	-	Chaud-Netto 1972, 1975
Bombus atratus	Lab	-	Garofalo & Kerr 1975
Bombus florilegus	Wild	2.7%	Takeshi *et al.* 2008
Bombus terrestris	Lab / Lab	-	Duchateau & Marien 1995 / Ayabe *et al*. 2004
Wasps			
Bracon sp. near *hebetor*	Lab	-	Holloway *et al*. 1999
Habrobracon juglandis (Bracon hebetor)	? / Lab / Lab / Lab	? / - / - / -	Bostian 1934, 1936 / Torvik-Greb 1931, 1935 / Whiting 1943, 1961 / Woyke & Skowronek 1974
Habrobracon pectiophorae	?	?	Inaba 1939 (cited in Whiting 1961).
Cotesia vestalis	Lab	-	de Boer *et al*. 2007
Diadromus pulchellus	?	?	Chauvin *et al*. 1987
Nasonia vitripennis	Lab / Lab	-	Whiting 1960 / Beukeboom & Kamping 2006
Polistes aurifer	Wild	0.6%	Liebert *et al.* 2004
Polistes dominulus	Wild	2.5 - 3.9%	Liebert *et al.* 2004
Polistes fuscatus	Wild	3.5 - 4.7%	Liebert *et al.* 2004
Ropalidia revolutionalis	Wild	1.9%	M. Henshaw (reported in Liebert *et al.* 2004).
Sawflies			
Athalia rosae ruficornis	Lab	-	Naito & Suzuki 1991
Neodiprion nigroscutum	?	?	Smith & Wallace 1971

N.B. To the best of my knowledge these are all the reports of triploid females in the Hymenoptera, and I provide the species names at the time of the study, i.e. some of the species names may have changed.

METHODS

Colony sampling

Spanish (OT & V) population

Individuals (n=1272) were genotyped from 106 colonies (*OT*: 73 MQ & 22 SQ; *V*: 10 MQ & one SQ). From these colonies, 710 workers (mean=7.3 per colony, n=96 colonies) and 409 larvae (mean=4.6 per colony, n=79 colonies) were genotyped. In addition, 153 queens were genotyped from 33 MQ colonies (*OT*: 124 queens from 27 colonies; *V*: 29 queens from six colonies).

Japanese population

Individuals (n=536) were genotyped from 36 colonies (24 MQ & 12 SQ). From these colonies, 271 workers (mean=7.5 per colony), 152 larvae (mean=5.2 per colony; n=29 colonies), and 113 queens (101 from MQ colonies: mean=4.21 per colony; 12 from SQ colonies) were genotyped.

Genetic analysis

To investigate ploidy, individuals were genotyped at four polymorphic microsatellite loci (see chapter 2 for details) to determine the number of alleles present at each locus per individual. Identification of alleles was carried out using a Beckman Coulter CEQ 8000 which produced chromatograms showing the size (bp) of alleles and the number of different alleles per locus per individual (Figure 2). The overall number of alleles at each locus in the population (allele diversity) ranged from five at the least polymorphic locus (L18) to 66 at the most polymorphic locus (LXAGT1). This means

that the probability of heterozygosity is lower at loci with low polymorphism, therefore, I should expect that the probability of detecting triploidy is higher in the highly polymorphic loci (see Results). This is particularly important when considering that the triploid individuals are the consequences of matched matings and that the probability of this is increased if inbreeding occurs, and therefore, mated partners are likely to share alleles across the genome. The expected level of heterozygosity per locus (Table 2) was calculated for the Spanish OT population and Japanese MF population (based on all diploid individuals) using the program Arlequin (Excoffier *et al.* 2005).

Determining ploidy

Adult individuals (workers & queens) possessing three different alleles at one or more microsatellite loci were considered triploid females (Figure 2C). Workers and queens not possessing three different alleles at any one locus were classed as diploid females (Figure 2B). Identifying triploid workers and queens is potentially subject to error because if individuals possess two identical alleles at all four loci they would appear diploid rather than triploid. Thus, genotyping at multiple loci with high polymorphism is important to minimise misclassification. Such error is considered low as only 1.98% (OT & V) / 0.39% (MF) of workers and queens were homozygous at three loci (n=17 individuals (OT & V) & 15 individuals (MF)), and importantly there were no workers or queens found to be homozygous at all four loci in both populations.

Larvae possessing three different alleles at one or more loci were also considered as triploid females. The presence of triploid males (Figure 1) could not be entirely ruled-out, but studies have shown that triploid males are rare (Naito & Suzuki 1991; Krieger *et*

al. 1999; Ayabe *et al.* 2004; de Boer *et al.* 2007), Furthermore, the detection of triploid individuals is used as a general indicator for the occurrence of matched matings in populations, so misclassification of triploid females and males would not undermine the objective of this study. Larvae found to possess two different alleles at one or more loci were classed as diploid female individuals, and those with only a single allele at all four loci were classed as haploid male individuals (Figure 2A). The likelihood of misclassifying diploid female larvae as haploid male larvae, as a result of sharing alleles at all four loci, again appears negligible as I found no workers or queens homozygous at all four loci. However, I could not distinguish between diploid female larvae and the presence of diploid male larvae, because I was unable to determine the sex based on larval morphology. In this chapter no adult males have been genotyped (see discussion), hence, the study focuses on the production and presence of triploid females.

Inbreeding coefficient (F_{IS})

Population genetics analysis using Genepop (available at http://kimura.univ-montp2.fr/~rousset/Genepop.htm; updated from Raymond & Rousset 1995) tested each locus in each population for departures from Hardy-Weinberg equilibrium (HWE) to calculate observed (Ho) and expected (He) heterozygosities and to perform tests for genetic linkage disequilibrium. Statistical significance of these tests was adjusted for multiple comparisons using sequential Bonferroni correction (Rice 1989). The population average inbreeding coefficient was calculated by obtaining an F_{IS} value per sample from 1000 re-samples with the average of these estimates taken, whereby for each sample the genotype of one diploid larvae or worker was randomly taken from each colony (n=88

OT & 31 MF colonies). The re-sampling method and F_{IS} calculation was done using the program *R* with a specifically designed script (written by Sam De Blasi), and was checked by using a smaller number of samples (x10) in the program FSTAT v 2.9.3 (available at www.unil.ch/izea/softwares/fstat.html; updated from Goudet 1995).

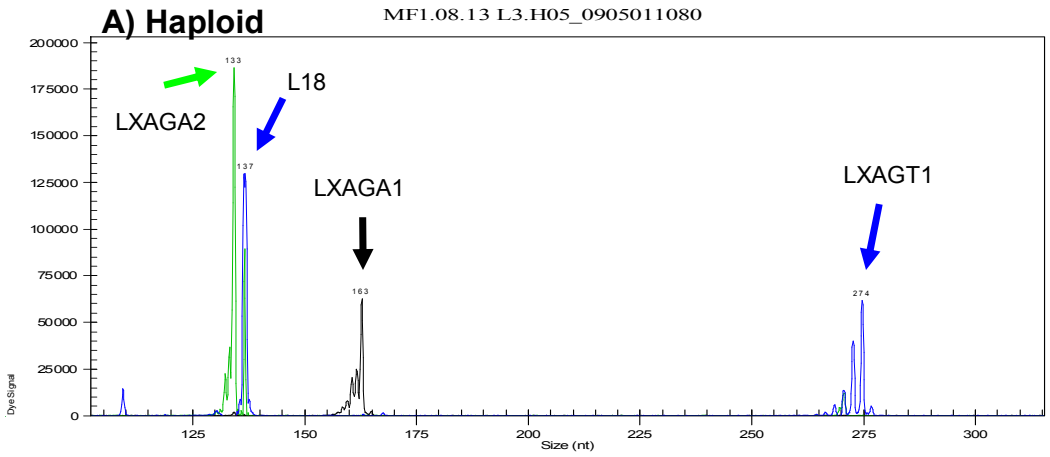
Sibship, queen mated and reproductive status, and evidence for diploid male production

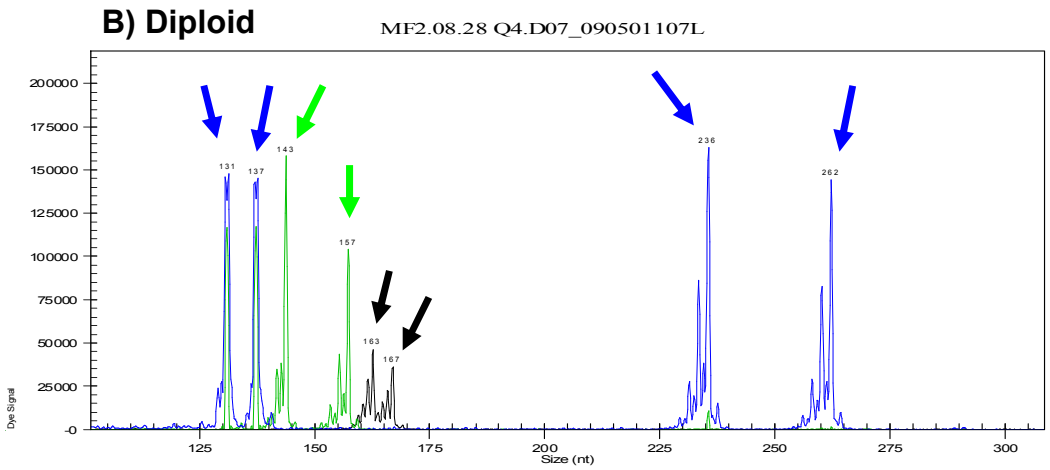
Previous sibship analysis, using the program COLONY (Wang 2004), accompanied with parental assignment was available for some of the colonies used in this study because resident queens had been genotyped (see chapter 2 for methods). This showed whether any resident queen(s) per colony was the mother of any colony members, hence it provided information on the reproductive history of each queen. Furthermore, knowledge of the maternal genotype means I am able to deduce the paternal genotype of triploid individuals, assuming that queens mate singly (Heinze *et al.* 1995b; Hammond *et al.* 2001). This was done by looking to see if all triploid members within the same fullsib family consistently possessed the same two alleles - the two inherited paternal alleles. The mated status was also known for the majority of genotyped queens.

RESULTS

Detecting triploidy

Triploid female individuals were detected in both populations. Chromatograph readings revealed three distinct allele peaks at one or more loci which were similar in size and shape (Figure 2), and triploidy could be found in multiple individuals within the same colony. The level of expected heterozygosity was lowest at locus L18, and increased respectively - GA1 to GA2 to GT1 (Table 2). As expected, triploid detection was positively associated with the expected level of heterozygosity (H_{exp}) at each locus (Figure 3, Table 3). Together these findings supports that the detection of three different alleles within an individual is unlikely to be the result of contamination. Furthermore, a particularly high proportion of all triploid individuals were found to show three distinct alleles even when only considering one locus (GT1; see Appendix 4). Together the high H_{exp} of at least two of the loci amplified and the high observed informative value of such loci supports that the error of misclassification of diploid and triploid individuals should be low.


A) Haploid
MF1.08.13 L3.H05_0905011080
LXAGA2
L18
LXAGA1
LXAGT1
133
137
163
274
Dye Signal
Size (nt)


B) Diploid
MF2.08.28 Q4.D07_090501107L
131
137
143
157
163
167
236
262
Dye Signal
Size (nt)

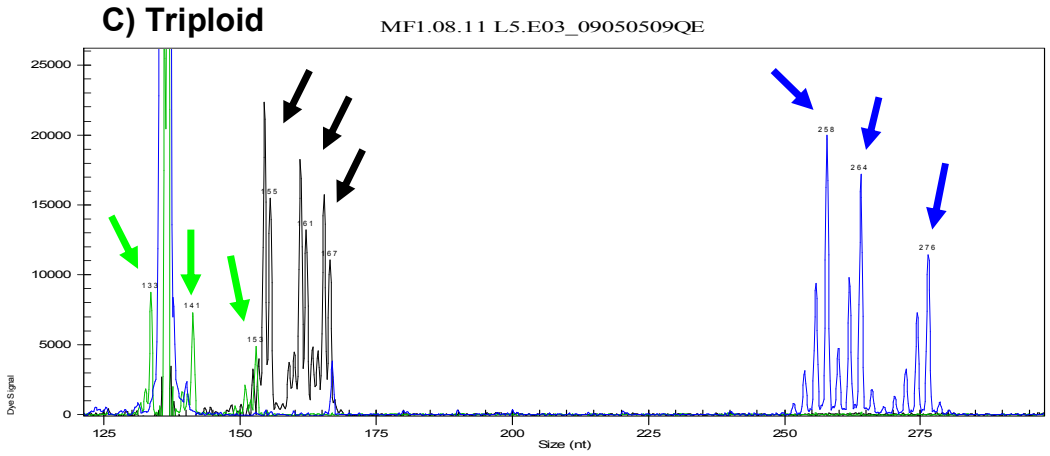

C) Triploid
MF1.08.11 L5.E03_09050509QE
133
141
153
155
161
167
258
264
276
Dye Signal
Size (nt)

Figure 2. Chromatograms showing the number and size of alleles at four polymorphic microsatellite loci in three separate individuals from the Japanese (MF) population. L18: blue peaks at the far left (<150bp); LXAGA1: black peaks; LXAGA2: green peaks; LXAGT1: blue peaks to the far right (>190bp). **A)** Haploid individual as there is only a single allele at all four loci. **B)** Diploid individual as there is two alleles at one or more loci, but importantly no locus with three alleles. **C)** Triploid individual as there is three alleles at one or more loci (the peak representing the L18 locus in the triploid chromatograph is not highlighted). In all chromatographs the number at the top of each peak shows the size (bp) of each allele.

