BREEDING STRATEGIES AND THE REPRODUCTIVE ECOLOGY OF *NASUTITERMES CORNIGER*

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Tamara R. Hartke

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Author:	Tamara R. Hartke
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Dissertation Committee

Rebeca Rosengaus, Advisor	Date
Colin Brent	Date
Veronica Godoy	Date
Geoffrey Trussell	Date
Steven Vollmer	Date

Head of Department

Günther Zupanc	Date

Graduate School Notified of Acceptance

Director of Graduate Student Services	Date

Abstract

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ABSTRACT OF DISSERTATION

in partial fulfillment of the requirements for the degree of
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Abstract

The phylogenetically-derived Neotropical termite *Nasutitermes corniger* is known for its facultatively polygamous mating strategy. It has been hypothesized that these associations of multiple unrelated queens and kings come about through pleometrosis, a process of group colony foundation well-studied in the social Hymenoptera, but virtually unexplored in the termites. To better understand the dynamics of colony foundation, and in particular the costs and benefits of pleometrosis compared to monogamy, experimental incipient colonies were established in conjunction with field observations and choice trials under semi-natural conditions. The results presented in this dissertation suggest no clear benefit of pleometrotic colonies over those established by monogamous pairs in founder survival, colony growth, disease resistance, or efficient division of labor. A genetic basis for tolerance of more than a single queen and king is suggested by the data, however. Under certain ecological circumstances, and for individuals from particular parental colonies, group colony foundation may carry a heavy survival cost, compared to the typical monogamous mating strategy. Low rates of

pleometrosis in the field, and potential costs highlighted by laboratory experiments, suggest that mature polygamous colonies of this species come about through fusion of incipient colonies after they have passed through a bottleneck of early mortality and before establishing a permanent arboreal nest.

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Chapter 1

Introduction

The vast majority of individuals in a colony of social insects forgo production of offspring, and instead take care of younger siblings to increase their inclusive fitness (Hamilton 1964*a*, *b*), relying on the breeding strategies of their parents to increase colony, and thus their own, reproductive fitness. This phenomenon has been likened to multicellularity, in that the majority of group members (workers and soldiers, the "soma") do not reproduce, but rather foster the reproduction of others (queens and kings, the "germline") (Boomsma 2009*b*). Extensive research on the social Hymenoptera (all ants, some bees and wasps) has illuminated some of the strategies that eusocial organisms may use to maximize overall reproductive success, including polyandry (one queen mating with multiple males), pleometrosis (cooperative colony founding by more than one queen), and sterile castes with no or limited worker reproduction.

However, while the Isoptera (termites) have converged with the social Hymenoptera in many attributes of their social organization, questions about the different mating strategies of termites have received relatively little attention. In spite of their similar eusocial life styles, the two groups have very different life history attributes (wood-eating vs omnivorous, hemimetabolous vs holometabolous development, diplo-diploid vs haplo-diploid) and phylogenetic origins. Strict lifetime monogamy has been proposed as the crucial condition for the development of eusociality (Boomsma 2007). Nearly all termite species have maintained this ancestral condition of monogamous mating systems, with colonies founded by a single queen and a single king (Nutting 1969).

One of the few described exceptions to this monogamy rule in the termites is the phylogenetically-derived species *Nasutitermes corniger*, in which 20-25% of mature colonies are headed by multiple unrelated reproductives (Fig 1.1, Table 1.1 and references therein). The ontogeny of these colonies is unknown, and it has been suggested by many authors that they arise through pleometrosis, colony foundation by groups of alate reproductives. Boomsma (2007) has recently suggested that this condition may simply represent the co-existence of multiple monogamous pairs within the same nest without remating promiscuity (i.e., reproductive monogamy within a socially polygamous group), although no empirical data exists to support or refute this hypothesis.

Figure 1.1 Multiple queens found in mature colonies of *Nasutitermes corniger* near Gamboa, Panama. Top: two queens and one king in the royal chamber. Bottom: Ten of the eleven queens and one king removed from a single nest. Photos: Casey Hamilton

Table 1.1 Number of mature polygamous colonies of *Nasutitermes corniger* recovered in the field, by founder relatedness and presence of multiple queens or kings. All polyandrous colonies were also polygynous, with the exception of three recovered by Thorne (1984) which contained recently de-alated (young) kings and were apparently undergoing king replacement. Pleometrosis is hypothesized to account for polygamy with unrelated queens and kings, and replacement of deceased founders for related reproductives.

Source	Founder Relatedness		Poly-		Total	%
	unrelated	siblings	gynous	androus	n	polygamous
Gamboa area (2009)*	unknown		4	0	27	15%
Sardinilla (2009)*	unknown		2	0	15	13%
Adams et al. (2007)	27	16	not determined		120	36%
Atkinson and Adams (1997)	6	7	16	13	44	36%
Roisin (1987)†	unknown		14	?	15	93%
Roisin and Pasteels (1986)†	unknown		27	20	28	96%
Thorne (1984)	unknown		25	10	76	34%

* Unpublished data from nest dissections by Hartke, Hamilton, and Seid (2009).
† *N. costalis* and *N. polygynus* have been synonymized with *N. corniger* (Scheffrahn et al. 2005*a*, *b*).
? Number of colonies with multiple kings was not reported in Roisin (1987), only that "polygyny is usually associated with polyandry".

All species in the family Termitidae, to which the genus *Nasutitermes* belongs, have fixed sterile castes. This characteristic of organisms that have passed through the so-called "monogamy window" theoretically allows further elaboration of eusociality, including non-monogamous mating systems (Boomsma 2009*b*). When helpers retain the ability to reproduce, the queens and kings are constrained to producing highly-related offspring, as an incentive for helpers to remain in the nest and forgo their own personal reproduction (Hamilton 1964*a*, *b*). When helpers are permanently sterile, however, the relatedness requirement may be relaxed and reproductives may produce broods of lower relatedness without the consequence of worker reproduction or dispersal.

Pleometrosis may evolve when the benefits of joint colony foundation are equal to or greater than the costs. Ecological pressures during colony foundation may foster pleometrosis if groups are better able to deal with particular insults from the environment. These challenges may include infection by pathogens and parasites, nutrient-deficient food resources, or competition for resources with colonies of the same or different species. While this topic remains under-studied in termites, such a group-founding advantage is manifested in other eusocial organisms through faster colony growth rates (Cole and Wiernasz 1999), or higher survival following pathogen exposure (Baer and Schmid-Hempel 1999; Schmid-Hempel and Crozier 1999; Schmid-Hempel and Schmid-Hempel 1993; Shykoff and Schmid-Hempel 1991). Behavioral polymorphisms may result in specialization efficiencies (Bekkevold

et al. 1999), allowing more effective division of labor among co-founders and better use of limited resources during colony foundation. Pleometrosis is not without costs, however, including competition for limited resources and reproductive rights within the group (Aron et al. 2009).

In this chapter I will describe what is currently known about the life history traits and reproductive strategies of the most phylogenetically derived clade of termites, the Termitidae, and in particular about the facultatively polygamous species *N. corniger*. I will then review non-monogamous mating strategies in eusocial insects, and potential costs and benefits of pleometrosis. The chapter will close with a discussion of the particular aims of my dissertation research, testing specific hypotheses for the evolution of pleometrosis in termites.

1.1 Natural history background

Termite castes and colony composition

Termites are hemimetabolous social insects in the order Isoptera, exhibiting morphologically and behaviorally specialized castes. They are phylogenetically nested within and derived from the roaches (Inward et al. 2007*a*). Heuristically, the termites can be divided into the social, primitive "lower" termites, and the truly eusocial, derived "higher" termites. Most basal termite species do not have true workers, but rather pseudergates or "false workers". These developmentally plastic individuals often reproduce at

low levels within the natal nest (Nutting 1969). They retain the ability to become alate reproductives and disperse from the nest, or may become secondary reproductives and inherit the nest upon the death of a parent (neotenic reproductives) (Korb and Hartfelder 2008; Noirot and Pasteels 1987).

The circumtropical Termitidae comprise around 70% of all extant termite species, and most likely diverged from the more basal lineages around 50 million years ago, in the late Paleocene or early Eocene (Engel et al. 2009). In contrast to the rest of the termites, the highly-derived family Termitidae is characterized by irreversibly sterile worker and soldier castes. Developmental division into a winged reproductive line and the apterous worker and soldier castes occurs very early, before the first molt is completed (Roisin 2000). Dispersal to found new colonies, or replacement of a deceased queen or king, is solely through the alate developmental line. In addition to fixed sterile worker and soldier castes, the Termitidae differ from the lower termites in their lack of flagellated gut protists and the adoption of a "separate piece" nesting habit, in which nests are constructed apart from, rather than within, their food source (Inward et al. 2007*b*).

In both primitive and derived termites, new colonies are generally established by alates following dispersal flights from the parent nest. Alates settle in suitable habitat, where they shed their wings and form tandem pairs to search for a nest site (Fig 1.2). Both the female and the male participate in excavating a chamber in the substrate and raising the first several broods,

after which time the helpers gradually take over nest construction, foraging, and brood care responsibilities (Nutting 1969). New colonies may also be established by budding, however there is evidence that this may not be as frequent or widespread as previously thought (Vargo and Husseneder 2009). In lower termites, primary reproductives are strictly monogamous and do not tolerate additional founders (Shellman-Reeve 1994; Thorne et al. 2003, pers. obs.), although multiple neotenic (helper-derived) reproductives are common even in colonies containing the original queen and king. Polygamy of primary reproductives has been reported throughout the Termitidae, and is discussed in more detail below.

Distribution and life history of *Nasutitermes corniger*

Within the large and highly diverse family Termitidae, the subfamily Nasutitermitidae and the genus *Nasutitermes* are large, diverse, and paraphyletic (Inward et al. 2007*b*; Miura et al. 2000; Scheffrahn et al. 2005*a*). *N. corniger* (Motschulsky) is type species of the genus. Numerous geographic species have been synonymized with *N. corniger*, including *N. morio*, *N. sanchezi*, *N. insularis*, and most recently *N. costalis* (Scheffrahn et al. 2005*a*), and *N. polygynous* (Scheffrahn et al. 2005*b*). Three other *Nasutitermes* species, *N. tatarendae*, *N. arauji*, and *N. globiceps*, are likely also synonyms (Scheffrahn et al. 2005*a*).

Figure 1.2 Typical tandem running courtship behavior following a dispersal flight of *Nasutitermes corniger*. Alates have broken off their wings. In the center, three males are following a female as she searches for a nest site. A tandem pair is running at the top of the photograph.

N. corniger is widely distributed throughout the New World tropics, from southern Mexico to southern Brazil and northern Argentina, including the West Indies (Scheffrahn et al. 2005*a*). It is very common and considered a pest species in much of its range (Nickle and Collins 1992). The study site is near the center of the species range, along the Panama Canal in and around the Parque Nacional Soberanía, Gamboa, and Galeta, Panamá. The experiments described here were done in cooperation with the Smithsonian Tropical Research Institute (STRI) in and near the sites of previous work on this species by Thorne, and Atkinson and Adams.

N. corniger constructs ellipsoidal dark brown to black carton-like arboreal nests from chewed wood and feces (Fig 1.3). Colonies are frequently polycalic, with a single colony constructing up to 37 separate nests connected by covered trails (Levings and Adams 1984). A mature colony may contain one million workers and soldiers (Thorne and Noirot 1982). This species feeds on dead wood, including fence posts and structural timber. It is important in nutrient cycling in tropical forests and savanna both for the breakdown of dead plant material and for its contribution of nitrogen, fixed by symbiotic gut bacteria (Prestwich et al. 1980), to nutrient-poor tropical soils (Nickle and Collins 1992). These termites are also vital food sources for other organisms (Jaffe et al. 1995; Lubin et al. 1977).

N. corniger workers are small (up to 3mm long) and soft-bodied, with well-sclerotized head capsules and mandibles. Workers perform foraging, nest construction and maintenance, and brood care duties of the colony

Figure 1.3 Arboreal carton nest of *Nasutitermes corniger* near Gamboa, Panama. Photo: Casey Hamilton

(Nutting 1969). They are able attackers in conflicts intra- and inter-specific contests, using their mandibles to disable or disembowel opponents (Levings and Adams 1984; Thorne 1982*c*). Laboratory observation suggests that most queen care is performed by older larger workers (pers. obs.).

Soldiers are smaller and slighter than workers in this species. They have reduced, vestigial mandibles, and must be fed by the workers. Soldiers' heads form a funnel-like nasus, source of the subfamily and genus names, through which highly sticky mono- and di-terpenoid defensive secretions are directed at enemies. These secretions combine entanglement with toxicity to ant predators, as well as serving as ant repellents (Mill 1983). Defensive compound composition is distinct between even closely-related species and is linked with lines of descent rather than climatic similarities (Gush et al. 1985; Howard et al. 1988). Up to 20% of the neuter population of a colony may be soldiers (Thorne 1985*a*). This relatively large proportion of soldiers may be an acceptable energetic investment for the colony due to their roles in scouting and organization of foraging (Traniello 1981), the anti-fungal activity of their defensive secretions (Fuller 2007; Rosengaus et al. 2000*a*), and their significant nitrogen fixation abilities, up to four times as much as a worker (Bentley 1984; Prestwich et al. 1980).

Alates mature at the end of the dry season. Only mature colonies with a neuter population greater than 50,000 will produce alates (Thorne 1983). Time from colony foundation to production of the first alate brood is not directly known, however polygamous colonies are thought to produce alates

sooner than monogamous colonies (Thorne 1983). Dispersal flights from the parent nest occur at the beginning of the rainy season, April–May in the study site.

Incipient colony development in the Termitidae

While colony foundation has been studied extensively in a number of the more basal termites lineages (particularly *Zootermopsis* and the economically important *Reticulitermes* and *Coptotermes*), understanding of colony foundation in the derived termites (Termitidae) is incomplete. The general sequence of events is known: dispersal of alates from the parent nest, tandem running and nest site selection, production of first brood, expansion of the colony and construction of the mature nest (Nutting 1969). However very little is known about the events between disappearance of the de-alated pair into their selected nest site and the sudden appearance of the maturing, arboreal, carton nest. Unsettled phylogenies and diverse habits within this group make it difficult to generalize from the few natural history studies (discussed below) focused on this part of the life cycle.

While *N. corniger* is arguably one of the best-studied of the Termitidae, the details of colony foundation and development have not previously been described for this species. However, the dynamics of colony development in *Nasutitermes ephratae*, as reported by Becker (1961), may extend in large part to *N. corniger*. The two species are closely related, perhaps sister species (Miura et al. 2000; Scheffrahn et al. 2005*a*). They have similar

ecology (Thorne 1980, and pers. obs.), and nests of the two species may be found within several meters of each other in the study site. Polygamous mature colonies of *N. ephratae* have also been reported (Becker 1961).

In laboratory observations, pairs of *N. ephratae* alates quickly constructed elliptical nest hollows (Becker 1961). They did not burrow directly into the unsuitable pine wood provided, but rather created hollows beneath and just into the wood. Tandem-running groups were observed, but only pairs were placed in experimental colonies and pleometrotic colony foundation was not investigated.

The first eggs were laid within 3–4 days, and Becker suggested that such a short delay between flight and egg-laying was characteristic of the genus *Nasutitermes* and its closest relatives. *Anoplotermes*, another member of the Termitidae, required 8 days before first oviposition, and the more basal Kalotermitids and Rhinotermitids even longer (references in Becker 1961). In *N. ephratae*, the first egg-laying bout lasted 3–6 weeks (longer at lower temperatures) and yielded 20–30 eggs, after which no eggs were laid while the parents cared for the first brood. The first worker completed development before the first soldier, and both were smaller than their counterparts from mature colonies. Development time was found to be temperature-dependent as well, and significantly slower at temperatures below 28°C. A new round of egg-laying began 6–10 weeks post-establishment, when the last larvae from the first batch of eggs were in their second instar and the first worker(s) were taking on brood and nest care.

The only other member of the Termitidae in which colony foundation has been studied in detail are the specialized African grass-harvesting mound-building termites *Trinervitermes*. Polygamous colonies of these species have never been found (J. Mitchell, pers. comm.). Some aspects of incipient colony development may, however, apply to *N. corniger* in spite of their quite different ecology and life history.

Egg laying was found to begin at 2–8 days post-pairing in 5 species of *Trinervitermes*, with 30–60 eggs produced during the first 12 days (Sands 1965). Incubation times differed between individual eggs in a colony and between colonies of a species, again depending on ambient temperature. Sands estimated mortality of eggs and larvae at 30% over the course of development, with most mortality occurring in the egg stage. The first workers and soldiers completed development at approximately the same time; caste proportions varied between species. Tunneling and foraging began three weeks after the first workers appeared, after a period of brood care behaviors. The sex ratio of both alates and sterile helpers in mature colonies of *Trinervitermes* was female-biased (36%♂:64%♀).

Recently, the first 25 weeks of incipient colony development were examined in laboratory-established pairs of *Trinervitermes trinervoides* (Adam and Mitchell 2009). Colony mortality was very low initially ($\approx 5\%$ over 16 weeks), then increased sharply; total mortality over 25 weeks was slightly less than 40%. Nest chambers were formed below the soil surface by the de-alated pairs, and eggs were produced within the first week after pairing,

continuing for four weeks, to a total of up to 60 eggs. Incubation time for the eggs to hatch into larvae was 6 weeks. Three to four weeks later, depending on caste, first instar larvae molted, and at 14 weeks post-pairing the first functional workers and soldiers appeared simultaneously. The first worker(s) enlarged the chamber and constructed a tunnel to the surface, as well as performing brood care. Foraging began at 20 weeks post-pairing, when those workers eclosed into the next instar of major workers, and apparently stimulated the second round of egg production at 22 weeks post-pairing. First workers and soldiers of the incipient colonies were smaller than their counterparts from mature nests.

These studies suggest that in *N. corniger*, eggs will be laid shortly after excavation of an initial chamber, followed by a period of intense parental care. Observations from these other species suggest that *N. corniger* will produce discreet broods, with no additional eggs laid until the first brood has completed development. Mortality under the most natural conditions tested was low until 16 weeks post-establishment, just before the initiation of foraging in *T. trivervoides*. Due to differences in ecology, specifically the need for *T. trivervoides* to collect grass as a substrate for fungus cultivation, that may not hold true for *N. corniger*. Based on results from *N. ephratae*, additional nutrition beyond the bodily reserves of the founders is required within eight weeks of colony foundation, so mortality may peak early for *N. corniger*. None of the previous detailed studies on the dynamics of colony foundation in the Termitidae examined pleometrosis, only colony founda-

tion by monogamous pairs. The research presented in this dissertation thus represents a valuable contribution to the literature in its examination of pleometrosis during the critical period of colony establishment.

1.2 Non-monogamy in Social Insects

Polygamy, including polygyny and polyandry, is considered to be a derived characteristic in eusocial organisms, with monogamy hypothesized as the primitive state (Boomsma 2009*b*). Monandry (females mating with only one male) has indeed been found to be the primitive condition in the social Hymenoptera (Hughes et al. 2008*a*). No such analysis has been done for the termites, however it has been suggested that the ancestor to the Termitidae went through this "monogamy window of opportunity" to become strictly eusocial, with castes irrevocably determined early in development and members of the worker cohort entirely losing the option of dispersing to establish their own colony (Boomsma 2009*b*). The more basal lineages of termites did not pass through this evolutionary bottleneck, and thus their "workers" maintain the ability to reproduce within the colony as well as molt into a dispersing form. This is consistent with the elaboration of polygamy within the Termitidae and the scarcity of it in the other termite families, as outlined below.

Pleometrosis in the Termitidae

The incidence of polygamy in the Termitidae was previously reviewed by Thorne (1985*b*), who found 38 documentated cases of alate-derived polygamy in 15 genera in all 4 families of the Termitidae [Fig.1.4, on the phylogeny of Inward et al. (2007*b*)]. Additional cases have been reported since that time (Roisin 1987; Thorne and Levings 1989, and Roisin, pers. comm), and it is likely that more remain to be discovered. Given the paucity of data on the reproductive habits of derived termites, this suggests that polygamy may be reasonably common in this group.

Hypotheses for the origin of polygamous groups include pleometrosis, colony fusion, adoption of unrelated alates, budding from the parental colony, and inheritance of the original nest by offspring of the founding queen and king (Roisin 1987; Thorne 1982*a*, 1984, 1985*b*). Pleometrosis has been considered the most plausible mechanism for the origin of unrelated polygamous groups in termites (Roisin 1993; Thorne 1985*b*), although it has remained largely speculative, with few empirical observations.

Relatedness of polygamous groups has now been explored using microsatellite markers in two phylogenetically distant species of Termitidae, to differentiate between situations in which the reproductives were related (secondary polygamy, replacement of deceased parents) or not. Secondary polygamy accounts for a one-third to one-half of non-monogamous mature *N. corniger* colonies (Table 1.1). In these cases, large numbers of reproductives are often located in the same nest, further indicating replacement

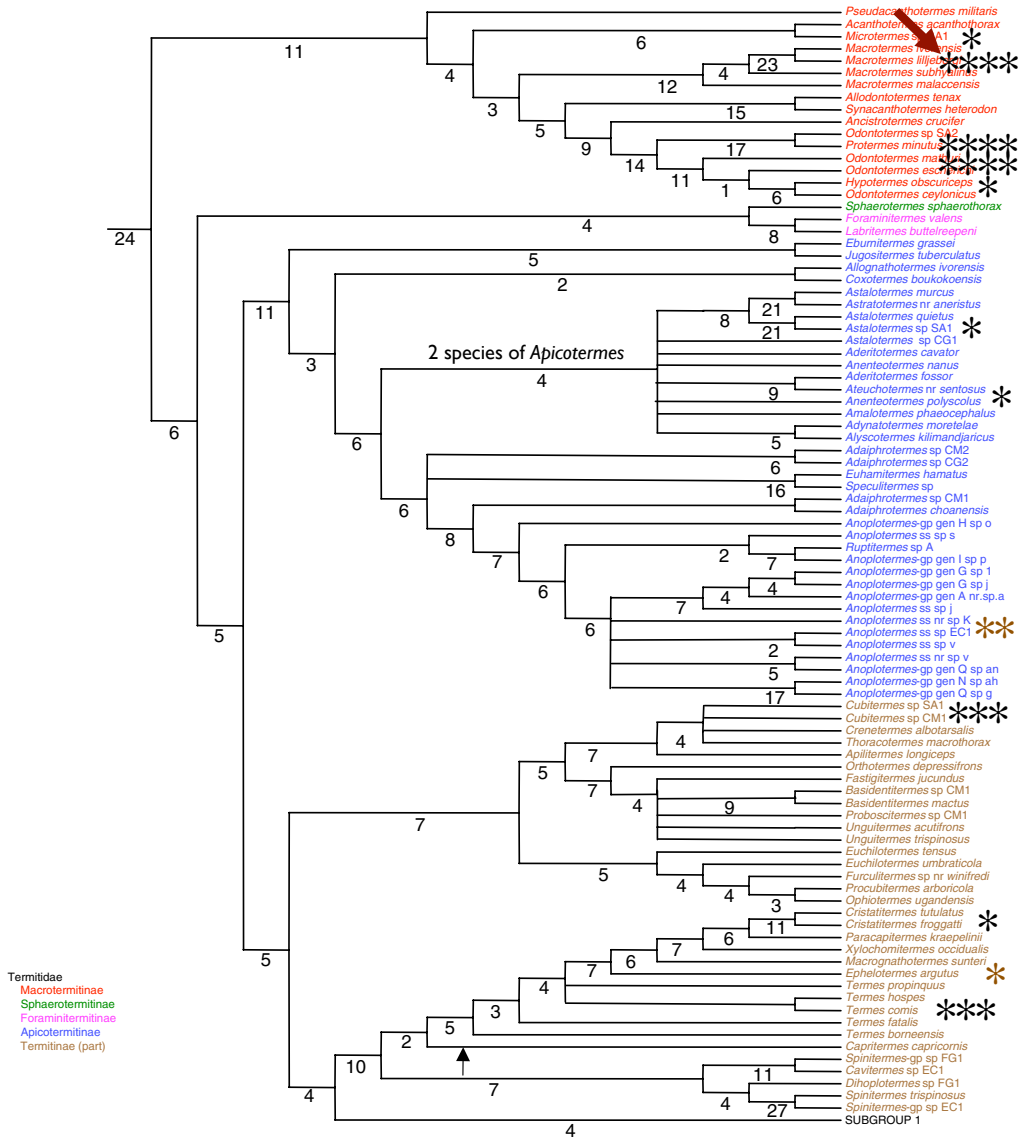


Figure 1.4 Reports of polygamy in mature colonies, on the phylogeny of (Inward et al. 2007*b*). Part A: Termitidae base group. Species not on the phylogeny are indicated on the branches or next to their closest relatives. Black stars = Thorne (1985*b*); brown stars = Roisin (1987) and Roisin (pers. comm., 2009). Arrow indicates *Macrotermes michaelseni*, in which polygamous reproductives are unrelated (Hacker et al. 2005).

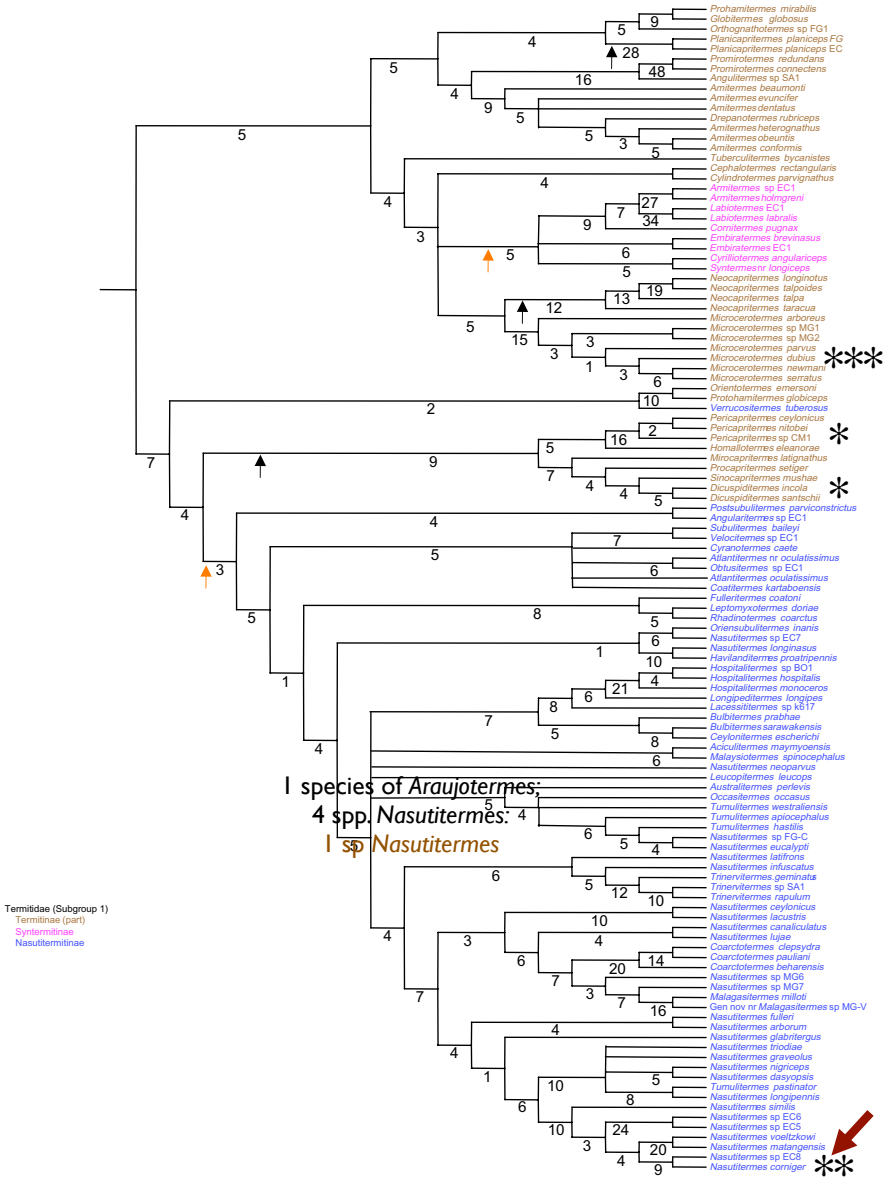


Figure 1.4, continued from previous page. Reports of polygamy in mature colonies, on the phylogeny of (Inward et al. 2007*b*). Part B: Termitidae crown group. Arrow indicates *Nasutitermes corniger*, in which polygamous reproductives are unrelated (Atkinson and Adams 1997).

of deceased founders (Atkinson and Adams 1997). This phenomenon is distinct from pleometrosis in that new reproductives derive from unflown sibling alate offspring of the founding pair, rather than being unrelated co-founders. No polygamous colonies with related queens and kings were reported by Hacker et al. (2005), suggesting that secondary polygamy does not occur in *Macrotermes michaelseni*.

Within mature *N. corniger*, unrelated reproductives were found in smaller numbers and in cases where reproductives were located in multiple nests, suggesting pleometrosis or fusion of independently established colonies (Atkinson and Adams 1997). No evidence was found of an association between polygamy and haplotype, or between polygamy and habitat. While Thorne (1985*b*) and Atkinson and Adams (1997) indicate that polygamous *N. corniger* queens are all the same size and therefore likely to be the same age, Roisin and Pasteels (1986) encountered polygamous queens of different sizes and pigmentation, suggesting they may not always be the same age. Similarly, Becker (1961) reported one nest of the closely-related *N. ephratae* containing 6 queens of different sizes (24–32mm), suggestive of multiple origins, together with "several" kings.

Multiple unrelated queens are also found in nests of the fungus-growing termite *Macrotermes michaelseni* (Hacker et al. 2005). All queens contributed to the production of sterile castes, although there was reproductive skew observed in half of the colonies studied that could not be accounted for by queen mass. Queen mass is correlated with egg production in ter-

mites (Adams and Atkinson 2008; Kaib et al. 2001), with heavier queens laying more eggs per unit time than lighter queens. Greater numbers of eggs were laid by groups even though per capita egg production was lower (Kaib et al. 2001). Polygamy rates increase near the border of the species' range (Brandl et al. 2001) and vary negatively with rainfall two years prior to the sampling (Brandl et al. 2004), suggesting that pleometrotic colony foundation is favored in this species when environmental conditions are more difficult.

Pleometrosis has been directly observed in *M. michaelseni* from excavation of incipient colonies following dispersal flights (Darlington 1984). The proportion of polygamous colonies in the population decreases over time, from 50% at founding to 23% of mature colonies. A sister species, *Macrotermes herus*, seems to manifest a number of the predicted advantages of pleometrosis, including more rapid colony growth in colonies with larger numbers of queens (Darlington 1988), which may allow colonies to move beyond a vulnerable incipient stage more quickly. Further, scarcity of nesting sites was found to foster group colony foundation, with supernumerary kings but not queens eliminated over time. Similar dynamics have been postulated for *N. corniger* (Thorne 1984), but have not been investigated until now.

Pleometrosis in other social insects

As mentioned previously, colony foundation is strictly monogamous in the more basal termites and monogamy is favored by the relatedness constraints imposed by reproductive plasticity of helpers. Secondary polygamy, resulting from inheritance of the parental colony by offspring of the founding reproductives or supplementation of parental reproduction by neotenic reproductives, is very common in the primitive termite families (Roisin 1993), but is physiologically and mechanistically distinct from primary polygamy. Generally, multiple primary reproductives (alates, future queens and kings) are not tolerated during colony foundation in the basal termite families (Nutting 1969, and pers. obs.), and queens and kings in excess of a monogamous pair are rapidly "assassinated" when incipient colonies meet (Thorne et al. 2003). Only one case of pleometrosis outside of the Termitidae is found in the literature, for *Reticulitermes kanmonenis* (Kitade et al. 2004). Out of 57 incipient colonies found in the field, one consisted of two kings and one queen while another contained two queens and one king. Incipient colonies of *Reticulitermes flavipes* in the lab can be formed with multiple alates and do persist with multiple primary reproductives (Grube and Forschler 2004, and pers. obs.), however there are no reports in the literature of this occurring in nature (Vargo and Husseneder 2009).

Because Hymenoptera males do not participate in colony foundation except as stored sperm, pleometrotic colony foundation in ants, bees, and wasps may be achieved either through colony foundation by more than

one queen (co-foundresses), or as a result of multiple mating by individual queens who go on to establish a colony either alone or in groups. Direct benefits of colony foundation by multiple hymenopteran queens has been shown in interspecific contests (Izzo et al. 2009) and in division of labor during colony foundation (Jeanson and Fewell 2008). Competition may be manifested in pleometrotic hymenopteran species that undergo queen reduction (Aron et al. 2009) or develop dominance heirarchies (Fanelli et al. 2008), although egalitarian foundress associations may persist throughout the lifetime of the colony in other species (Johnson 2004). In some ants, mature multiple-queen colonies are larger, but may produce fewer alates in proportion to the number of workers than do mature single-queen colonies (Rosset and Chapuisat 2007), demonstrating a fitness cost to polygny.

The vast majority of social Hymenoptera mate only once, and those that do mate multiply often have internal mechanisms to minimize sperm mixing and the production of multiple-paternity broods (Strassmann 2001). Thus, high relatedness is generally maintained within the colony, minimizing conflicts of interest between the queen and the workers. Mating frequency and founding group size are inversely correlated in army ants, such that group-founding queens tend to be singly-mated (Kronauer and Boomsma 2007), again constraining relatedness of the workers. Similar results have been found in a meta-analysis of the social Hymenoptera (Hughes et al. 2008*b*), suggesting that genetic diversity per se is not a factor driving non-monogamy in that clade. Interestingly, initial studies on colony foundation

in social thrips indicate similar costs and benefits to pleometrosis as those found for the social Hymenoptera (Bono and Crespi 2008).

Costs and benefits of group colony foundation

The decision to pursue a non-monogamous mating strategy clearly requires balancing the costs and benefits associated with it. Previous reviews on the causes, costs, and benefits of polygyny (Keller 1995) and polyandry (Hughes et al. 2008*b*) have focused on the social Hymenoptera. With so little investigation of non-monogamous mating strategies in termites, no such comparative analysis is possible. The theoretical framework does generally apply, however, and a summary of the main hypotheses and predictions relevant to pleometrosis in termites is presented in Table 1.2. Below I discuss specific evidence for each of these hypotheses.

Group living in general confers a survival benefit in many gregarious, social and cooperative organisms, including social spiders (Bilde et al. 2007), salamanders (Banning et al. 2008), birds (Jullien and Clobert 2000), and moth larvae (Wilson et al. 2001). The benefits of group living may be even greater for eusocial organisms (Hughes et al. 2002; Walker and Hughes 2009; Wilson-Rich et al. 2009), which live in even more highly integrated societies. For example, in the primitive termite *Z. angusticollis*, group living increases survival of workers following exposure to a fungal pathogen (Rosengaus et al. 1998*b*; Traniello et al. 2002) and a parasitic nematode (Wilson-Rich et al. 2007).

Table 1.2 Hypotheses on the evolution of non-monogamy in termites.

Hypothesis	Predictions
Polygamy reduces the risk of colony extinction due to founder death.	Individuals in larger groups survive longer. Colonies founded by more individuals persist longer than those founded by pairs.
Dispersal is risky. Predation or competition for nest sites severely limit chances for success.	Polygamous colonies should be more common in some areas. Fewer nest sites or higher alate densities result in larger founding groups.
Benefits of increased genetic diversity: behavioral polymorphisms lead to greater colony efficiency.	More effective division of labor in polygamous colonies.
Benefits of increased genetic diversity: greater resistance to parasites and pathogens.	Higher survival in polygamous colonies when exposed to disease. Greater variability in immune response of workers and soldiers in polygamous colonies compared to monogamous colonies.
Intraspecific parasitism.	Some reproductives should be "lazy" during initial colony foundation, then take over the colony after the first helpers have matured.
Increased reproductive output and colony growth.	Groups have more offspring/sooner than pairs. Polygamous colonies mature (produce alates) faster than monogamous colonies.
Phylogenetic constraints.	Unrelated primary kings and queens should only be found in mature colonies of certain clades.

Although some authors conclude that group colony foundation is ultimately a matter of "making the best of a bad job" (Aron et al. 2009), group living during colony foundation has generally been found to increase survival in the social Hymenoptera. In ants, foundress groups have higher survival than solitary founding queens (Bartz and Hölldobler 1982) and produce larger broods more quickly (Adams and Tschinkel 1995). In the only investigations to date in termites, survival of groups was not higher than that of monogamous pairs (Darlington 1984).

The benefit of more rapid colony growth in polygamous colonies may be offset by lower individual reproductive fitness. Studies of mature polygamous *N. corniger* colonies found higher total egg production by multiple queens than single queens (Adams and Atkinson 2008), and little reproductive skew between co-occurring queens. Egg production was also found to be higher in groups than in pairs for the facultatively polygamous *M. michaelseni* (Kaib et al. 2001), although per queen egg production was lower. More rapid colony growth was inferred in a study of young *M. herus* colonies (Darlington 1988), however no previous studies have directly compared offspring production between pleometrotic and monogamous colonies during incipient colony development.

Severe ecological conditions have been suggested as factors fostering pleometrosis in other termite species (Brandl et al. 2001, 2004). Colony establishment under adverse conditions, including inter- and intra-specific competition for nest sites, high risk of desiccation, or predation hazards,

may be so difficult that a monogamous pair cannot successfully found a colony alone. Specific hypotheses regarding the impact of ecological factors have not been experimentally tested, even for the relatively easy to manipulate factors such as nest site availability and intraspecific competition or mate availability.

Non-monogamous colonies may benefit from increased levels of overall genetic diversity in their offspring (Hughes and Boomsma 2004; Reber et al. 2008). Genetic variability of workers (due to multiple matrilines and patrilines) may increase either a) colony resilience against disease and parasitism due to variation in innate immune response or production of antimicrobial compounds (Wilson-Rich et al. 2009), or b) the variability of the worker castes behavioral repertoire (Smith et al. 2008), ultimately enhancing energetic efficiency through division of labor, colony growth rates and fitness. Results to date have been mixed. In a re-analysis of previous studies while controlling for phylogeny, Brown and Schmid-Hempel (2003) found no support for the efficiency hypothesis although the pathogen resistance hypothesis was supported by one study as well as simulation models. The benefits of within-colony genetic variability have been reported for certain social Hymenoptera species (Oldroyd and Fewell 2007; Seeley and Tarpy 2006), but have been understudied in termites (Calleri et al. (2006*a*); Issa and Rosengaus, unpublished data).

Costs and benefits of pleometrotic colony foundation (Table 1.3) should be particularly acute during the earliest stages of incipient colony develop-

ment, as this is the most hazardous period of the termite colony life cycle, and most energetically costly to the founding reproductives. Cheaters, who contribute less than their co-founders do to nest construction, egg production, or brood care, may bide their time and take over the reproductive role following the emergence of the first workers. Pleometrotic colonies may have an advantage over monogamous pairs in division of labor and more efficient use of bodily reserves during colony establishment. Both the additional capacity for parental care and larger pool of bodily reserves could benefit pleometrotic colonies, however, these resources may be squandered if founders are competing with one another rather than cooperating. Such competition may range from subtle agonism to death or injury incurred during aggressive encounters. Similarly, the increased potential for exposure to pathogenic micro-organisms brought by a co-founder or rapid spread of infections through the group may increase the risk of death or require diversion of resources from colony establishment activities and reproduction to mounting an immune response.

1.3 Central Aims of this Study

My research expands the current knowledge of termite colony development, and is the first study to systematically examine particular hypotheses regarding pleometrotic colony foundation in termites. This study provides comparative data on monogamous and polygamous colony foundation, the

Table 1.3 Costs and benefits of pleometrotic colony foundation

Costs	Benefits
Shared paternity/maternity of brood.	Larger initial broods than could be produced by a single individual, resulting in more rapid production of a large workforce.
Competition for reproductive hegemony.	Additional parental care capacity.
Greater exposure to pathogens carried by additional founders.	More partners for allogrooming.
"Lazy" individuals that do not contribute costly eggs or nest construction and brood care behaviors.	Greater pool of available resources in the form of individual fat stores.
Death or injury during aggressive encounters between co-founders.	Behavioral polymorphism or specialization in nest construction and brood care.
Energy wasted in agonistic or antagonistic behaviors.	Economies of scale, e.g. one nest for four individuals is less costly to construct than two separate nests.
Rapid spread of disease throughout a group	Insurance against death of a mate.

lack of which has been lamented for decades (Roisin 1987). First I tested whether pleometrotic colonies do survive better or grow more quickly than colonies founded by monogamous pairs (Chapter 2), and could therefore reach a critical mass vital for colony survival sooner and more reliably than those founded by pairs. Although this hypothesis was postulated by Thorne (1984), no empirical studies have tackled this issue. These demographic studies in the laboratory were supplemented by observations of naturally-established incipient colonies in the field.

Behavioral observations of reproductives during nest construction and brood care were undertaken to test both the intraspecific parasitism hypothesis and the suggestion that behavioral polymorphism among co-founders may result in higher success rates (Chapter 3). To explore any genetic propensity to form groups rather than monogamous pairs, alates were allowed to choose their own mates and founding group size in microcosm and mesocosm choice trials (Chapter 4).

If the genetic diversity and social facilitation of immunity hypotheses were applicable to this system, colonies founded by multiple primary queens and kings should be better able to combat disease than colonies founded by monogamous pairs. To explore this possibility, alates were exposed to an entomopathogenic fungus or nematode immediately prior to colony establishment with either nestmates or non-nestmates (Chapter 5). To examine the effect of number and relatedness of parents on immune potential of offspring, workers from mature field colonies and from experimental labora-

tory colonies were tested for differences in survival, fat content, and fungal inhibitory properties (Chapter 6).

Throughout this study, I have made extensive observations of the natural history of colony foundation in this species under natural, semi-natural, and laboratory conditions. Previously, little was known about the invisible incipient colony stage, between pairing of the founders and appearance of a conspicuous, densely-populated arboreal carton nest. Stemming from these explorations into the natural history and ecology of the *Nasutitermes*, was an experiment on the potential for hybridization between *N. corniger* and its sympatric sister species *N. ephratae* (Chapter 7).

A final summary and overall conclusions of this work are presented in Chapter 8. The research presented in this dissertation is the first detailed examination of colony foundation and development in a derived facultatively-polygamous termite. In addition to previously-unknown aspects of its natural history, the comparative observations of monogamous and polygamous incipient colonies reported here provide the first comprehensive tests in termites of hypotheses regarding the advantages and disadvantages of group colony foundation. This fills glaring holes in the extant literature and theory, and will aid in the synthesis of a unified understanding of the evolution of advanced eusociality and of non-monogamous mating systems in eusocial organisms.

Chapter 2

Survival and Reproductive Output of Incipient Colonies

Abstract

Most termite colonies are founded by a monogamous pair (king and queen) following the dispersal flight, but a few species, such as the phylogenetically-derived *Nasutitermes corniger*, have variable breeding strategies whereby colonies may be headed by multiple unrelated queens and/or kings. While costs and benefits of group living have been documented for a variety of organisms, little is known about the incipient stage of colony development, or the costs and benefits of polygamous versus monogamous founding, in termites. In the present study, laboratory and field studies were undertaken to understand the effects of founding group size and relatedness on

the early stages of colony development. Five thousand experimental incipient *N. corniger* colonies were established in the laboratory and censused daily for 90 days to record demographic information: deaths of founders, first appearances of eggs, larvae, workers and soldiers, and numbers of each offspring class at 15, 30, 60 and 90 days post-establishment. Demographics of naturally-established incipient colonies were found to be similar to those of laboratory-established colonies, indicating that these laboratory results accurately reflect colony growth dynamics of natural colonies. Survival of incipient colonies founded by nestmates was higher than survival of those headed by non-nestmates, however non-nestmates produced more offspring earlier. Increased founding group size was detrimental to both individual and colony survival, as well as offspring production. Groups larger than trios are not beneficial at this stage of colony development. Given the lower success of larger founding groups in the laboratory and the fact that incipient colonies found in the field are either queen-king pairs or male-biased trios it seems unlikely that true pleometrosis is a common method for the formation of polygamous mature colonies. The presence of female-biased royals in mature nests, and evidence of low aggression and experimental fusion of mature nests, further suggests that incipient colonies merge at an intermediate stage of development.

2.1 Introduction

Reproductive strategies are influenced by many factors, including predation, disease, nutrition, and competition (Choe and Crespi 1997). Social insects experience these challenges at both the individual and the colony, or super-organism, levels (Wilson 1971). Extensive research on the social Hymenoptera (bees, ants, wasps) has focused on the strategies that eusocial organisms may use to maximize overall reproductive success. The vast majority of the social Hymenoptera practice strict monogamy (Strassmann 2001), however the alternative strategies of polyandry (one queen mating with multiple males) and pleometrosis (cooperative colony founding by more than one queen) are successful in the clades in which they occur

While the Isoptera have converged with the social Hymenoptera in many attributes of their social organization, questions about the mating strategies of termites have received relatively little attention. Nearly all termites have monogamous mating systems, with colonies founded by a single queen and a single king (Nutting 1969). One of the few studied exceptions is the phylogenetically-derived species *Nasutitermes corniger*, in which 20–25% of mature colonies are headed by multiple unrelated primary reproductives (Table 1.1 and references therein).

To date, little is known about the ontogeny and development of these polygamous termite colonies. Although pleometrosis has been the favored explanation for the origin of multiple unrelated queens and kings, it has

never been documented in *N. corniger*. Pleometrosis has been directly observed in another derived termite lineage, however (Darlington 1984, 1988). It is hypothesized in both of these cases that ecological pressures may favor colony foundation in groups because of an improved ability to deal with particular insults from the environment, including infection by pathogens and parasites, predation, nutrient-deficient food resources, and/or competition for unevenly distributed resources with colonies of the same or different species (Brandl et al. 2001; Hacker et al. 2005; Thorne 1984).

Group living itself has been found to confer a survival benefit in many gregarious, social and cooperative organisms [e.g., social spiders, Bilde et al. (2007); salamanders, Banning et al. (2008)]. Indeed, some research suggests that the benefits of group living may be even greater for eusocial organisms (Hughes et al. 2002; Walker and Hughes 2009; Wilson-Rich et al. 2009). For example, in the primitive termite *Zootermopsis angusticollis*, group living increases worker survival following exposure to a fungal pathogen (Rosengaus et al. 2000*b*; Traniello et al. 2002) and a parasitic nematode (Wilson-Rich et al. 2007). Relatedness of founders also affects incipient colony survival in termites, with nestmate pairs surviving better than non-nestmates pairs in *Z. angusticollis* (Calleri et al. 2005; Rosengaus and Traniello 1993) and the Rhinotermitid *Coptotermes formosanus* (Fei and Henderson 2003).

Although untested in termites, survival benefits of pleometrosis have also been found in ants. Mortality of foundress queens is lower in pleometrotic groups than for solitary queens or pairs of queens in many ant species

(Bernasconi and Strassmann 1999), although there do seem to be limits to the number of co-founders (Deslippe and Savolainen 1995). These associations may persist through the lifetime of the colony without conflict (Trunzer et al. 1998), or competitive interactions between founders can reduce the mutualistic benefit of group colony foundation leading to elimination of all but one queen (Aron et al. 2009). While there is an extensive literature on the costs and benefits derived from pleometrosis in the social hymenoptera, no such analysis is possible for the termites.

The experiments reported here directly test the effects of group size and relatedness on survival and colony growth in the neotropical termite *Nasutitermes corniger*. While reproductive effort of individual queens within mature polygynous and monogynous *N. corniger* colonies has been investigated (Adams and Atkinson 2008), this study is the first concerted investigation into the initial stages of colony development. By manipulating founding group size and relatedness in Petri dish microcosms this study has recorded the critical events early in the life history of a termite colony.

2.2 Methods

Experimental colony establishment and monitoring

Field-collected *N. corniger* alates (winged reproductives) were collected from mature (parent) nests in and around Gamboa and Galeta, Panamá, just prior to dispersal flights in April–June 2006, 2007, 2008, and 2009.

They were sorted by sex, then maintained in moist filter paper-lined Petri dishes until placement in experimental incipient colonies.

Experimental colonies were formed by placing alates in moist filter paper-lined 30mm petri dishes with a fragment of dead mango wood. From each parent nest, 20 replicate incipient colonies were established for each combination of relatedness of founding individuals (nestmates, non-nestmates), and founding group size (pairs, trios, and quintets, Table 2.1). Although the genetics and underlying mating strategy of the parent nests was unknown, nestmates were assumed to be more closely related (inbreeding treatment) than non-nestmates (outbreeding treatment). Sex ratios of founding groups were: pairs (1♂:1♀), trios (2♂:1♀or 2♀:1♂), quintets (3♂:2♀or 3♀:2♂). Female-biased groups were initially chosen to reflect the queen:king ratio of dissected mature nests (Thorne 1984, 1985*a*), however the majority of colonies in 2008 and all in 2009 were established with a male-biased sex ratio, reflecting the natural sex ratio of alates in the field at the time of dispersal and of newly-established incipient colonies in the field.

Experimentally-established incipient colonies were stacked in boxes in an environmental chamber set to 28° C and 80% humidity (2006 and 2007) or in ambient conditions in Panamá (2008 and 2009). All colonies were observed daily for the first 90 days post-establishment to record deaths of founders and significant events related to reproductive success and colony growth, including the first appearances of eggs, larvae, workers, and soldiers. In 2006 and 2007, observations extended to 220 days post-establishment.

Table 2.1 Total number of individuals used (top) and number of colonies established (bottom) in the experiment, by group size and relatedness, with percent survival to the census points. NM: nestmates, NNM: non-nestmates.

Individuals

			Percent survival			
		Total n	d15	d30	d60	d90
Pairs	NM	1408	54.3	43.8	35.1	10.7
	NNM	2160	43.7	32.4	22.6	9.0
Trios	NM	1743	69.8	57.7	39.0	10.8
	NNM	3102	47.4	34.5	18.6	5.9
Quintets	NM	2865	62.0	48.1	23.4	3.9
	NNM	5140	42.0	28.9	10.4	2.0

Colonies

			Percent survival			
		Total n	d15	d30	d60	d90
Pairs	NM	704	56.4	45.5	36.4	10.9
	NNM	1080	45.6	34.1	23.6	9.4
Trios	NM	581	75.7	66.3	46.8	13.9
	NNM	1034	56.4	42.3	22.9	8.3
Quintets	NM	573	70.5	59.5	35.1	7.0
	NNM	1028	55.7	41.3	17.2	3.5

Complete censuses of every colony took place at 60, 120, and 180 days post-establishment in 2006, and at 15, 30, 60, and 90 days in 2007, 2008, and 2009. A total of 5000 colonies were established for this experiment, tracking 16,418 individual alates and their progeny (Table. 2.1).

Natural incipient colony collection

To compare the survival and colony growth rates of experimental colonies with field-established colonies, a total of 100 naturally-occurring incipient colonies were field-collected in June–August 2008 and 2009, by intensive searching of the habitat at 30, 60, and 90 days after the main dispersal flights. Number and sex of founders, and number and class of offspring were determined for each natural incipient colony. Each colony was preserved in 90% alcohol for future genetic analysis.

Statistical analysis

Less than 1% of incipient colonies established as trios or quintets survived the experiment intact, with all founding members alive; for the analyses presented here, colonies were retained in their original founding group size category following the death of one or more founders. Although the colony was no longer headed by a trio or quintet, the historical fact of having been established by three or five individuals made such "reduced" colonies qualitatively more similar to each other, than to colonies originally established

as pairs or trios. Removing reduced colonies from the analysis entirely was not desirable, because we wanted to explicitly test whether larger groups were reducing to an optimal group size and whether mortality was affected by group size. Colonies were considered "dead" when less than two founders remained. Widowed founders may survive for some time following loss of their partner, particularly if offspring are present, however, the reproductive potential of the colony is lost.

Individual and colony survival, and time courses of reproductive milestones, were analyzed using Cox Proportional Regression (Therneau and original R port by Thomas Lumley 2009). All possible covariates (founding group size, relatedness, sex) and their interactions, as well as clustering variables (year, establishment date, parental colony), were used in the initial model, which was simplified in a step-wise manner, removing variables and interactions that did not contribute significantly to the fit of the model. The proportional hazards assumption, that there is no difference in the hazard rate of death over time, was checked on the final model. Interactions with time were added as necessary, and step-wise simplification again attempted. Curves were plotted for each group size by relatedness category.

Kruskall-Wallis tests (KW) were performed to determine differences in offspring production by each group size by relatedness category at each census point. Significant tests were followed by non-parametric multiple comparisons (Giraudoux 2009). All statistical analysis was performed in R (R Development Core Team 2009, v. 2.9.2).

2.3 Results

Experimental colonies: founder survival

Mortality was high during the first 90 days of colony establishment, in spite of adequate food and moisture, and a predator-free environment. Roughly 10% of individuals and incipient colonies survived to 90 days post-establishment (Table 2.1). Individuals placed with nestmates survived better than those placed with non-nestmates for all founding group sizes (Fig. 2.1). The interaction of founding group size and relatedness was a significant predictor of mortality of individuals. Both founding group size and relatedness interacted non-linearly with time (i.e. their influence on survival changed over time) for individual survival.

About one-third of alates placed in nestmate pairs survived to 90 days and one-quarter survived to 220 days post-establishment, significantly more than any other treatment. Individuals in nestmate trios died at 1.09 times the rate of those in nestmate pairs ($p = 0.01$). Individuals in nestmate quintets and non-nestmate pairs survived equally well, dying at 1.43 (hazard ratio, $p = 0.0001$) and 1.49 ($p = 0.0001$) times the rate of nestmate pairs, respectively. Individuals placed in non-nestmate trios and quintets died at 1.86 ($p = 0.0001$) and 2.47 ($p = 0.0001$) times the rate of nestmate pairs.

When considered at the colony level, groups composed of all nestmates survived better than those composed of non-nestmates (Fig. 2.2). While both founding group size and relatedness were significant predictors of

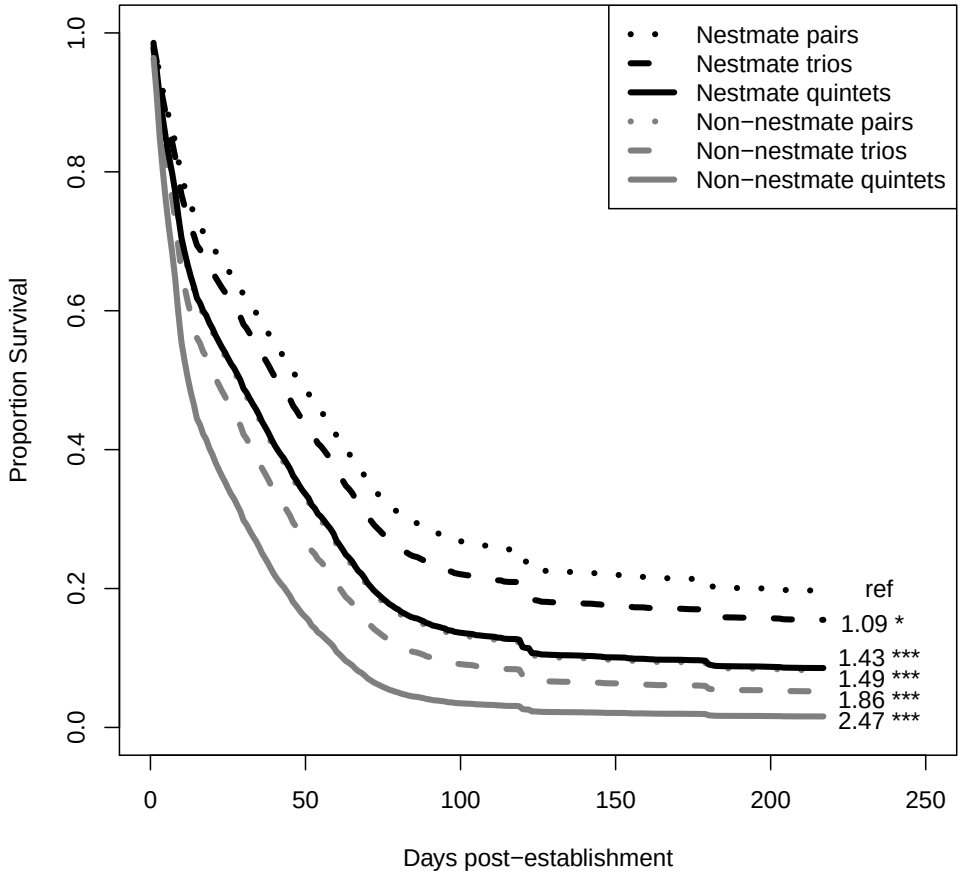


Figure 2.1 Survival curves of individuals, by founding group size and relatedness over the first 220 days of colony establishment. Pairs are represented by dotted lines, trios dashed lines, quintets solid lines. Nestmates are black, non-nestmates grey. Numbers to the right of each curve are hazard ratios relative to the reference. As compared to the reference, * p = 0.01, *** p = 0.0001. Note that curves describing survival of individuals in nestmate quintets and non-nestmate pairs are nearly overlapping.

colony-level mortality, their interaction was not. Considered at the colony level, only relatedness demonstrated an interaction with time in its effect on colony mortality. Colonies founded as nestmate pairs and trios survived equally well (reference group pairs, hazard ratio for trios 1.09, $p > 0.05$), and significantly longer than nestmate quintets (hazard ratio 1.22, $p = 0.001$). Colonies established by non-nestmate pairs and trios died at 1.60 and 1.71 times the rate of those founded by nestmate pairs, respectively, while non-nestmate quintets died 1.95 times as quickly as the reference group ($p = 0.0001$).

Experimental colonies: reproductive milestones and colony growth

Founding group size was a significant predictive factor in time to production of all four offspring classes (eggs, larvae, workers, and soldiers), while relatedness was significant for all offspring classes except workers (Cox Proportional Regression). No interaction was found between the two factors, however the effect of both group size and relatedness interacted non-linearly with time to first appearance of larvae, while relatedness showed a significant interaction with time for soldier production. In other words, the impact of group size and relatedness on reproduction changed over the course of the experiment. Variation in days to each reproductive milestone was large for each group size by relatedness treatment.

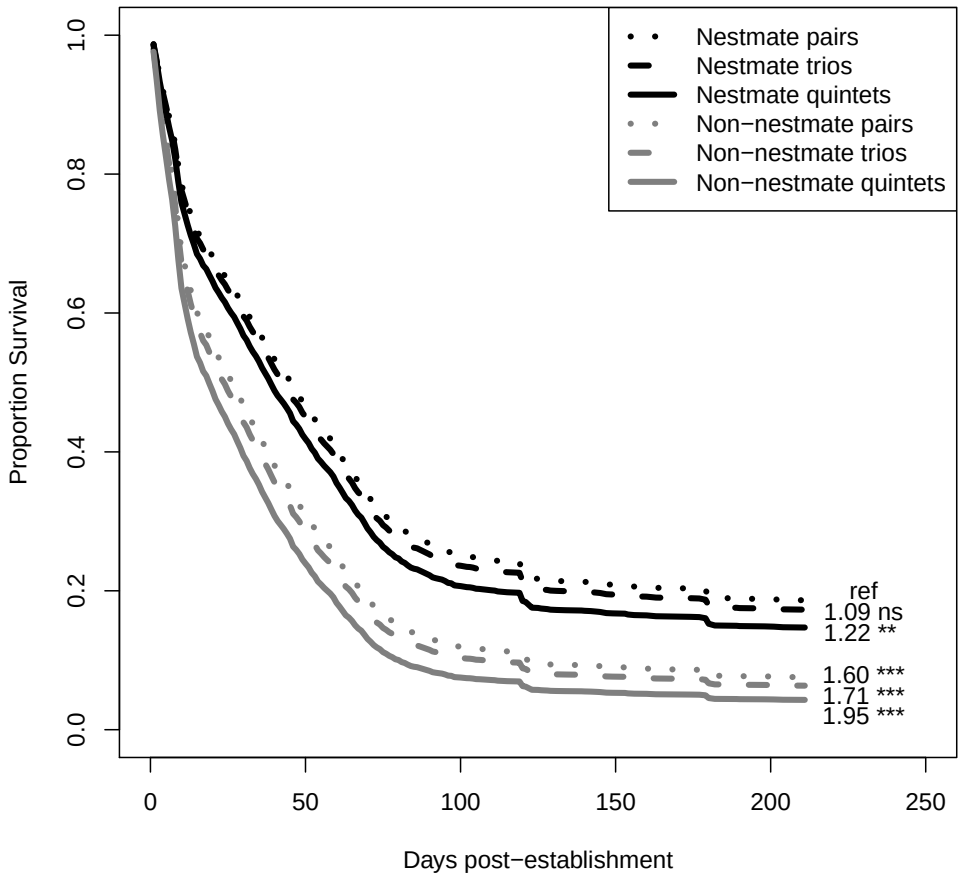


Figure 2.2 Survival curves of colonies by founding group size and relatedness over the first 220 days of colony establishment. Pairs are represented by dotted lines, trios dashed lines, quintets solid lines. Nestmates are black, non-nestmates grey. Numbers to the right of each curve are hazard ratios relative to the reference. As compared to the reference, ns = no significant difference, ** p = 0.001, *** p = 0.0001.

Quintets produced eggs slightly faster than smaller founding groups, but they were less successful in developing all other offspring classes. Overall, non-nestmates produced eggs sooner than nestmates, and quintets sooner than the other group sizes (Fig 2.3). There were no significant differences in time to production of first eggs between non-nestmate quintets (reference group), trios (hazard ratio 0.92, $p > 0.05$), and pairs (0.91, $p > 0.05$). Nestmates were less likely to produce eggs than non-nestmates, with egg production of nestmate quintets (hazard ratio 0.78), trios (0.75), and pairs (0.66) significantly lower than that of non-nestmate quintets ($p = 0.0001$ for each).

Larvae were produced most quickly and by the highest proportion of colonies in the non-nestmate pairs treatment (Fig 2.4). Overall, more non-nestmate colonies produced larvae than did nestmates. Quintets lost the advantage of earlier egg production, and produced larvae later and at lower rates than the other founding group sizes. Larva production did not differ significantly between non-nestmate pairs and trios and nestmate pairs. Nestmate trios and non-nestmate quintets were, respectively, 0.65 and 0.64 times as likely to produce larvae as the reference group (non-nestmate pairs). Nestmate quintets were 0.49 times as likely to produce larvae as the reference group.

Production of helpers followed a similar pattern to that seen for larvae. Non-nestmates produced soldiers sooner than nestmates, however relatedness was not a significant predictor of time to the appearance of the first

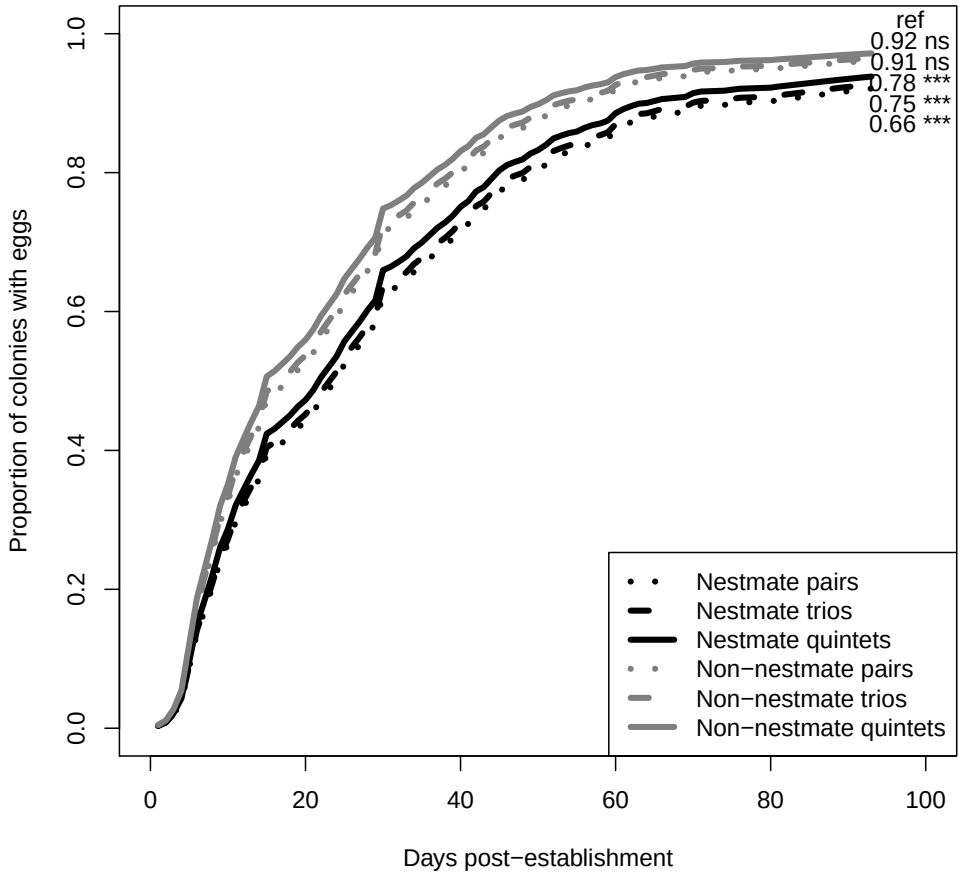


Figure 2.3 Time course to first appearance of eggs, by founding group size and relatedness. Numbers to the right of each curve are hazard ratios relative to the reference. As compared to the reference, ns = not significantly different, *** p = 0.0001.

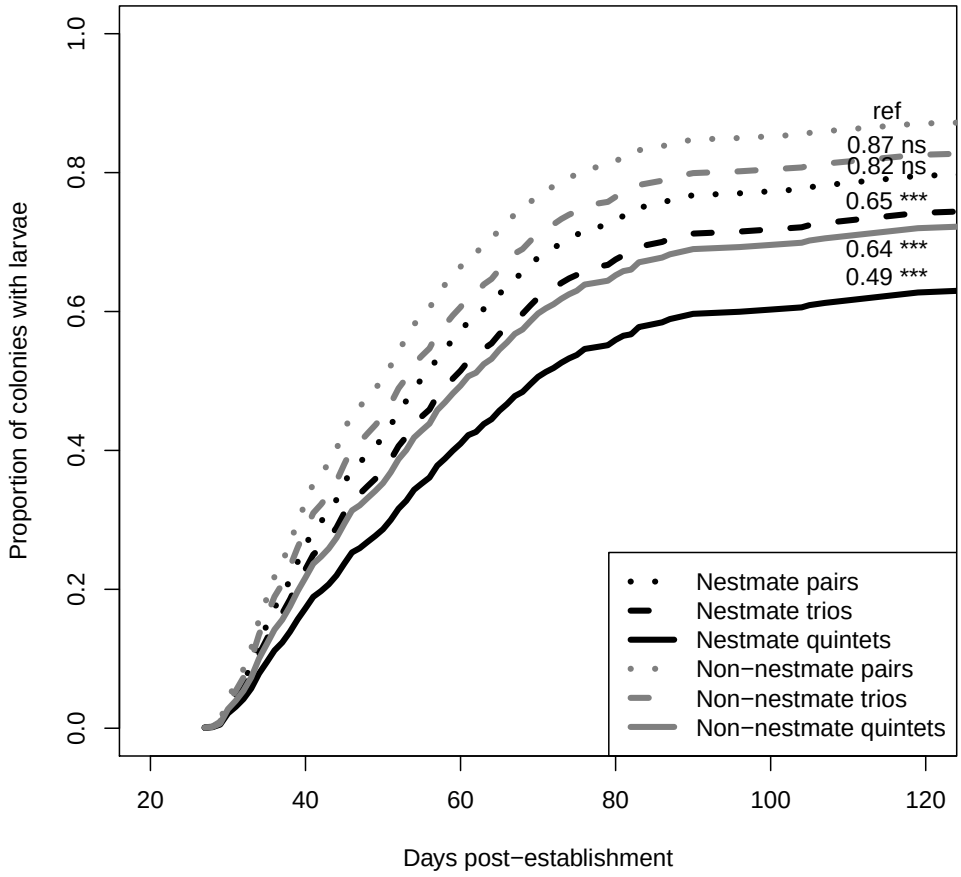


Figure 2.4 Time course to first appearance of larvae, by founding group size and relatedness. Numbers to the right of each curve are hazard ratios relative to the reference. As compared to the reference, ns = not significantly different, *** p = 0.0001.

worker. Smaller founding groups produced soldiers (Fig 2.5) and workers (Fig 2.6) more quickly, with a larger proportion of surviving colonies achieving each milestone compared to quintets. Quintets were half as likely to produce workers (hazard ratio 0.49) as pairs.

Soldier production was not significantly different between non-nestmate and nestmate pairs and non-nestmate trios, while nestmate trios were 0.47 times as likely to produce soldiers as the reference group, non-nestmate pairs. Non-nestmate and nestmate quintets were 0.34 and 0.30 times as likely to produce soldiers as the reference group. Of the 450 colonies that produced at least one worker and/or soldier in 2007, 2008, and 2009, significantly more colonies (proportion test, $p < 0.0001$) produced a worker before they produced a soldier (206, 45.8%). While 31.1% of colonies (140) produced a soldier first, the remaining 23.1% (104) matured both on the same day.

Larger founding groups showed the most variation in total colony size (sum of eggs, larvae, workers and soldiers) at each census point. Distributions are much tighter for pairs than for the other group sizes, with the number of offspring per colony falling within a relatively small range. Within the trios and quintets, some colonies had very few offspring, while others greatly exceeded the mean. The distribution is more uniform for trios, and much less even for quintets.