Table 2. Allelic diversity in the Spanish (OT) and Japanese (MF) populations, and the expected heterozygosity at each microsatellite locus assuming random mating.

	No. Alleles	H_{exp}
Spanish (OT)		
L18	6	0.36
GA1	9	0.76
GA2	31	0.92
GT1	61	0.98
Japanese (MF)		
L18	5	0.66
GA1	8	0.77
GA2	17	0.87
GT1	33	0.94

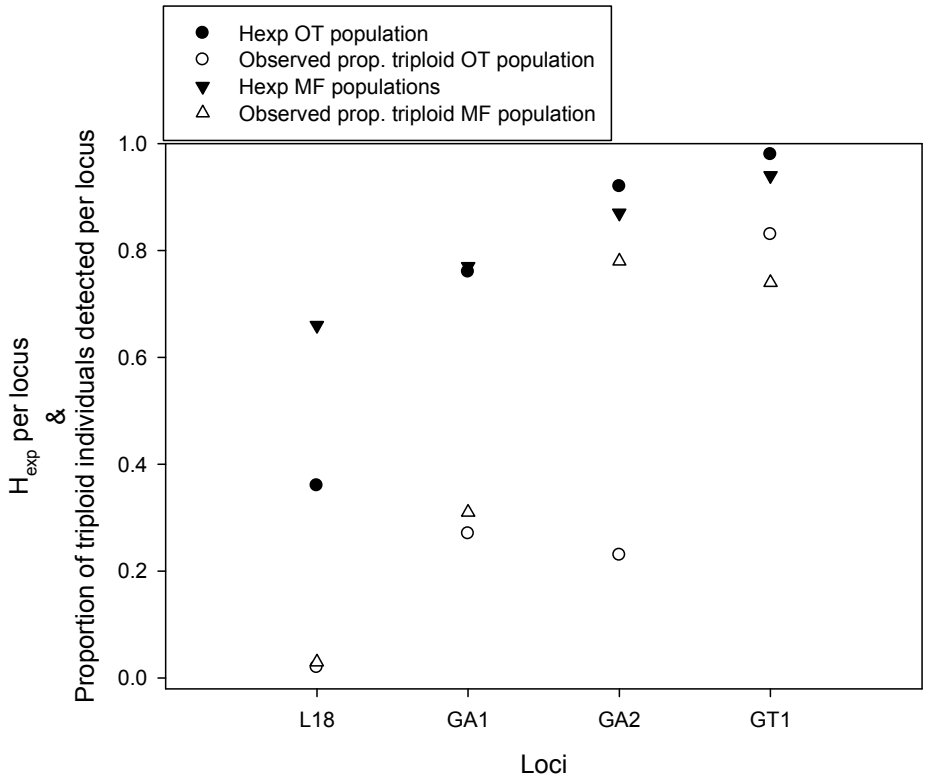


Figure 3. Paralleled trend between the expected heterozygosity (H_{exp}) per locus (Spanish OT population (black filled circle) and Japanese MF population (back filled triangle) and the proportion of detected triploid individuals found to possess three distinct alleles at each locus (Spanish OT population (open circle) and Japanese MF population (open triangle). As expected, the proportion of individuals possessing three distinct alleles at each locus, was higher at loci with a high H_{exp}. This figure accompanies Table 3.

Table 3. The proportion (%) of individuals found to be triploid per locus out of all detected triploid individuals in each population (the number in brackets next to the percentage is the number of individuals amplified successfully at each locus). This table accompanies Figure 3.

	Spain (OT)	Spain (V)	Spain (OT & V)	Japan (MF)
No. triploid ind. detected	62	20	82	76
L18	2% (58)	60% (20)	17% (78)	3% (76)
GA1	27% (59)	80% (20)	41% (79)	31% (75)
GA2	23% (57)	80% (20)	38% (77)	78% (72)
GT1	83% (60)	90% (20)	85% (80)	73% (73)

Level of triploidy detected

Spanish population (OT & V)

Eighty-two individuals (6.5%; n=23 colonies) out of all individuals genotyped were identified as triploid at an average of 1.7 loci per individual (Figure 4, Tables 4 & 5; OT=5.5%, n=19 colonies; V=14.6%, n=4 colonies). Specifically, triploidy was identified in 50 workers (7.0% of all workers genotyped), 28 larvae (6.8% of all larvae / 8.9% of all female larvae genotyped (excluding haploid male larvae)), and four queens (2.6% of all queens genotyped) (Figure 5).

In the 23 colonies possessing triploid individuals, the average number of triploid individuals per colony was 3.6 (28.9%; range=1-13 (4.5-100%)). There was variation between cohorts (workers, larvae and queens) in the proportion of triploid individuals detected within each genotyped cohort (Figure 6). The average proportion of triploid workers per colony was 38.1±11.3% which was not significantly different from the average proportion of triploid larvae per colony (32.5±6.2%; Mann Whitney: U=148, n_1=20, n_2=18, p=0.35). The average proportion of larvae, but not workers, was

significantly higher than the proportion of triploid queens per colony (10.4±7.2%; Mann Whitney: U=34, n_1=18, n_2=9, p=0.016) (Figure 6).

All remaining individuals constituted: 660 diploid workers, 149 diploid queens, and 381 larvae of which 285 were diploid females and 96 were haploid males (diploid:haploid ratio=2.97:1) (Figure 4).

Japanese population (MF)

Seventy-six individuals out of all those genotyped (14.2%; n=17 colonies) were identified as triploid at an average of 1.8 loci per individual (Figure 4, Tables 4 & 5). Specifically, triploidy was identified in 56 workers (20.7% of all workers genotyped), 14 larvae (9.2% of all larvae / 11.4% of all female larvae genotyped) and six queens (5.3% of all queens genotyped) (Figure 5).

In the 17 colonies possessing triploid individuals, the average number of triploid individuals per colony was 4.5 (27.6%; range=1-12 (4.2-85.7%)). Similar to that found in the Spanish populations there was variation between cohorts in the proportion of triploid individuals detected within each genotyped cohort (Figure 6). The average proportion of triploid workers per colony was 41.8±9.6% which was not significantly different from the average proportion of triploid larvae per colony (19.1±7.5%; Mann Whitney: U=72, n_1=17, n_2=14, p=0.062). In this population the proportion of workers, but not larvae, was significantly higher than the proportion of triploid queens per colony (6.9±3.9%; Mann Whitney: U=50, n_1=17, n_2=17, p=0.0012) (Figure 6).

All remaining individuals constituted: 215 diploid workers, 107 diploid queens, and 138 larvae of which 109 were diploid females and 29 were haploid males (diploid:haploid ratio=3.76:1) (Figure 4).

The variation between cohorts, in the proportion of triploid individuals detected within each genotyped cohort, for each population appears to show a general trend with a higher proportion of triploid workers and larvae found compared with triploid queens. However, in the Spanish population only the larvae had a significantly higher proportion than queens, and in Japan population only workers had a significantly higher proportion than queens, but in both populations workers and larvae were not significantly different from each other. Therefore, pooling the data, given that there is no significant difference between cohorts from each population (Mann Whitney: all $U>79$, $p>0.05$), increases sample size and statistical power. As found previously, the average proportion of triploid workers per colony (38.2±6.9%) was not significantly different from the average proportion of triploid larvae per colony (26.6±4.8%; Mann Whitney: $U=549$, $n_1=37$, $n_2=32$, $p=0.61$). However, in this case both the proportion of triploid workers and larvae were significantly higher that the proportion of triploid queens per colony (8.1±3.5%; W vs Q: Mann Whitney: $U=248$, $n_1=37$, $n_2=26$, $p=0.001$; L vs Q: Mann Whitney: $U=209$, $n_1=32$, $n_2=26$, $p=0.0012$) (see Figure 7).

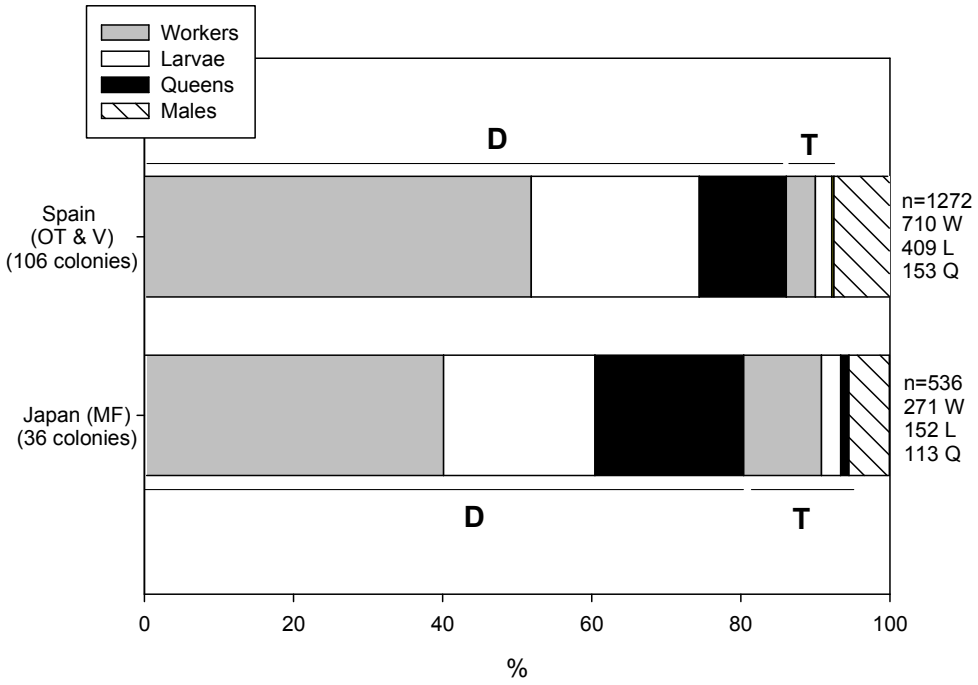


Figure 4. Proportion of individuals identified as being diploid females (D), triploid females (T) and haploid males (striped) out of all individuals genotyped (n) in both the Spanish and Japanese populations. The grey bars show the proportion of workers (W), the white bars the proportion of larvae (L) and the black bars the proportion of queens (Q).

Table 4. Frequency of triploidy per locus detected among all individuals genotyped from the Spanish and Japanese populations.

Populations	No. ind. genotyped (no. cols).	No. ind. triploid per locus (no. of cols) L18	GA1	GA2	GT1	No. ind. triploid at 1+ loci (no. cols)	No. alleles per locus
Spain (OT & V)	1269 (106)	13 (3)	32 (6)	28 (11)	69 (15)	82 (23)	L18=6; GA1=12; GA2=31; GT1=66
		1.0%	2.5%	2.2%	5.4%	6.5%	
Japan (MF)	536 (36)	2 (2)	23 (6)	57 (13)	53 (13)	76 (17)	L18=5; GA1=8; GA2=19; GT1=35
		0.4%	4.3%	10.6%	9.9%	14.2%	
Spain & Japan	1805 (142)	15	55	85	122	163 (40)	L18=9; GA1=16; GA2=33; GT1=72
		0.8%	3.0%	4.7%	6.8%	9.0%	

Table 5. The number of individuals within each cohort, workers (W), queens (Q), and female larvae (L(f)) detected as triploid across all the 40 colonies containing triploid individuals in both the Spanish and Japanese populations. Each colony is classified as being single queen (SQ) or multiple queen (MQ) and the number of workers, queens, female larvae and male larvae (L(m)) genotyped per colony is shown.