At 15 days post-establishment, highly significant differences were found in total offspring number between different relatedness and founding group

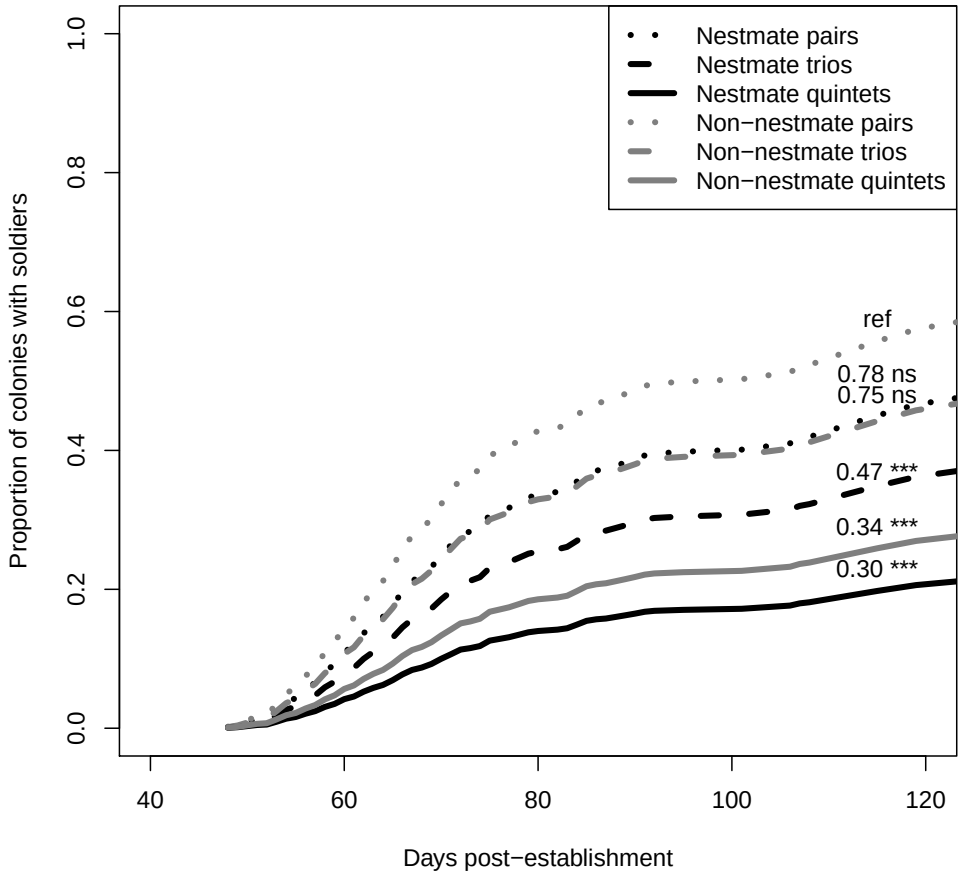


Figure 2.5 Time course to first appearance of soldiers, by founding group size and relatedness. Numbers above each curve are hazard ratios relative to the reference. As compared to the reference, ns = not significantly different, *** p = 0.0001.

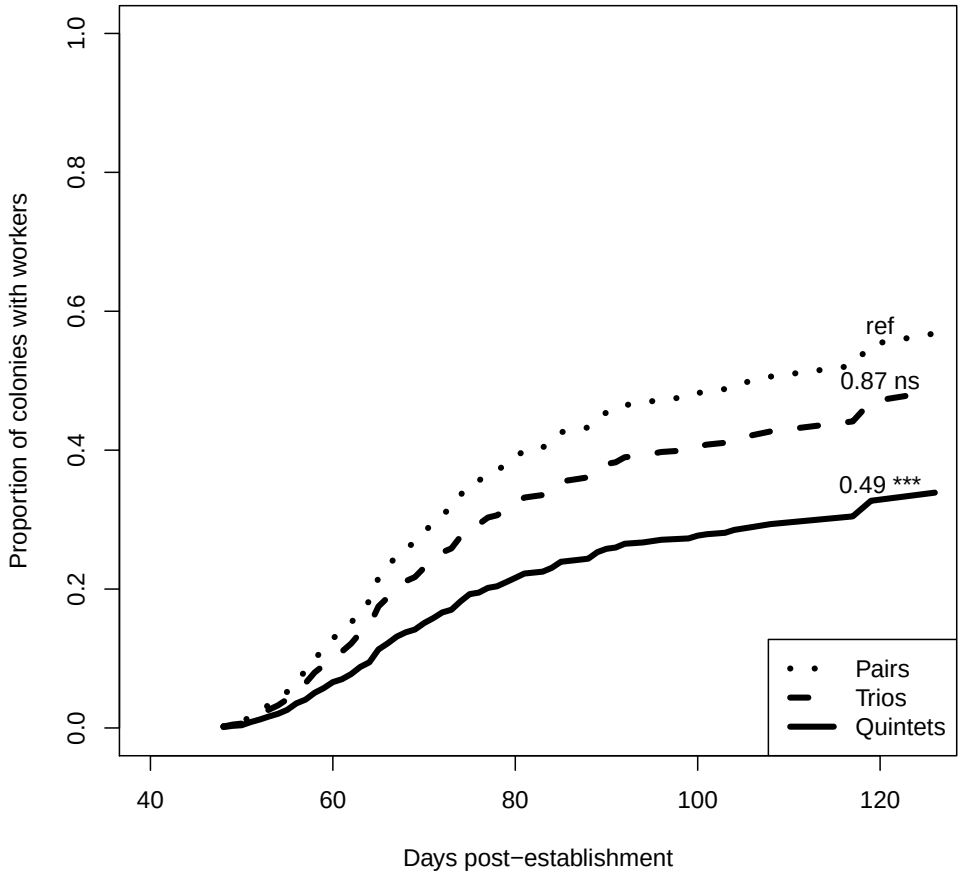


Figure 2.6 Time course to first appearance of workers, by founding group size. Relatedness of founders did not influence time to first workers, so nestmates and non-nestmates were pooled. Numbers above of each curve are hazard ratios relative to the reference. As compared to the reference, ns = not significantly different, *** p = 0.0001.

size combinations (Fig 2.7, KW p <0.001). Thirty percent of surviving colonies had produced eggs by 15 days, and no larvae had yet hatched (Fig 2.3). Non-nestmate quintets laid significantly more eggs than non-nestmate pairs, but no other groups were found to be significantly different from each other.

Significant differences between relatedness and group size combinations were again found at 30 days post-establishment (Fig 2.8). By this time, over 50% of surviving colonies had laid eggs and a few had hatched larvae (Fig 2.4). Non-nestmate trios were found to have produced significantly more total offspring (eggs and larvae) than nestmate pairs or trios.

At 60 days (Fig 2.9) and 90 days (Fig 2.10) post-establishment, no significant differences were found in the total number of offspring (sum of all eggs, larvae, workers and soldiers). Founding group size influenced offspring production more than relatedness at 60 days, and means were nearly the same for nestmate and non-nestmate colonies of each group size. Means were not significantly different, for nestmates and non-nestmates at 90 days, but as mentioned above, the shape of the distributions is very different for the three founding group sizes, approaching a normal distribution for pairs and a bimodal distribution for quintets.

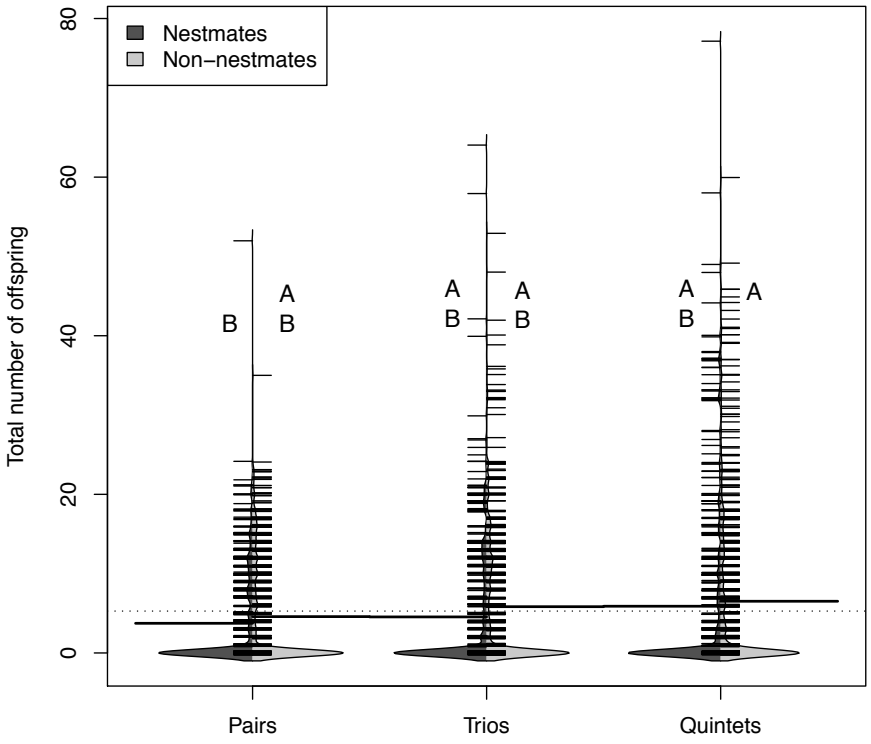


Figure 2.7 Total number of offspring at 15 days post-establishment, by founding group size and relatedness of founders. Beanplot shows the total number of offspring for all colonies that survived to that census point. The overall mean is the horizontal dotted line. Category means are long solid lines, and the short lines are individual observations within the density trace (shaded area). Individual beans are split by relatedness. Overall Kruskal-Wallis p = 0.0004. Homogeneous subgroups are indicated by different letters, as determined by non-parametric multiple comparisons.

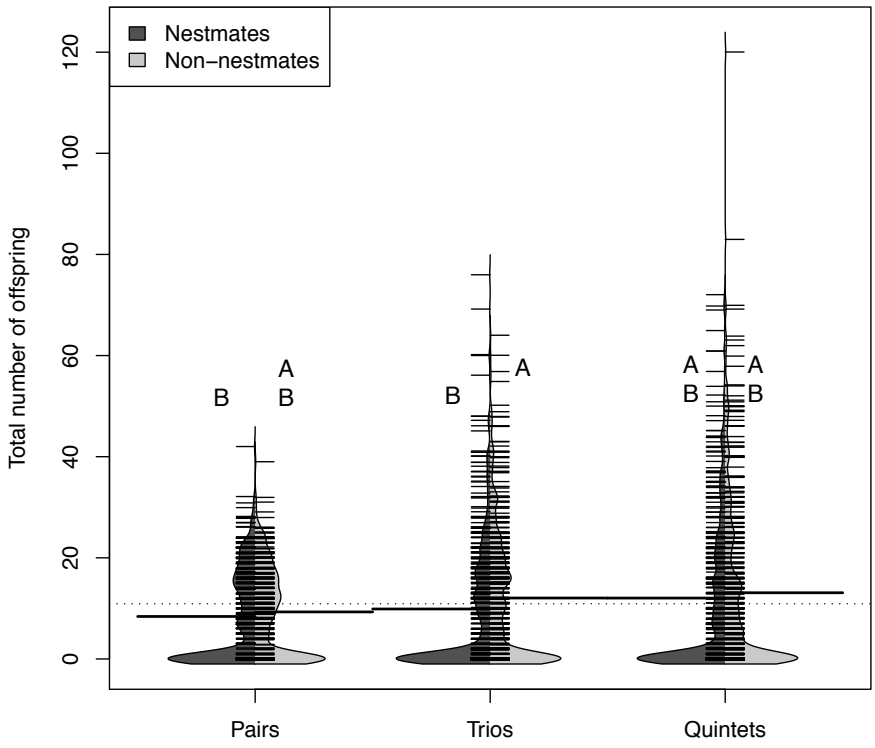


Figure 2.8 Total number of offspring at 30 days post-establishment, by group size and relatedness of founders. Beanplot shows the total number of offspring for all colonies that survived to that census point. The overall mean is the horizontal dotted line. Category means are long solid lines, and the short lines are individual observations within the density trace (shaded area). Individual beans are split by relatedness. Overall Kruskal-Wallis $p = 0.002$. Homogeneous subgroups are indicated by different letters, as determined by non-parametric multiple comparisons.

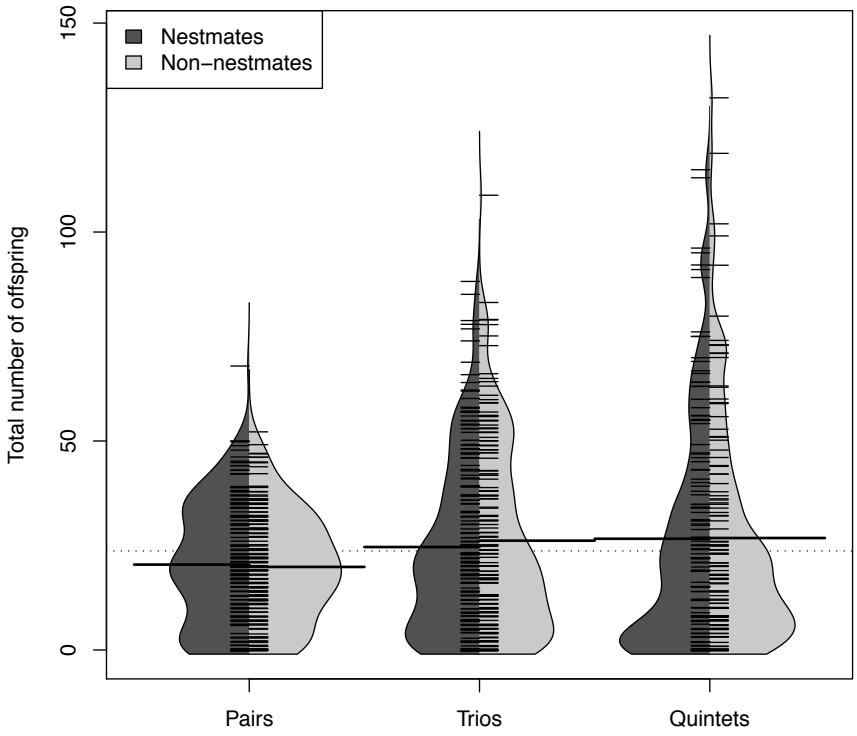


Figure 2.9 Total number of offspring at 60 days post-establishment, by founding group size and relatedness of founders. Beanplot shows the total number of offspring for all colonies that survived to that census point. The overall mean is the horizontal dotted line. Category means are long solid lines, and the short lines are individual observations within the density trace (shaded area). Individual beans are split by relatedness. Overall Kruskal-Wallis p = 0.51.

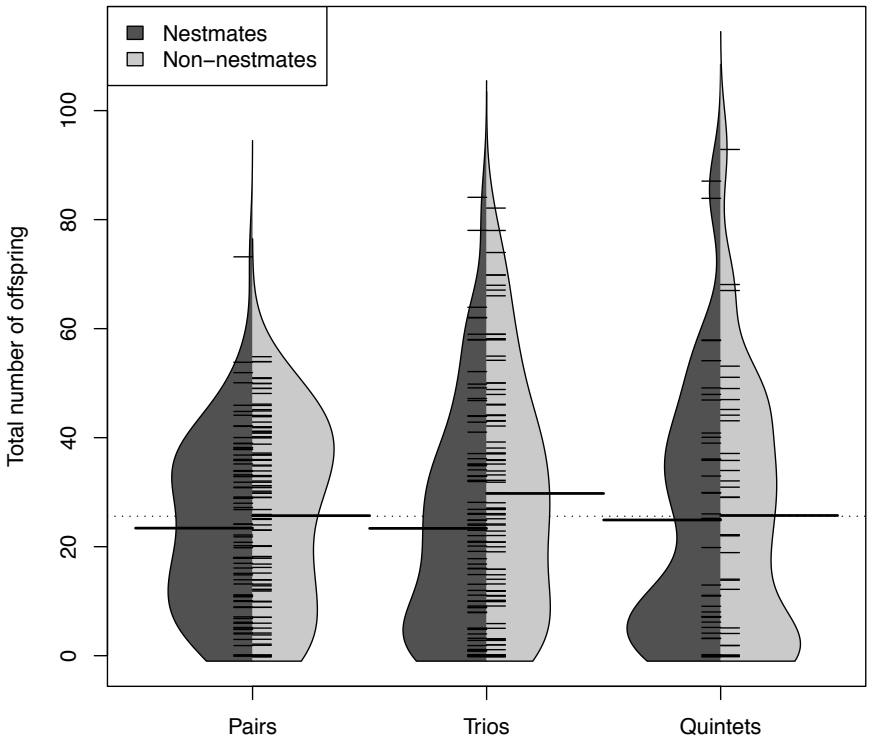


Figure 2.10 Total number of offspring at 90 days post-establishment, by founding group size and relatedness of founders. Beanplot shows the total number of offspring for all colonies that survived to that census point. The overall mean is the horizontal dotted line. Category means are long solid lines, and the short lines are individual observations within the density trace (shaded area). Individual beans are split by relatedness. Overall Kruskal-Wallis p = 0.34.

Comparison between natural and experimental incipient colonies

Although days post-establishment are only approximate for field-collected colonies, total populations and offspring classes found in these colonies were within the ranges found in the experimental data (Fig 2.11). Recovery of incipient colonies containing offspring at all stages of development (eggs, larvae, pre-soldiers), indicate that new eggs are laid before the first workers mature. This supports the observation in the laboratory that eggs are laid continuously rather than in discrete broods.

Note that in the field, nearly all colonies recovered approximately 30 days after the dispersal flights had offspring, while half of all surviving lab colonies did not. No colonies were collected between 60 and 90 days post-flight without offspring (Fig 2.12). The two trios, both composed of two kings and one queen, that were collected in the second sampling had 37 and 26 total offspring each. There is not sufficient data to statistically compare the effect of founding group size, however these two colonies are in the upper 50% of the distribution for field-collected colonies at that time point.

2.4 Discussion

Under laboratory conditions, pleometrotic founding in *N. corniger* is costly compared to monogamy in terms of both survival and reproduction. Potential queens and kings died more quickly when placed in 5-member founding

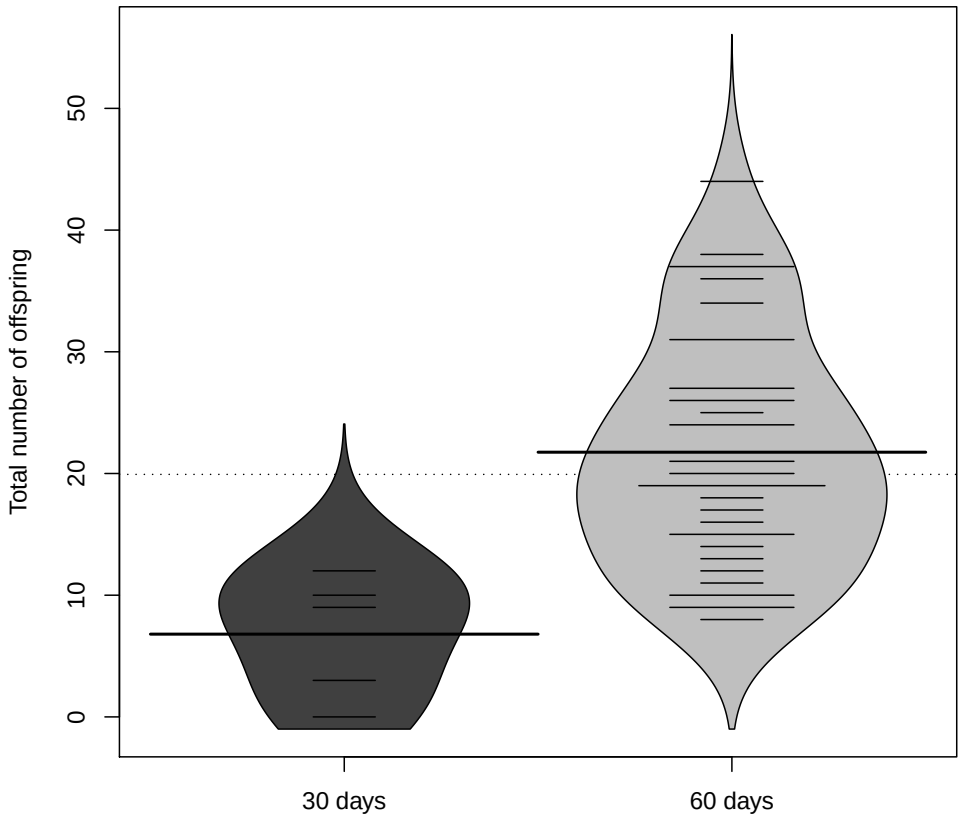


Figure 2.11 Total number of offspring from field-collected incipient colonies at approximately 30 and 60–90 days after the main dispersal flights. Beanplot shows the total number of offspring for each colony recovered. Means are long solid lines, and the short lines are individual observations within the density trace (shaded area).

Figure 2.12 Naturally-established colonies with eggs (top) and larvae (bottom) recovered August 3, 2008, 60-90 days post-flight. Photos: Casey Hamilton

groups as compared to individuals in smaller groups. No other similar studies have been done in termites, however, this is similar to the results of Deslippe and Savolainen (1995), who found that larger (8–16 queen) founding associations had lower survival, but not reproduction, compared to lone foundresses or small (2–4 queen) groups. The decreased reproductive output with increased group size found in this experiments is in stark contrast to facultatively polygynous ants (Adams and Tschinkel 1995; Bernasconi and Strassmann 1999; Johnson 2004).

The high mortality rate seen in this experiment is seldom, if ever, due to overt aggression. This indicates that there is no active elimination of supernumerary reproductives to achieve an optimal group size at this stage of colony development in *N. corniger*, as seen in more basal termites, which kill or repel additional reproductives during colony foundation (Shellman-Reeve 1999; Thorne et al. 2003). No fighting was ever observed (see also Chapter 3); in the few cases in which a deceased founder was found in a state of partial dismemberment, it was not possible to determine whether this was due to fighting, or to incomplete hygienic or nutritional cannibalism post-mortem.

The higher mortality rates of individuals in the largest founding groups translated into lower colony-level survival. If greater mortality of individuals in quintets was due to reduction of large groups to an "ideal" group size of a monogamous pair, colony-level survival should have been equivalent across group sizes. On the contrary, colonies initiated with five founders

survived less well than groups founded as either pairs or trios, indicating that death of individuals in these larger groups was not solely attributable to elimination of excess founders. Rather, it may be due to intrinsic hazards of group living such as spread of disease and competition for resources or breeding opportunities (Pulliam and Caraco 1984), costs which are theoretically lower for eusocial insects due to behavioral adaptations to group life (Hughes et al. 2002). Pairs and trios were recovered from the field, but no larger pleometrotic associations, further suggesting that multiple reproductives are not necessarily an advantage during the initial stages of colony foundation in *N. corniger*.

Not only survival, but also reproductive success was lower for quintets compared to pairs and trios in the laboratory. While more oviposition occurred slightly sooner in quintets than in smaller founding groups, quintets hatched fewer larvae and lagged behind smaller founding groups in the production of helpers (workers and soldiers). Behavioral differences in nest maintenance, parental care, and interactions between co-founders within pairs, trios and quintets may explain these results, and will be discussed further in Chapter 3.

The mean number of offspring in field-collected incipient colonies was similar to what was found in the laboratory, indicating that laboratory experiments accurately reflect the early stages of colony development. Laboratory conditions did, however, seem to allow more variation in offspring production. Notably, no field colonies were recovered after 30 days that

did not have offspring, while 10–20% of laboratory colonies surviving to 90 days post-establishment never laid eggs.

By forcing alates into pairs, trios and quintets rather than allowing them to choose their own mate(s), these barren colonies may highlight incompatibilities, either physiological or behavioral. Physiological incompatibilities could include *Wolbachia*, known to induce cytoplasmic incompatibility (Bourtzis and O'Neill 1998) and found in other termite species (Bordenstein and Rosengaus 2005; Matsuura et al. 2004). If there is a genetic component to pleometrotic colony foundation, this could also result in more cooperative behaviors between founders that choose to establish a colony pleometrotically than between individuals randomly placed into such a group.

In the experimental colonies, workers were produced before soldiers, indicating that brood care, nest expansion, and feeding the growing colony are more pressing needs than defense. The roles of soldiers may also be less necessary, as external scouting and foraging does not occur at this early stage of colony development. Pathogen pressure during this claustral stage is likely low, so the fungistatic properties of soldier secretions (Rosengaus et al. 2000*a*) are not as vital. Ratio of workers to soldiers is highly variable in field colonies, but the recovery of 4 natural incipient colonies with 2–15 workers and no soldiers suggests that workers are also produced first in the field, and may sometimes not be produced until the third clutch of eggs.

Relatedness of founders significantly impacted both survival and reproduction. While nestmates survived better than non-nestmates, non-

nestmates began producing offspring sooner and had greater overall reproductive success. Delay in initiation of oviposition by nestmates could indicate a preference for non-nestmate partners, or may be an artifact of experimental conditions (nestmates not "realizing" that they have dispersed from the natal nest). Thus, delayed oviposition may be an adaptive trait, fostering dispersal rather than staying in the parent nest.

Workers and soldiers actively exclude or kill remaining alates in the queen- and king-right parent colony after the normal dispersal flights have occurred (Thorne 1982*a*, and pers. obs.). Oviposition and other reproductive behaviors may be delayed in the presence of nestmates until the alate is either in a queen-less satellite nest of the parent colony or it is clear that the previous queen or king is dead. Preferences for founding group size and relatedness in *N. corniger* are further explored through choice experiments reported in Chapter 4.

These findings directly contradict the widely-accepted view, supported by research on the social Hymenoptera (Bartz and Hölldobler 1982), that pleometrotic colony foundation increases individual and colony-level survival rates compared to colony foundation by a single reproductive unit (termite queen and king; inseminated ant queen). Clearly, pleometrotic colony foundation is not directly comparable between ants and and termites, as termites colony foundation literally requires the presence of at least two individuals (Matsuura et al. 2002), while ant foundresses are not required to cooperate during this time. Interestingly, these results also contradict the

evidence of faster colony growth under pleometrosis seen in another facultatively polygamous termite lineage, *Macrotermes* (Darlington 1984, 1988). Previously, faster growth and earlier alate production compared to monogamous pairs, had been suggested as potential advantages of pleometrosis in *N. corniger* (Roisin 1993; Thorne 1983).

While not tested empirically, observations made in these experiments suggests that disease spreads more quickly in quintets. All members of a quintet often died within one or two days of just one individual showing signs of infection. This contradicts research on lower termites, in which larger groups survived disease exposure rather than spreading it throughout the group (Pie et al. 2005; Traniello et al. 2002), and the general idea of density-dependent prophylaxis (Wilson et al. 2002). This is further explored in the experiments reported in Chapter 5.

Regardless of founding group size, mortality is extremely high during the first 30 days of colony establishment, essentially a survival bottleneck. Beyond that point, groups with additional founders, beyond a monogamous pair, could take advantage of the benefits of group size as suggested by other authors (Keller 1995; Roisin 2000; Thorne 1983). From this perspective, the outliers in offspring production among trios and quintets are of most interest, demonstrating the real potential for pleometrotic groups to achieve faster colony growth than monogamous pairs. Density of pairs may be very high under natural conditions, with encounters between developing colonies inevitable. Joining forces with other survivors of the post-flight bottleneck

might be a way to eliminate the risks of pleometrosis demonstrated here, but still take advantage of larger group size for rapid colony growth

Chapter 3

Behavior During Colony Establishment

Abstract

As eusocial organisms, termites cooperatively construct and maintain their nest, and rely on interactions with nestmates for hygiene and nutrition. The importance of social interactions and cooperative behavior during colony foundation, however, has not been thoroughly studied. The facultatively polygamous neotropical termite *Nasutitermes corniger* is particularly appropriate to investigate this question, as it allows direct comparison of monogamous pairs and pleometrotic founding groups. Theoretically, pleometrotic groups should have an advantage over monogamous pairs due to economies of scale in nest construction and maintenance, and the presence of

additional partners for allogrooming, trophallaxis, care of developing offspring, and colony defense. This study tracked the behavior during colony establishment of two cohorts of experimental colonies established as pairs, trios, and quintets. In 2007, pleometrotic colonies performed fewer parental behaviors overall, and exhibited lower survival and reproductive success compared to monogamous pairs. Individuals in larger groups interacted more frequently with one another than did founding pairs, but for shorter durations. In 2008, however, there were no significant differences in parental care based on founding group size, and individuals in pairs and trios interacted more frequently with co-founders than did those in quintets. No differences were found in the amount of time individuals in pairs, trio, or quintets spent interacting with co-founders. The contradictory results between the two cohorts suggests that group size alone is not an indicator of the degree of cooperation between co-founders or the amount of parental care provided during colony foundation.

3.1 Introduction

Termite reproductives rely on the assistance of their co-founder to successfully excavate an initial nest site and care for offspring. Cooperation with a mate is vital to the development of the initial worker broods, and ultimately to successful colony establishment (Matsuura et al. 2002; Rosengaus and Traniello 1991; Shellman-Reeve 1994, 1997). Survival is enhanced by

mutual grooming (Rosengaus and Traniello 1991) and cooperative nutrition, including sharing of nutritional reserves via trophallaxis (Shellman-Reeve 1990).

Cooperative associations of more than a single reproductive unit (monogamous pair in termites, inseminated queen in social Hymenoptera) may be favored when such collective actions improve incipient colony success (Lehmann et al. 2006). Success may be measured by competitive ability against conspecifics or heterospecifics for the same resources (Izzo et al. 2009), or survival of predator, parasite, or pathogen attack (Clouse 2001), resulting in faster colony growth or larger colony size. Competitive interactions within the group or selfish behavior of individuals, however, may reduce incentives for group membership or make such associations unstable (Hamilton 1964*a*, *b*).

For pleometrosis to evolve, the benefits of cooperating must be equal to or greater than the costs of cooperating. Sharing investment in sterile workers during this vegetative state of colony growth could benefit an individual if they can expend fewer personal resources by sharing offspring care and production of eggs destined to become sterile helpers. Individual reproductives and the colony as a whole may thus survive better or reach a critical mass more quickly than colonies established by monogamous pairs. This has been found to occur in the facultatively polygynous species of social Hymenoptera, in which foundress associations are more competitive and survive better than single-queen colonies (Strassmann 1989).

Pleometrosis has been demonstrated to result in stable primary polygyny in some ants (Trunzer et al. 1998). In other cases, the costs of sharing the reproductive role in the incipient colony may eventually outweigh the benefits of pleometrosis. Initially-cooperative associations may break down after emergence of the first workers, with competitive interactions leading to active aggression between co-founders and elimination of all but one queen (Aron et al. 2009; Bernasconi and Keller 1996). While the dynamics of cooperation during colony establishment in facultatively polygamous termites has not been explored, it seems likely that some competitive interactions, particularly reduction in the number of kings, may occur between termite reproductives as pleometrotic colonies mature (Darlington 1988).

The 23% of mature *Nasutitermes corniger* colonies that contain multiple unrelated reproductives (Atkinson and Adams 1997) have been hypothesized to arise through pleometrosis, cooperative colony foundation by multiple queens and/or kings. Pleometrotic groups are found in the field far less frequently (around 5%) than would be expected from the proportion of polygamous mature colonies, and were found to have lower survival and reproductive output under laboratory conditions than monogamous pairs (see Chapter 2). This may be the result of differences in parental care behaviors and interactions between co-founders in pairs as compared to larger founding groups.

To understand the balance of cooperation and competition between co-founders, we analyzed the behavior of monogamous pairs and pleometrotic

trios and quintets established in the laboratory. Differences in nest construction behaviors, effectiveness of disease prevention via allogrooming, and/or quality of parental care to developing offspring, due to founding group size, should be evident during the first 90 days of colony development, when cooperation between mates is most critical for colony establishment. Aggressive or competitive interactions between co-founders, or lack of cooperation, could arise early in the association, or manifest after the production of sterile helpers.

3.2 Methods

Laboratory colonies were established in May–June 2007 and 2008 from wild-caught alates as described elsewhere (Chapter 2). Each individual was marked with a dot of enamel paint to facilitate observation. Preliminary studies indicated that the paint did not affect courtship behavior or interactions between individuals. In each year, 3 replicates each of nestmate and non-nestmate pairs (1♀1♂), trios (2♀1♂), and quintets (3♀2♂) were videotaped at 10 day intervals between 30 and 80 days post-establishment. Each videotaped session was 20 minutes long, beginning after a 15-minute acclimation period to minimize behavioral effects of moving the colony. This resulted in a total of 230 hours of video footage from 6 replicates each of nestmate pairs, trios, and quintets, and 6 replicates each of non-nestmate pairs, trios, and quintets.

Video was analyzed using J-Watcher (Blumstein and Daniel 2007) behavior-analysis software with behavior definitions standardized to a training video (Table 3.1). The beginning and ending of each activity or interaction was noted, resulting in both frequency and duration data for every behavior. Recipients of behaviors (offspring, mates, wood) were noted as appropriate for each instance. All timepoints between 30 and 70 days were analyzed in 2007. Only 50, 70, and 80 days post-establishment were analyzed in 2008, to focus more specifically on parental care behaviors. Only non-resting behaviors were included in the statistical analysis. Ethograms and time budgets were developed in SPSS. Differences in frequency and duration of the most common behaviors were analyzed using Kruskal-Wallis non-parametric tests in R (R Development Core Team 2009, v 2.9.2), followed by non-parametric multiple comparisons (Giraudoux 2009) when indicated (R Development Core Team 2009, v 2.9.2).

3.3 Results

Survival and reproductive success differed between the two years, so data from each year was analyzed separately. In the larger experiment from which the observed colonies were drawn (Chapter 2), 21.0%, 20.8%, and 6.8% of pairs, trios, and quintets, respectively, survived to 90 days post-establishment in 2007, while 9.5% of pairs, 9.9% of trios, and 5.7% of quintets survived to 90 days post-establishment in 2008. Out of all colonies

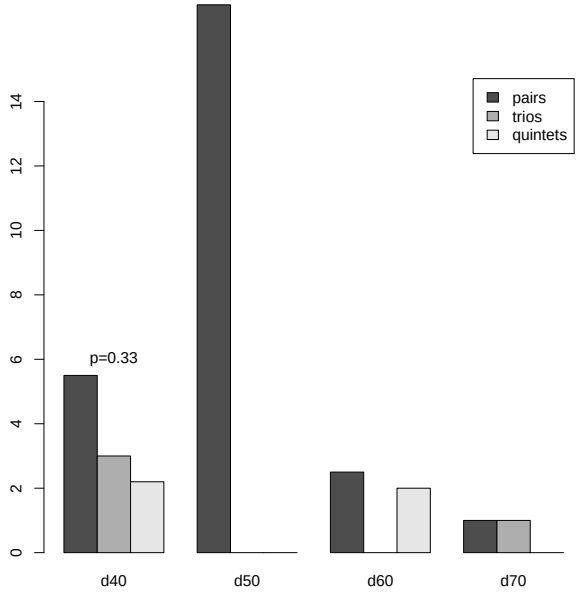
Table 3.1 Behavioral definitions

Behavior	Description
Antennating	Stroking an object or other termite with the antennae
Chewing	Picking up an object, such as wood, with the mandibles and consuming it
Defecating	Excretion of feces from the posterior end of the termite
Licking	Touching an object, such as an egg, with the mandibles but not consuming it
Resting	The termite is motionless and is not performing any other behaviors
Rocking	Moving the body slowly backwards and forwards while standing
Running	Forward movement approximately twice the speed of walking
Scratching	Rubbing one leg rapidly against another part of the focal individual's own body
Vibrating	Similar to rocking, but rapidly
Walking	The termite takes more than two steps in the same direction
Waving	Standing completely still, moving the antennae in the air without touching another object or termite.

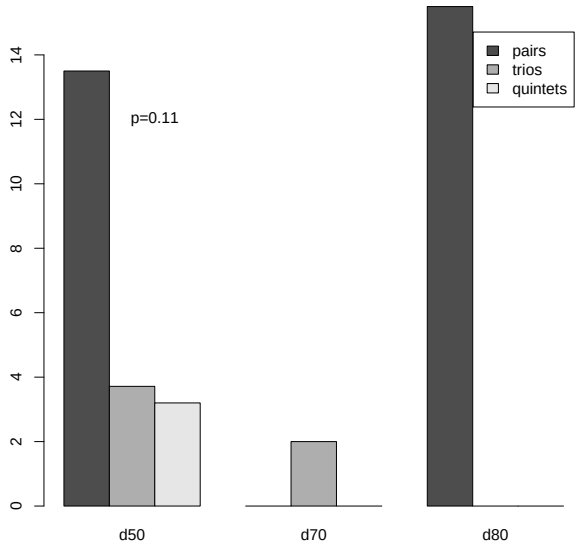
established in 2007, 67.3% of pairs, 74.5% of trios, and 67.5% of quintets ever produced eggs. In 2008, 47.2%, 52.7%, and 59.2% of pairs, trios, and quintets, respectively, ever laid eggs. Thus, there were greater differences in survival between quintets and the smaller groups in 2007, and generally lower survival and reproductive success for all group sizes in 2008 compared to 2007.

Trends in the behavioral data were also different between years, further prohibiting pooling the two years. Frequencies of the behaviors of interest were low and mortality decreased the sample size over time, so nestmates and non-nestmates were pooled within each year. Between 85–90% of behavior was walking or resting, activities which were excluded from the statistical analysis. The remaining behaviors were grouped into interactions with each offspring class (antennating or licking eggs, larvae, workers or soldiers), feeding and nest construction, self-grooming, and adult-adult interactions, such as antennating or mutual grooming.

Care of eggs was observed in all group sizes at 40 days in 2007 and at 50 days in 2008 (Fig 3.1), but was infrequently observed at the other time points. No significant differences could be detected in the frequency or proportion of time individuals in pairs, trios and quintets spent interacting with their eggs (Table 3.2). The amount of time spent on egg care was not significantly different between individuals of different group sizes at 40 days in 2007 ($p = 0.36$) or at 50 days in 2008 ($p = 0.50$). No cannabalism of eggs or larvae was observed in the videos.



2007



2008

Figure 3.1 Frequencies of interactions with eggs in 2007 and 2008 by founding group size. Kruskal-Wallis p-values are above each time point. KW tests were not done on time points that did not have observations in all three founding group sizes.

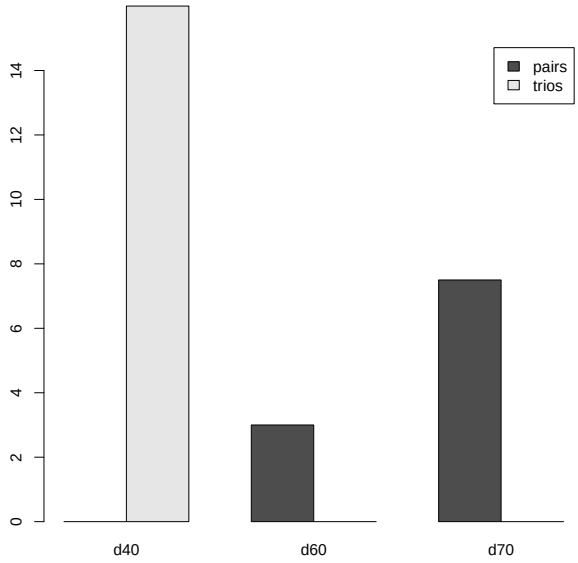
Table 3.2 Proportion of time (top) and frequencies (bottom) of key behaviors at 30–70 days post-establishment, 2007. Letters indicate homogeneous subgroups determined by non-parametric multiple comparisons. KW p= p-value of Kruskal-Wallis test. na = no observations

Proportions		30d	40d	50d	60d	70d
Adult	Pairs	47.4% a	17,1% a	13.1% a	12.2%	11.3%
interactions	Trios	42.0% a	25.4% a	19.4% a	18.0%	21.1%
	Quintets	50.1% b	31.9% b	23.5% b	11.2%	3.9%
	KW p =	0.04	0.001	0.04	0.07	0.18
Egg	Pairs	na	0.2%	0.2%	0.4%	0.5%
care	Trios	na	9.2%	na	na	0.8%
	Quintets	na	11.4%	na	4.0%	na
	KW p =	na	0.24	na	na	0.32
Larva	Pairs	na	na	na	7.7%	9.9%
care	Trios	na	30.2%	na	na	na
	Quintets	na	na	na	na	na
	KW p =	na	na	na	na	na
Frequencies						
Adult	Pairs	36.2 c	14.7 c	11.9 c	14.6	17.0
interactions	Trios	46.2 b	22.7 b	20.0 b	20.8	17.7
	Quintets	58.9 a	33.0 a	29.4 a	13.8	4.5
	KW p =	0.001	0.001	0.007	0.11	0.28
Egg	Pairs	na	5.5	17.0	2.5	1.0
care	Trios	na	3.0	na	na	1.0
	Quintets	na	2.2	na	2.0	na
	KW p =	na	0.33	na	na	na
Larva	Pairs	na	na	na	3.0	7.5
care	Trios	na	16.0	na	na	na
	Quintets	na	na	na	na	na
	KW p =	na	na	na	na	na

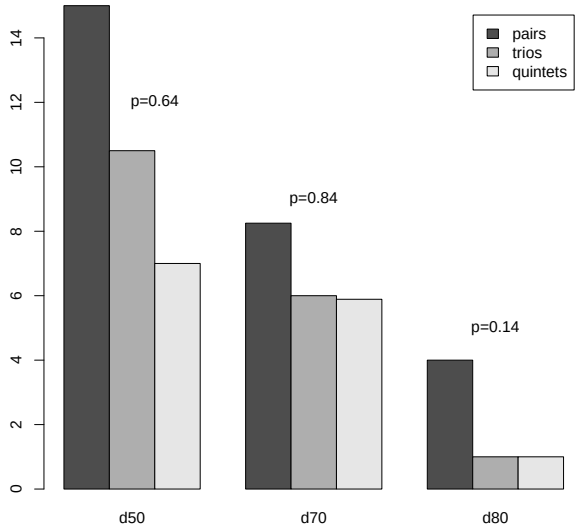
Care of larvae was only observed in pairs and trios in 2007 (Fig. 3.2), and there was too little data for statistical analysis. In 2008, individuals in all founding group sizes were observed caring for offspring at 50, 70, and 80 days post-establishment (Table 3.3). There were no significant differences in the frequency of interactions with larvae or the proportion of time spent on offspring care based on founding group size. Neither were there significant differences in the total amount of time spent by individuals of different founding group sizes on interactions with larvae at 50 ($p = 0.20$), 70 ($p = 0.68$), and 80 ($p = 0.17$) days post-establishment.

In 2007, the majority of observed brood care was done by females. Males were only observed performing brood care twice in 2007, once in a nestmate pair and once in a non-nestmate quintet. In contrast, males and females were both observed caring for offspring in 2008, and there were no significant differences between either the frequency or proportion of time spent by males and females on brood care at any time point.

Interaction rates between adults in pairs, trios and quintets were significantly different at the earliest three time points in 2007, and at all time points in 2008 (Fig 3.3). Trends were different between the two years. In 2007, individuals in quintets exhibited higher frequencies and proportions of total non-walking, non-resting behavior directed toward co-founders than did pairs and trios (Table 3.2). The frequency of inter-adult interactions was significantly higher within trios than between individuals in pairs, but the proportion of total non-walking, non-resting behavior spent on these adult



2007



2008

Figure 3.2 Frequencies of interactions with larvae in 2007 and 2008 by founding group size. KW tests were not performed on 2007 data, as there were insufficient observations. For 2008, Kruskal-Wallis p values are above each time point.

Table 3.3 Proportion of time and frequencies of key behaviors at 50, 70, and 80 days post-establishment, 2008. Letters indicate homogeneous subgroups determined by non-parametric multiple comparisons. KW p= p-value of Kruskal-Wallis test. na = no observations

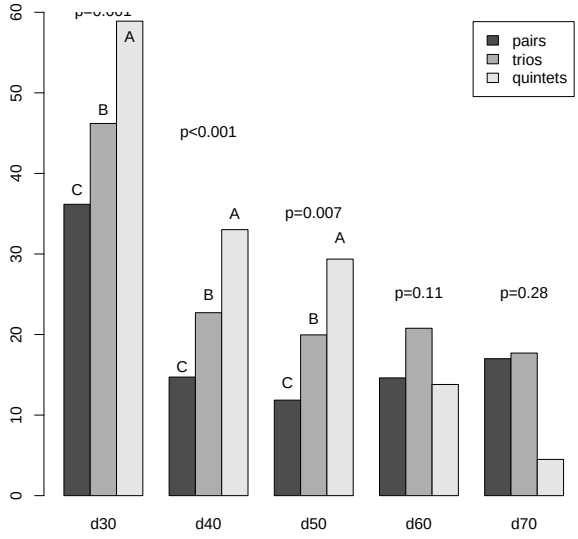
Proportions		50d	70d	80d
Adult	Pairs	17.6% a	11.8% a	11.8% a
interactions	Trios	11.2% a	10.3% a	8.5% a
	Quintets	7.3% b	5.6% b	6.0% b
	KW p =	0.0001	0.0001	0.0001
Egg	Pairs	19.1%	na	18.5%
care	Trios	13.3%	3.2%	na
	Quintets	17.3%	na	na
	KW p =	0.86	na	na
Larva	Pairs	16.2%	14.1%	3.9%
care	Trios	36.9%	7.4%	7.3%
	Quintets	12.3%	6.8%	0.6%
	KW p =	0.12	0.66	0.37

Frequencies				
Adult	Pairs	11.5 a	8.6 a	10.7 a
interactions	Trios	7.7 a	6.6 a	7.0 a
	Quintets	6.0 b	4.6 b	5.8 b
	KW p =	0.02	0.003	0.004
Egg	Pairs	13.5	na	15.5
care	Trios	3.7	2.0	na
	Quintets	3.2	na	na
	KW p =	0.11	na	na
Larva	Pairs	15.0	8.3	4.0
care	Trios	10.5	6.0	1.0
	Quintets	7.0	5.9	1.0
	KW p =	0.64	0.84	0.14

interactions was not different between the pairs and trios. Total amount of time spent by individuals in different founding group sizes on adult interactions was not significantly different in 2007 at 30 ($p = 0.07$), 60 ($p = 0.11$), or 70 ($p = 0.21$) days post-establishment. The amount of time spent interacting with co-founders was significantly different at 40 ($p = 0.001$) and 50 ($p = 0.009$) days post-establishment, with quintets spending significantly more time on these behaviors than trios, and trios significantly more than pairs.

The opposite pattern was seen in 2008, with individuals in pairs and trios interacting with each other significantly more frequently than individuals in quintets (Table 3.3). The total time spent by individuals in pairs, trios, and quintets in 2008 on inter-adult interactions was not significantly different at 50 ($p = 0.99$), 70 ($p = 0.40$), and 80 ($p = 0.44$) days post-establishment. No aggression between cofounders was seen in either year.

Sex differences in interaction frequency were further examined in the 2007 quintets between 40 and 60 days post-establishment. Males frequently interacted with females and rarely with other males. In quintets, 15% of non-resting behaviors, up to one-half of all adult-adult interactions, involved a male stroking a female with his antennae or grooming her, while only 3–5% of non-resting behaviors were interactions between two males. Females interacted with males or other females equally (10% of non-resting behaviors).



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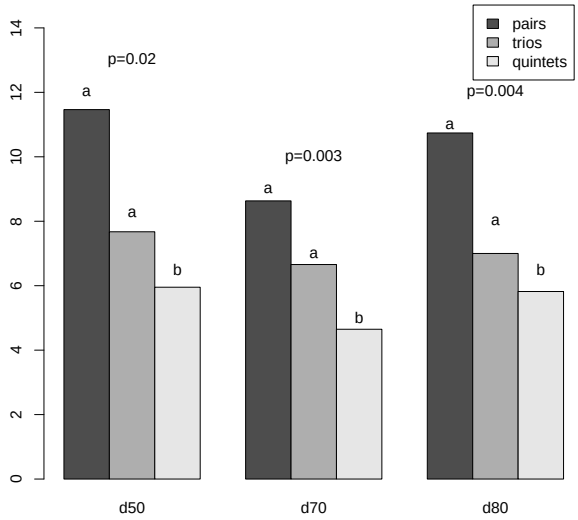


2008

Figure 3.3 Frequencies of adult interactions in 2007 and 2008 by founding group size. Kruskal-Wallis p-values are above each time point. Different letters within each time point indicate significant differences between groups (non-parametric multiple comparisons).

3.4 Discussion

Differences between the two years in both the frequencies of parental behavior and the patterns of co-founder interactions make it difficult to generalize about the affect of founding group size on behavior during colony establishment. The colonies observed in 2008 produced more offspring than those in 2007, and spent more time on care of eggs and larvae. The proportion of time spent interacting with co-founders was similar in the two years. This underscores the importance of cooperative brood care to incipient colony success, even though group size did not affect the frequency of parental behaviors or the amount of time spent on them.

Brood care behaviors, such as grooming of eggs and larvae on the part of both parents, are required for successful termite colony establishment (Rosengaus and Traniello 1991; Shellman-Reeve 1997). In *N. corniger*, these interactions with eggs and larva are similarly vital to the survival and development of a colony since untended eggs do not hatch and often succumb to pathogen infection (pers. obs.). The lack of parental care behavior by males in this study in 2007 could reflect sex-based differences in behavior, as found for replacement reproductives of *Kalotermitidae flavicollis* (Maistrello and Sbrenna 1999). However, that seems unlikely in light of the equal division of labor during brood care in the 2008 cohort in this study and in previous studies of with primary reproductives (Machida et al. 2001; Rosengaus and Traniello 1991).

Another important consideration is the fact that alates in these experiments were forced into pairs, trios, and quintets, without the opportunity to select from a pool of potential partners. The greater levels of parental care in 2008 may indicate more compatible co-founder combinations than those in the colonies observed in 2007. It is possible that cooperative behavior would have been more prevelant if individuals in the experiment had been allowed to choose their own mate and founding group size. Social context alters behavior during colony foundation in facultatively polygynous ants (Cahan and Fewell 2004; Jeanson and Fewell 2008), a process that could be more complicated in facultatively polygamous termites. Because typical termite colony foundation behavior already requires extensive cooperation between mates, the choice of co-founders in pleometrotic associations could disrupt or enhance behavioral interactions vital to colony establishment.

Chapter 4

Group Choice Experiments

Abstract

While ecological and genetic factors are known to impact founding group size in some facultatively polygamous social insects, their effects on founding group size decisions in termites has not been explored. The few previous studies have been natural experiments suggesting a strong role for ecological limitations imposed by weather and habitat suitability. To understand the effect nest site availability, abundance of potential mates, or colony of origin may have on founding group size, the number of nest sites and the pool of potential mates was manipulated in experimental ecologically-simple microcosms and larger, more complex mesocosms of the facultatively polygamous Neotropical termite *Nasutitermes corniger*. Alates from particular parental colonies were more likely to establish and maintain colonies in larger groups than were alates from other parental

colonies. Nest site availability did not factor into group size decisions in microcosm or mesocosm experiments. High alate density initially resulted in large founding groups in the microcosms, which decreased in size with time and mortality, although alate density did not impact group size choice in mesocosms. Queen and king weight was not a significant predictor of group size, but was a significant predictor of offspring production. Together, these results suggest that nest site and mate availability have minimal impact on the decision to found colonies pleometrotically, and that genetic factors may impact choice of founding group size as well as success in a pleometrotic group.

4.1 Introduction

Little is known about the factors affecting facultative polygamy in termites, in spite this phenomenon being phylogenetically widespread throughout the Termitidae (Thorne 1985*b*). In the derived fungus-growing African termite *Macrotermes michaelseni*, Brandl et al. (2001) found more polygamous colonies near the ecologically less-suitable edge of the species' range. Further, they found an impact of rainfall on the proportion of established colonies that had multiple queens two years later (Brandl et al. 2004), supporting the view that ecological factors impact group size choice and possibly the relative success of polygamous and monogamous colonies.

Group size choice in other facultatively-polygamous eusocial organisms may also be mediated by ecological pressures. Limitations on the availabil-

ity of nest sites (Herbers 1986) and food sources (Herbers and Banschbach 1998) facilitates polygyny in ants, however the effects are not consistent across habitats, and the influence of ecological factors on social organization can vary over time (Deheer et al. 2001). Variation in social structure may result in different life history attributes, including nest density, colony lifespan, and investment in and dispersal of reproductives (Rosset and Chapuisat 2007). In the case of the fire ant *Solenopsis invicta*, polygyny has a proven genetic basis (Gotzek and Ross 2007).

To date, no studies have directly examined the factors that may impact founding group size choices in termites. To remedy this lack, the derived, facultatively-polygamous Neotropical termite *Nasutitermes corniger* was used to investigate group size choices by termite reproductives during colony foundation. To assess the impact of ecological factors, namely nest site availability and alate density, as well as genetic background on founding group size choice, marked alates from multiple parental colonies were released into micro- and mesocosms at different nest site:alate densities. Their founding group size choices and resulting reproductive success were followed for 80 days.

4.2 Methods

Alate collection

Pieces of carton nest material were excised from mature parent colonies in Gamboa, Panamá in April and May 2009, and the alates extracted using forceps. For use in microcosm experiments, alates were sorted by sex. To create more natural conditions, alates used in mesocosm experiments were not sorted by sex but used in the natural sex ratio of the sample without selection. Individuals in both experiments were color-coded with a dot of enamel paint, and maintained on moist Whatman #1 filter paper in petri dishes until use, within 24 hours. To maintain as much correspondence between the two experiments as possible, ten parental colonies supplied alates to both microcosm and mesocosm experiments. One additional colony supplied alates to the microcosms (total parental colonies = 11), and two additional parental colonies supplied alates to the mesocosms (total parental colonies = 12). Dead mango wood used in microcosms and mesocosms was from the same tree, and all in a similar state of decay.

Microcosms

To examine the effect of different alate densities, simple arenas were designed with a fixed number of nest sites. Microcosms were constructed by gluing two 30 mm dishes into a 100 mm petri dish lined with Whatman #1 filter paper (Fig. 4.1). The smaller dishes were also lined with filter

paper, and had one opening in the side through which alates could enter and exit. Chips of dead mango wood, approximately 1.0 cm x 2.5 cm x 0.5 cm, were placed in each dish and in the spaces between dishes to create 4 potential nesting sites per microcosm (A–D, Fig. 4.1). This design was chosen to minimize accidental movement of wood pieces during handling, yet give termites ample room for tandem running courtship behavior, in which the male closely follows the female as she searches for a nest site. The small dishes were labeled to facilitate consistent position tracking over the course of the experiment. Filter paper and wood were moistened before alates were placed in the microcosms, and remoistened as needed over the course of the experiment.

A total of 320 replicates were established as nestmate (all from the same parent colony; n = 120) or non-nestmate (alates from multiple colonies; n = 200) groups of 5, 7, 10, or 14 alates. Sex ratios were male-biased, 2♀:3♂or 3♀:4♂, to reflect the sex ratios of alates found in the field. Microcosms were examined at 24 and 48 hours post-establishment, every 5 days from day 5 through day 30, then every 10 days from day 30 through day 80 post-establishment. Alates were marked with a dot of enamel paint to facilitate tracking (see Chapter 3). The position of each individual was noted at each time point, as well as deaths, and presence and number of eggs, larvae, workers, and soldiers.

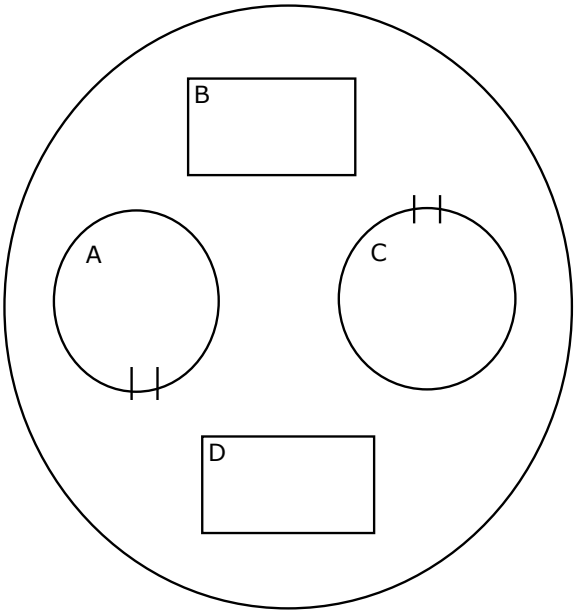


Figure 4.1 Top: Schematic of group choice microcosm (100 mm diameter). Wood chips were placed within 30 mm petri dishes A and C that termites could access through a door cut into the side wall. B and D are additional wood nesting sites, equal in size to those inside dishes A and C. Drawing is not to scale. Bottom: Microcosm experiment in progress.

Mesocosms

As a snapshot of initial founding group size choices under more natural conditions, short-term mesocosms were established in shallow plastic boxes, 25.0 cm x 30.5 cm x 5.0 cm, filled with 2 cm of soil from which arthropods and stones had been removed. Fragments of dead mango wood (up to 3.0 cm x 10.0 cm x 1.0 cm) covering 50% of the soil surface area were lightly pressed into the soil. A total of 138 marked alates from seven parent colonies were released into five replicates (n = 34, 34, 34, 19, 17) on three start dates complementary to the other experiments. Experimental arenas were destructively sampled at 72-hours post-establishment.

Longer-term mesocosms were designed to examine the interaction between alate density and nest site availability under similar, more natural conditions (Fig 4.2). Mesocosm arenas were established in deep plastic boxes, 20.0 cm x 30.5 cm x 15.0 cm, filled with 6 cm of soil from which arthropods and large stones had been removed. Dead mango branches, cut to length to standardize both surface area and volume, were randomly chosen to be placed in the mesocosms whole (1 piece), cut in half then split in two (4 pieces), or cut in half and split four times (16 pieces). Soil and wood were moistened 24 hours prior to the initiation of each replicate, and as needed throughout the experiment.

Color-coded alates, marked with a dot of enamel paint, were released into each mesocosm at high (150 alates, 30/parent colony) or low (75 alates, 15/parent colony) densities (Fig 4.2). One replicate of each treatment was

Figure 4.2 Top: Mesocosm arenas were developed to mimic natural conditions in sites where *N. corniger* alates had previously been observed settling during dispersal flights. Photo: Casey Hamilton. Bottom: Alates were marked with enamel paint by colony of origin, then released en masse into each mesocosm replicate.

established on each of five start dates. Due to differences in alate production by parent colonies, and their use in other contemporaneous experiments, combinations of parent colonies were different in each replicate. Lids were placed tightly on the boxes for the first 24 hours to encourage experimental alates to remain in the mesocosm, then placed loosely on the boxes until the end of the dispersal flights to prevent ingress of non-experimental conspecific alates. Once alates were no longer dispersing from their natal nests, the lids were removed entirely. The arenas remained undisturbed under ambient (Panamá) conditions until destructive sampling at 60 days post-establishment.

When destructively sampling the mesocosm experiments, the wood pieces were carefully lifted and broken apart, and the soil was systematically removed to recover, intact, both live and dead incipient colonies. Recovered queens and kings were weighed, and all offspring were counted.

Statistical analysis

The effects of parental colony of origin were tested using Kruskal-Wallis tests (KW), followed by non-parametric multiple comparisons as indicated (Giraudoux 2009). The binomial proportion test or chi-squared test was used for comparisons between proportions. Correlations involving chosen group size, mass, and offspring number utilized Kendall's rank correlation, as appropriate for data that do not necessarily come from a bivariate normal distribution.

A generalized linear mixed model (GLMM) was applied separately to each data set (Bates and Maechler 2009). The initial model for microcosms included the number of alates originally placed in the arena, their relatedness, the interaction between number of alates and relatedness, and sex as fixed factors. Fixed factors in the initial 72-hour mesocosm model were sex, weight, and their interaction. Alate density, number of nesting sites, and the interaction between them were included as fixed factors in the initial 60 day mesocosm model, in addition to those used in the 72-hour model. Colony of origin was included as a random factor for all three analyses. The model was simplified in a stepwise manner, beginning with interactions, and each new model compared with the previous using ANOVA. All statistical analysis was conducted in R (R Development Core Team 2009, v 2.9.2).

4.3 Results

Microcosms

Mortality was high during the course of this experiment, consistent with results presented in Chapter 2, and group sizes generally decreased over time. Few alates from parent colonies 820 and 901 were used in the experiments, and many of them died before 5 days post-establishment, so they were excluded from the statistical analysis. Independent, non-interconnecting, incipient colonies were occasionally found within the same piece of wood (Fig 4.3), and were treated as separate colonies in the analysis.

Figure 4.3 Independent colonies, separated by constructed walls, found in the same piece of wood in a microcosm.

In most mesocosm replicates, group composition was stable by 5 days, and changed only due to mortality. Following the death of one or more founders, colonies would occasionally change locations, break up, or accept additional member(s). Most individuals who had lost their mate(s) joined another group, although some remained alone. However some large founding associations in the higher alate density treatments did break up into smaller groups after 5 days post-establishment without the impetus of mortality. No sex differences in founding group size preference were detected at any time point or starting density analyzed (for each, KW $p > 0.05$).

The number of alates originally placed in the microcosm, and their relatedness (Table 4.1), significantly predicted founding group size at all four time points (KW $p < 0.01$). As would be expected, in microcosms with fewer individuals, smaller groups formed at 5 days than in those with more individuals. However, as time and mortality continued, the number of individuals originally placed in the arena became less a predictor of chosen group size, and relatedness a stronger predictor of chosen group size.

In general, alates initially formed larger founding groups in all-nestmate treatments than when placed in arenas with non-nestmates (Table 4.1 and Fig 4.4). Except for arenas initiated with 7 individuals, by 30 days larger founding groups were found in non-nestmate treatments than in the corresponding all-nestmate replicates. By 60 days, chosen founding group size was significantly higher in non-nestmate arenas established with 5, 10, or 14 individuals than in the all-nestmate arenas.

Table 4.1 Chosen founding group sizes in microcosms. Treatment combines relatedness (nm, nestmate; nnm, non-nestmate) and number of individuals originally in the arena. Total = number of surviving individuals from all arenas. Significant differences (KW p <0.01) were detected at each census; heterogeneous subgroups are indicated by different letters within each time point.

	treatment	total	mean group size	
day 5	nnm5	193	3.5	C
	nm5	138	4.1	C
	nnm7	256	4.8	C
	nm7	192	6.0	C
	nnm10	375	6.9	B
	nm10	273	8.6	B
	nnm14	479	10.1	A
	nm14	390	11.6	A
day 15	nnm5	151	3.4	G
	nm5	126	4.1	F
	nnm7	173	4.2	E
	nm7	161	5.3	D
	nnm10	213	5.7	C
	nnm14	279	7.5	B
	nm10	207	7.5	B
	nm14	290	9.7	A
day 30	nm14	28	3.1	F
	nm5	75	3.1	E
	nnm5	92	3.1	D
	nnm7	91	3.4	D
	nm7	53	3.7	C
	nm10	37	3.6	C
	nnm10	106	4.4	B
	nnm14	92	5.6	A
day60	nm10	6	2.0	F
	nnm7	35	2.3	E
	nm5	23	2.5	D
	nm14	5	2.6	D
	nm7	15	2.6	D
	nnm10	26	2.8	C
	nnm5	47	3.1	B
	nnm14	20	3.8	A

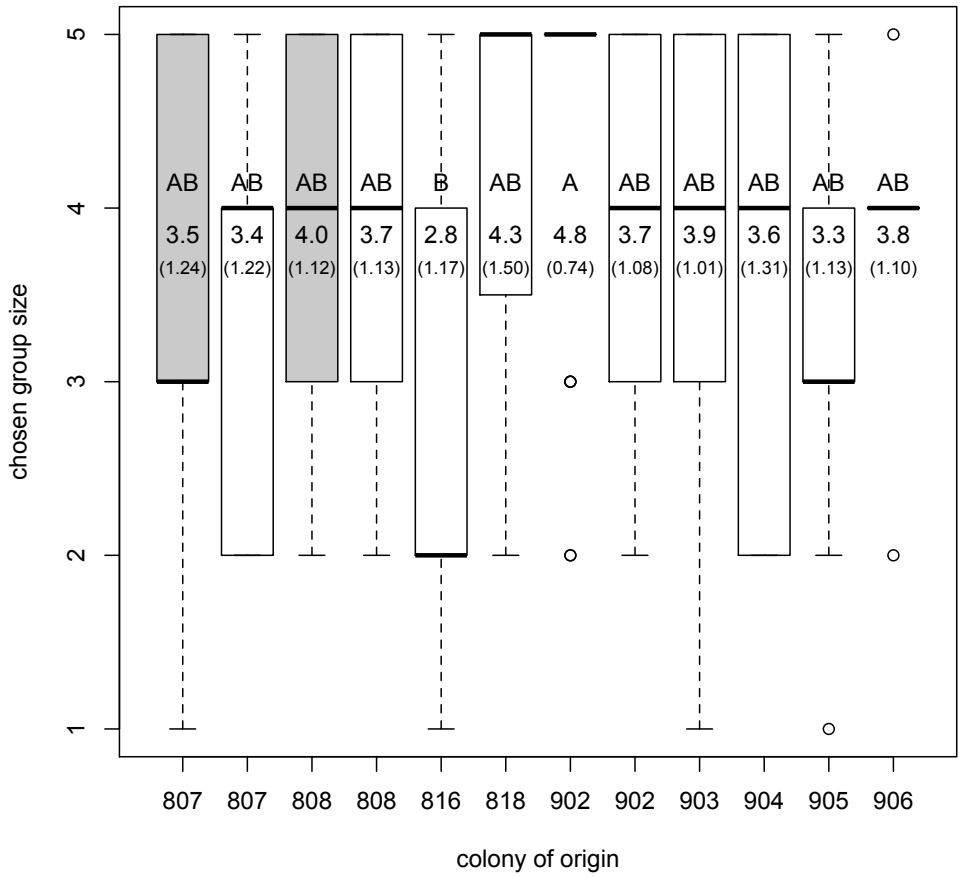


Figure 4.4 Boxplot of group sizes chosen by individuals in microcosms initiated with 5 members, at 5 days post-establishment. Dark horizontal lines within each box are median group sizes, by colony of origin. Shaded boxes represent the all-nestmate treatment, and open boxes the non-nestmate treatment. Outliers are represented by open circles. Numbers within each column indicate the mean (standard deviation). Overall mean group size is 3.8 (1.20). Chosen founding group size is significantly different based on colony of origin (Kruskal-Wallis $p < 0.001$); heterogenous subgroups determined by non-parametric multiple comparisons are indicated on the figure by different letters. Males and females did not chose different founding group sizes (KW $p = 0.63$).

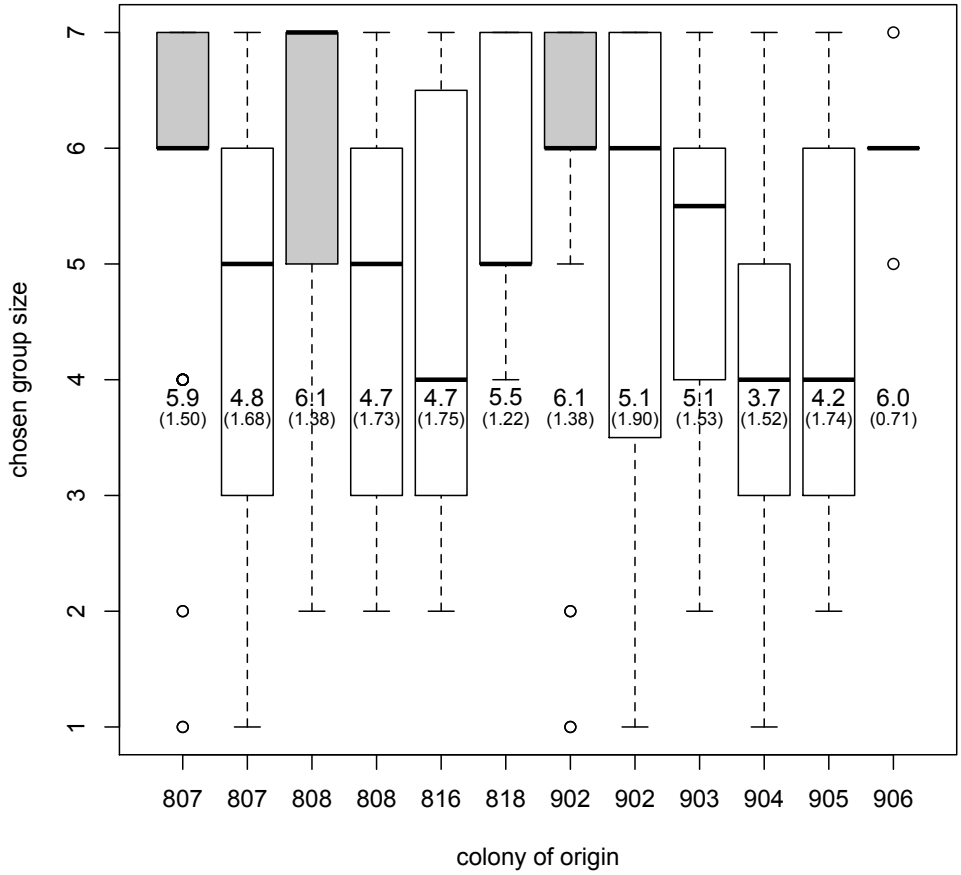


Figure 4.4, continued from previous page. Boxplot of group sizes chosen by individuals in microcosms initiated with 7 members, at 5 days post-establishment. Overall mean group size is 5.3 (1.72). Chosen founding group size is significantly different based on colony of origin (Kruskal-Wallis $p < 0.001$), however no heterogenous subgroups could be detected using non-parametric multiple comparisons. Males and females did not chose different founding group sizes (KW $p = 0.80$).