Colony	SQ/	Genotyped					Identified as Triploid			
	MQ	All	W	Q	L(f)	L(m)	All	W	Q	L
Spain (OT)										
A03_1810	SQ	3	-	-	3	1	1	-	-	1
A06_1910	SQ	14	8	-	6	2	1	-	-	1
A07_1910	MQ	12	9	-	3	0	1	0	-	1
A08_1810	MQ	8	8	-	-	0	8	8	-	-
A09_1910	MQ	22	8	8	6	1	1	0	0	1
A10_1710	MQ	12	8	-	4	0	1	0	-	1
A13_1910	MQ	10	8	2	-	0	8	8	0	-
A14_1910	MQ	17	8	4	6	2	1	0	0	1
A21_1810	MQ	17	12	-	5	0	2	2	-	0
B01_1810	MQ	8	4	-	4	0	8	4	-	4
B03_1710	MQ	16	12	-	4	1	3	3	-	0
B10_1810	SQ	5	5	-	-	0	5	5	-	-
B11_1910	MQ	9	5	-	4	0	3	0	-	3
B12_1810	MQ	9	9	-	-	0	1	1	-	-
B13_1810	SQ	4	4	-	-	0	1	1	-	-
B13_1910	MQ	21	9	3	9	5	1	0	0	1
B14_1710	MQ	14	8	-	6	1	1	0	-	1
B18_1710	MQ	19	8	5	6	1	4	2	0	2
OT4.09	MQ	16	8	2	6	3	11	8	0	3
Totals:		**236**	**141**	**24**	**72**	**17**	**62**	**42**	**0**	**20**
Averages:		**12.4**	**7.8**	**4.0**	**5.1**	**0.9**	**3.3**	**2.5**	**0.0**	**1.4**
Spain (V)										
V.07	MQ	10	6	-	4	0	1	0	-	1
V.08	MQ	9	-	3	6	0	5	-	1	4
V.11	MQ	18	8	5	5	2	13	8	3	2
V.12	MQ	11	-	7	4	0	1	-	0	1
Totals:		**48**	**14**	**15**	**19**	**2**	**20**	**8**	**4**	**8**
Averages:		**12.0**	**7.0**	**5.0**	**4.8**	**0.5**	**5.0**	**4.0**	**1.3**	**2.0**

Japan (MF)										
MF1.02	MQ	23	8	10	5	1	1	0	0	1
MF1.07	SQ	12	8	1	3	3	8	8	0	0
MF1.11	SQ	14	8	1	5	1	12	8	0	4
MF1.13	MQ	15	6	3	6	1	2	1	0	1
MF1.16	SQ	14	8	1	5	0	2	2	0	0
MF1.18	MQ	21	8	7	6	0	2	1	1	0
MF1.19	MQ	19	8	11	-	0	1	0	1	-
MF2.24	MQ	14	8	2	4	1	2	2	0	0
MF2.25	MQ	17	8	3	6	0	2	2	0	0
MF2.36	MQ	19	7	6	6	1	3	3	0	0
MF2.37	SQ	13	7	1	5	0	5	1	0	4
MF2.39	SQ	15	8	1	6	4	9	7	0	2
MF2.41	MQ	15	8	2	5	4	5	4	0	1
MF2.43	MQ	11	8	3	-	0	9	8	1	-
MF2.44	MQ	24	8	10	6	0	1	1	0	0
MF2.46	MQ	16	8	2	6	1	1	0	0	1
MF2.48	MQ	13	8	5	-	0	11	8	3	-
Totals:		**275**	**132**	**69**	**74**	**17**	**76**	**56**	**6**	**14**
Averages:		**16.2**	**7.8**	**4.1**	**5.3**	**1.0**	**4.5**	**3.3**	**0.4**	**1.0**

Figure 5. Proportion of triploid individuals detected within each cohort (workers, larvae and queens) out of the total individuals genotyped (numbers at the top of each bar) in the Spanish (OT & V) and Japanese (MF) populations.

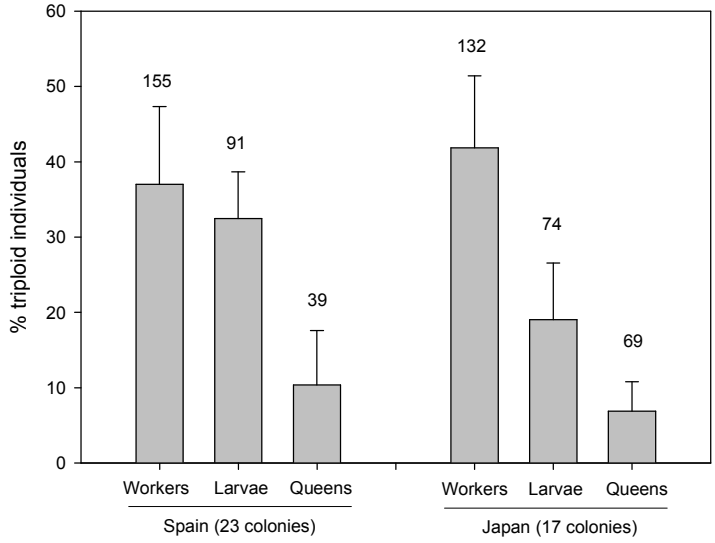


Figure 6. The mean (±s.e.m.) proportion per colony of triploid individuals detected in each cohort (workers, larvae and queens) in the 40 colonies containing triploid individuals (the numbers at the top of each bar represent the total number of individuals genotyped) from the Spanish (OT & V) and Japanese (MF) populations.

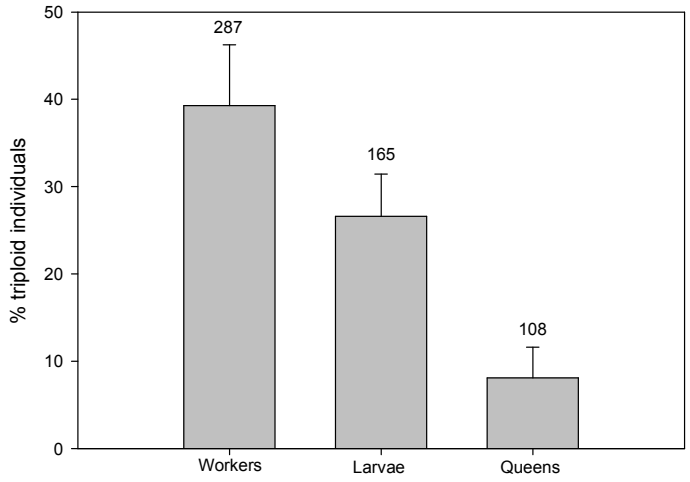


Figure 7. The mean (±s.e.m.) proportion per colony of triploid individuals detected in each cohort (workers, larvae and queens) in the 40 colonies containing triploid individuals, in which the data for both populations has been pooled (the numbers at the top of each bar represent the total number of individuals genotyped).

Inbreeding coefficient (F_{IS})

The inbreeding coefficient among colony diploid workers and larvae for the OT population was low (F_{IS}=0.006, n=88 colonies, 95% CI=-0.029 – 0.044) showing no significant difference from zero. The low inbreeding coefficient value may have been somewhat biased by the lower F_{IS} value found at one locus (GA1) which showed a higher than expected heterozygosity particularly in comparison to all other loci (see Table 5.5), but over all loci there was no significant deviation from the expected heterozygosity. In contrast the inbreeding coefficient for the MF population was relatively high (F_{IS}=0.107 n=31 colonies, 95% CI=0.050 – 0.1667) suggesting a significant heterozygote deficit and importantly was significantly higher than zero ($p<0.001$). However, the level of homozygosity at one locus (L18) was particularly high which contributed to the high estimated F_{IS} value (see Table 5.5). Importantly, when only accounting for three of the loci (excl. L18) the inbreeding coefficient was low (FIS=0.032, 95% CI=-0.027 – 0.091) and was not significantly different from zero.

Table 5.5. Inbreeding coefficient (F_{IS} values) provided for the Spanish (OT) and Japanese (MF) populations. The FIS values are shown per locus and the overall value for each population.

Population	F_{IS} values per locus				Average F_{IS} value
	L18	GA1	GA2	GT1	
OT (n=88)	0.0147	-0.070	0.063	0.017	0.006
MF (n=31)	0.332	0.092	-0.068	0.073	0.107

Sibship, queen mated and reproductive status, and evidence for diploid male production

Of the 40 colonies from both populations containing triploid individuals, 26 colonies had sibship and parentage data available (as resident queens were genotyped). Nine of these colonies were from the Spanish population (all MQ colonies; mean=4.3 queens per colony; range=2-8), and 17 were from the Japanese population (five SQ & 12 MQ colonies; MQ: mean=5.3 queens per colony; range=2-11). In the Spanish population, in six colonies no resident queen was compatible with being the mother of any detected triploid females ('non-maternal' colonies from hereon), although the number of detected triploid individuals in these colonies was particularly low (mean=2.2 ind. per colony, range=1-5; av. 17.3% of genotyped female colony members per colony). In three colonies there was a single resident queen who was compatible with being the mother ('maternal' colonies from hereon), and in these colonies, in contrast to the non-maternal colonies, the majority of colony members were detected as triploid (mean=10.7 ind. per colony, range=8-13; av. 84% of genotyped female colony members per colony).

Similarly, in the Japanese population, in 13 colonies no resident queen was compatible with being the mother of any detected triploid females, and again in these non-maternal colonies the number of detected triploid individuals was on average low (mean=3.2 ind. per colony, range=1-11; 23.8% of genotyped female colony members). In contrast, in four colonies there was a single resident queen who was compatible with being the mother, and in these maternal colonies the majority of colony members were detected as triploid (mean=8.5 ind. per colony, range=5-12; 77% of genotyped female

colony members). In no colonies from either population was there evidence that a triploid queen had been reproductive.

In the seven maternal colonies from both populations there was clear evidence to support that triploidy is the result of fertile diploid males producing diploid sperm. It was also evident in two non-maternal colonies, because the majority of colony members were triploid so the maternal and paternal genotypes could be predicted (presented in Table 6). Within each colony all triploid individuals (except one individual in one colony) were assigned to the same fullsib family, and in every case, all individuals possessed two of the same alleles (the inherited paternal alleles) and only ever differed by one allele (the inherited maternal allele) (see Table 6). Dissection data was available for all 10 detected triploid queens (Spain: four queens from two colonies; Japan: six queens from four colonies). Ovary dissection showed that only five queens were mated and the remaining five queens were unmated, this was significantly higher than expected based on the overall proportion of unmated queens detected in both populations (Fisher exact test: df=1, p=0.014).

Table 6. Seven maternal and two non-maternal colonies with the majority of colony members being triploid. In all cases the genotypes (at each locus) for all individuals per colony are shown. The 'maternal queen' is compatible with being the mother of the triploid individuals, and the 'predicted paternal' is the genotype with the highest likelihood of being the father to the triploid individuals. Loci that did not amplify (•) are shown for each individual. Note: in every case the predicted paternal genotype is diploid.

	L18	GA1	GA2	GT1
Spain (OT)				
A13.19 (MQ)				
Maternal Queen	131/131	151/151	127/131	240/320
Queen	131/131	151/169	129/163	296/320
Worker	131/131	151/169	127/131	236/240/252
Worker	131/131	151/169	127/131	236/252/320
Worker	131/131	151/169	•	236/240/252
Worker	131/131	151/169	127/131	236/240/252
Worker	131/131	151/169	127/131	236/240/252
Worker	131/131	151/169	127/131	236/240/252
Worker	131/131	•	127/131	236/252/320
Worker	131/131	151/169	127/131	236/252/320
Predicted paternal:	131/131	151/169 or 169/169	127/131	236/252
OT4.09 (MQ)				
Maternal Queen	139/139	151/173	131/131	260/308
Unrelated Queen	131/139	167/173	133/143	226/278
Male Larvae	139	151	131	308
Male Larvae	139	151	131	260
Male Larvae	139	151	131	308
Female Larvae	131/139	167/171/173	131/139	250/282/308
Female Larvae	131/139	151/167/171	131/139	250/282/308
Female Larvae	131/139	151/167/171	131/139	250/282/308
Worker	131/139	151/167/171	131/139	250/282/308
Worker	131/139	151/167/171	131/139	250/282/308
Worker	131/139	151/167/171	131/139	•
Worker	131/139	167/171/173	131/139	250/282/308
Worker	131/139	151/167/171	131/139	250/260/282
Worker	131/139	167/171/173	131/139	250/282/308
Worker	131/139	167/171/173	131/139	250/260/282
Worker	131/139	167/171/173	131/139	250/260/282
Predicted paternal:	131/131 or 131/139	167/171	131/139 or 139/139	250/282

*** In the first example colony above (A13.19), if we focus on the GT1 locus, the maternal queen's genotype is 240 / 320. In all the triploid workers they either possess the 240 allele or the 320 allele, therefore, we know that these are the maternally inherited alleles. All workers always possess the 236 allele and the 252 allele, therefore, these are the paternally inherited alleles and this shows that the father was diploid and it is not the result of the mother passing on two alleles.**

V.11 (MQ)