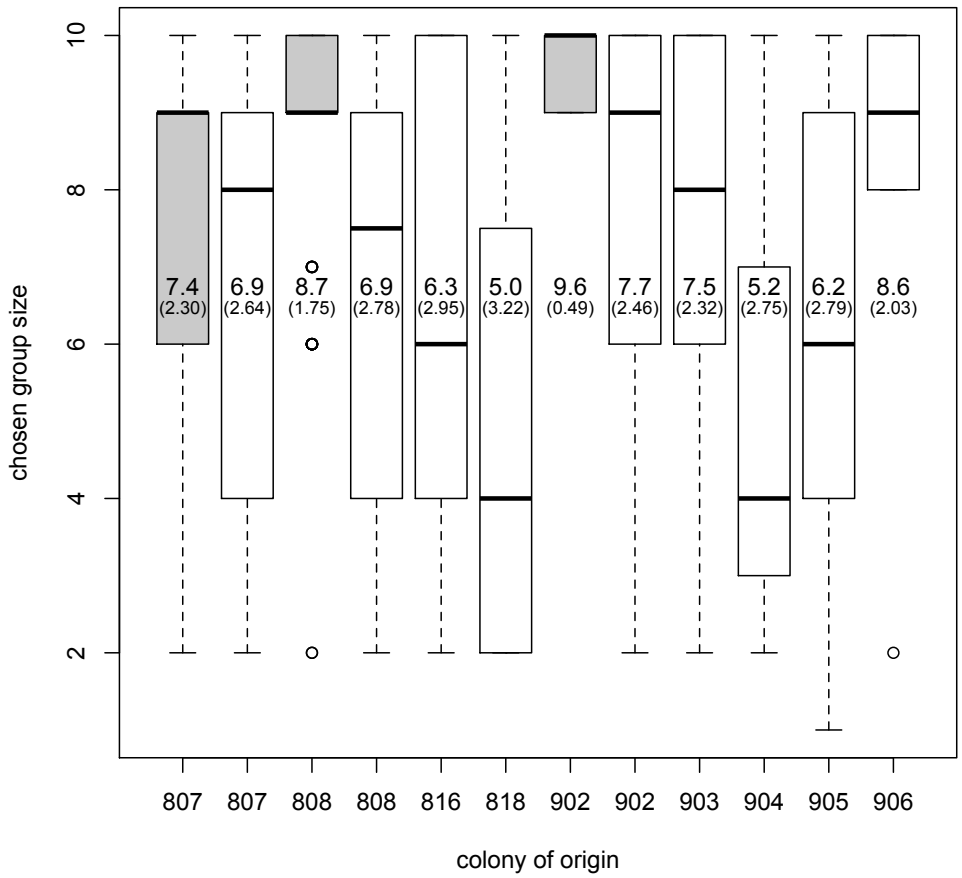


Figure 4.4, continued from previous page. Boxplot of group sizes chosen by individuals in microcosms initiated with 10 members, at 5 days post-establishment. Overall mean group size is 7.6 (2.56). Chosen founding group size is significantly different based on colony of origin at both time points (Kruskal-Wallis $p < 0.001$), however no heterogenous subgroups could be detected using non-parametric multiple comparisons. Males and females did not chose different founding group sizes (KW $p = 0.77$).

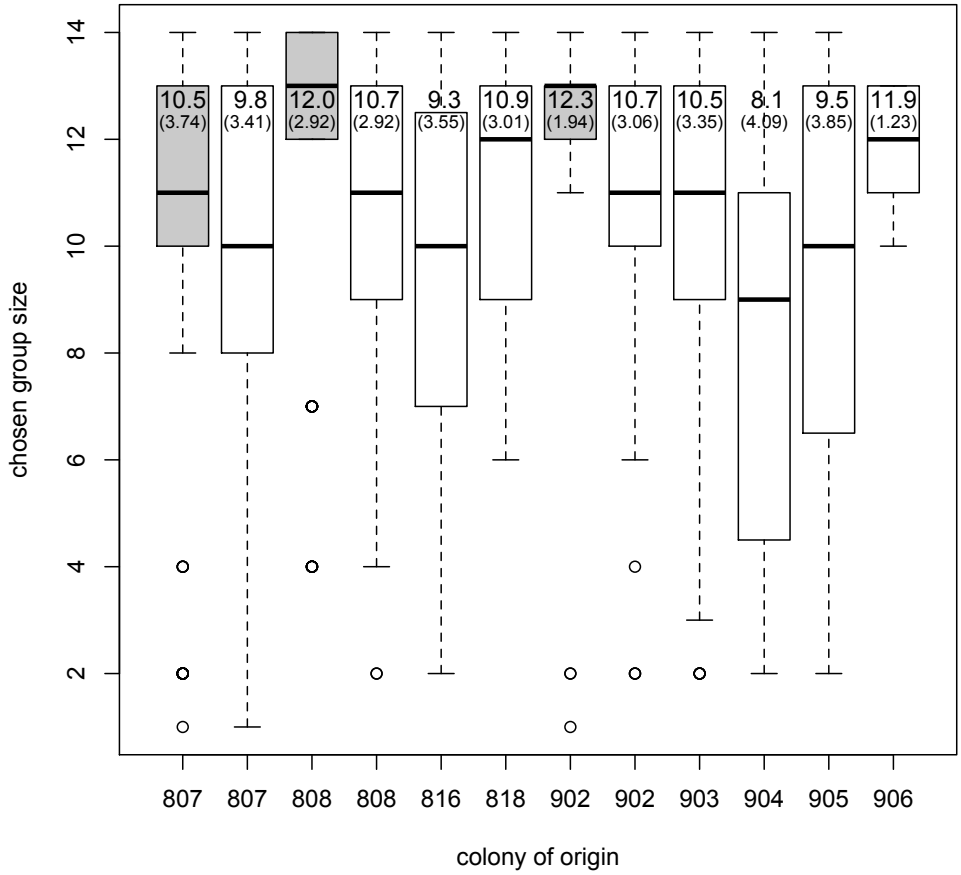


Figure 4.4, continued from previous page. Boxplot of group sizes chosen by individuals in microcosms initiated with 14 members, at 5 days post-establishment. Overall mean group size is 10.8 (3.34). Chosen founding group size is significantly different based on colony of origin (Kruskal-Wallis $p < 0.001$), however no heterogenous subgroups could be detected using non-parametric multiple comparisons. Males and females did not chose different founding group sizes (KW $p = 0.90$).

Mortality differed between individuals of the same parental colony placed in all-nestmate or non-nestmate treatments, so when assessing the impact of colony of origin, individuals from those colonies (807, 808, and 902) were analyzed as two separate groups. Individuals from different parental colonies chose significantly different founding group sizes at the beginning of the experiment (Fig 4.4, KW $p<0.001$ for each alate density), although heterogenous subgroups could only be detected for arenas initiated with 5 individuals. Similarly, founding group size was significantly different based on parental colony 15 days post-establishment for each initial alate density (Fig 4.5, KW $p<0.001$ each), however significant differences could only be detected for the treatment with initial alate density of 14 individuals.

While all individuals in the arena could be found together in some replicates at 5 and 15 days post-establishment, that was no longer the case at 30 days (Fig 4.6). Individuals from parent colonies 818 and 902 were observed in the largest founding groups at day 30 in the replicates with initial densities of 7, 10, and 14 individuals, while individuals from parent colonies 807 and 808 placed in all-nestmate arenas were found in the smallest groups. Colony of origin was not a significant predictor of group size in microcosms initiated with five individuals at 30 days (Fig 4.6, KW $p = 0.41$).

By 60 days post-establishment, no groups with more than five founders remained in replicates of any starting density (Fig 4.7). No effect of parental colony of origin was found in microcosms initiated with 5 (KW $p = 0.57$) or 14 alates (KW $p = 0.14$) at 60 days post-establishment. The largest

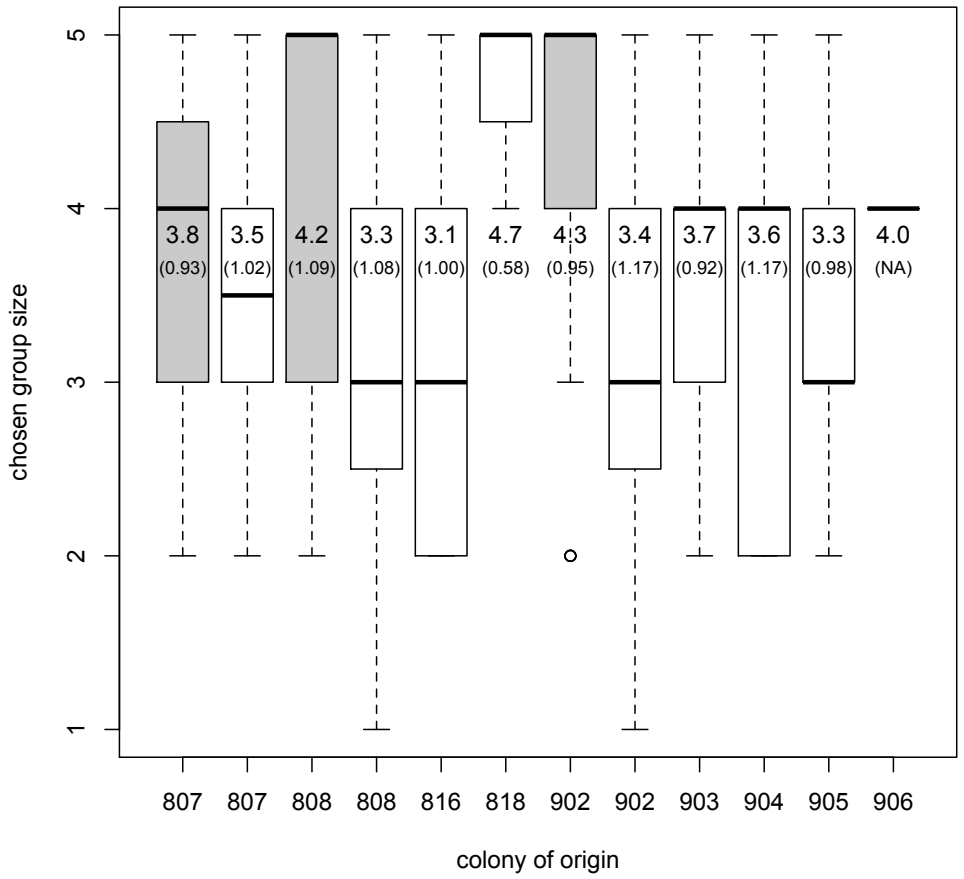


Figure 4.5 Boxplot of group sizes chosen by individuals in microcosms initiated with 5 members, at 15 days post-establishment. Dark horizontal lines within each box are median group sizes, by colony of origin. Shaded boxes represent the all-nestmate treatment, and open boxes the non-nestmate treatment. Outliers are represented by open circles. Numbers within each column indicate the mean (standard deviation). Overall mean group size is 3.7 (1.08). Chosen founding group size is significantly different based on colony of origin (Kruskal-Wallis $p < 0.001$), however no heterogenous subgroups could be detected using non-parametric multiple comparisons. Males and females did not chose different founding group sizes ($p = 0.95$).

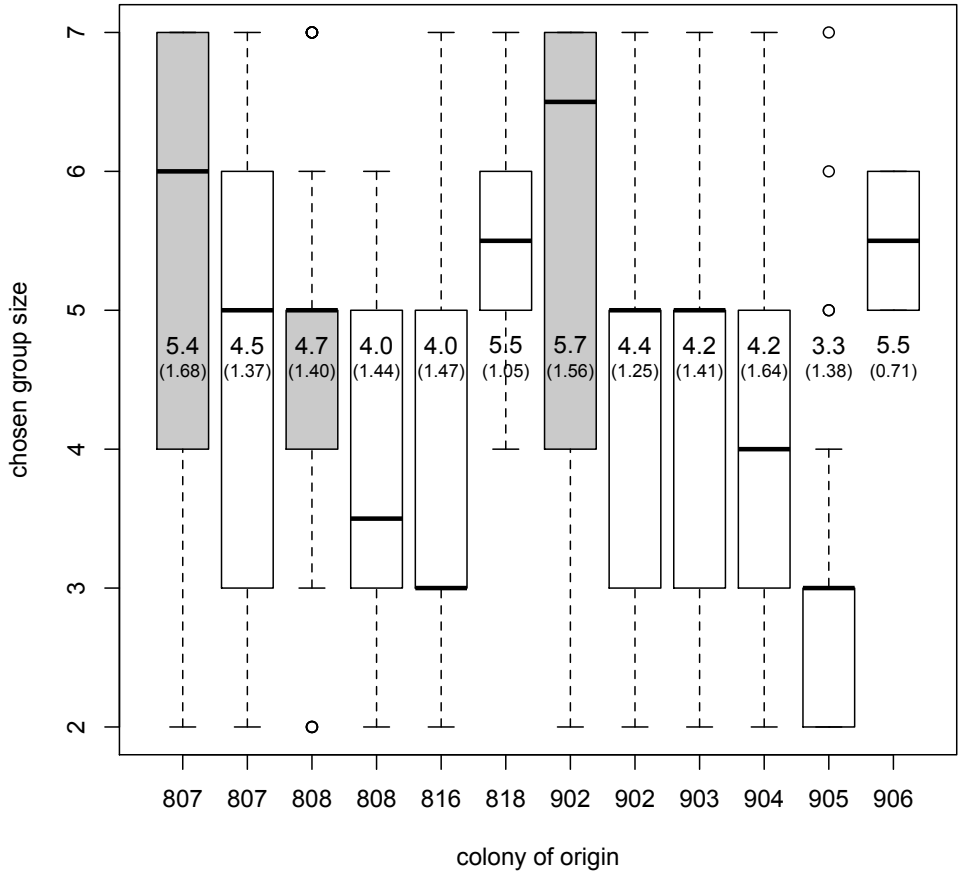


Figure 4.5, continued from previous page. Boxplot of group sizes chosen by individuals in microcosms initiated with 7 members, at 15 days post-establishment. Overall mean group size is 4.7 (1.59). Chosen founding group size is significantly different based on colony of origin (Kruskal-Wallis $p < 0.001$), however no heterogenous subgroups could be detected using non-parametric multiple comparisons. Males and females did not chose different founding group sizes at 5 days (KW $p = 0.80$) or at 15 days ($p = 0.86$).

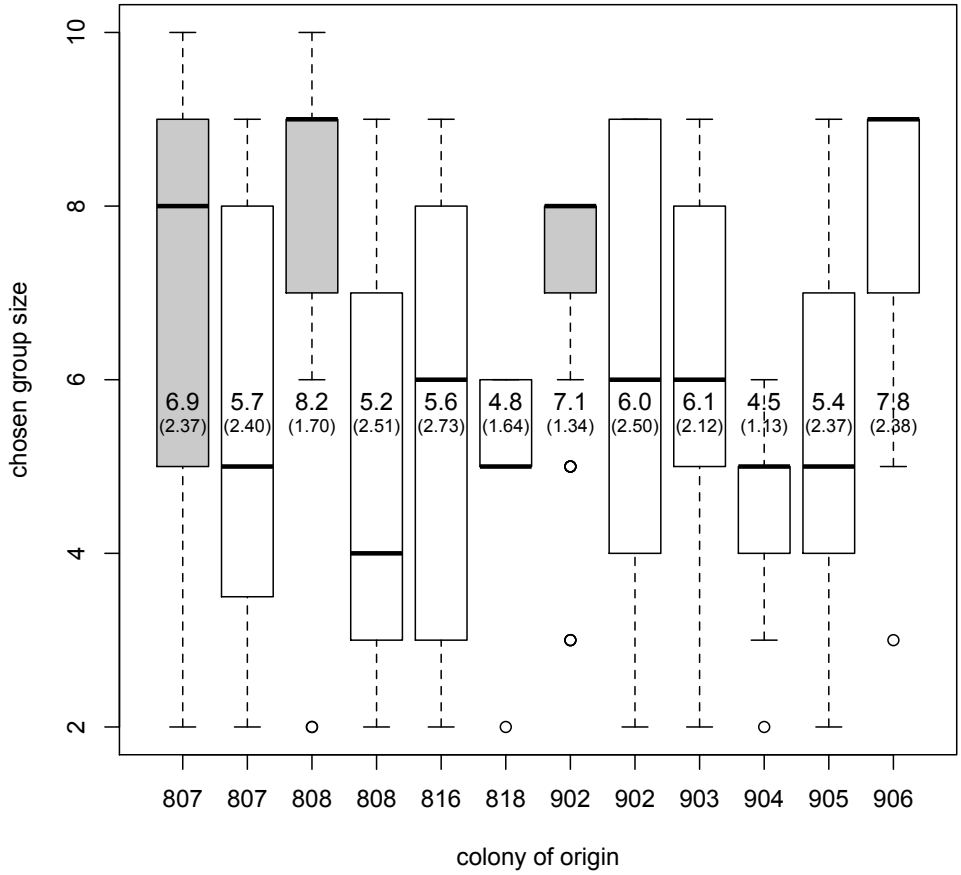


Figure 4.5, continued from previous page. Boxplot of group sizes chosen by individuals in microcosms initiated with 10 members, at 15 days post-establishment. Overall mean group size is 6.5 (2.33). Chosen founding group size is significantly different based on colony of origin (Kruskal-Wallis $p < 0.001$), however no heterogenous subgroups could be detected using non-parametric multiple comparisons. Males and females did not chose different founding group sizes (KW $p = 0.54$).

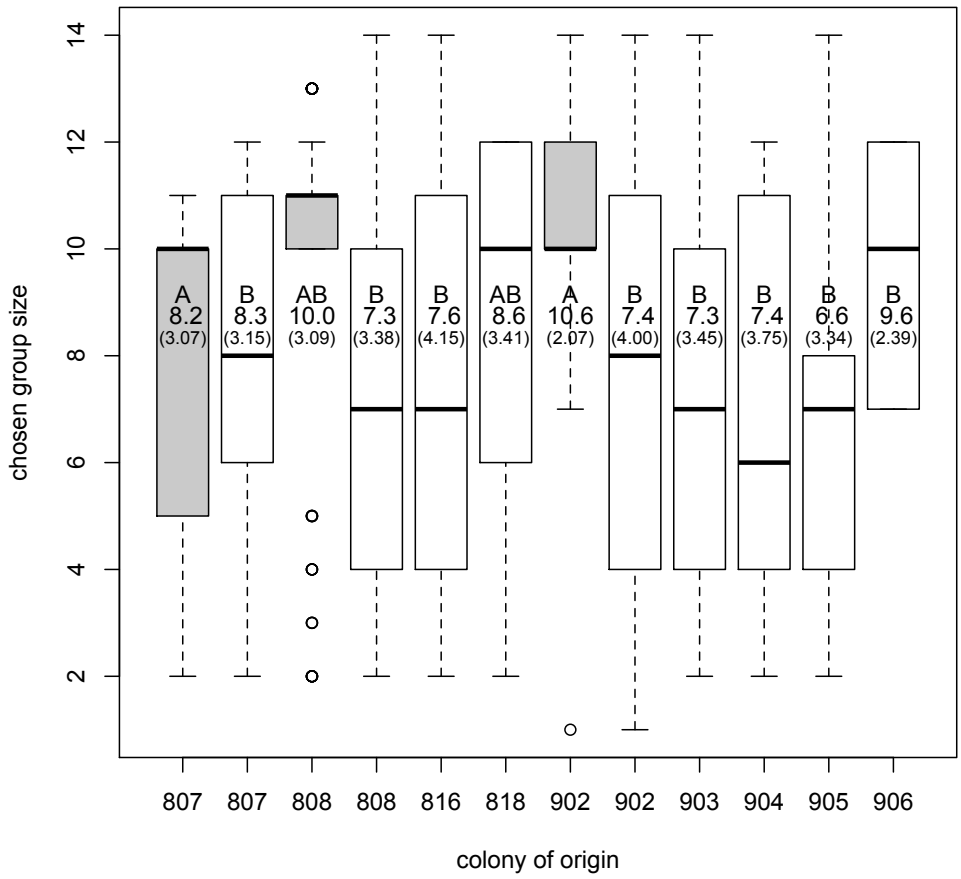


Figure 4.5, continued from previous page. Boxplot of group sizes chosen by individuals in microcosms initiated with 14 members, at 15 days post-establishment. Overall mean group size is 8.7 (3.39). Chosen founding group size is significantly different based on colony of origin (Kruskal-Wallis $p < 0.001$ for each); heterogenous subgroups determined by non-parametric multiple comparisons are indicated on the figure by different letters. Males and females did not chose different founding group sizes at 5 days (KW $p = 0.90$) or at 15 days ($p = 0.82$).

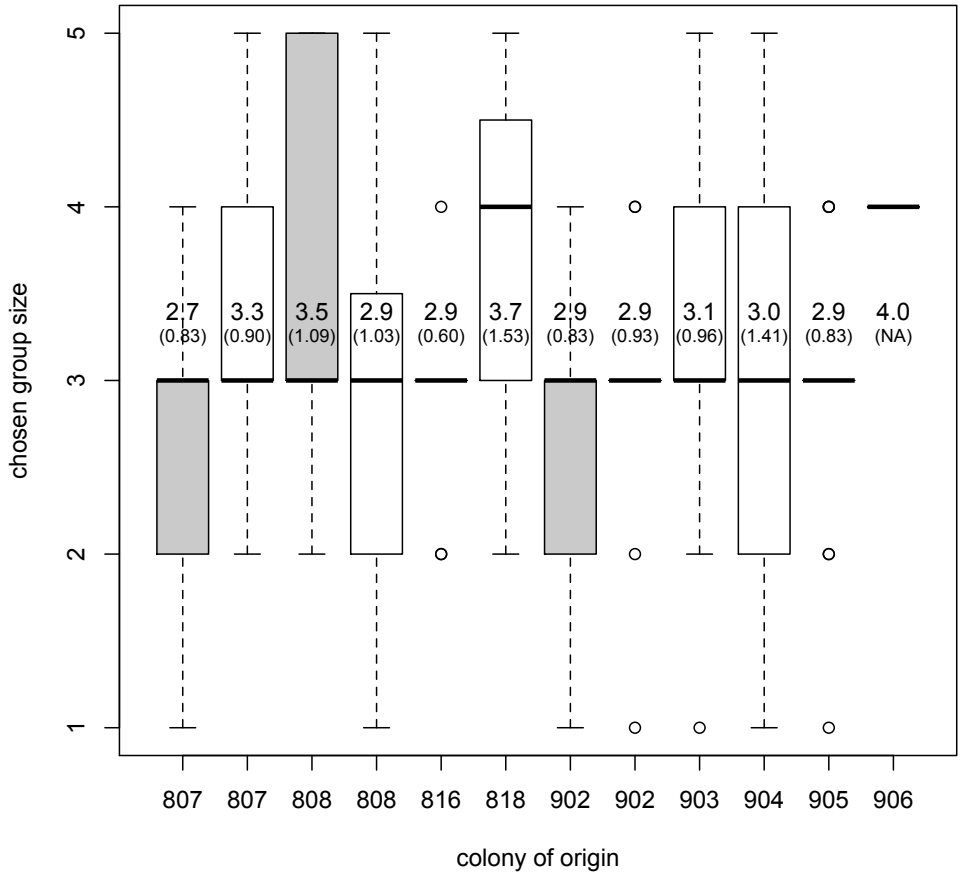


Figure 4.6 Boxplot of group sizes chosen by individuals in microcosms initiated with 5 members, at 30 days post-establishment. Dark horizontal lines within each box are median group sizes, by colony of origin. Shaded boxes represent the all-nestmate treatment, and open boxes the non-nestmate treatment. Outliers are represented by open circles. Numbers within each column indicate the mean (standard deviation). Overall mean group size is 3.1 (0.98). Chosen founding group size is not significantly different based on colony of origin (Kruskal-Wallis p = 0.41). Males and females did not chose different founding group sizes (KW p = 0.95).

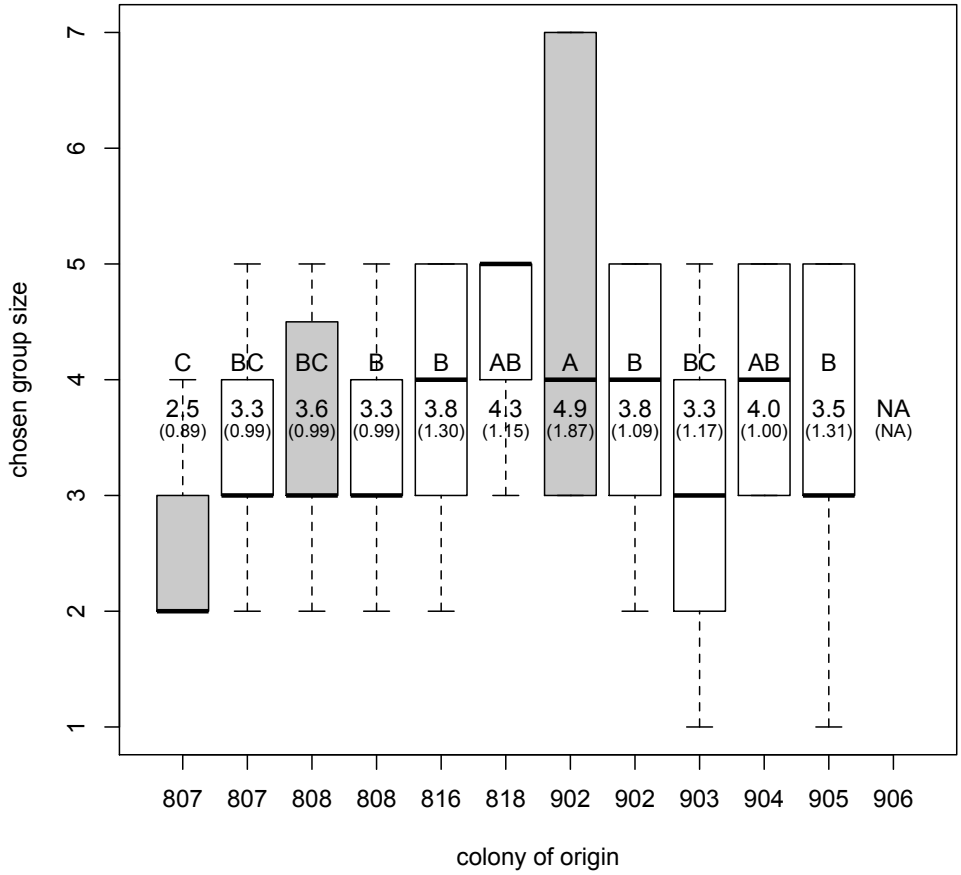


Figure 4.6, continued from previous page. Boxplot of group sizes chosen by individuals in microcosms initiated with 7 members, at 30 days post-establishment. Overall mean group size is 3.6 (1.31). Chosen founding group size is significantly different based on colony of origin (Kruskal-Wallis $p = 0.003$); heterogenous subgroups determined by non-parametric multiple comparisons are indicated on the figure by different letters. Males and females did not chose different founding group sizes (KW $p = 0.92$).

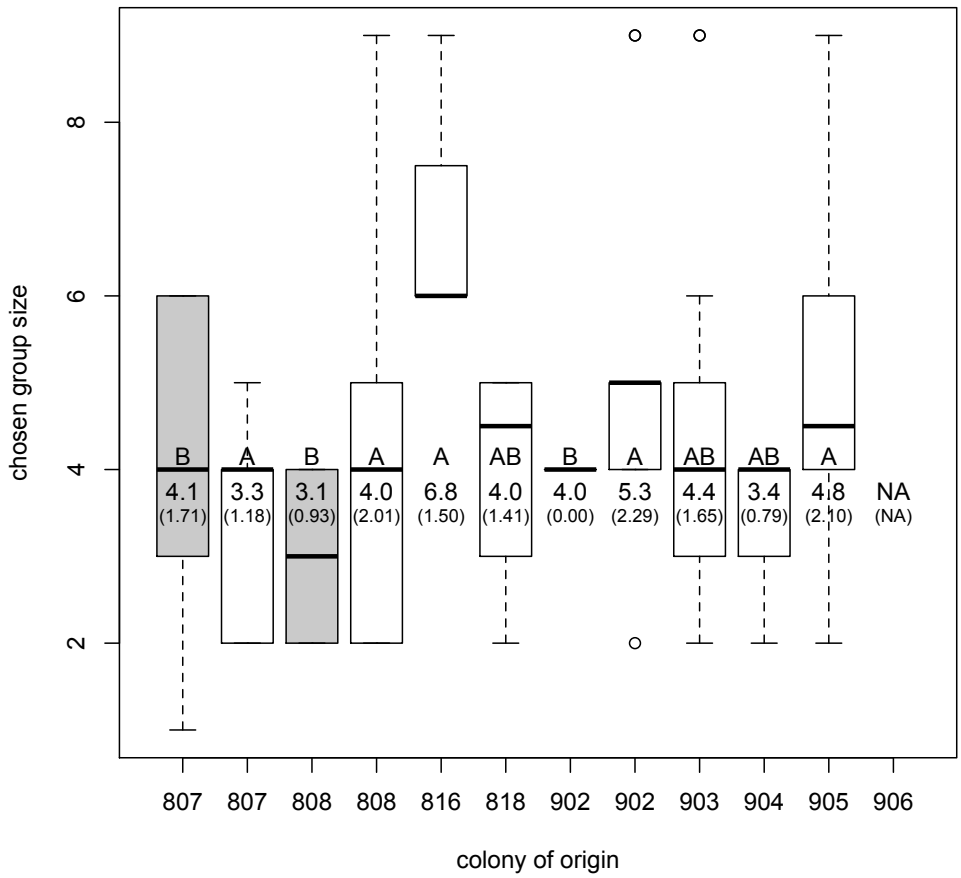


Figure 4.6, continued from previous page. Boxplot of group sizes chosen by individuals in microcosms initiated with 10 members, at 30 days post-establishment. Overall mean group size is 4.2 (1.77). Chosen founding group size is significantly different based on colony of origin (Kruskal-Wallis $p = 0.004$); heterogenous subgroups determined by non-parametric multiple comparisons are indicated on the figure by different letters. Males and females did not chose different founding group sizes (KW $p = 0.90$).

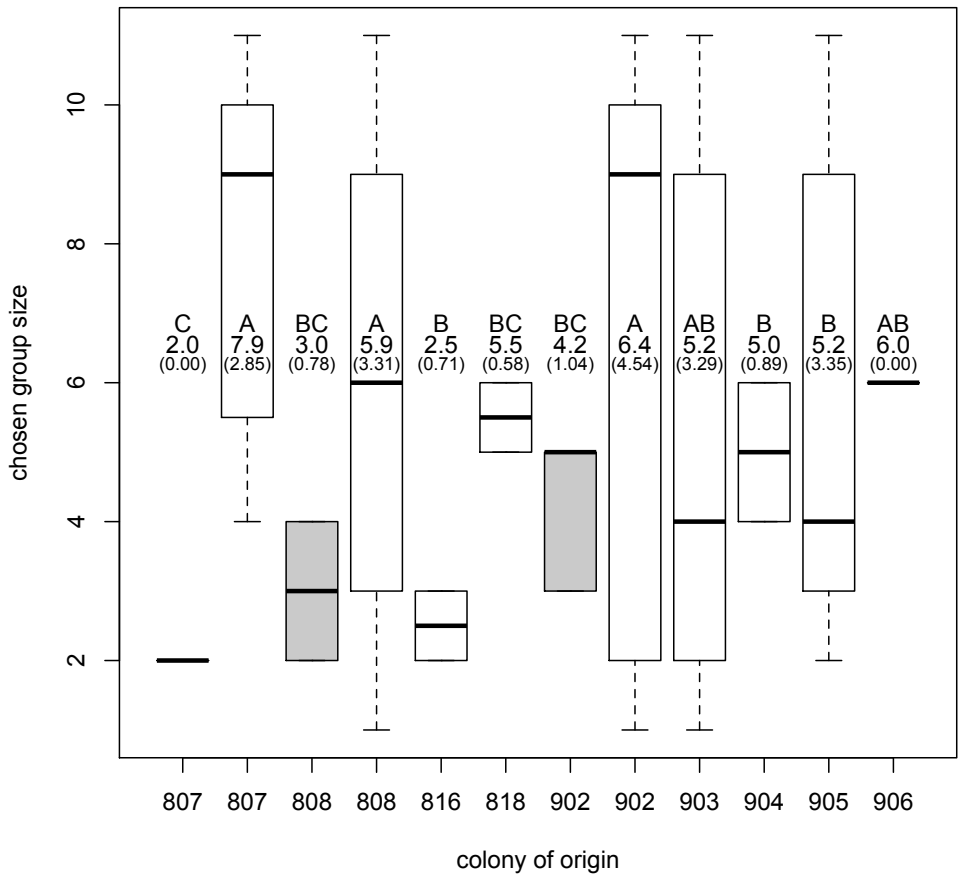


Figure 4.6, continued from previous page. Boxplot of group sizes chosen by individuals in microcosms initiated with 14 members, at 30 post-establishment. Chosen founding group size is significantly different based on colony of origin (Kruskal-Wallis p = 0.004); heterogenous subgroups determined by non-parametric multiple comparisons are indicated on the figure by different letters. Males and females did not chose different founding group sizes (KW p = 0.45).

founding groups observed at 60 days contained individuals from parent colonies 904, 905, and 808.

Individuals from particular parent colonies were more likely to be found in smaller groups throughout the experiment, while individuals from certain other parent colonies were more likely to be found in larger groups. Reproductives from parent colonies 818 and 903 were generally found in larger-than-average groups, and those from 816 in smaller-than-average groups. Individuals from parent colony 902 were in large founding groups at 5 days, but were found only in smaller groups by 60 days post-establishment.

When this data was analyzed in a Generalized Linear Mixed Model (GLMM), it confirmed that sex was not an important predictive factor for founding group size at any time point tested. The interaction between number of alates in the treatment and their relatedness contributed significantly to the model at 5 and 30 days. Both factors were retained at 15 days, but their interaction was not. At 60 days, only the number of alates originally placed in the microcosm as well as the random factor of colony of origin were retained in the model, and relatedness no longer had a significant impact on founding group size.

Mesocosms

Recovery of alates (now de-alate queens and kings of incipient colonies) was 100% at 72 hours, but only 14% (475 out of 3375 total) at 60 days post-establishment (Fig 4.8). While this is lower than survival in the no-choice

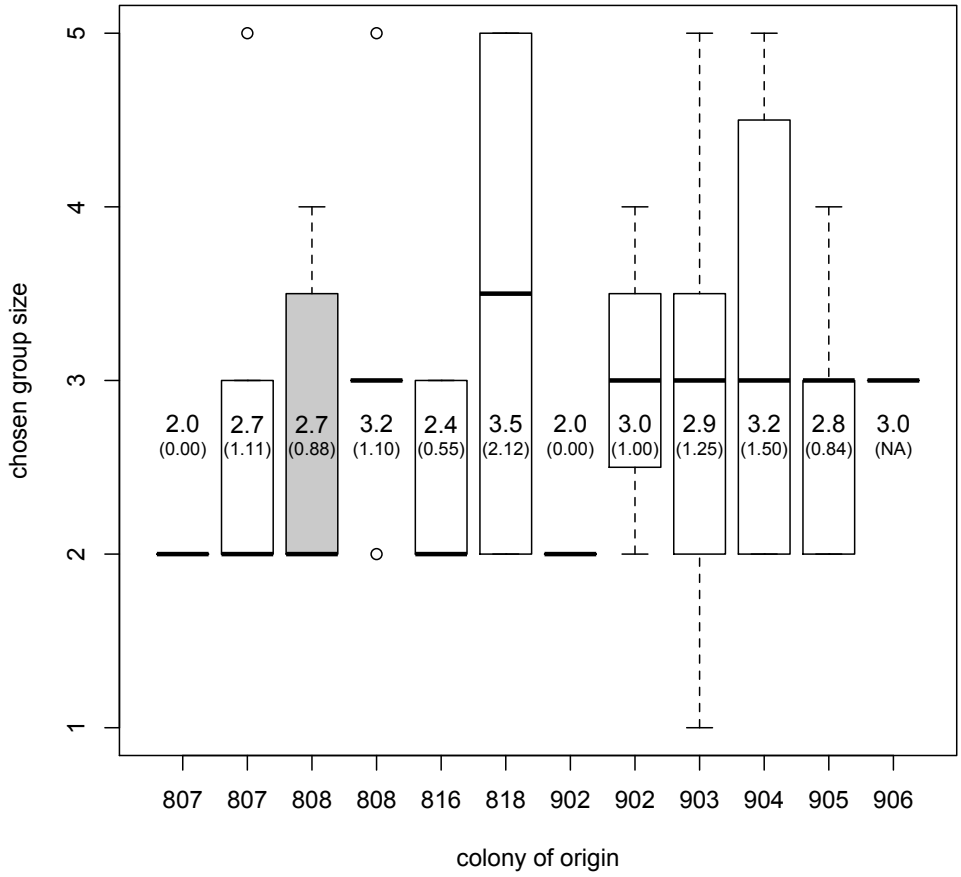


Figure 4.7 Boxplot of group sizes chosen by individuals in microcosms initiated with 5 members, at 60 days post-establishment. Dark horizontal lines within each box are median group sizes, by colony of origin. Shaded boxes represent the all-nestmate treatment, and open boxes the non-nestmate treatment. Outliers are represented by open circles. Numbers within each column indicate the mean (standard deviation). Overall mean group size is 2.7 (0.98). Chosen founding group size is not significantly different based on colony of origin (Kruskal-Wallis p = 0.57). Males and females did not chose different founding group sizes (KW p = 0.67).

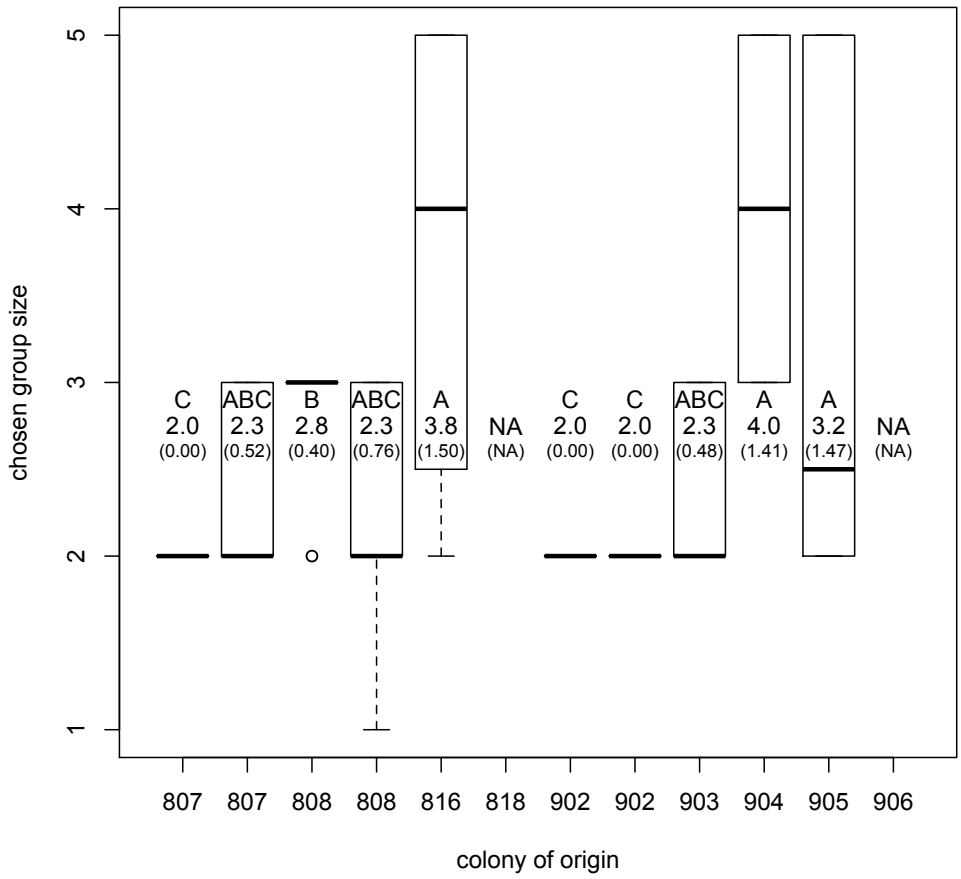


Figure 4.7, continued from previous page. Boxplot of group sizes chosen by individuals in microcosms initiated with 7 members, at 60 days post-establishment. Overall mean group size is 2.6 (0.90). Chosen founding group size is significantly different based on colony of origin (Kruskal-Wallis $p = 0.03$); individuals from parental colony 902 and from parental colony 807 placed with all nestmates were found in significantly smaller groups than those from all other parental colonies. Males and females did not chose different founding group sizes ($p = 0.91$).

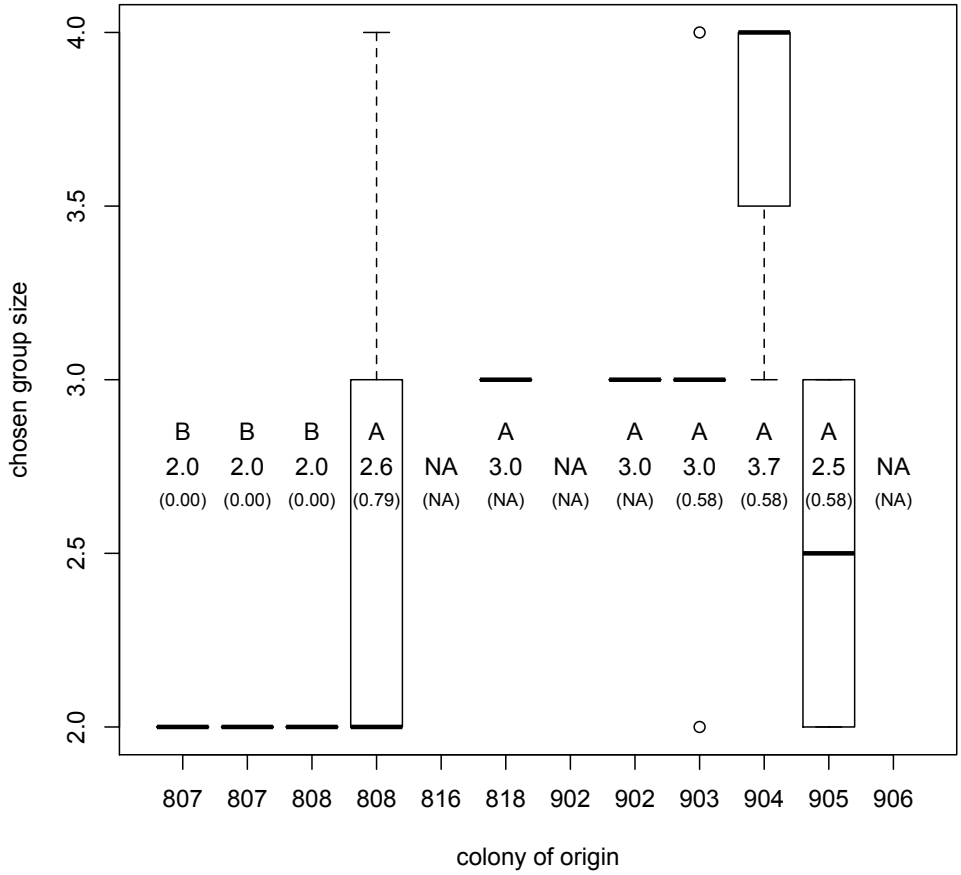


Figure 4.7, continued from previous page. Boxplot of group sizes chosen by individuals in microcosms initiated with 10 members, at 60 days post-establishment. Overall mean group size is 2.6 (0.71). Chosen founding group size is significantly different based on colony of origin (Kruskal-Wallis $p = 0.03$); heterogenous subgroups determined by non-parametric multiple comparisons are indicated on the figure by different letters. Males and females did not chose different founding group sizes (KW $p = 0.86$).

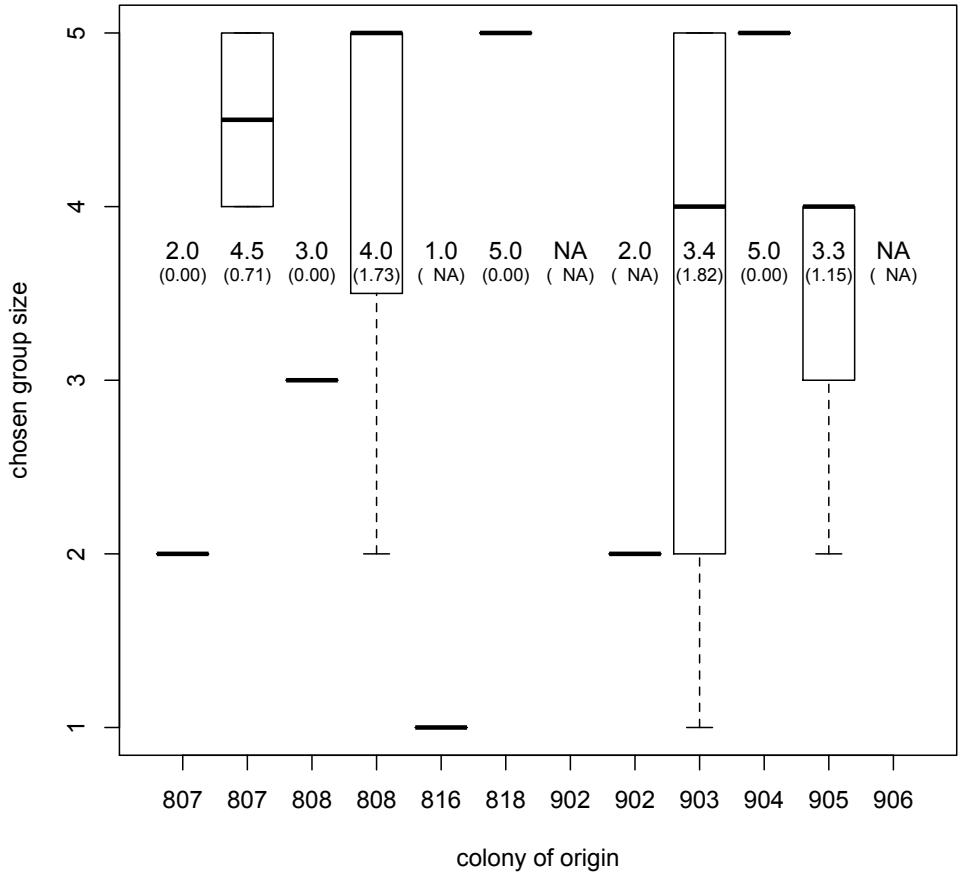


Figure 4.7, continued from previous page. Boxplot of group sizes chosen by individuals in microcosms initiated with 14 members, at 60 days post-establishment. Chosen founding group size is not significantly different based on colony of origin (Kruskal-Wallis $p = 0.14$). Males and females did not chose different founding group sizes (KW $p = 0.73$).

experiments (Chapter 2), it was expected that mortality could be higher under these semi-natural conditions (Fig 4.9). Recovery rates at 60 days ranged from 0–47%. There were significant differences in recovery rates at 60 days, but they were not systematically related to alate density, nest site availability, or replicate.

Chosen group size at 72 hours was not significantly different based on parent colony of the individual (Fig 4.10, KW $p = 0.92$). At 60 days, no relationship was found between chosen founding group size and initial alate density for all of the individuals recovered (Table 4.2, KW $p = 0.70$), nor when considering only those found alive (KW $p = 0.28$). No correlation was found between group size and nesting site availablity (all: $p = 0.41$; alive only: $p = 0.87$). There was also no significant difference between chosen group size (KW test, $p = 0.55$) or total offspring ($p = 0.59$) at 60 days as a function of colony of origin (Fig 4.11), so for the remaining analysis all 60 day data were pooled.

The majority of colonies recovered at both time points were monogamous pairs (Fig 4.12). At 72 hours, 29 out of 46 (63%) of recovered colonies were pairs. At 60 days, 103 out of 116 (89%) of colonies found alive were pairs, as were 135 out of the total 252 (54%) colonies recovered. The relative proportions of each colony size were significantly different at the two time points (chi-squared = 21.1, df = 4, $p<0.001$).

The proportion of non-nestmate groups was not significantly different between the two time points (binomial proportion test, $p = 0.50$). Non-

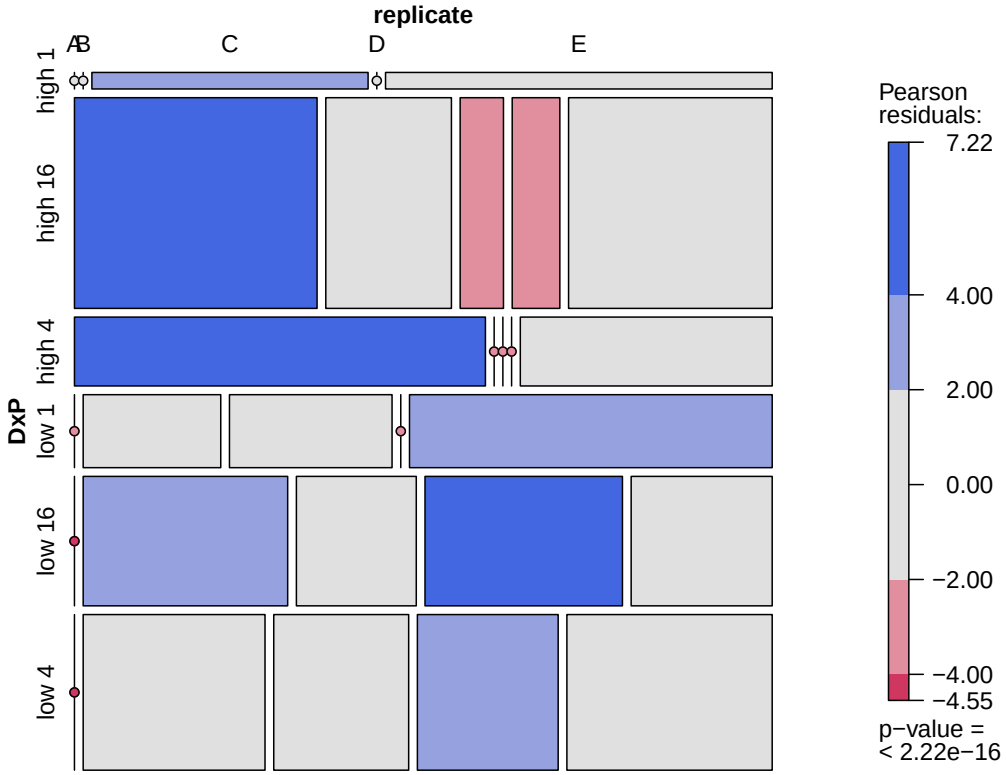


Figure 4.8 Mosaic plot of number of individuals recovered dead or alive in 60-day mesocosms. Each tile represents the number of individuals recovered from each replicate of each density by nest site (DxP) treatment. Colors indicate values of the standardized residuals; shades of blue are positive (larger number of individuals than expected in the case of independence), shades of red are negative (fewer individuals than expected). The p-value (chi-squared test), indicates that there are significant differences in recovery, however they are not attributable to alate density, nest site availability, or establishment date (replicate).

Figure 4.9 Incipient colonies recovered from mesocosms at 60 days post-establishment. The other part of each colony, including mates and offspring, remained on the soil surface when this piece of wood was lifted. Note the proximity of neighboring colonies. Photo: Casey Hamilton

Table 4.2 Correlations with group size in mesocosms at 72 hours and 60 days post-establishment. No correlations are available between group size and alate density or nest site availability at 72 hours because there was only one treatment level. For day 60, correlations between group size and alate density or nest site were examined for all individuals recovered (all) and for only those found alive (alive). Total weight is the sum of the masses of all reproductives found in an incipient colony.

	Group Size, 72h		Group Size, 60d	
	Kendall's τ	p-value	Kendall's τ	p-value
alate density (all)			-0.02	0.70
nest sites (all)			0.04	0.41
alate density (alive)			-0.07	0.28
nest sites (alive)			0.01	0.87
total offspring	0.12	0.64	-0.05	0.50
female weight	0.01	0.91	-0.15	0.05
male weight	-0.15	0.13	-0.003	0.97
total weight	0.73	< 0.001	0.43	< 0.001

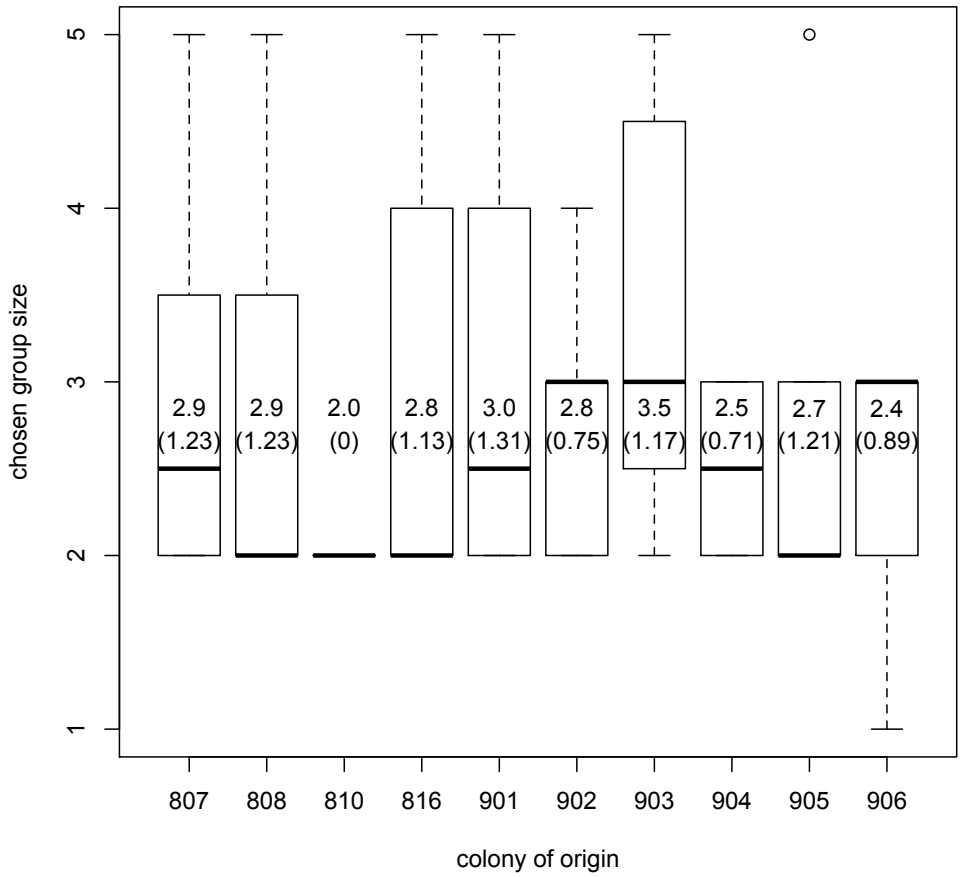


Figure 4.10 Boxplot of group sizes chosen by individuals in mesocosms at 72 hours post-establishment. Dark bars are median group sizes, by colony of origin. Outliers are represented by open circles. Numbers within the boxes indicate the mean (standard deviation). Kruskal-Wallis tests did not detect significant differences in chosen group size between individuals of different colonies of origin, however colony of origin was found to significantly impact chosen group size by the GLMM.

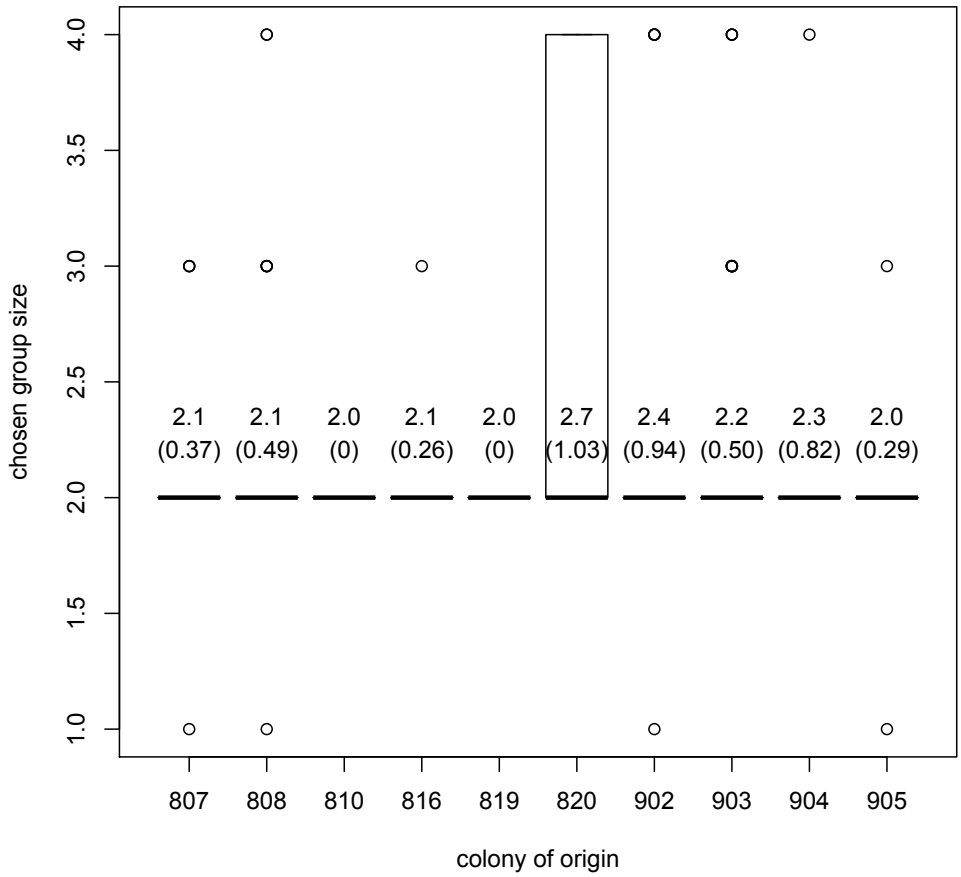


Figure 4.11 Boxplot of group sizes chosen by individuals in mesocosms at 60 days post-establishment. Dark bars are median group sizes, by colony of origin. Outliers are represented by open circles. Numbers within the boxes indicate the mean (standard deviation). Kruskal-Wallis tests did not detect significant differences in chosen group size between individuals of different colonies of origin, however colony of origin was found to significantly impact chosen group size by the GLMM.

nestmates made up 35 out of the 44 (80%) recovered colonies in which relatedness could be determined at 72 hours, and 83 of 114 (73%) at 60 days. There was no difference between the proportion of surviving monogamous pairs containing nestmates and non-nestmates at 72 hours (binomial proportion test, p = 0.51) or 60 days (p = 1), and the proportion did not differ between the two time points (p = 0.43).

Individual mass of kings did not differ significantly at 72 hours (p = 0.10, Fig 4.13), however, out of seven colonies providing female alates to the short-term study, the queens from one colony were significantly lighter than queens from the three heaviest colonies (p<0.001). Neither the light nor the heavy queens differed significantly from queens from the remaining three colonies. At 60 days, individual masses of queens (p = 0.18) and kings (p = 0.13) was not different between parental colonies.

No correlation was found between individual weight and group size for queens or kings at 72 hours (Table 4.2; p = 0.91 and p = 0.13, respectively) or at 60 days (p = 0.05, p = 0.97), although total weight of all individuals in a group was correlated with group size (72 hours: $tau = 0.73$, p = 0.001; 60d: $tau = 0.43$, p <0.001). Mass of mates was positively correlated at 72 hours ($tau = 0.33$, p = 0.002), and even more strongly correlated at 60 days ($tau = 0.50$, p <0.001).

At 72 hours, 10 of 12 (84%) of colonies with eggs were established by non-nestmates, 9 of 12 (75%) of which were pairs. Only 12 of 46 (26%) of incipient colonies recovered at 72 hours had produced eggs. Colonies with eggs

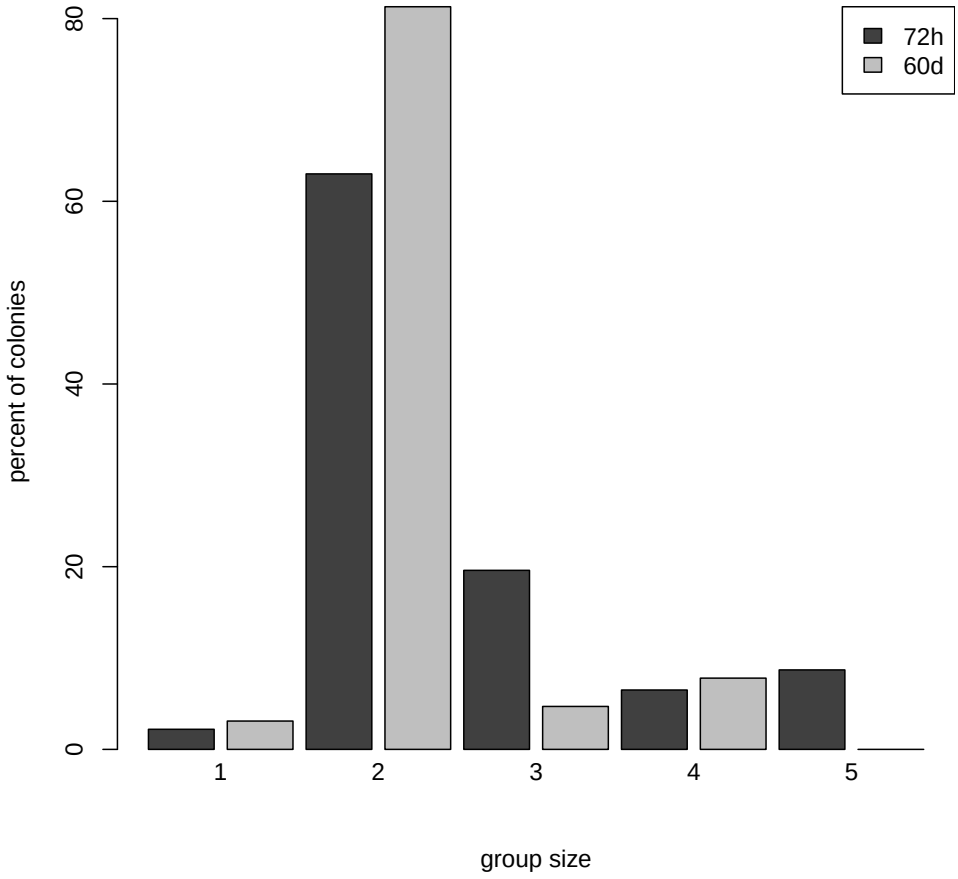
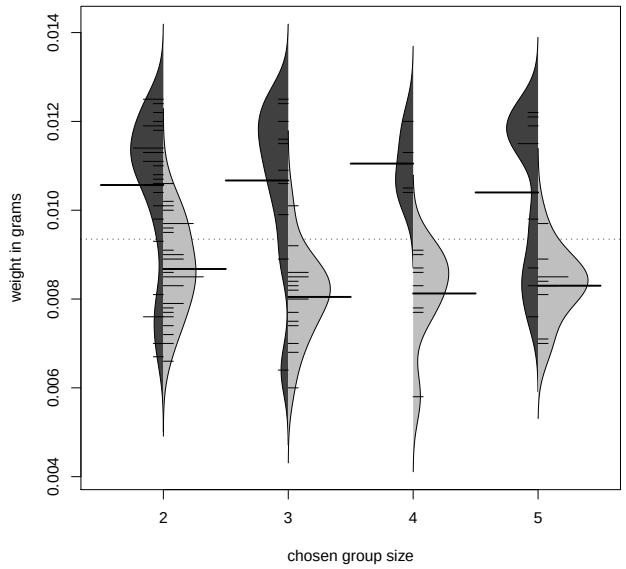
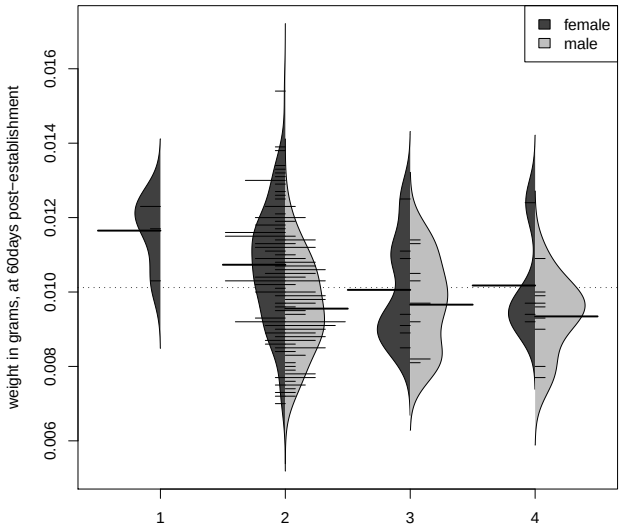


Figure 4.12 Group size of colonies recovered from mesocosms at 72 hours (dark bars) and 60 days (light bars) post-establishment. Relative proportions of the different founding group sizes were different between the two time points (chi-squared = 21.1, df = 4, $p<0.001$).



72 hours



60 days

Figure 4.13 Masses of individuals recovered from mesocosms at 72 hours (top) and 60 days (bottom) post-establishment as a function of founding group size. Note that the x-axis, chosen founding group size, ranges from 2–5 at 72 hours and 1–4 at 60 days.

contained queens from the two heaviest colonies, suggesting a relationship between female weight and time to oviposition. No correlation was found, however, between number of eggs and queen weight (Table 4.3, p = 0.31), king weight (p = 0.10), or total weight of reproductives (p = 0.53). No correlation was observed between chosen group size and number of eggs (p = 0.64) at 72 hours or between group size and total offspring (p = 0.50) at 60 days. Total number of offspring was weakly correlated with queen weight (tau = 0.22, p<0.001), king weight (tau = 0.15, p = 0.03), and total weight of all queens and kings (tau = 0.17, p = 0.01) at 60 days. These factors were also weakly correlated with number of eggs (p<0.001 for each; tau: queen weight 0.29, king weight 0.24, total weight 0.30). No correlations were found for further offspring stages.

Similar to the pattern suggested by the correlations, the 72-hour GLMM simplified to weight as a fixed factor and colony of origin as a random factor predicting chosen group size. The final simplified model for day 60 included weight, and the interaction of initial alate density and number of nesting sites, as fixed factors. The random factor colony of origin also significantly impacted chosen founding group size.

Table 4.3 Correlations with offspring production in mesocosms at 72 hours and 60 days post-establishment. τ = Kendall's τ, p = significance of the correlation. Total weight is the sum of the masses of all queens and kings in a colony.

		groupsize		queen weight		king weight		total weight	
	Number of	τ	p	τ	p	τ	p	τ	p
72 hours	Eggs	0.12	0.64	-0.21	0.31	-0.34	0.10	-0.15	0.10
60 days	Eggs	0.08	0.31	0.29	< 0.001	0.24	< 0.001	0.30	< 0.001
	Larvae	-0.12	0.13	0.11	0.10	0.02	0.80	0.05	0.45
	Workers	-0.16	0.06	0.05	0.50	-0.05	0.49	-0.05	0.51
	Soldiers	-0.18	0.34	0.09	0.24	-0.06	0.42	-0.01	0.91
	Total offspring	-0.05	0.50	0.22	< 0.001	0.15	0.03	0.17	0.01

4.4 Discussion

Alate density, which can influence both intraspecific competition and availability of mates, is not a significant factor fostering pleometrosis in this termite species. When given a choice, *N. corniger* alates will establish colonies in pairs and in larger pleometrotic groups, often in close proximity.

The early influence of alate density on chosen founding group size in microcosms was likely an artifact of experimental conditions. Founding pairs or groups did not cloister themselves as quickly or effectively as they did in the mesocosms. It is possible that weaker individuals could join groups and survive longer in microcosms than in mesocosms, as the arena was much smaller and more uniform, and less energy expenditure was necessary to construct a nest chamber.

Further, the initial clumping of alates in the microcosms suggests that the alates did not "realize" that they had dispersed. Such aggregation is common in unflown termite alates (pers. obs.), and may be mediated by the frequency of body-antenna contact (Hewitt et al. 1972). Additionally, the small space and filter-paper substrate in the microcosms may have initially suggested that they had not dispersed and did not need to begin colony-establishment activities in earnest. The fact that initial clumping was more pronounced in nestmate than in non-nestmate microcosms further supports the idea that colony-establishment behavior was delayed. Lack of nestmate recognition cues, via cuticular odors (Kaib et al. 2002, 2004), could have

been an additional signal to non-nestmates that they should begin colony foundation.

The movement of alates between nest sites and re-organization of founding groups in microcosms is not a natural behavior. Alates were not observed moving around in the mesocosms beyond one hour post-"flight"; dead co-founders were discovered entombed in the walls or floor of the chamber, and did not trigger movement to a new nest site.

Efforts were made to simulate natural conditions in the mesocosms, using materials found in the habitat from which the alates were taken, while excluding ants and other predators and competitors for nest sites. Some beetle larvae, which do not feed on the termites but may harm incipient colonies by their burrowing activities, were found in the wood when the mesocosms were dissected. These simplified ecological conditions tested factors that could not be specifically addressed in previous studies of another pleometrotic termite (Brandl et al. 2001, 2004). The results from these mesocosm experiments are more qualitatively similar to what is found in nature (reported in Chapter 2) than the decay in founding group size seen in the microcosms.