Maternal Queen	139/143	171/175	131/143	234/258
Daughter Queen	131/139/143	167/169/171	139/143/157	234/252/310
Daughter Queen	131/139/139	167/169/175	139/143/157	234/252/310
Daughter Queen	131/139/139	167/169/171	131/139/157	252/258/310
Queen	131/131	167/175	131/137	240/264
Male Larvae	139	171	143	234
Male Larvae	131	169	139	258
Male Larvae	139	171	131	234
Female Larvae	131/139/143	167/169/175	139/143/157	252/258/310
Female Larvae	131/139/144	167/169/175	131/139/157	252/258/310
Worker (non-fullsib)	131/139	175/177/181	129/143/159	242/252/310
Worker	139/139/143	167/169/175	139/143/157	252/258/310
Worker	131/139/143	167/169/171	131/139/157	234/252/310
Worker	131/139/143	167/169/171	131/139/157	234/252/310
Worker	131/139/143	167/169/175	139/143/157	252/258/310
Worker	131/139/143	167/169/175	139/143/157	252/258/310
Worker	131/139	167/169/171	131/139/157	234/252/310
Worker	131/139/143	167/169/175	131/139/157	234/252/310
Predicted paternal:	131/139	167/169	139/157	252/310

Japanese population (MF)

MF1.07 (SQ)

Maternal Queen	131/141	155/165	141/161	230/264
Male Larvae	141	155	141	264
Male Larvae	131	155	141	230
Male Larvae	131	155	•	264
Worker	131/141	165/165	133/141/143	264/268
Worker	131/141	165/165	133/143/161	230/264/268
Worker	131/141	165/165	133/141/143	264/268
Worker	131/141	155/165	133/141/143	•
Worker	131/141	165/165	133/143/161	264/268
Worker	131/141	165/165	133/143/161	230/264/268
Worker	131/141	165/165	133/141/143	264/268
Worker	131/141	165/165	133/143/161	230/264/268
Predicted paternal:	131/141	165/165	133/143	264/268

MF1.11 (SQ)

Maternal Queen	137/137	•	141/143	252/276
Male Larvae	137	167	•	252
Female Larvae	137/137	155/163/167	133/143/153	252/258/264
Female Larvae	137/137	155/163	133/141/153	252/258/264
Female Larvae	137/137	155/163/167	133/141/153	258/264/276
Female Larvae	137/137	155/163	133/143/153	258/264/276
Worker	137/137	155/163	133/141/153	252/258/264
Worker	137/137	•	133/143/153	258/264/276
Worker	137/137	155/163	133/141/153	252/258/264
Worker	137/137	155/163	133/143/153	258/264/276
Worker	137/137	155/163/167	133/143/153	252/258/264
Worker	137/137	155/163	•	258/264/276
Worker	137/137	155/163	133/141/153	252/258/264
Worker	137/137	155/163/167	133/141/153	252/258/264
Predicted paternal:	137/137	155/163	133/153	258/264

MF2.39 (SQ)

Maternal Queen	137/137	163/167	157/157	238/258
Male Larvae	137	•	157	258
Male Larvae	137	167	157	258
Male Larvae	137	167	157	238
Male Larvae	137	167	157	238
Female Larvae	131/137	163/165/169	157/167	238/264
Female Larvae	131/137	165/167/169	157/167	238/264
Worker	131/137	165/167/169	157/167	258/264
Worker	131/137	163/165/169	157/167	258/264
Worker	131/137	163/165/169	157/167	258/264
Worker	131/137	•	157/167	258/264
Worker	131/137	163/165/169	157/167	238/264
Worker	131/137	165/167/169	157/167	258/264
Worker	131/137	163/165/169	157/167	238/264
Worker	131/137	163/165/169	157/167	238/264
Predicted paternal:	131/131	165/169	167/167	264/264

MF2.43 (MQ)

Daughter Queen	137/137	163/167	141/165/169	266/272/274
Queen	131/137	163/165	157/167	252/254
Queen	137/137	163/169	133/133	262/264
Worker	137/137	163/167	141/165/169	266/272/274
Worker	137/137	163/167	143/165/169	254/266/274
Worker	137/137	163/167	141/165/169	254/266/274
Worker	137/137	163/167	141/165/169	254/266/274
Worker	137/137	163/167	141/165/169	266/272/274
Worker	137/137	163/167	141/165/169	266/272/274
Worker	137/137	163/167	141/165/169	266/272/274
Worker	137/137	163/167	143/165/169	254/266/274
Predicted maternal:	137/137	163/163 or 167/167	141/143	254/272
Predicted paternal:	137/137	163/167	165/169	266/274

MF2.48 (MQ)

Daughter Queen	131/137	163/165	133/141/163	252/272/398
Daughter Queen	131/137	163/165	141/157/163	252/272/398
Queen	131/137	165/169	161/169	262/272
Queen	137/137	163/167	143/161	266/276
Worker	131/137	163/165	141/157/163	272/376/398
Worker	131/137	163/165	133/141/163	272/376/398
Worker	131/137	163/165	141/157/163	252/272/398
Worker	131/137	163/165	133/141/163	252/272/398
Worker	131/137	163/165	141/157/163	252/272/398
Worker	131/137	163/165	133/141/163	252/272/398
Worker	131/137	163/165	133/141/163	252/272/398
Worker	131/137	163/165	141/157/163	272/376/398
Daughter Queen	131/137	163/165	141/157/163	272/376/398
Predicted maternal:	131/131*	163/163 or 165/165	133/157	252/376
Predicted paternal:	131/137	163/165	141/163	272/398

* This genotype could also be 131/137 or 137/137.

DISCUSSION

Genetic analysis revealed a substantial level of triploidy in two functionally monogynous populations of the ant *Leptothorax acervorum*. Microsatellite genotyping identified triploidy in 6.5% of all genotyped individuals (incl. workers, queens, and larvae) in the Spanish population, and 14.2% in the Japanese population. The level of triploidy detected in this study is relatively high in comparison to other studies (see Table 1). To my knowledge the highest level of triploidy previously documented was in a population of the fire ant *Solenopsis invicta* where between 7.7-9.8% was detected among workers and non-reproductive queens (Krieger *et al.* 1999). Therefore, the level of triploidy found in the Japanese population appears to be the highest found to date, and this study is the first to show the production of triploid individuals in *L. acervorum*. Moreover, these are likely underestimates of the level of triploidy because of the potential error of misclassifying triploid individuals as diploid individuals because of identical alleles at all loci (see Krieger & Keller 1998).

Sibship and parentage analysis revealed that in some colonies (n=12) the majority of genotyped colony members were triploid and were members of the same fullsib family (fullsisters). Those with genotyped queens and sibship data available showed that in most of these colonies a single resident queen was genetically compatible as being the mother of the triploid individuals (Table 6). This supports a single locus CSD mode of reproduction, because the female offspring of a queen who is singly mated (Heinze *et al.* 1995b; Hammond *et al.* 2001) to a diploid male should all be triploid. We would expect a substantially lower level of triploidy if the mode of reproduction was multi locus or was not dependent on sex alleles (Buschinger 1989) Cook 1993; (Keller & Passera 1993; also

see Fjerdingstad *et al.* 2003; Schrempf *et al.* 2006). Furthermore, based on the known maternal genotype the predicted paternal genotype was diploid supporting that the production of triploid females was a consequence of fertile diploid males (Table 6).

In many colonies, however, the number of triploid individuals detected was particularly low (as few as one individual per colony; see Table 5), and no resident queen was compatible with being the mother. There may be a number of possibilities why only a minority of triploid individuals were found in such colonies: i) There is potential for misclassification of diploid and triploid individuals because parents share alleles at all four microsatellite loci. Although this should be uncommon (i.e. H_{exp} for microsatellite loci is high), this may be confounded by the fact that matched matings are the result of sharing alleles at the CSD locus which may also be associated with sharing alleles throughout the genome, as is the case when inbreeding. ii) Caused by queen turnover where the minority of triploid individuals detected are the offspring of a previous or new reproductive queen. The former can only occur if either the new reproductive queen is not the offspring of the previous queen, or that she is an immigrant, because all re-adopted daughter queens will be triploid. On the otherhand, there is little support for the latter as sibship and parental data showed that in colonies with few triploid individuals there was no resident queen compatible with being the mother. iii) The majority of triploid individuals (incl. the maternal queen and offspring) have been evicted or killed but some are still present in the nest (see below). iv) Triploid workers and larvae have come from another colony (discussed in previous chapters).

In both the Spanish and Japanese populations, the proportion of triploid individuals within each cohort was higher among workers and among larvae than that

found among queens. Similarly, this has also been reported in other ant species such as *S. invicta* (Krieger *et al.* 1999) and *Tapinoma erraticum* (Cournault & Aron 2009). Krieger *et al.* (1999) proposed two primary hypotheses to explain the lower level of triploidy in queens, suggesting either: that triploid queens may die from endogenous causes before they have the opportunity to reproduce, or that workers may eliminate triploid queens before they have the opportunity to reproduce. In relation to both hypotheses, there is much evidence to suggest that triploid queens are generally infertile (refs in Table 1), therefore, we cannot assume that triploid queens will ever have the opportunity to reproduce. Indeed, in this study I found no evidence to suggest that triploid queens have had a history of reproduction (although based on a relatively small sample). The possibility that triploid queens are short lived has little supportive evidence, and in this study five triploid queens were mated and therefore may have been resident in the nest for an extended period of time.

With respect to the second hypothesis the reasoning underlying worker discrimination may not necessarily be to prevent triploid queens from reproducing but to ensure that infertile triploid queens do not impose a load on colony resources as queens do little or no work. This is plausible in *L. acervorum,* as workers are known to possess the power and information to police and discriminate among queens (see chapters 3 & 4). In addition, in the honey bee *Apis mellifera* workers can recognise and kill diploid males based on cuticular hydrocarbons (Woyke 1963; Santomauro *et al.* 2004), suggesting that workers in other species, including *L. acervorum*, may be able to recognise triploid individuals in other species. If such mechanisms are in place why are triploid queens found? In this study, dissections showed that half of the triploid queens were unmated

(5/10 incl. both populations) which may suggest that these queens have only recently developed and have not yet been eliminated by workers. That said, the detection of mated triploid queens could mean that such worker regulation is prone to error, perhaps because of recognition errors. The higher incidence of worker triploidy may be because triploid workers can still undertake an important role in rearing and protecting the colony so are therefore still valued colony members and are not eliminated from the colony. Another possible explanation is that if workers are able to detect triploidy at the larval stage then there is the potential when rearing larvae to coerce their development towards the fate of workers instead of queens rather than destroying all triploid larvae (see Hammond *et al.* 2002).

Link between social organisation and female triploid production

There have been a number of laboratory experiments, producing artificial lines of inbred colonies to investigate the fitness consequences of diploid males & triploid females (e.g. Plowright & Pallett 1979; Cowan & Stahlhut 2004; Schrempf *et al.* 2006; Zhou *et al.* 2007; Whitehorn *et al.* 2009) (also see Table 1). However, there are fewer studies looking to explain the causes and consequences of matched matings in natural populations which integrate population genetics with data on the sociogenetic, life-history, and ecological components of social organisation (e.g. Krieger *et al.* 1999; Antolin *et al.* 2003; Cournault & Aron 2009). The high level of triploidy found in both functionally monogynous *L. acervorum* populations is a contrast to that found in the polygynous populations of the same species. For example, triploidy in polygynous populations of *L. acervorum* has not been reported, and the level of diploid male

production seems particularly rare; only 1.3% of diploid males was reported in a UK population (Hammond *et al.* 2001), and none were found in a German population (Heinze *et al.* 1995b). In addition, studies on other Leptothorax species have also shown relatively low levels of diploid male production (Loiselle *et al.* 1990; Herbers & Grieco 1994; Foitzik & Heinze 2000). The evidence presented in this study is in accordance with the original prediction stated in this study, namely that the functionally monogynous population of *L. acervorum* is at a higher risk of matched matings than compared with studied polygynous populations.

An indicative feature of the functionally monogynous *L. acervorum* populations is that of a restricted and patchy distribution of colonies, suggesting that constraint on dispersal may be high. Such constraint may affect the distance that mating occurs away from the natal area, indeed laboratory observations of the Spanish population suggest that queens signal and mate outside the natal nest (female calling) and are then re-adopted, but little is known about the distance that males may travel. If, for example, males also have low dispersal then the probability of matched matings is likely to increase as neighbouring colonies may be related and therefore share CSD alleles (i.e. inbreeding). However, the average population inbreeding coefficient appeared low in the OT population suggesting that inbreeding in this population is rare. That said, the inbreeding coefficient in the MF population was particularly high in comparison to the OT population and other studied ant populations (e.g. Haag-Liautard *et al.* 2009). The large difference in the level of homozygosity between the OT and MF population was unexpected, although this does correlate with the high triploidy level found in the MF population. My estimation of inbreeding coefficients, however, should be handled with

caution for two reasons: firstly, they are based on only four neutral markers which may be imprecise (e.g. Slate *et al.* 2004), and secondly in the MF population one locus (L18) contributed largely to the high F_{IS} value. Therefore, further investigation into inbreeding in these populations should attempt to use more neutral markers, and to investigate whether the high F_{IS} value at specific loci could be due to the presence of a null allele (Microchecker designed by: van Oosterhout *et al.* 2004). In addition, a population measure of heterozygosity does not provide information on the inbreeding level of the individual or colony and looking at the relatedness between maternal and paternal alleles is likely to provide a better estimation (Liautard & Sundstrom 2005). If inbreeding was a factor underlying the high triploidy level we might expect a higher inbreeding coefficient in the OT population. Therefore, perhaps the constraint on dispersal is a major factor in maintaining patchily distributed small (effective) populations which are susceptible to drift (e.g. Kukuk & May 1990; Chapman & Stewart 1996; Roubik *et al.* 1996; Zayed & Packer 2001). This in combination with a highly skewed social organisation can lead to low population genetic diversity and can increase the probability of matched matings, all of which is supported by the findings of this study.

In polygynous populations there is evidence to suggest that nuptial mating flights away from the natal area do occur (Franks *et al.* 1991; see Bourke & Heinze 1994) which is likely to decrease the probability of matched matings because individuals are less likely to mate with related individuals. Hammond *et al.* (2001) also showed in a polygynous UK population that males mated to resident queens within the same colony were unrelated, suggesting that males disperse widely. Such out-crossing limits the detrimental impact of diploid male and triploid female production. However, such wide male

dispersal in the functionally monogynous populations may not be feasible if the nearest population is either too distant or the constraints are too high selecting for limited dispersal. Polygyny also leads to multiple lineages within colonies producing greater genetic diversity, which could be an adaptation to negate the detrimental impact of matched matings (see Page & Marks 1982; Pamilo *et al.* 1994; Tarpy & Page 2001). This supports that social organisation is an important component when considering the causes of diploid male and triploid male production (the frequency of matched matings).

The impact of matched matings may be particularly detrimental when establishing a new colony because the production of workers is fundamental for successful establishment, yet half of all intended female offspring will develop as diploid males who carry out no work. In *L. acervorum*, however, there is some evidence to suggest that colony founding is achieved by budding, where a cohort of workers and queen(s) disperse together and establish a new colony. This may be an important adaptation to counter the potential detrimental effect of matched mating and solitary colony founding by a single queen. Selection to alleviate the genetic load caused by the production of diploid males and triploid females also might be achieved in a number of ways: i) Avoidance of inbreeding (e.g. Ode *et al.* 1995). ii) Selection for diploid males to produce viable offspring, for example, a recent study in the vespid wasp *Euodynerus foraminatus,* which has regular sib mating and thus high levels of inbreeding, showed that fertile diploid males could sire viable diploid female offspring (Cowan & Stahlhut 2004). iii) In the opposite direction, selection may favour mechanisms to prevent mating with diploid males or even to prevent matched matings occurring. This may rely on a mechanism by which individuals can recognise either ploidy, or the expression of particular alleles at the

CSD locus. iv) The evolution of multi locus sex determination (Cook & Crozier 1995; Schrempf *et al.* 2006; de Boer *et al.* 2008).

High diploid male and triploid female production in the Hymenoptera has implications on theoretical treatments and empirical investigations of reproductive skew. If either diploid males or triploid females are infertile (dead end individuals) this can distort the true degree of reproductive skew and make measures of skew potentially erroneous (Liebert *et al.* 2005a). For example, when considering a two-queen colony, complete monopolisation of reproduction (high skew) may be an artefact if one of the queens is triploid and therefore infertile as there is no spectrum for skew among queens when there is only one potentially functional queen. Furthermore, little is understood regarding whether individuals are able to detect the ploidy level of individuals (but see Santomauro *et al.* 2004), therefore, we cannot say whether individual ploidy is a relevant factor in the 'decision' over how reproduction is skewed among group members ('social contract' between members) when considering who holds the power to determine skew.

Additional Data

See Appendix 4.

Overall Discussion

The objective of the work presented in this thesis was to contribute to the current understanding of the proximate and ultimate causes for variation in reproductive skew. Many empirical studies that set out to test skew theory have found little variation in skew to explain, and comparative studies between species are potentially confounded by differences in life-history and phylogeny (see Magrath & Heinsohn 2000). The polymorphic social organisation in *L. acervorum*, however, provides an ideal and novel system to investigate the genetic, ecological, and social basis for a marked contrast in skew because it potentially overcomes such problems.

There are also fundamental differences in colony behaviour between the polygynous and functionally monogynous populations. The behavioural studies undertaken in chapter 3 support that high skew in the Spanish functionally monogynous population is maintained by worker policing of queens. Interestingly, this behaviour is not observed in polygynous populations and tellingly multiple queens reproduce. The finding of worker control over queen reproduction has fundamental implications on the assumption of skew theory and our understanding of intra-colony conflicts. Furthermore, worker control contradicts a primary assumption of most skew models - that control lies with individuals in direct competition over reproduction (queens) - and shows that third party influence is important. Genetic analyses further revealed that workers favour the queen who meets their fitness interest, showing that workers posses the information required to act in accordance with their best interests. This also shows that we can not underestimate the power of the collective. Rather than control by specific members, it

seems that control over skew in *L. acervorum* may be determined by all, or the majority of, group members which act as a collective genetic interest (see Reeve & Jeanne 2003).

My findings also highlight the importance in gaining knowledge surrounding the proximate control of skew before we can address the ultimate causes (West *et al.* 2007; Saltzman *et al.* 2009). Textbook explanations of cooperation often focus on the degree of relatedness between individuals, but in social insects with a haplodiploid reproductive system, specific queens may be favoured because of relatedness to her offspring rather than relatedness to the queen *per se*. The work presented here supports relatedness as an important factor shaping a highly skewed social organisation, and this has implications on the recent debate over the importance of relatedness in social interactions (e.g. Wilson 2005; Wilson & Holldobler 2005; Lehmann *et al.* 2007; Wilson & Wilson 2007).

An evolutionary ecology perspective of the divergence in social organisation in L. acervorum

The polymorphic social organisation in *L. acervorum* provides a good testing ground for kin selection theory. Functional monogyny in *L. acervorum* maintains high relatedness and therefore provides indirect fitness benefits to group members. This in mind: why does polygyny persist when such relatedness benefits are reduced? Polygyny is a common social organisation among the ants, therefore, factors that influence the benefit and cost in Hamilton's rule (b & c) must also be important, and so require investigation (Keller 1995; Herbers 2009). Thus to address this we must investigate the ecological factors responsible for the divergence in social organisation.

Ecological factors inevitably play an important role in shaping social organisation (e.g. Holldobler & Wilson 1977; Herbers 1993). In particular, are the factors constraining the success of individual dispersal and colony founding after mating ('ecological constraints': Emlen 1982; Vehrencamp 1983b), which is considered to be fundamental to the evolution of MQ societies (Crozier 1979; Rosengren & Pamilo 1983; Pamilo 1991; Herbers 1993; Keller 1993; Rosengren *et al.* 1993; Bourke & Franks 1995). Bourke & Heinze (1994) combined the ecological constraints hypothesis (Emlen 1982) with models of game theory on dispersal (Hamilton & May 1977) and skew theory, and incorporated them within a kin selective framework, in an attempt to explain variation in skew among MQ societies. Using this approach functional monogyny was predicted to be associated with high ecological constraints caused by a patchy habitat promoting a mixed dispersal strategy but with a low number of dispersers, high relatedness, and high aggression among queens (Table 9). In contrast, polygyny (multiple queens reproducing relatively equally) is predicted to be associated with moderate ecological constraints due to a more extensive and uniform habitat subsequently promoting a mixed dispersal strategy with high number of dispersers, low relatedness, and low aggression among queens.

In support of Bourke & Heinze's predictions on habitat patchiness, both Japanese and Spanish populations are restricted to high altitudes (approx. above 1500m), suggesting a dependence on a specific habitat and climate. Furthermore, within these restricted areas, colonies were found in dense and patchy aggregations where the ground appeared to be moist yet in a sunny opening on the forest floor. At the population scale, dispersal to neighbouring populations may be severely constrained because the habitat is not uniformly ditstributed at this altitude. In addition, there may also be constraint on the

ability to successfully disperse between each patch of colonies. In comparison, polygynous populations are not restricted altitudinally, and the habitat is described as more extensive and uniform (see Bourke & Heinze 1994). However, colonies are also found in dense and patchy aggregations suggesting that again a specific habitat is required, making it unclear as to whether there is a major difference in constraint on dispersal caused by habitat patchiness between functionally monogynous and polygynous populations.

A potential indicator of the constraint on dispersal is to compare the mating strategies between populations. There is evidence to show that there is variation in the mating and dispersal strategy between polygynous and functionally monogynous populations. In polygynous colonies it appears that there is a mixed mating strategy with evidence for both queen re-adoption and large nuptial mating swarms in the field (Collingwood 1958; Buschinger 1971; Douwes *et al.* 1987; Franks *et al.* 1991). In contrast, lab observations suggest that in the functionally monogynous Spanish population (Felke & Buschinger 1999; Gill *et al.* 2009), mating occurs close to the nest involving queens walking out of the nest and lifting their abdomens to release a chemical signal in order to attract males (referred to as the 'female calling syndrome' Holldobler & Bartz 1985), a strategy that has been reported in other functionally monogynous ant species (Heinze & Buschinger 1987, 1989; Heinze & Smith 1990; Heinze 1993b). This female calling behaviour means queens do not disperse far from the natal nest/area which may suggest that constraint on dispersal is higher in functionally monogynous populations.

Limitation on dispersal is likely to lead to nest site saturation within each suitable habitat patch, which is predicted to favour a high retention of daughter queens after mating. Nest site limitation in *Leptothorax* sp. may be common as they are generally dependent upon the extrinsic natural processes (i.e. weathering and wood boring organisms) to create appropriate nest sites (i.e. cavities between and under rocks and within twigs). For example, an experimental study on the ant *Leptothorax longispinosus* showed that increasing the number of available nest sites in the field led to a decrease in the number of queens per colony (Herbers 1986), suggesting that by increasing nest site availability you decrease the constraints of dispersal and colony founding. Bourke and Heinze (1994) predicted that functional monogyny should be associated with high nest site limitation, leading to higher daughter queen re-adoption than that found in polygynous populations and further leading to high relatedness among queens and high skew. However, from field collections, although subjective, it appeared that available nest sites within each patch were relatively saturated not only in the Japanese and Spanish functionally monogynous but also the UK polygynous populations (RG & RH pers. obs; Dr. Satoh pers. comm.). In addition, in both functionally monogynous and polygynous populations, daughter queen re-adoption occurs (chapters 2 & 3; Hammond *et al.* 2001; Gill *et al.* 2009), and although relatedness among queens in the functionally monogynous populations is higher this may be a consequence of high skew rather than a cause as all dispersing queens are from the same matriline (Gill *et al.* 2009). According to Bourke & Heinze's prediction we should expect a greater proportion of MQ colonies and higher numbers of queens per colony. However, there was no distinct variation in the number of MQ colonies between populations (see chapter 2; Gill *et al.* 2009), nor in the mean

number of queens found per colony (FM pop.s: 2.8 - 15.4; Ito 1990; Felke & Buschinger 1999; Gill *et al.* 2009; current study, vs, Poly pop.s: 1.8-9.8; Heinze 1995; Heinze *et al.* 1995a; Chan & Bourke 1994).

Bourke & Heinze's last prediction is that low aggression among queens should be exhibited in low skew colonies, and high aggression among queens in high skew colonies (Reeve & Ratnieks 1993; Bourke & Heinze 1994). This does not appear to be supported in *L. acervorum* as observations of functionally monogynous colonies show little or no aggression among queens (Chapter 3), as is also found in the polygynous populations (Bourke 1991; Heinze *et al.* 1997). However, little is known regarding aggression in the Japanese populations, although a previous report indicated that among queen aggression can be found (Ito 2005).

Table 9. Overview of the factors predicted to be associated with polygyny and functional monogyny according to Bourke & Heinze (1994), and how my findings and that of others concerning the polymorphism in social organisation in *L. acervorum* fits with these predictions (this table has been taken and modified from Bourke & Heinze (1994) Phil. Trans. Roy. Soc Lond. B.).