In all three experiments, pleometrotic groups formed when unused nest sites were available, indicating that pleometrosis is not simply a function of crowding or lack of nest sites. In facultatively polygamous ants, group colony foundation is promoted by crowding and competition between incipient colonies (Herbers 1986; Herbers and Banschbach 1998; Sommer and

Hölldobler 1995), although the importance of each of these factors may vary between sites and over time (Deheer et al. 2001). A key difference between the ants and the termites, however, is that termite founders are not predators, and live within their relatively-abundant, readily-available food source. Competition within a founding group, and even between neighboring incipient colonies, is thus less acute for termites than for ants, and mutualistic benefits could theoretically more easily accrue to co-founders.

Colony of origin had a complex effect on group size choice and survival of individuals in pleometrotic groups. Individuals from certain parent colonies more frequently formed larger founding groups. Interestingly, individuals from some parent colonies survived better in larger founding groups of their own choice than did individuals from other parent colonies that have also chosen large founding group sizes. A case point is that of parent colony 902. Individuals from this colony had the second-highest mean rank of founding group size at day 5, but were found in significantly smaller groups, on average, at later time points than individuals from all other parental colonies. Colony of origin was found to be a significant predictor of group size in mesocosms at both 72 hours and 60 days post-establishment when using a GLMM approach, but not in the less-sensitive mean rank method of the Kruskal-Wallis test.

While a correlation was found between the masses of mates, the choice of founding group size was not correlated with weight. In other words, the data presented here suggest that lighter individuals do not choose larger

founding groups. The lack of correlation between group size and number of eggs in these experiments indicates that early egg-laying by larger founding groups observed in Chapter 2 may be an artifact of the less-natural experimental conditions. Group size and number of workers at 60 days were nearly negatively correlated, which could reflect individuals in groups dealing with stressors unique to increased group size and delaying reproduction (Boyer 1978). It would be interesting to see whether significant differences could be detected beyond that point.

Heavier individuals with more bodily reserves probably do survive better than smaller alates, hence the stronger correlation between masses of mates at 60 days than at 72 hours. If co-founders are heavy and have larger fat reserves, weight loss during colony foundation may be minimal, resulting in the observed correlation between total number of offspring and the mass of the reproductives. If greater founder mass results not only in increased survival but also in better nutrition for the offspring, there may be cascading effects leading to earlier development of helpers and ultimately faster colony growth.

The significant colony of origin effects indicate that group size choice during colony foundation may have some genetic basis in *N. corniger*. The genetic role in founding group size choice is not so strong as to effect a clear life history dichotomy as seen in fire ants (Gotzek and Ross 2007). Indeed, as most termite founders do not tolerate additional co-founders (Nutting 1969), this trait may more properly be viewed as a lack of intolerance rather

than a true preference for pleometrosis. Still, alates from particular families (parent colonies) do choose pleometrotic colony foundation more frequently, and survive better in such groups than do individuals from other families. Incipient colonies founded pleometrotically by fortuitous groups of these individuals could then take advantage of the benefits of mutualism (see Chapter 1) for faster colony growth, or improved defense against predators, parasites, or pathogens.

Chapter 5

Pathogen Exposure During Colony Foundation

Abstract

Social facilitation of disease resistance has been considered a benefit of group living for social organisms. Such pathogen-survival benefits may stem from behavioral defenses, such as increased levels of allo-grooming, or from molecular defenses, such as higher concentrations of antimicrobial substances. To test whether these benefits foster pleometrotic colony foundation in the facultatively-polygamous Neotropical termite *Nasutitermes corniger*, alates were exposed to either the generalist entomopathogen *Metarhizium anisopliae* or the endoparasitic nematode *Steinernema carpocapsae*, then placed in monogamous pairs or three- or five-member pleometrotic founding

groups. Survival of the founders and reproductive milestones in these experimental incipient colonies were monitored for up to 180 days. As expected, fungus- or nematode-exposed individuals died more quickly than control individuals of the same relatedness and group size. Survival was lower in quintets compared to pairs and trios, at both the individual and colony levels. Relatedness was also a significant predictor of survival following pathogen exposure. In general, non-nestmates of a given group size survived fungal exposure better than nestmate groups. Reproductive output was affected by group size, but not by relatedness or pathogen exposure. These results indicate that group facilitation of resistance to parasites and pathogens is not a benefit of increasing group size during colony foundation in this species. On the contrary, pathogen exposure may actually be more detrimental to pleometrotic founders than to monogamous pairs.

5.1 Introduction

Previous chapters of this dissertation, comparing survival and reproduction in pleometrotic and monogamous founding groups of *N. corniger*, have found little general advantage to larger founding group size. In particular, individuals in quintets died significantly faster than those in pairs and trios (Chapter 2), in contradiction to the increase in founder survival predicted by theory (Keller 1995) and observed in some other eusocial organisms

(discussed in Chapter 1). As no survival advantage to pleometrotic colony foundation was found under "normal" conditions, the experiments in this chapter explored the hypothesis that pleometrotic groups have an advantage over monogamous pairs under more stressful conditions, such as when resisting pathogen or parasite attack.

Social facilitation of disease resistance is well known in ants and termites (Cremer et al. 2007), reducing susceptibility to infection through allogrooming or social transmission of immunity. Increasing group size has been found to enhance survival of termites following exposure to poison (DeSouza et al. 2001), and a fungal pathogen (Traniello et al. 2002). It has been suggested that this group size effect may also effected by increasing concentrations of termite-produced anti-microbial substances (Rosengaus et al. 1998*a*, 2000*a*, 2004).

During colony foundation, males and females of the primitive dampwood termite *Zootermopsis angusticollis* transmit infections to each other (Rosengaus et al. 2000*b*). Such disease exposure significantly reduces survival and reproduction during colony establishment (Calleri et al. 2006*b*), and may impact non-nestmate founding pairs more than nestmates (Calleri et al. 2005). Similarly, non-nestmate pairs of *Coptotermes formosanus* were also found to be more likely to die during colony foundation than nestmate pairs (Fei and Henderson 2003).

The effect of group size on social facilitation of disease resistance has not previously been tested during termite colony establishment. Previous

research would suggest that reproductives in larger founding groups should survive better than those in monogamous pairs, and that non-nestmates will not accrue the same benefit of pleometrotic colony foundation as nestmate founding groups. Neither of the species in the studies outlined above are pleometrotic, so the facultatively-polygamous derived termite *N. corniger* was used to explore this possibility.

Mature alates of *N. corniger* were exposed to one of two different generalist entomopathogens immediately prior to establishment in experimental incipient colonies. The fungus *Metarhizium anisopliae* is naturally associated with several termite species (Rosengaus et al. 2003; Zoberi and Grace 1990) and has been isolated in Panama from soil near *N. corniger* nests (M. Bulmer, pers. comm.). *M. anisopliae* has been tested as a potential biocontrol agent (Culliney and Grace 2000; Lenz 2005), and used extensively to investigate the costs and benefits of sociality with respect to disease and disease resistance (Calleri et al. 2005; Rosengaus et al. 1998*a*, 2000*b*; Traniello et al. 2002; Ugelvig and Cremer 2007; Yanagawa and Shimizu 2007; Yanagawa et al. 2009). *S. carpocapsae* belongs to a widely-distributed class of entomopathogenic nematodes, obligate parasites of soil-dwelling insects (Stock et al. 1999) harboring bacteria symbionts that actively break down insect tissues. They have also been investigated as biocontrol agents (Denno et al. 2008; Liu et al. 2000; Wang et al. 2002) and in studies of parasitism and parasite resistance (Jarosz 1998; Wilson-Rich et al. 2007).

5.2 Methods

Experimental procedures

Wild-caught, unflown *N. corniger* alates (winged reproductives) were collected in and around Gamboa and Galeta, Panamá, and established in experimental laboratory colonies in May – June 2006 and 2008 as described previously (Chapter 2). Alates were sorted by sex then maintained in moist filter-paper lined Petri dishes until use in these experiments. Individuals from two parent colonies were used in 2006, and from 10 different parent colonies in 2008 to establish a total of 627 experimental colonies.

From each parent colony, 20 replicate incipient colonies were established in a full factorial design with the following factors and levels (Table 5.1): relatedness of founding individuals [nestmates (inbred), non-nestmates (outbred, 2008 only)], pathogen exposure (*M. anisopliae* or *S. carpocapsae* exposed, control solution-exposed), and founding group size (pairs, trios, and quintets). Sex ratios of founding groups were: pairs 1♀:1♂, trios 2♀:1♂, quintets 3♀:2♂. Female-biased groups were chosen to reflect the queen:king ratio found in dissected mature nests (Thorne 1982*b*). Samples of workers from each parent colony in 2008 were also collected for microsatellite analysis to determine the degree of relatedness within and between each colony of origin, however, in the field the assumption was made that nestmates are more closely related to each other (inbred treatment) than non-nestmate individuals originating from different colonies (outbred treatment).

Table 5.1 Number of colonies established in each experiment, by relatedness and founding group size. Ma = fungus *Metarhizium anisopliae*, Sc = nematode *Steinernema carpocapsae*. NM = nestmates, NNM = non-nestmates.

		Rel	pairs 1♀:1♂	trios 2♀:1♂	quintets 3♀:2♂
2006 Ma	control	NM	26	24	22
	exposed		25	25	24
2008 Ma	control	NM	90	90	90
	exposed		90	90	90
	control	NNM	80	80	80
	exposed		80	80	80
2006 Sc	control	NM	8	9	9
	exposed		8	10	11

Fungal exposure protocols follow those of Rosengaus et al. (2000*b*). Termites were forced to walk for one hour on Whatman #5 filter paper moistened with fungal conidia solution or a conidia-free control solution (1 mL/100 mm petri dish). Fungus-exposure experiments were carried out in 2006 using 10^5 conidia/ml, and in 2008 using 10^6 (high dose), 10^5 (medium dose), and 10^4 (low dose) conidia/ml, in a 0.1% Tween 80 solution. Germination rates of all conidia solutions were 98%.

Nematode exposures follow the protocols of Wilson-Rich et al. (2007). The nematode exposure experiment was performed in 2006 only. Alates were subjected, via a one-hour walking exposure in filter paper-lined Petri dishes, to 200 infective juveniles per termite or a nematode-free distilled water control.

In all experiments, randomly-chosen individuals were alternately placed in pairs, trios, and quintets following exposure. Incipient colonies were observed daily for 90 days post-pairing then bi-weekly through 180 days post-pairing, to record deaths of founders and significant events related to reproductive output and colony growth, such as the first appearances of eggs, larvae, workers, and soldiers. Complete censuses of every colony took place at 60, 120, and 180 days post-establishment in 2006, and at 15, 30, 60, and 90 days post-establishment in 2008.

Deceased alates were surface sterilized by submersion in 3% sodium hypochlorite solution, then gently rinsed in sterile water. To confirm infection, they were placed on potato dextrose agar plates (fungus experiments)

or moist filter paper (nematodes experiment), and incubated for up to 14 days at 25°C in closed containers lined with moist paper towel to maintain humidity.

Statistical analysis

Statistical analysis was performed using Cox Proportional Regression for individual survival and time courses of reproductive milestones (Therneau and original R port by Thomas Lumley 2009). All possible covariates (factors: treatment, group size, relatedness, sex; clustering variables: colony of origin, year, and establishment date) as well as the interaction between treatment and group size, were included in the initial model. The model was simplified in a step-wise manner to eliminate factors that did not significantly influence mortality. Kruskall-Wallis tests were performed to determine differences in offspring production by each group size by relatedness category at each census date. Significant tests were followed by non-parametric multiple comparisons (Giraudoux 2009). All statistical analysis was performed in R (R Development Core Team 2009, v 2.9.2).

5.3 Results

Fungus exposure: Survival

Colony of origin, year, and establishment date were not significant predictors of mortality and were eliminated from the model, so data from the low dose exposures in 2008 and their Tween controls were combined with the 2006 data for further analysis. Treatment, group size, relatedness, and sex were all retained in the model. No non-nestmates were tested in 2006, so in the combined analysis, data for female and male nestmates were analyzed separately from the non-nestmates established in 2008. The proportional hazards assumption was violated for group size, indicating that the affect of founding group size on mortality changed over the course of the experiment.

Nestmate females survived best in trios (Fig 5.1), regardless of exposure. There was no significant difference in the survival of control or exposed females in trios, and both survived as well as control pairs. Quintets had the lowest survival, whether exposed to the fungus or not. In comparison, nestmate males survived best in control pairs and trios, which were significantly different from all exposed treatments and the control quintets (Fig 5.2).

Medium and high doses of fungal conidia in 2008 resulted in more rapid death rates than those observed in 2006, so combined analysis of all 2008 data focused on the first 12 days post-establishment. During this time period, there were significant differences in survival attributable to the sex of the individual, so males and females were analyzed separately. Relat-

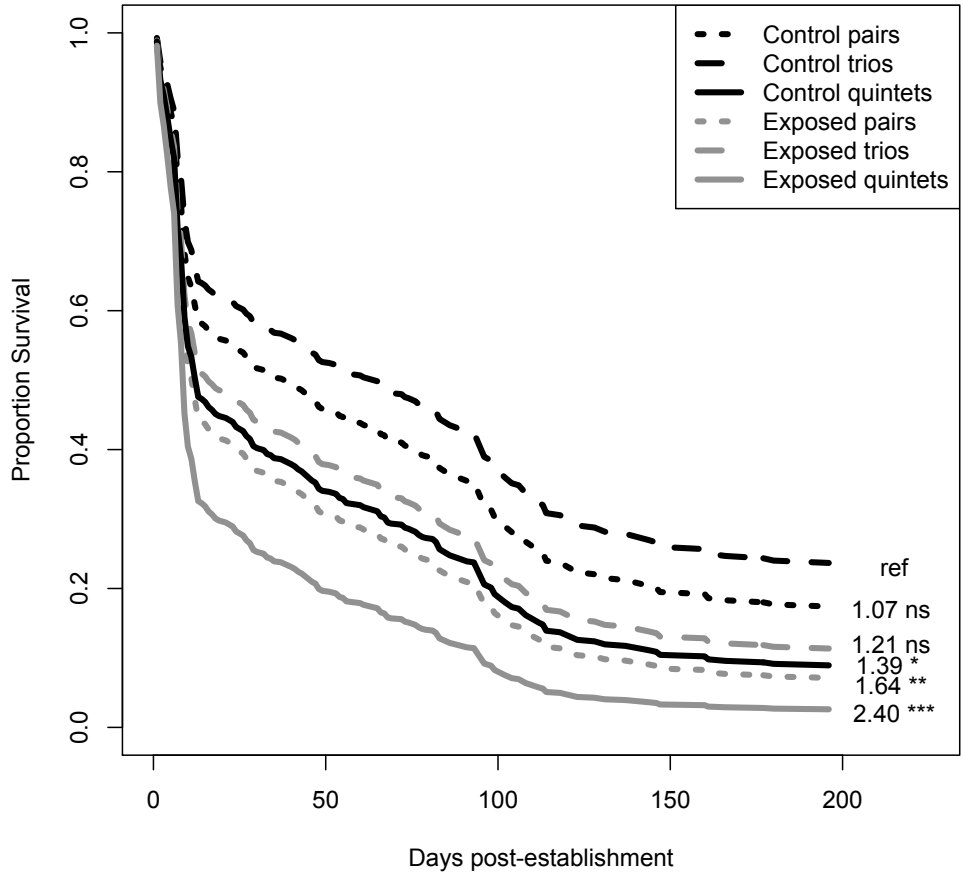


Figure 5.1 Survival of nestmate females exposed to *Metarhizium anisopliae*. Year was not a significant predictor of mortality, so data from 2006 was combined with data from the lowest exposure level of 2008. Hazard ratio of death, compared to the reference group with highest survival, is to the right of each line. ns = not significantly different from the reference, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

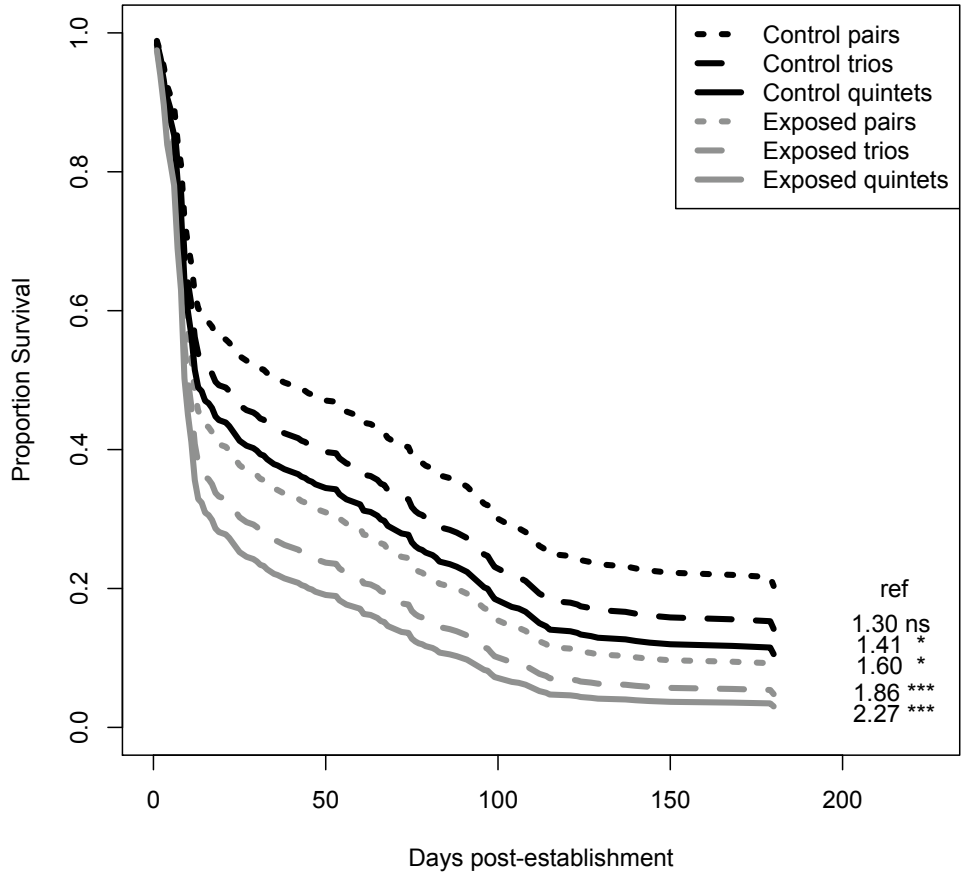
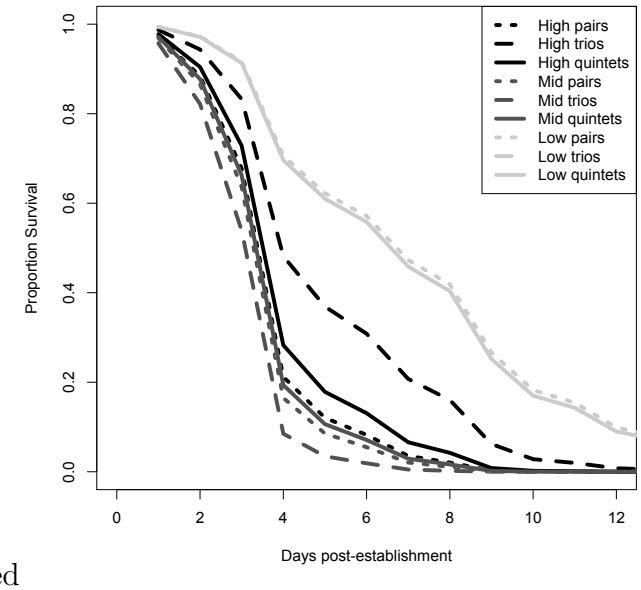


Figure 5.2 Survival of nestmate males exposed to *Metarhizium anisopliae*. Year was not a significant predictor of mortality, so data from 2006 was combined with data from the lowest exposure level of 2008. Hazard ratio of death, compared to the reference group with highest survival, is to the right of each line. ns = not significantly different from the reference, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

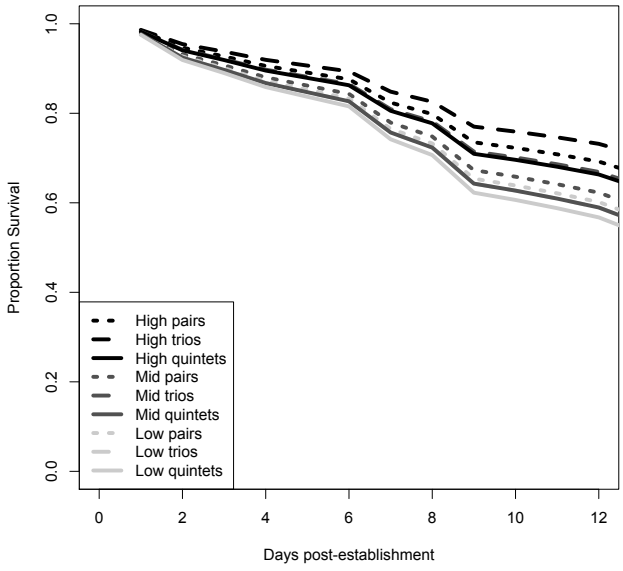
edness of co-founders was also a significant factor for females and control males, but not for males exposed to the pathogen. Colony of origin and establishment date were not significant factors overall, or for the sub-models presented below. Thus, the following paragraphs will discuss nestmate and non-nestmate females separately from males, by dosage level and pooled for colony of origin and establishment date.

Within the nestmate females (Fig 5.3), exposed individuals died significantly sooner than controls, except for those in trios exposed to the lowest dose of the pathogen (Table 5.2). As expected, differences in the hazard ratio of death between control and exposed treatments increased with dosage. Interestingly, the death rate of trios exposed to the highest dose was much smaller, compared to their controls (hazard ratio = 5.01), than that of pairs (hazard ratio = 87.02) or quintets (hazard ratio = 51.65), although all were significantly different from their respective controls ($p = 0.001$). Death rates were similar for all group sizes at the medium dose, and for all pairs and quintets at the low dose.

Non-nestmate females (Fig 5.4), were not as dramatically impacted by pathogen exposure as the nestmate females. Survival of control individuals was more variable between the non-nestmate trials than it was for nestmates. While colony of origin was not a significant factor in the model, different combinations of colonies were used for each exposure, so it is possible that differences in survival of controls may thus reflect suitability of the combination of the two colonies, which we do not have sufficient statisti-



exposed



control

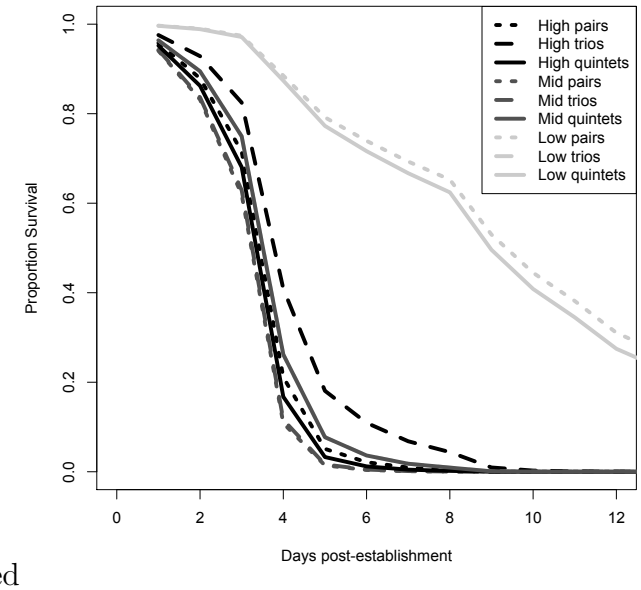
Figure 5.3 Survival of nestmate females exposed to different concentrations of *Metarhizium anisopliae* conidia (top) and their controls (bottom). Hazard ratios of death between control and exposed individuals for each group size by exposure treatment can be found in Table 5.2.

Table 5.2 Hazard ratios of death for exposed individuals compared to their matched controls by exposure level and group size. As compared to the reference (matched control), ns = not significantly different, ** p = 0.001, *** p = 0.0001.

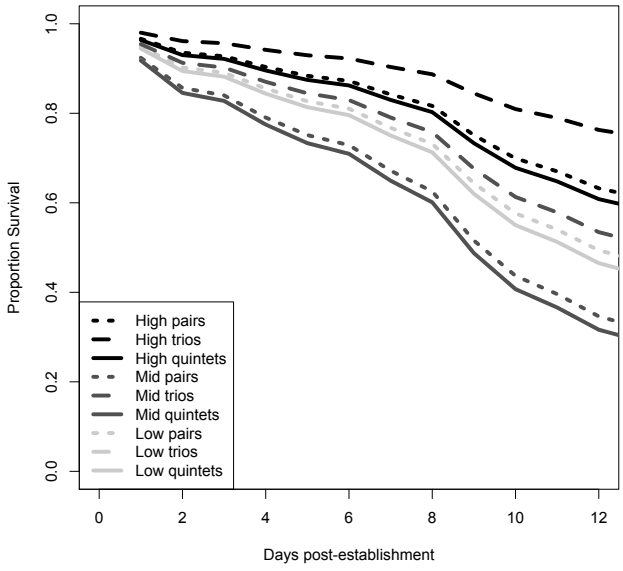
		Dose		
		high	medium	low
Female nestmates	Pairs	87.02 ***	12.59 **	2.18 **
	Trios	5.01 ***	15.76 ***	1.02 ns
	Quintets	51.65 ***	15.73 ***	2.03 ***
Female non-nestmates	Pairs	15.66 ***	8.53 ***	0.98 ns
	Trios	30.36 ***	15.70 ***	1.14 ns
	Quintets	74.30 ***	7.57 ***	1.16 ns
Males	Pairs	36.42 ***	8.72 ***	1.52 **
	Trios	32.98 ***	12.99 ***	1.46 **
	Quintets	66.57 ***	8.85 ***	1.41 **

cal power to detect. Death rates again increased with exposure level (Table 5.2). The low dose did not significantly affect survival of females placed in non-nestmate groups as compared to their controls, but survival did significantly decrease in the medium and high exposure treatments. At the high exposure level, death rates compared to controls increased as group size increased, such that the hazard ratio for trios compared to their controls was twice that of pairs, and that of quintets compared to their controls was more than twice that of trios. This pattern was not seen at the medium dosage level.

Survival of males was highest in trios at all doses of the fungus (Fig 5.5), although group size did not significantly impact the survival of males exposed to the control solution. Relatedness did not affect survival rates of individuals exposed to the fungus, but was a significant predictor of mortality in the controls. Group size did not predict mortality of control males but it was predictive of mortality for exposed males. Exposure to the pathogen significantly reduced survival compared to the matched control at all dosage levels (Table 5.2). Individuals in trios were impacted more severely at the medium dose than were those placed in pairs and quintets, while the death rates of males in quintets compared to their matched controls were roughly twice those of pairs and trios compared to their respective controls.

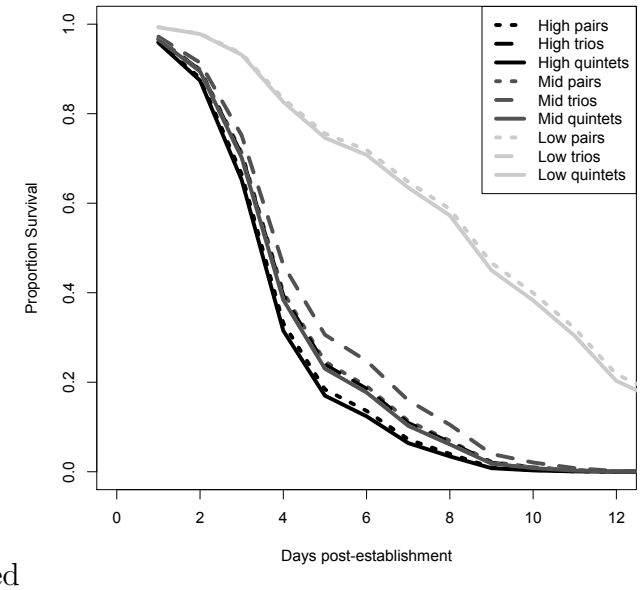


exposed

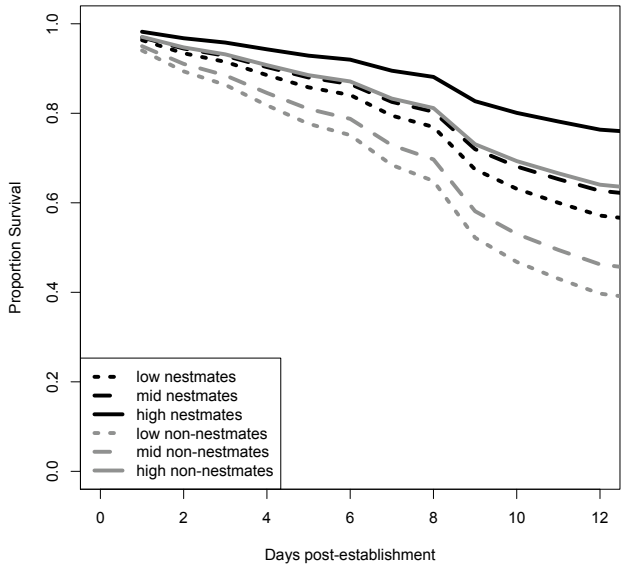


control

Figure 5.4 Survival of non-nestmate females exposed to different concentrations of *Metarhizium anisopliae* conidia (top) and their controls (bottom). Hazard ratios of death between control and exposed individuals for each group size by exposure treatment can be found in Table 5.2.



exposed



control

Figure 5.5 Survival of males exposed to different concentrations of *Metarhizium anisopliae* conidia (top) and their controls (bottom). Relatedness was not a significant predictor of mortality for males exposed to the pathogen. For control males, relatedness was a significant predictor of survival but not founding group size. Hazard ratios of death can be found in Table 5.2.

Fungus exposure: Reproduction

Due to differences in census points, offspring production for 2006 and 2008 were analyzed separately. For 2006, significant differences in total offspring number (sum of eggs, larvae, workers, and soldiers) attributable to group size and pathogen exposure were found at 60 days post-establishment (Fig 5.6, KW $p = 0.01$). At that time, both control and exposed pairs had produced significantly fewer total offspring than control trios. No significant differences were found in offspring number at 120 days (KW $p = 0.39$). Only 8 control quintets and 3 exposed quintets survived and had offspring at 120 days. Significant differences in offspring production were not detected at 180 days post-establishment (KW $p = 0.27$). Only 30 colonies out of the original 147 had survived and produced offspring, and no exposed quintets survived and had offspring at this time point.

In 2008, offspring production was analyzed for the lowest exposure only. Colonies exposed to the medium and high doses of the pathogen generally did not produce offspring or survive to the 15-day census point. Relatedness was found to significantly predict offspring (egg) number at 15 days (Fig 5.7), with non-nestmates more likely to produce offspring than nestmates. No significant effects of founding group size and fungus exposure were found for either nestmates (KW $p= 0.17$) or non-nestmates (KW $p= 0.77$).

Relatedness did not significantly predict total offspring number at 30 days post-establishment or beyond. Less than half as many fungus-exposed colonies produced offspring compared to the controls at 30 days (Fig 5.8).

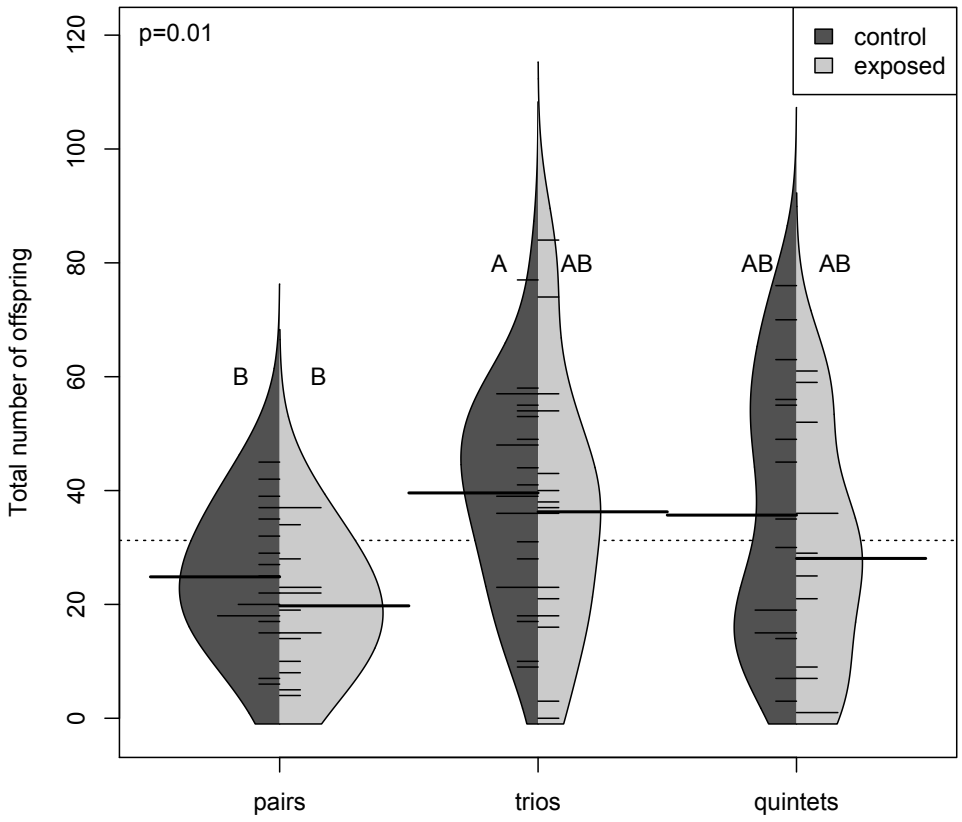
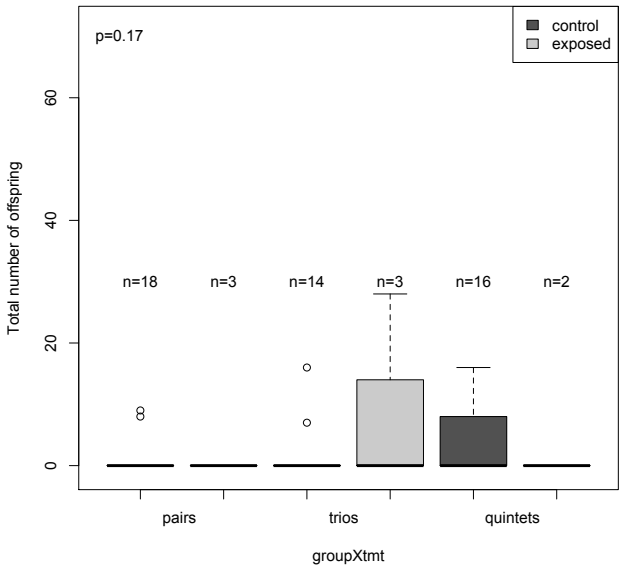
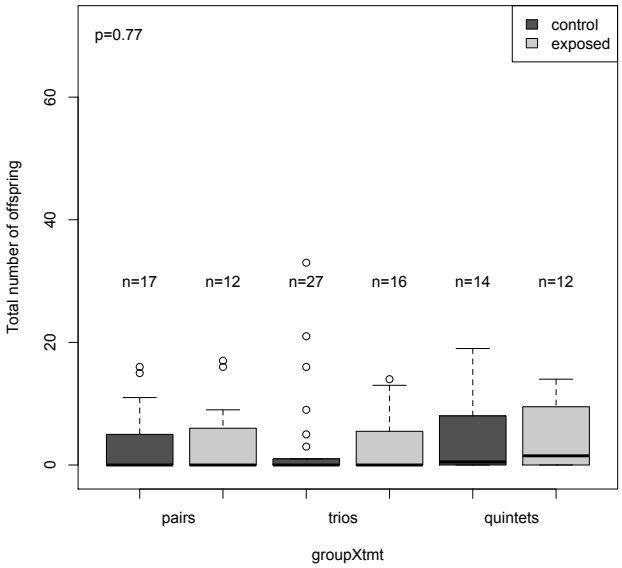


Figure 5.6 Reproductive output at 60 days post-establishment of colonies exposed to *Metarhizium anisopliae* in 2006. Number of offspring are significantly different between group size by exposure treatments (KW p = 0.01). Letters above each bar indicate homogeneous subgroups, as determined by non-parametric multiple comparisons.



nestmates



non-nestmates

Figure 5.7 Reproductive output at 15 days post-establishment of colonies exposed to *Metarhizium anisopliae* conidia and their controls in 2008. Relatedness was a significant predictor of offspring number. Number of offspring are not significantly different between treatments (nestmates, top, KW p = 0.17; non-nestmates, bottom, KW p = 0.77).