Polygyny	***Functional monogyny***	***Evidence in* L. acervorum**
Extensive uniform habitat with moderate costs of solitary colony foundation	Habitat of small, scattered patches (leading to high costs of solitary colony foundation)	Polygynous habitat appears to be relatively more extensive, yet colonies are often found in patchy aggregations. Functionally monogynous habitat appears restricted altitudinally and colonies are found in small and scattered patches. Little known about the direct costs of solitary foundation.
Mixed dispersal strategy with relatively many dispersers	Mixed dispersal strategy with relatively many non-dispersers	Daughter queen re-adoption occurs in both polygynous and functionally monogynous populations. However, some reports suggest mixed dispersal strategy with higher dispersal (nuptial mating swarms) in polygynous populations, whereas mating may be close to the nest via 'female calling' in functionally monogynous populations.
Queen – queen relatedness above zero, but moderate.	High queen-queen relatedness	As found, but this may be a consequence of low and high skew rather than a cause. In a polygynous society dispersing queens are from different matrilines, so even if there is high daughter queen re-adoption queens will be less related to each other than daughter queens that are re-adopted into a functionally monogynous society as queens are form a single matriline.
Colony budding	More frequent colony budding	Lack of information on the presence of colony budding in polygynous and functionally monogynous populations.
Low to absent queen-queen aggression	High queen-queen aggression	Low aggression among queens in both polygynous and functionally monogynous populations.

Queen turnover in the Spanish and Japanese populations is comparatively lower than that found in polygynous populations (Bourke *et al.* 1997; Hammond *et al.* 2006; Gill *et al.* 2009) (Figure 5). The level of queen turnover may, therefore, be a particularly important determinant of skew among queens. For instance, if queen turnover was high this would counteract the high indirect relatedness benefits gained from a high skew social organisation (i.e. functional monogyny), because frequent replacement of queens would result in multiple matrilines within the colony. This means that polygyny may not necessarily suffer large costs due to lowered indirect fitness benefits to group members if turnover is high. In fact, although the per capita queen reproduction is known to decrease with increased queen number (reproductivity effect Wilson 1971; Vargo & Fletcher 1987; see Keller 1988), the potential benefit of polygyny is that multiple reproductive queens as a collective may have a higher productivity than that of a single reproductive queen, because the rate of egg-laying per queen has a physiological limit (although it is also limited to the size of the workforce). For example, in a UK population of *L. acervorum* Chan *et al.* (1999) reported higher productivity in MQ colonies than in SQ colonies. Further support has been found in other facultative polygynous species where queen number positively correlates with colony size (e.g. Rissing & Pollock 1988; Elmes & Keller 1993; Evans 1996; Buschinger & Heinze 2001). High queen turnover may therefore promote polygyny over functional monogyny. In contrast, if queen turnover is low, functional monogyny may be favoured over polygyny because the long term production of fullsibs (or kin) outweighs the cost of lower productivity in the short term. A further avenue of work would be to investigate the direct cause(s) for variation in turnover

which would allow a better understanding of its importance in shaping social organisation.

Group productivity is considered a fundamental factor in the success and survival of social groups/colonies (e.g. Vehrencamp 1983a; Johnstone 2000; Magrath *et al.* 2004; Vehrencamp & Quinn 2004). An increase in productivity may provide benefits such as a more rapid production of a workforce, the opportunity to produce a high number of sexuals, an increased success in the ability to disperse and found new colonies, and greater establishment success and competitive ability (see Ito 1993; Keller 1993; Bourke & Franks 1995). Such traits may be fundamentally important for colonisation into newly available habitats and hence increasing a species range. If, for instance, we assumed that functional monogyny was the ancestral trait (see Bourke & Heinze 1994), the logical next step would be to understand how the transition to polygyny occurred. Using the Spanish population as an example, the Iberian peninsula is a known refuge during the last ice age (during Pleistocene: retreated ~18,000ya) (see Hewitt 2000; Pusch *et al.* 2006). Therefore, polygyny may be an adaptation to allow successful postglacial expansion into newly available habitats. In *L. acervorum*, as previously mentioned, the benefit of polygyny is that it could increase group productivity allowing colonies to have a higher success of establishment (i.e. dispersal and colony founding) and competitive ability. In addition, an increase in genetic diversity could possibly be important if experiencing new parasites (Reber *et al.* 2008), and possibly other social parasitic ant species which are known to occur in *L. acervorum* populations in central Europe (Buschinger & Alloway 1979; Rosengren & Pamilo 1983; Buschinger 1986). Although speculative, the above proposal is plausible (also see Foitzik *et al.* 2009), and the phylogenetic relationship

between the Spanish and northern European populations shows that the evolutionary divergence in social organisation is relatively recent.

There is also evidence to suggest that the ancestral mating strategy of the Leptothoracines is female calling outside the nest (possibly exhibited in the Spanish functionally monogynous population), and that the nuptial flights reported in the polygynous European *L. acervorum* populations is a recently evolved trait as the behaviour of female calling is still observed at the mating site (Collingwood 1958; Buschinger 1971; Buschinger & Alloway 1979; Douwes *et al.* 1987; Franks *et al.* 1991; Lipski *et al.* 1992). Mating further away from the natal area in polygynous populations of *L. acervorum* may have been a recent adaptation allowing greater queen dispersal, and therefore aiding range expansion as discussed. In support, the frequency of polygyny among many ant species is known to increase with increasing latitude (Heinze & Buschinger 1988). Furthermore, large scale migration or introduction events have been shown to cause changes in social organisation in other species as well, for example in the fire ant *S. invicta* (Ross *et al.* 1996), the Argentine ant *Linepithema humile* (Tsutsui *et al.* 2000; Giraud *et al.* 2002) and yellow jacket wasp (Goodisman *et al.* 2001a; Goodisman *et al.* 2001b).

The importance of life-history traits and ecological factors is highlighted by my finding of a particularly high level of triploid females in the functionally monogynous populations. This supports that the level of matched matings at the complementary sex determination locus is high among mating sexuals in the population (see Cook 1993). In functionally monogynous populations it seems like there is a likelihood of limited dispersal and a lack of gene flow between populations - due to being restricted to high

altitudes. In addition, to high skew and low population size, these can all be causes for low genetic diversity within the population (Zayed & Packer 2005). Consequently, this increases the susceptibility to matched matings which results in a genetic load at the colony level and can undermine the high relatedness benefits of high skew. Therefore, the increased risk of matched matings may also be an important factor in shaping skew, for example polygyny may lower such risk.

In this thesis, I often refer to Hamilton's inclusive fitness theory by using its adopted name - kin selection theory (Maynard Smith 1964). However, it is arguably this naming that has led to misguidance when empirically testing it. Recently, the importance of kin selection theory has been questioned, based on the fact that relatedness alone may not explain observed cooperative behaviour or the evolution of eusociality (e.g. see Wilson & Wilson 2007), but this does not necessarily mean that Hamilton's rule does not apply. Relatedness is a fundamental component, but we must also consider the benefit and cost components in Hamilton's rule and the roles of ecological and life-history factors.

Caveats and future directions/studies

i) Exclusion of triploid individuals in specific analyses (associated with chapters 2, 4 and 5)

In chapters 2 and 4 I only used haploid and diploid individuals and did not include triploid individuals in the analyses because of software restrictions (e.g. COLONY and Relatedness). Specifically, colonies possessing a majority proportion of triploid individuals were not included in the analysis, whereas for colonies possessing a minority proportion all individuals bar the triploid individuals were still used in the analyses. This means that I will have underestimated the average number of matrilines per colony. A possible consequence of this is that I have not accounted for supplementary layers giving rise to these triploid individuals. This seems unlikely, however, given that both the dissection data and genetic (sibship) data for the vast majority of colonies not possessing triploid individuals provides evidence for functional monogyny. That said, it is important to question where these individuals came from? (see below).

ii) Explaining the presence of unrelated group members and investigating local population structure (associated with chapters 2, 4 and 5)

In the discussion of chapter 4 I attempt to provide a number of explanations into why there are unrelated individuals (workers and larvae) present in colonies. A possible explanation for adult individuals could be that individuals from neighbouring colonies drift into others. Certainly this can be tested by using local population mapping of colonies collected (spatial data) and the available genetic microsatellite data both of which are available. Subsequently, tests for isolation by distance can be done by

comparing pairwise FST values between colonies. Such analysis would also be useful in identifying where unrelated larvae have come from, particularly if investigating whether they stem from neighbouring colony queen eggs (i.e. intra-specific social parasitism) or larvae themselves originated from neighbouring colonies. It also could provide valuable information colony life history such as queen dispersal and/or budding events.

iii) Heritability of social organisation (associated with chapter 2)

Common garden experiments supported a genetic basis for the polymorphism in social organisation, although I could not rule out complex effects during colony development. Therefore, further investigation into the heritable nature of social organisation is needed. For instance, cross breeding experiments using newly developed alate queens and males (drones) from polygynous and functionally monogynous populations may allow identification of a genetic effect on skew and/or behaviour in future generations. This would provide valuable and novel information on the genetic basis underpinning social behaviour and/or whether the social environment can play an important role. Furthermore, these bred lines can be kept for genetic analysis and investigation into the potential gene(s) responsible for the observed variation in behaviour (i.e. genetic mapping). Controlling for social plasticity, such as environmental imprinting at an early life stage, would require colonies being kept and reared in the lab (in controlled and consistent conditions) over multiple generations to see if newly reared brood (queens and workers) alter their behaviour and colony organisation over an a more extensive period of time (i.e. multiple seasons).

Further investigation into the genetic basis for the polymorphic social organisation would be to indentify the specific gene(s) (the genetic architecture) responsible for variation in behaviour. A possible method to achieve this could be to use restriction-site associated DNA (RAD) tags in order to map multiple (potentially thousands) SNP sites within the genome (Baird *et al.* 2008). This RAD sequencing approach would allow identification of possible markers linked with the region(s) of interest underlying the genetic basis for variation in social organisation and even allow detection of quantitative trait loci (QTL mapping). Furthermore, it would also allow a powerful population genetic analysis between polygynous and functionally monogynous populations, providing a more precise estimate of genetic differentiation (F_{ST}) between populations and an insight into the ancestral social phenotype (Hohenlohe *et al.* 2010). Importantly, however, before for this avenue of research can progress I must provide: i) further confirmation that social organisation is stable in the lab over a more extended period of time; ii) the possible importance of the social environment when individuals are reared; iii) that breeding experiments are viable in order to produce crosses; iv) and strong evidence of a heritable basis for social organisation.

iv) Worker vs queen control (associated with chapter 3)

In chapter 3 I show that workers play a role in regulating high skew in functionally monogynous colonies, but further tests of worker and queen control can be achieved which would shed further light on the complexities of intra-colony conflict over skew. For example, queens and workers from different populations could be reared together to understand the social influence on behaviour. Within colony aggression (i.e.

worker policing of queens) appears absent in polygynous colonies, therefore, if multiple queens from the functionally monogynous population were reared with workers from a polygynous colony, we might expect different outcomes on whether queens or workers are in control. Queen control would predict that still only a single queen would become reproductive, however, if all were to reproduce this would support the role of worker control in my previous studies. Similarly, multiple queens from a polygynous colony can be reared with workers from a functionally monogynous colony.

In addition, I showed that a new queen becomes reproductive when colonies have been split, and that worker aggression predicts which queen establishes reproductive dominance. Re-introduction of these new-reproductive queens into the group 1 colonies containing the original reproductive queen would allow observations of the response of workers and queen to the presence of multiple reproductive queens (an artificially polygynous state). Do workers still maintain high skew by killing or evicting one of the queens, and if so which queen do they discriminate against, and does between queen interactions such as sting smearing occur?

v) Worker policing of queens in the Japanese population (associated with chapters 3 and 4)

Worker policing of queen behaviour has not yet been investigated in the functionally monogynous Japanese population. This would be an intriguing study considering that a previous report suggests that among queen aggressive interactions may be important in establishing reproductive dominance (Ito 2005). This raises the possibility that functional monogyny is regulated through different behavioural

mechanisms which questions whether high skew (functional monogyny) has evolved independently multiple times.

vi) Identifying the ecological factors associated with social organisation (associated with chapter 4)

Comparing the features of functionally monogynous and polygynous populations in *L. acervorum* provides a great opportunity to isolate the fundamental factors promoting variation in skew. The evolution of polygyny is important in understanding kin selection theory, yet still relatively little is understood concerning the benefits of polygyny, calling for further investigation. For example, studies looking at the differences in productivity, dispersal and establishment success, and benefits of genetic diversity between the polygynous and functionally monogynous populations will contribute to our understanding of why polygyny persists. For instance, a previous study on another Leptothorax species showed that queen number per colony was dependent on newly available nest sites (Herbers 1986). A similar experiment could be undertaken in *L. acervorum* in both polygynous and functionally monogynous populations to provide a better measure of the proportion of dispersing queens that are re-adopted into the natal nest and the level of nest site saturation in the field. In addition, little is known about what causes variation in queen turnover; whether it is extrinsic and directly caused by demands of the environment which would suggest that turnover plays a role in shaping social organisation, or whether it is endogenous (genetically predetermined).

The evolutionary history of social organisation in *L. acervorum* should be further investigated including additional phylogenetic analyses using other coding and non-

coding regions. In particular would be to amplify additional neutral (microsatellite) markers that are not highly mutable and do not suffer from homoplasy to provide an accurate and powerful analysis of genetic distance between *L. acervorum* populations. This should hopefully provide information on the ancestral social organisation of the species and whether the transition was from functional monogyny to polygyny, vice versa, or something more complex. For example, is polygyny a result of a recent post glacial expansion from a refugia population exhibiting functional monogyny as I have previously suggested.

vii) Diploid males and variation in triploid cohorts (associated with chapter 5)

Genotyping of males in the functionally monogynous populations will provide definitive evidence for the production and frequency of diploid males. This would also allow modelling of the expected level of triploidy in the population which can then be compared with that detected (Krieger & Keller 1998). In addition, further investigation can be undertaken to see why there is variation in the number of workers and queens that are triploid. Are larvae coerced, and can adults detect ploidy level at the egg, larval or adult stages? This could be investigated by rearing colonies in the lab and sampling some larvae and workers from each colony to see whether triploidy is present. If so, then these colonies can be observed to see whether larvae are culled, whether there is a bias in female caste fate, and/or whether colony functioning is affected.

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APPENDICES

APPENDIX 1

Related to chapter 3 part 1.

Additional behavioural observations.

i) Aggression

Observed queen aggression towards workers (Q→W) was low (0.7% of all aggressive interactions), with the average rate of Q→W aggression carried-out (1.99±1.0 sec/hr) being significantly lower than W→Q aggression (Wilcoxon signed rank test: $Z=-6.3$, $n=69$, $p<0.001$), which was also significantly lower than the per capita rate of W→Q aggression (Wilcoxon signed rank test: $Z=-3.46$, $n=69$, $p<0.001$). However, the average rate of Q→W aggression was significantly higher than the average rate of Q→Q aggression carried-out (Wilcoxon signed rank test: $Z=2.58$, $n=69$, $p=0.005$) or received (Wilcoxon signed rank test: $Z=2.25$, $n=69$, $p=0.013$). There was also no significant difference in the amount of Q→W aggression carried out by future reproductive and non-reproductive queens (0.69±0.56 vs 2.59±1.44; Mann Whitney: $U=431$, $p=0.27$; Figure A).

ii) Grooming

Reproductive queens carried out a significantly lower rate of Q→W grooming than non-reproductive queens (3.53±1.63 sec/hr vs 97.5±18.5 secs/hr; Mann-Whitney:

U=152, n_1=22, n_2=47, P<0.001). Interestingly, although there was no difference in the amount of grooming received between reproductive and non reproductive queens (see main text), reproductive queens carried-out a significantly lower rate of Q→Q grooming than non-reproductive queens (0.27±0.27 sec/hr vs 19.0±5.9 sec/hr; Mann-Whitney: U=301, n_1=22, n_2=47, P<0.01). In addition, reproductive queens showed a higher rate of larval grooming compared with non-reproductive queens (293±61.4 vs 68.7±12.8; Mann-Whitney: U=167, P<0.001) (Figure B).

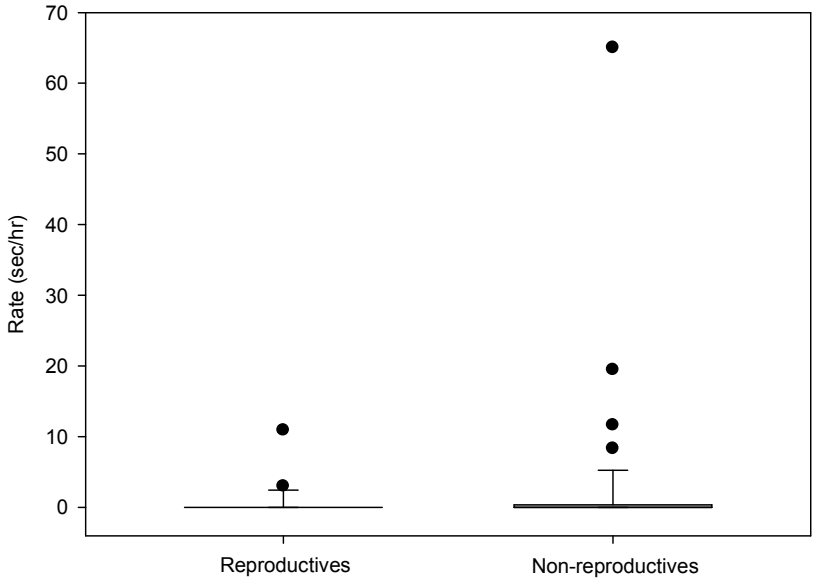


Figure A. The rate of Q→W aggression carried-out by queens that became either reproductive (n=22) or non-reproductive (n=47). The p-value was calculated using a Mann-Whitney U test. Box-plots show the median (line), mean (dashed line), quartiles (box limits) and 10th and 90th percentiles (error bars).

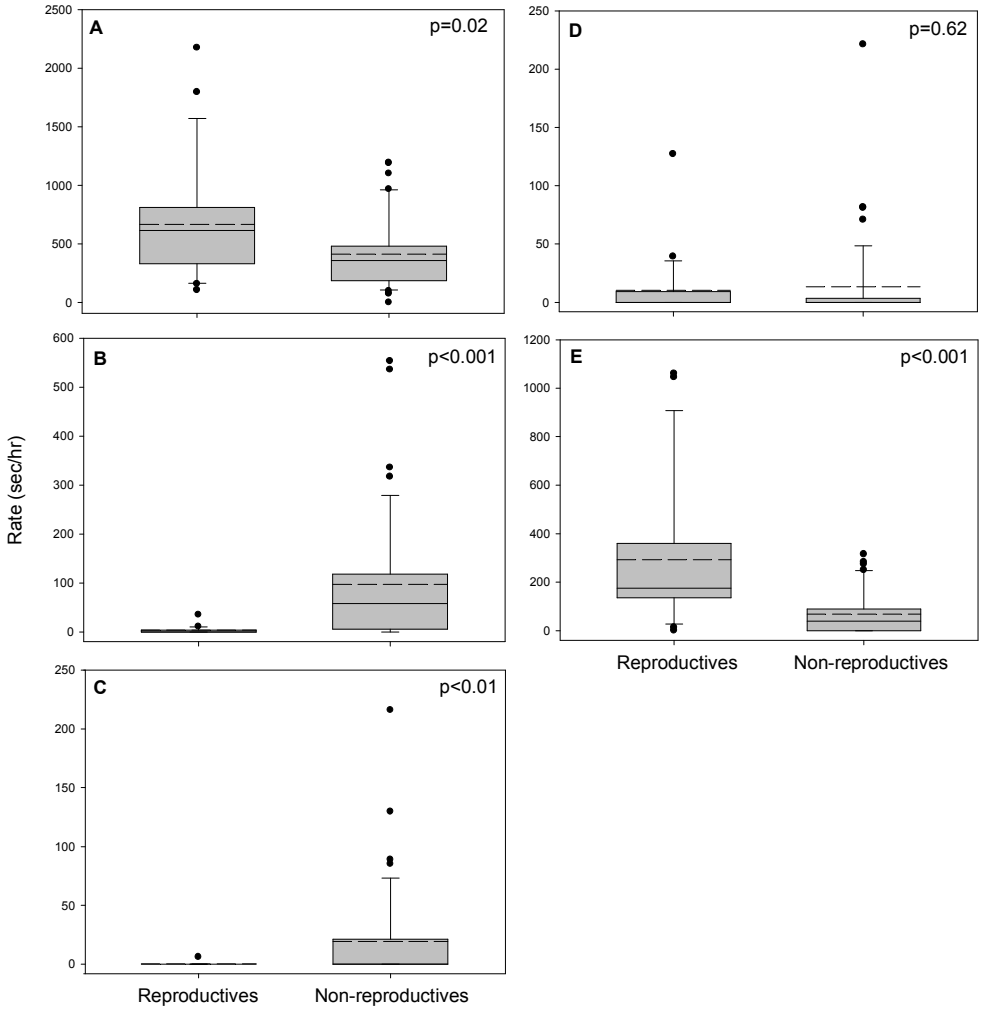


Figure B. Comparison of the rate of grooming behaviour received and carried-out by future reproductive (n=22) and non-reproductive (n=47) queens. **A)** W→Q; **B)** Q→W; **C)** Q→Q carried-out; **D)** Q→Q received; **E)** Q→L; p-values were calculated using a Mann-Whitney U statistical test. Box-plots show the median (line), quartiles (box limits) and 10^{th} and 90^{th} percentiles (error bars).

APPENDIX 2

Related to chapter 3 part 2.

Additional behavioural observations.

i) Aggression

The average rate of Q→W aggression per queen (9.7±3.1 sec/hr; 3.3%) was significantly lower than the rate of W→Q aggression (Wilcoxon signed rank test: Z=-3.98, n=32, p<0.001), which was still found when accounting for the numerical superiority of workers (mean no. workers=31 per colony, range=8-65; Wilcoxon signed rank test: Z=-, n=32, p=). The rate of Q→W aggression was significantly higher than both Q→Q aggression carried-out (Wilcoxon signed rank test: Z=2.27, n=32, p=0.013), and received (Wilcoxon signed rank test: Z=2.12, n=32, p=0.018). I further found no significant difference in the rate of Q→W aggression between the new-reproductive queen and non-reproductive queens (means=15.22±5.88 vs 5.37±2.82; Mann-Whitney: U=83, p=0.11; Figure A).

There appears to be little evidence to suggest queen manipulation of worker behaviour through physical means as both the previous and current studies (chapter 3 parts 1 & 2) show little Q→W aggression and no significant difference in the rate between the new-reproductive and non-reproductives.

ii) Grooming

In group 2 colonies there was no significant difference in the rate of Q→W and Q→L grooming between new-reproductive and non-reproductive queens (Q→W: means=41.0±19.1 vs 91.0±21.7; Mann-Whitney: U=108, p=0.49; Q→L: means=71.8±17.8 vs 48.7±15.2; Mann-Whitney: U=89, p=0.16) (Figure B).

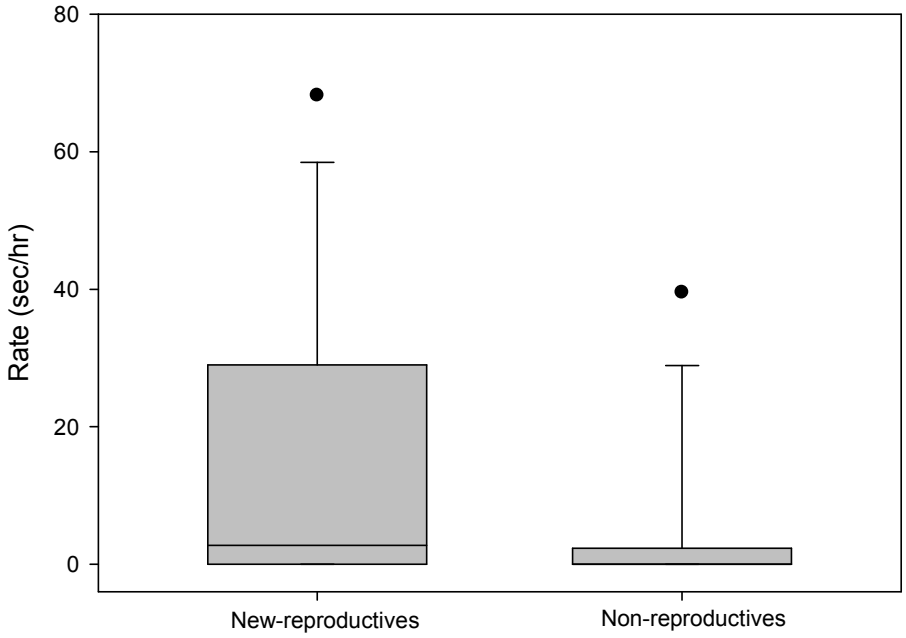


Figure A. Rate of Q→W aggression observed in the group 2 MQ colonies (n=14) carried-out by queens that became either the new-reproductive (n=14) or remained non-reproductive (n=18). The p-value was calculated using a Mann-Whitney U test. Box-plots show the median (line), mean (dashed line), quartiles (box limits) and 10^{th} and 90^{th} percentiles (error bars).

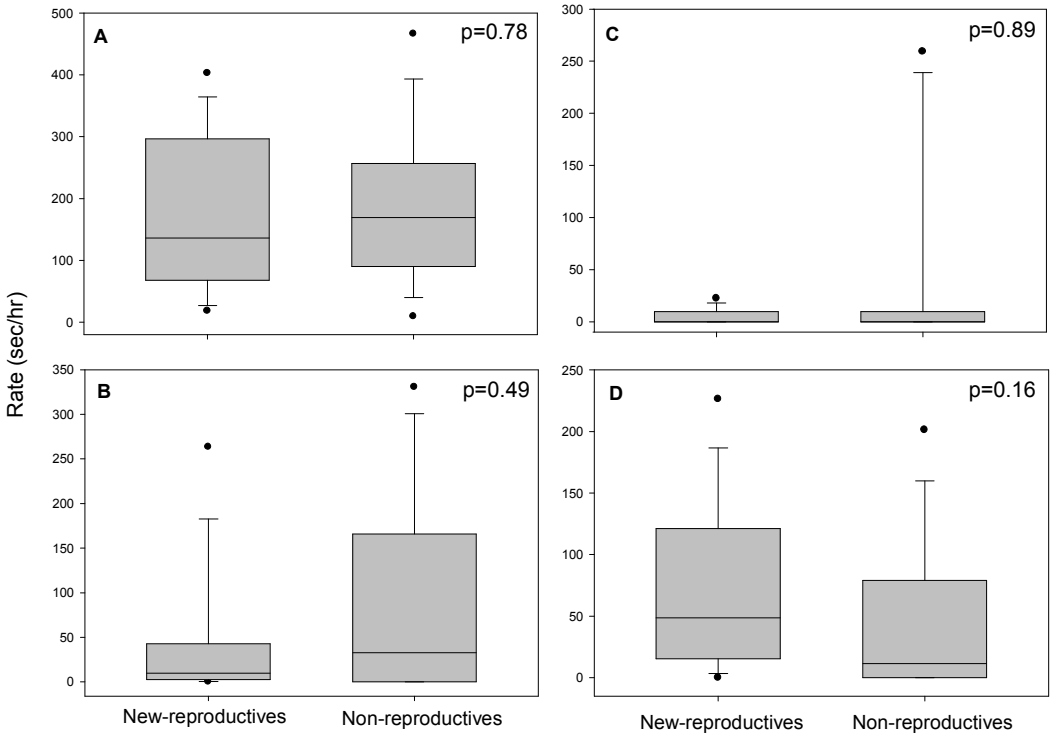
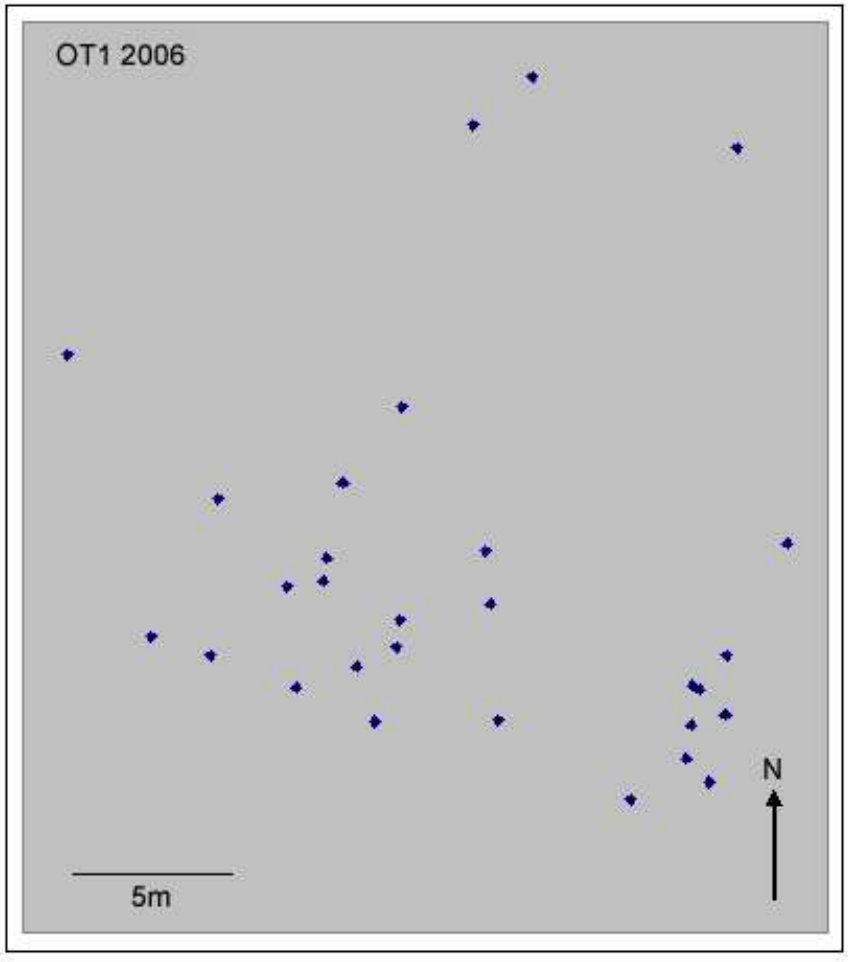


Figure B. Rate of grooming observed in the group 2 MQ colonies (n=14) involving queens who became the new-reproductive (n=14) and the queens who remained non-reproductive (n=18). **A**) W→Q grooming; **B**) Q→W grooming; **C**) Q→Q grooming; **D**) Q→L grooming. The p-values were calculated using a Mann-Whitney U statistical test. Box-plots show the median (line), quartiles (box limits) and 10^{th} and 90^{th} percentiles (error bars).

APPENDIX 3

Related to chapter 4.

Local distribution of L. acervorum *colonies within a patch located in the OT population (the scale measures 5 meters).*



APPENDIX 4

Related to chapter 5.

Table A. Record of all individuals detected as triploid at one or more loci, showing for each individual which loci amplified successfully and which loci had three distinct alleles. Individuals were either workers (W), queens (Q) or female larvae (L), and each row represents a separate individual.

Colony	Cohort	Successfully Genotyped				Triploidy per loci			
(no. ind. geno.)		L18	GA1	GA2	GT1	L18	GA1	GA2	GT1
Spain (OT)									
A03.1810 (3)	L	•	•	•	•			X	
A06.1910 (14)	L	•	•	•				X	
A07.1910 (12)	L		•		•		X		
A08.1810 (8)	W	•	•	•	•				X
	W	•	•	•	•				X
	W	•	•	•	•				X
	W	•	•	•	•				X
	W	•	•	•	•				X
	W	•	•	•	•				X
	W	•	•	•	•				X
	W	•	•	•	•				X
A09.1910 (22)	L	•	•	•	•				X
A10.1710 (12)	L	•	•	•	•	X	X	X	X
A13.1910 (10)	W	•	•	•	•				X
	W	•	•	•	•				X
	W	•	•		•				X
	W	•	•	•	•				X
	W	•	•	•	•				X
	W	•	•	•	•				X
	W	•			•				X
	W	•	•	•	•				X
A14.1910 (17)	L		•	•	•				X

A21.1810 (17)	W	•	•	•	•				X
	W	•	•	•	•				X
B01.1810 (8)	L	•	•	•	•				X
	L	•	•	•	•				X
	L	•	•	•	•				X
	L	•	•	•	•				X
	W	•			•				X
	W	•	•	•	•				X
	W	•	•	•	•				X
	W	•	•	•	•				X
B03.1710 (16)	W	•	•	•	•			X	
	W	•	•	•	•			X	X
	W		•		•			X	
B10.1810 (5)	W	•	•	•	•			X	X
	W	•	•	•	•			X	X
	W	•	•	•	•			X	
	W	•	•	•	•			X	X
	W		•	•	•			X	
B11.1910 (9)	L	•	•	•	•		X		
	L	•	•	•	•		X		
	L	•	•	•	•		X		
B12.1810 (9)	W	•	•	•	•			X	X
B13.1810 (4)	W	•	•	•	•			X	
B13.1910 (21)	L	•	•	•	•				X
B14.1710 (14)	L	•	•	•	•				X
B18.1710 (19)	L	•	•	•	•				X
	L	•	•	•	•				X
	W	•		•	•				X
	W	•	•	•	•				X
OT4.09 (16)	L	•	•	•	•		X		X
	L	•	•	•	•		X		X
	L	•	•	•	•		X		X
	W	•	•	•	•		X		X
	W	•	•	•	•		X		X
	W	•	•	•			X		
	W	•	•	•	•		X		X
	W	•	•	•	•		X		X
	W	•	•	•	•		X		X
	W	•	•	•	•		X		X
	W	•	•	•	•		X		X

Subtotal		**58**	**59**	**57**	**60**	**1**	**16**	**13**	**50**
Spain (V)									
V.07 (10)	L	•	•	•	•	X		X	
V.08 (9)	L	•	•	•	•				X
	L	•	•	•	•		X		X
	L	•	•	•	•		X		X
	L	•	•	•	•		X		X
	Q	•	•	•	•			X	X
V.11 (18)	L	•	•	•	•	X	X	X	X
	L	•	•	•	•	X	X	X	X
	W	•	•	•	•	X	X	X	X
	W	•	•	•	•		X	X	X
	W	•	•	•	•	X	X	X	X
	W	•	•	•	•	X	X	X	X
	W	•	•	•	•	X	X	X	X
	W	•	•	•	•	X	X	X	X
	W	•	•	•	•		X	X	X
	W	•	•	•	•	X	X	X	X
	Q	•	•	•	•	X	X	X	X
	Q	•	•	•	•	X	X	X	X
	Q	•	•	•	•	X	X	X	X
V.12 (11)	L	•	•	•	•			X	
Subtotal		**20**	**20**	**20**	**20**	**12**	**16**	**16**	**18**
Japan (MF)									
MF1.02 (23)	L	•	•	•	•		X		
MF1.07 (12)	W	•	•	•	•			X	
	W	•	•	•	•			X	X
	W	•	•	•	•			X	
	W	•	•	•				X	
	W	•	•	•	•			X	
	W	•	•	•	•			X	X
	W	•	•	•	•			X	
	W	•	•	•	•			X	X
MF1.11 (14)	L	•	•	•	•		X	X	X
	L	•	•	•	•			X	X
	L	•	•	•	•		X	X	X
	L	•	•	•	•			X	X
	W	•	•	•	•			X	X
	W	•		•	•			X	X
	W	•	•	•	•			X	X
	W	•	•	•	•			X	X
	W	•	•	•	•		X	X	X
	W	•	•		•				X

	W	•	•	•	•			X	X
	W	•	•	•	•		X	X	X
MF1.13 (15)	L	•	•	•	•			X	X
	W	•	•	•	•		X		X
MF1.16 (14)	W	•	•	•	•				X
	W	•	•	•	•	X	X		X
MF1.18 (21)	Q	•	•	•	•			X	X
	W	•	•	•	•				X
MF1.19 (19)	Q	•	•	•				X	
MF2.24 (14)	W	•	•	•	•			X	
	W	•	•	•		X		X	
MF2.25 (17)	W	•	•	•	•		X	X	X
	W	•	•	•	•		X	X	X
MF2.36 (19)	W	•	•	•	•			X	X
	W	•	•	•	•			X	X
	W	•	•	•	•			X	X
MF2.37 (13)	L	•	•	•	•		X	X	
	L	•	•		•		X		
	L	•	•		•		X		
	L	•	•		•		X		
	W	•	•	•	•		X	X	
MF2.39 (15)	L	•	•	•	•		X		
	L	•	•	•	•		X		
	W	•	•	•	•		X		
	W	•	•	•	•		X		
	W	•	•	•	•		X		
	W	•	•	•	•		X		
	W	•	•	•	•		X		
	W	•	•	•	•		X		
	W	•	•	•	•		X		
MF2.41 (15)	L	•	•	•	•			X	X
	W	•	•	•	•				X
	W	•	•	•	•			X	X
	W	•	•	•	•			X	X
	W	•	•	•	•			X	X
MF2.43 (11)	Q	•	•	•	•			X	X
	W	•	•	•	•			X	X
	W	•	•	•	•			X	X
	W	•	•	•	•			X	X

	W	•	•	•	•			X	X
	W	•	•	•	•			X	X
	W	•	•	•	•			X	X
	W	•	•	•	•			X	X
	W	•	•	•	•			X	X
MF2.44 (24)	W	•	•	•	•				X
MF2.46 (16)	L	•	•	•	•			X	X
MF2.48 (13)	Q	•	•	•	•			X	X
	Q	•	•	•	•			X	X
	W	•	•	•	•			X	X
	W	•	•	•	•			X	X
	W	•	•	•	•			X	X
	W	•	•	•	•			X	X
	W	•	•	•	•			X	X
	W	•	•	•	•			X	X
	W	•	•	•	•			X	X
	W	•	•	•	•			X	X
	Q	•	•	•	•			X	X
Subtotal		**76**	**75**	**72**	**73**	**2**	**23**	**56**	**53**
Totals:		**154**	**154**	**149**	**153**	**15**	**55**	**85**	**121**