Control quintets produced significantly more offspring than control pairs at 60 days post-establishment in (Fig 5.9, KW $p = 0.04$). Half as many exposed as control colonies were found with offspring, but the numbers of total offspring they had was not significantly different from any of the controls. Only 12 colonies with offspring survived to 90 days post-establishment, out of 480 originally established, and no significant differences were detected between them based on founding group size or pathogen exposure (KW $p = 0.93$)

Control pairs produced their first eggs more slowly and at an overall lower rate than the other treatments in 2008 (Fig 5.10). Over the 90 days of the experiment, however, this rate was not significantly different from that of control trios or quintets, or the pathogen-exposed pairs. Pathogen-exposed trios and quintets produced eggs sooner than the other groups, and all surviving fungus-exposed trios and quintets produced eggs prior to 60 days post-establishment. Some surviving colonies of pathogen-exposed pairs, and control colonies of all founding group sizes that survived to 90 days, were not observed to have produced eggs.

Founding group size and relatedness were not significant predictors of larvae hatching (Fig 5.11). While exposure was a significant predictor of the time to larvae production, the overall hazard ratio was not significantly different between colonies exposed to the pathogen and those that were not. A total of 11 out of the 1020 colonies in 2008 produced workers, while 12 colonies produced soldiers in the first 90 days of the experiment. Because

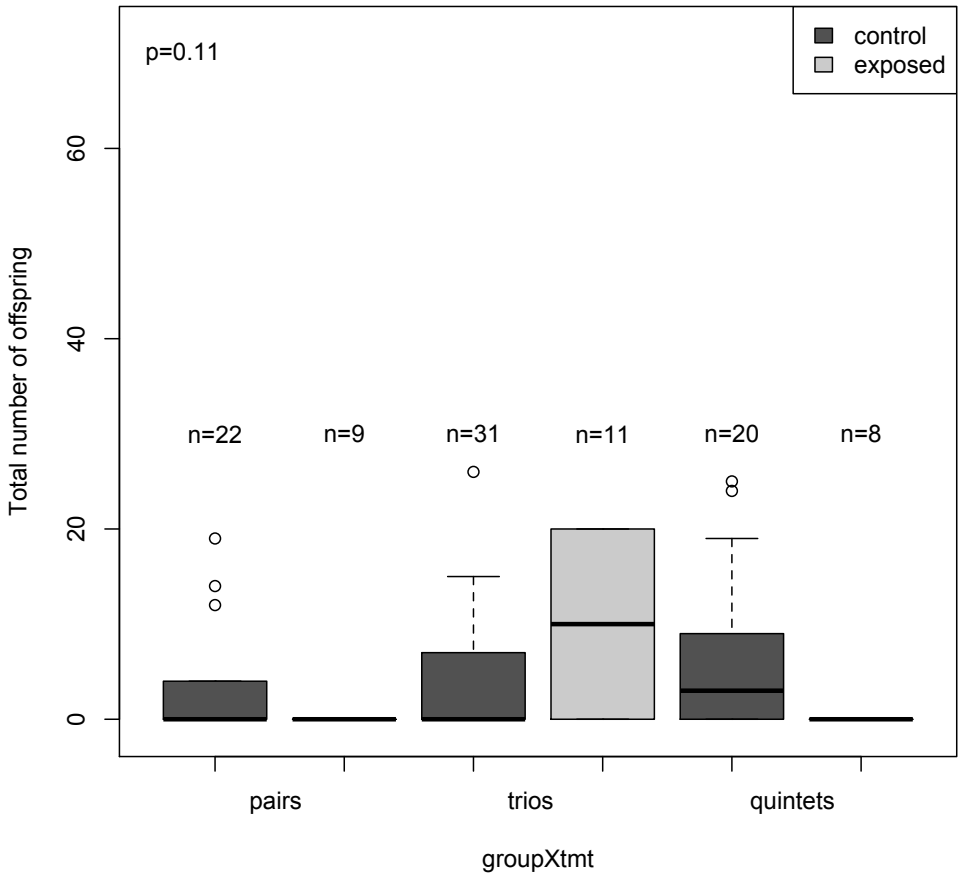


Figure 5.8 Reproductive output at 30 days post-establishment of colonies exposed to *Metarhizium anisopliae* in 2008. Relatedness was not a significant predictor of offspring number, so nestmates and non-nestmates are pooled. Number of offspring are not significantly different between group size by exposure treatments (KW p = 0.11).

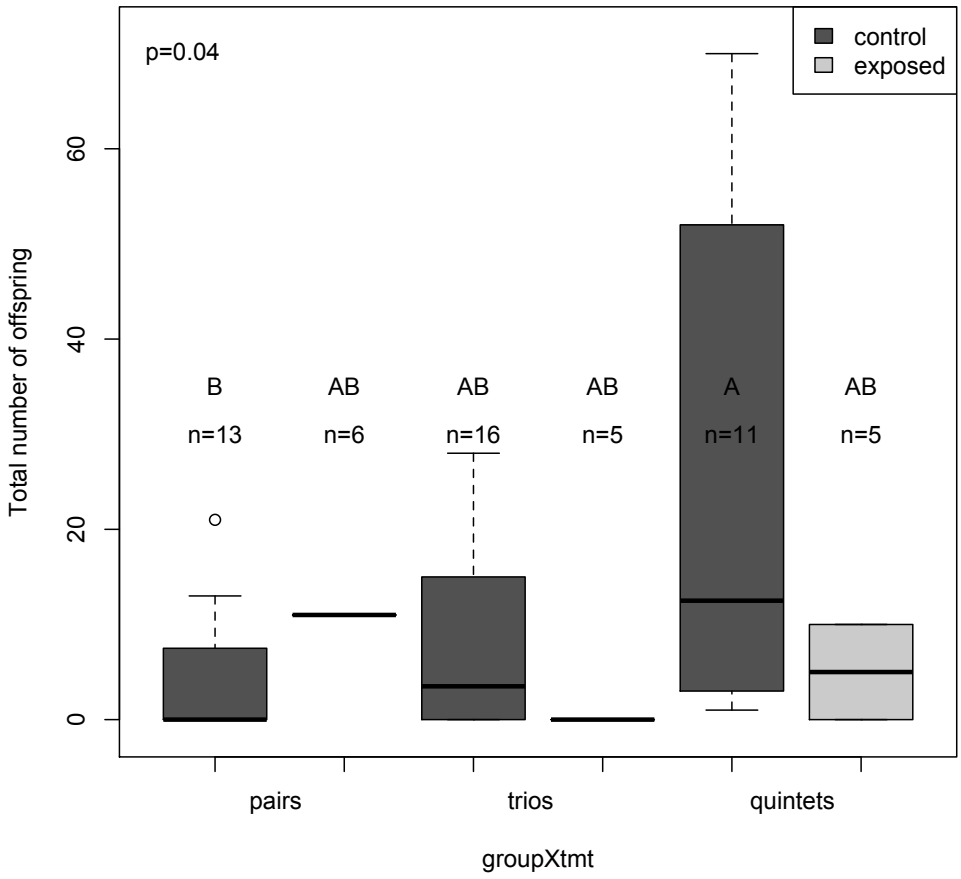


Figure 5.9 Reproductive output at 60 days post-establishment of colonies exposed to *Metarhizium anisopliae* in 2008. Relatedness was not a significant predictor of offspring number, so nestmates and non-nestmates are pooled. Number of offspring are significantly different between group size by exposure treatments (KW p = 0.04). Letters above each bar indicate homogeneous subgroups, as determined by non-parametric multiple comparisons.

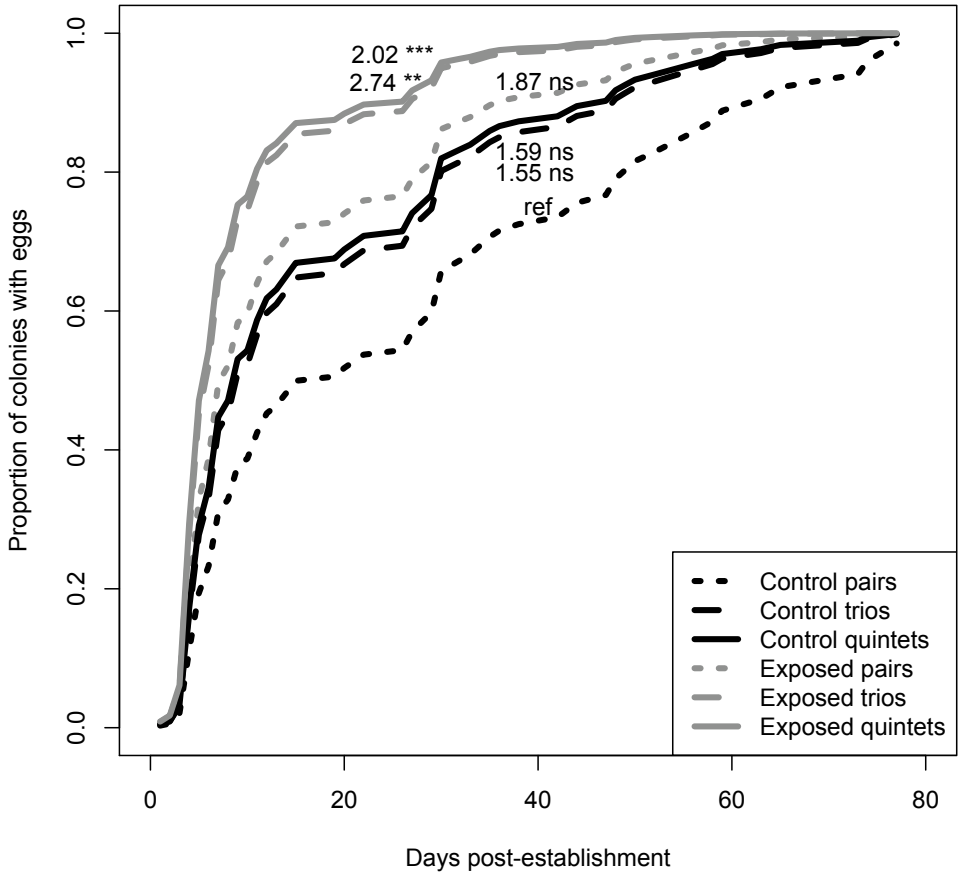


Figure 5.10 Time course to first appearance of eggs in 2008, by group size and treatment. Relatedness was not a significant predictor of oviposition. Numbers to the right of each curve are hazard ratios relative to the reference. A total of 131 colonies produced eggs during the experiment. As compared to the reference, ns = not significantly different, ** p = 0.001, *** p = 0.0001.

of inadequate sample size (too few observations per category), no analysis could be performed on time to first workers and soldiers.

Nematode exposure

Survival rates and reproductive output were not significantly different between nematode-exposed and control individuals, so the treatments were pooled for further analysis. Individuals in pairs (reference group, Fig 5.12) survived significantly better than individuals placed in trios (hazard ratio of death 2.34 compared to the reference) and quintets (hazard ratio of death 3.68 times that of the reference). There were also no significant differences between colonies established by pairs, trios, and quintets in total numbers of offspring (sum of eggs, larvae, workers and soldiers) at 60 days (KW $p = 0.97$, mean = 40.7), 120 days (KW $p = 0.68$, mean = 33.3), or 180 days post-establishment (KW $p = 0.40$, mean = 46.9).

Number of offspring tended to be higher in larger groups at 60 days, with greater variability in offspring number in larger founding groups compared to a tight, normal distribution of offspring number for colonies founded by pairs. Only 25 out of 56 colonies survived to 180 days and produced offspring. The ranges are similar to those in the 2006 fungus exposure experiment, and at 60 days both are slightly higher than offspring numbers in 2008.

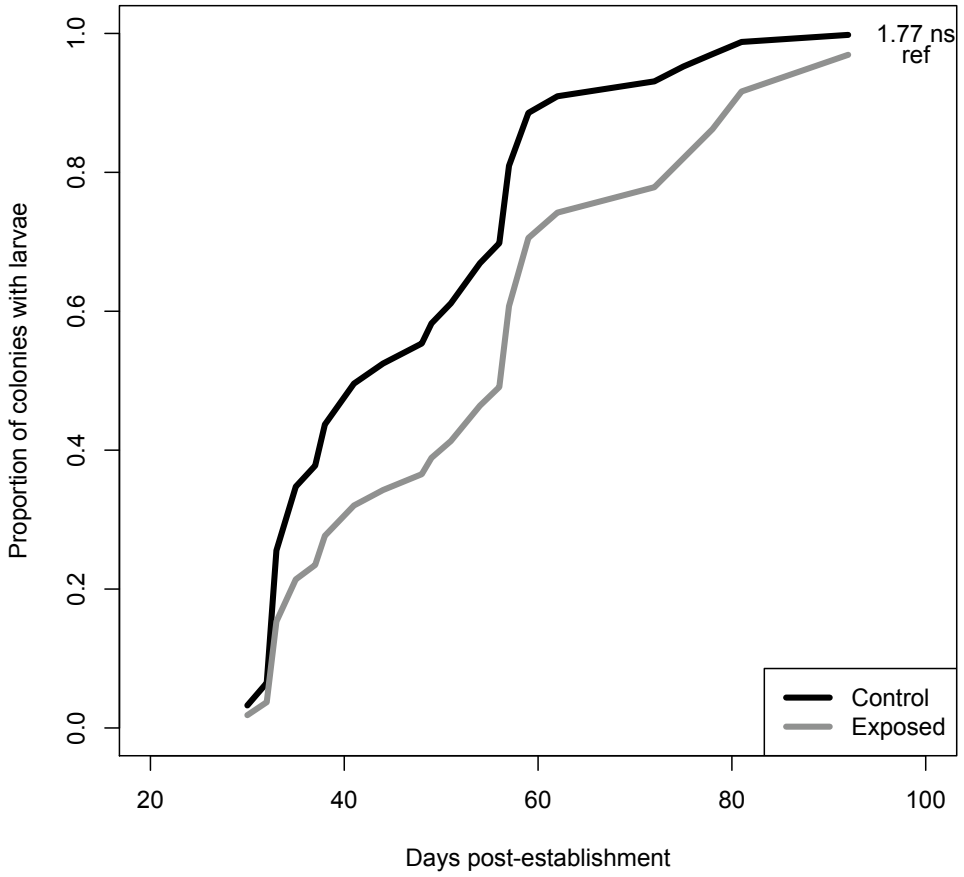


Figure 5.11 Time course to first appearance of larvae in 2008, by treatment. Group size and relatedness were not significant predictors of larvae hatching. A total of 36 colonies produced larvae during the experiment. Numbers to the right of each curve are hazard ratios relative to the reference. As compared to the reference, ns = not significantly different.

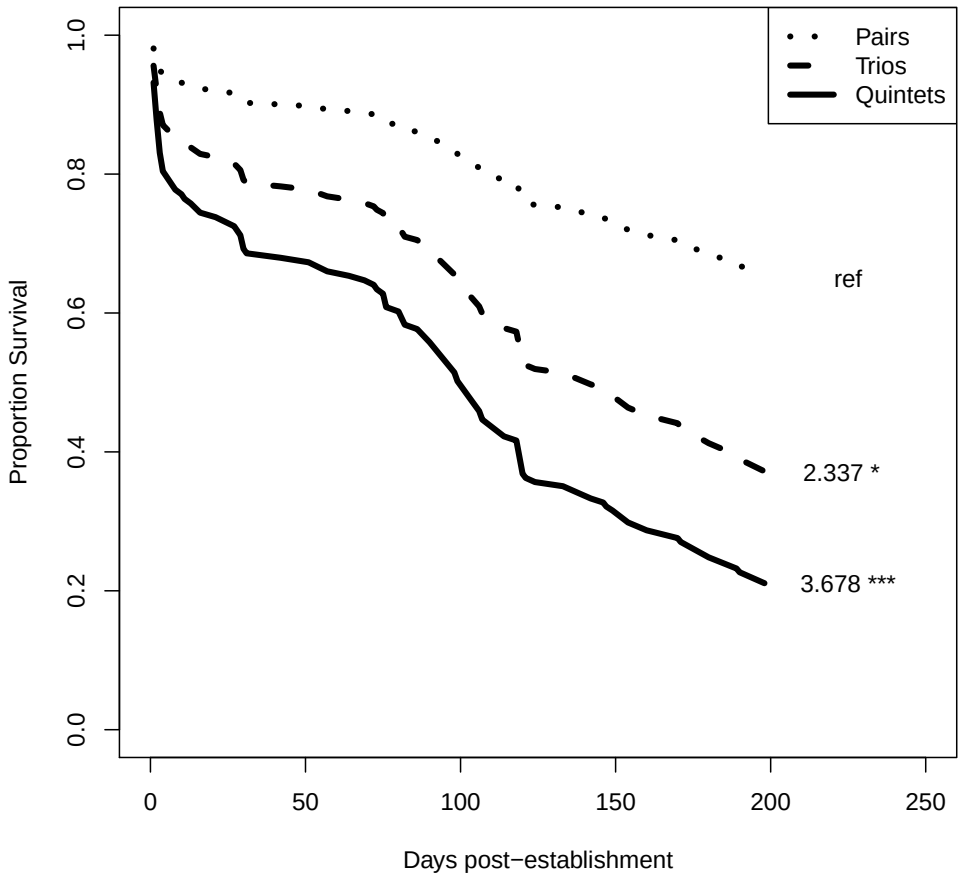


Figure 5.12 Survival of individuals in the *Steinernema carpocapsae* exposure experiment. Exposure to the parasite did not impact survival, so control and exposed individuals are pooled for each group size. Hazard ratio of death, compared to the reference group with highest survival, is to the right of each line. ns = not significantly different from the reference, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

5.4 Discussion

The impact of parasites and pathogens on survival may be different for different types of immune challenges, and may be context-dependent (Viney et al. 2005). In ants, the benefits of group living, and genetic diversity in particular, have also been found to vary with exposure rate (Hughes and Boomsma 2004). In this study, fungal exposure decreased survival while nematode exposure had no effect on mortality. Both the dose of fungal conidia and the founding group size in which the individual was placed affected survival.

As expected, exposure to fungal conidia increased the death rates of individuals, as compared to their controls. Increasing rates of death with higher exposure to the pathogen was also a logical and expected result. However, in light of previous work on termites (DeSouza et al. 2001; Pie et al. 2005) and the theory of density-dependent prophylaxis (Wilson et al. 2002), the finding that death rates increased as founding group size increased was unexpected. This contradicts the results of previous research using non-reproductive groups of primitive termites, in which larger groups survived pathogen exposure rather than spreading the disease throughout the group (Rosengaus et al. 1998*b*; Traniello et al. 2002). Indeed, increased susceptibility and rapid spread of disease, seen here under controlled conditions and observed anecdotally in Chapter 2, may be attributable to the additional stresses of reproduction and colony foundation activities, or to

a lack of cooperative behavior in larger pleometrotic groups (also discussed in Chapter 3).

Males and females responded differently to fungus exposure in 2006, but not in 2008. Sex differences in response to immune challenges are common (May 2007), and may be linked to trade-offs necessitated by differential investment in offspring production as suggested for reproductives of the basal termite *Z. angusticollis* (Calleri et al. 2007). In particular, immune defenses may be lower when demand for resources is high, an individual is investing heavily in a certain activity, or during reproductive bouts (Lee 2006). Colony foundation in termites meets all of those criteria, so this may be the time when parasite and pathogen exposure has the greatest effect.

Oviposition and offspring development occurred in both years, so sex differences in response in 2006 but not 2008 cannot be attributed to lack of reproductive investment in one year. Male investment in the first broods of eggs extends beyond sperm production and parental care, but may also include feeding the female via trophallaxis from his stored bodily resources (Nutting 1969). This might suggest that termites should show fewer sex differences in survival during incipient colony development, but could also support the idea that males can more "selfishly" choose how much to invest in offspring than females can. Alates originated from different parental colonies in the two years, and individual body mass or condition was not scored in this study. Both factors may have contributed to observed differences between the years.

The differences between nestmates and non-nestmates, and higher variability in the impact of fungal exposure within the non-nestmates, could be influenced by incompatibilities due to lack of mate choice. Similarly, some of the effect of group size may be due to different degrees of tolerance or affinity for group colony foundation (see also Chapter 4). The apparently non-linear effect of pathogen dosage is somewhat confounded by the use of individuals from different colonies of origin, which may have had different innate suspectibilities to fungal infection.

The lack of effect in the nematode experiment is not due to the dose of infective juveniles to which the alates were exposed. When preparing to re-run the nematode exposures in 2008, alates were exposed to a wide range of doses of infective juveniles of *S. carpocapsae*. If the problem had been too low of a dose, higher doses (including immersion in a concentrated nematode solution) should have resulted in infection. If mortality and morbidity was inhibited by infection with too many competing nematodes, the lowest doses should have been more effective in causing infection. However, no tested dose resulted in *S. carpocapsae* infection of *N. corniger* alates in 2008. It may be that the dark alates are too heavily sclerotized for effective penetration of this nematode compared to the soft, light-colored workers and nymphs used in previous studies with other termite species (Denno et al. 2008; Jarosz 1998; Liu et al. 2000; Wang et al. 2002; Wilson-Rich et al. 2007). Other nematodes have been found naturally parasitizing workers of *N. corniger* (S. Issa, pers. comm., and pers. obs.) and other

Nasutitermes species (Fuller and Jeyasingh 2004). The limited number of *N. corniger* workers tested concurrently with the alates in 2008 suggest that this species or strain of nematode may not be able to parasitize this termite.

Differences in reproductive output could not be connected directly with pathogen exposure in this experiment, and when they were found depended more strongly on founding group size. The observed differences, although transient, could be due to larger founding groups possessing a larger pool of resources, both as bodily reserves and in terms of greater numbers of egg-layers and/or care-givers. This may allow them to avoid trading-off pathogen resistance and reproduction as dramatically as pairs, which begin with fewer "personal" resources.

Empirical results regarding tradeoffs between immunocompetence and fitness are limited (Viney et al. 2005), and rely heavily on the ecological context (French et al. 2009). Not only do conditions during earlier stages of an individual's life affect immunocompetence during reproduction (Love et al. 2008), but social conditions experienced by parents impact the survival and disease resistance of their offspring (Miller et al. 2009). This pattern of significant differences in time to oviposition, but no differences in time to the appearance of the first larvae, is similar to what was observed in Chapter 2, although the magnitude of the difference between oviposition in trios and quintets and that of pairs is much greater in this study. In both studies, it was observed that eggs would often be laid 1–2 days before death, perhaps a form of reproductive compensation (Boyer 1978).

There is no clear advantage to pleometrosis in resistance to the two pathogens tested here. There may be a dosage-dependent effect on survival in different group sizes, as exposure to a high dose of the fungal pathogen generally decreased survival of individuals in quintets compared to their controls more than it did for those founding colonies in pairs or trios. The impacts of pathogen exposure on reproduction and colony growth, separate from survival, are few if any. The quintet disadvantage manifested in Chapter 2 is maintained under disease pressure, when it was hypothesized that larger groups could survive and/or reproduce more effectively than pairs due to social facilitation of disease resistance. The suggestion that social insects may not pay the cost of sociality in increased disease risk, and may actually benefit from increased group size in relation to disease dynamics (Hughes et al. 2002) does not seem to hold true for colony foundation in *N. corniger*.

Chapter 6

Effect of Pathogen Exposure on Offspring of Experimental Colonies

Abstract

Survival following pathogen exposure depends on the ability of the organism to mount an immune defense. To understand the array of constitutive immune defenses in the derived neotropical termite *Nasutitermes corniger*, *in vivo* and *in vitro* assays were performed on workers from laboratory colonies of known pedigree, as well as workers from field colonies. First, fat body mass, an indicator of resource reserves and possible source of immune factors, was estimated. Next, the fungistatic properties of whole-body extract were measured

as a proxy for circulating antimicrobial peptides. Finally, susceptibility to the generalist entomopathogen *Metarhizium anisopliae* was determined. Significant differences were found between colonies in fat mass, fungal inhibition, and survival after pathogen exposure. Contrary to expectation, however, none of these measures were correlated with one another. Variation was high within colonies for each of the three assays. A generalized linear mixed model approach suggests that other unmeasured factors impact survival following pathogen exposure more than either measure of immune potential used in this study. It is possible that individual variation in fat content and fungistatic activity cannot be generalized and compared at the colony level, particularly in light of the efficacy and efficiency of social insects' behavioral defenses against disease.

6.1 Introduction

Termites, foraging in soil and decaying wood, live under conditions that should select for high investment in immune function. Pathogen pressure is very high in soil environments, with up to 98% of soil insects exhibiting melanotic nodules characteristic of parasite or pathogen infection (Tunaz and Stanley 2009). Both basal and derived termites, as well as their close relatives the roaches, typically have cuticular microbial loads of around 200 colony-forming units (CFU)/mm^3 (Rosengaus et al. 2003, and references therein). In addition, the genetic similarity of nestmates, due to high levels

of relatedness within social insect colonies, fosters the spread of disease (Schmid-Hempel 1994), which may be further exacerbated by close quarters within the nest and frequent interactions with conspecifics.

In response to these pressures, social insects have evolved behavioral, physiological, and biochemical mechanisms for mitigating risk of pathogen exposure and infection. At the colony level, these mechanisms include alarm responses, pathogen avoidance, mutual grooming, and nest hygiene behaviors such as fumigation, waste management, and morgues (Choe et al. 2009; Evans and Spivak 2010; Mburu et al. 2009). The effectiveness of these mechanics has led to the suggestion that social insects may not pay the cost of sociality in increased disease risk, and may actually benefit in this respect from increased group size (Hughes et al. 2002). Genetic diversity within a colony may also decrease the impact of disease (Hughes and Boomsma 2004; Reber et al. 2008; Seeley and Tarpy 2006).

Once the insect cuticle has been breached, individual cellular and humoral immune responses are triggered (Gillespie et al. 1997; Schmid-Hempel 2005). Cellular defenses result in phagocytosis or encapsulation of the invading microorganisms, believed to eliminate 95.5% of invading microorganisms (Haine et al. 2008). Humoral processes lead to melanization, coagulation, and the synthesis of antimicrobial peptides, which are considered to be more important in clearing chronic infections (Haine et al. 2008).

Specific molecular and chemical immune mechanisms have recently been reviewed for ants (Schlüns and Crozier 2009) and bees (Wilson-Rich et al.

2009). Less is known about the specific immune processes in hemimetabolous social insects, however antimicrobial peptides (AMPs) have been identified in termites (Bulmer et al. 2009; Bulmer and Crozier 2004; Lamberty et al. 2001). Termite AMPs have low sequence homology with those of holometabolous insects (such as bees and ants). Further, termite AMPs seem to be produced constitutively in hemocyte granules and salivary glands whereas AMPs described in the Holometabola are produced in the fat body and released into circulation following induction by injury or infection (Lamberty et al. 2001).

This study investigates the relationship between survival after pathogen exposure and two indicators of individual immune potential in the derived Neotropical termite *Nasutitermes corniger*. It was hypothesized that if mass of the fat body is important in termite immune function, as appears to be the case in bees (Wilson-Rich et al. 2008), then survival should be correlated with overall fat content. Alternatively, if production of AMPs outside of the fat body, and their circulation in the hemolymph, are key to pathogen defense, survival should correlate positively with inhibition of fungal growth by whole body-extracts, but not necessarily with fat content. The unique opportunity afforded by laboratory colonies of known pedigree raised under standardized conditions, compared with results from natural colonies, allows us to examine the contributions of termite fat body mass and AMP production to survival.

6.2 Methods

Termite colonies

Incipient colonies were established in May–June 2006 and 2007 from wild-caught alates as described elsewhere (Chapter 2) and maintained in the laboratory on an *ad libitum* diet of moist birch wood at constant conditions of 28°C and 80% humidity. The parent colonies of all alates used to establish these laboratory colonies is known, however the degree of inbreeding within each parent colony and relatedness among the parent colonies are not known.

Laboratory colonies were originally established as either nestmate or non-nestmate pairs, trios, or quintets. In spite of their pleometrotic origins, however, all laboratory colonies had been reduced to monogamous pairs more than one year before sample collection. Of the 14 laboratory colonies used in this experiment, two colonies were established by non-nestmates (#62 and #142). The relatedness of the founders of one colony (#17) could no longer be determined due to a labeling error in the environmental chamber. The remaining eleven colonies were founded by nestmates.

The 22 field colonies tested were parental colonies of alates used in colony-establishment experiments in 2008 and 2009 (Chapters 2–5). Samples were collected from these naturally-established mature nests in July 2009. These colonies are located in and around Gamboa, Panama, the same site from which parent colonies of the queens and kings in the labo-

ratory colonies were taken. Relatedness of these nests to each other or to the parental colonies used to establish laboratory incipient colonies is not known, nor is their degree of inbreeding.

Collection of termites in both laboratory and field colonies was achieved by placing a loose ball of moistened paper towel in a break on the nest surface, or by gently removing them with forceps from an excised section of carton nest material. Termites were used in experiments immediately upon collection, with less than two hours holding time. Although both workers and soldiers were collected, only workers were used in this study to avoid any confounding anti-fungal effects of soldier defensive secretions (Rosengaus et al. 2000*a*).

Survival following pathogen exposure

Preliminary trials indicated that worker survival was lower in the absence of soldiers (data not shown), so to quantify susceptibility under the most natural conditions possible, survival was monitored in mixed groups containing both castes. Survival of each caste was tracked separately, and only survival of the workers was used in the statistical analysis. Workers and soldiers from each colony were randomly divided into two groups (control and fungus-exposed), each containing 35 workers and 35 soldiers.

Exposure protocols followed those of Rosengaus et al. (2000*b*). Each group was exposed to either 1.0 x 10^5 *Metarhizium anisopliae* conidia suspended in 0.1% Tween 80 or a conidia-freeTween 80 control solution, by

walking for one hour on #5 Whatman filter paper moistened with 300µl of the solution per 60 mm Petri dish. Dishes were tapped periodically during exposure to encourage walking and uniform exposure to conidia.

Following exposure, randomly-chosen workers and soldiers were alternately placed in one of three sterile 60mm petri dishes lined with water-moistened Whatman #1 filter paper. All replicates from all colonies were maintained at 28°C and 80% humidity, or ambient conditions in Panamà, for 20 days. Living termites were counted daily. Dead termites were removed and surface sterilized with 3% sodium hypochlorite solution, then plated on potato dextrose agar plates (lab) or moist filter paper (field) for confirmation of *M. anisopliae* infection.

Fat content

Fat content of workers was determined using a procedure modified from Wilson-Rich et al. (2008). This method measures total body lipids, including the mass of the fat body, and thus provides a measure of overall body condition as well as the potential for induced immune response. It has previously been used as an indirect measurement of humoral immunocompetence (Doums et al. 2002; Ellers 1995; Wilson-Rich et al. 2008).

From each colony, 50 randomly-chosen *N. corniger* workers were decapitated under semi-sterile conditions using a standard razor. Decapitation ensured good penetration of the solvent to the abdomen where the fat body resides. Abdomens were first dried for 36 hours at 25°C inside a fume hood

(lab) or drying oven (field). Dried abdomens were weighed (mg) in groups of five, then each group placed in a separate 17 x 60 mm glass vial and covered with 3 mL of lipid-dissolving diethyl ether.

Vials were capped, then sealed with Parafilm and incubated on a rotating platform at 90 rpm for 24 hours to dissolve the fat tissue. Following dissolution, the diethyl ether was removed by pipetting. The de-fatted abdomens were dried on Whatman #1 filter paper in a fume hood for 24 hours, then each group of five was again weighed. The difference between initial mass and final mass, divided by the initial mass, represents the percent of body mass that was made up of fat tissue.

Fungal inhibition

To test the anti-fungal activity of termite extracts, 24 workers were randomly selected from each colony. Workers from field colonies were individually weighed (mg). To eliminate the cuticular microbiota, each worker was individually surface-sterilized in 3% sodium hypochlorite solution. Termites were rinsed twice in sterile water, then placed whole into a BioMasher disposable homogenizer (Omni International, Kennesaw, Georgia, USA).

Following the protocol of Bulmer et al. (2009), termites were ground in 5µl of sodium acetate buffer, filtered to remove debris and fungal and bacterial contaminants, and centrifuged at 14000 rpm at 4°C for 1 minute to extract only the low molecular-weight fraction. Extracts were mixed with 5µl of additional buffer, 10µl of 2 mg/mL ampicillin to limit bacterial

growth, and 10µl of a 1.0 x 10^4 solution of *M. anisopliae* conidia. Control tubes (eight per colony) were established in the same manner, substituting 5µl of sodium acetate buffer for the termite extract. These preparations were incubated at 25°C, shaking at 50 rpm. After 24 hours, the solution was diluted with 60µl sterile water and plated on ampicillin-containing potato dextrose agar plates. Fungal colonies were counted after incubating plates for 4 days at 25°C.

Statistical analysis

Because of the destructive nature of these tests, different individuals were used for each assay; results were aggregated at the colony level. Field and lab colonies were analyzed separately due to differences between the data sets and the availability of a broader set of explanatory variables for laboratory colonies. Differences in fat content and fungal inhibition between colonies were analyzed using Kruskal-Wallis (KW) non-parametric tests, followed by non-parametric multiple comparisons as indicated (Giraudoux 2009). Logistic regression was applied to elucidate any direct relationship between the fat content and fungal inhibition, as well as individually between each measure of immune potential and survival.

To understand the relationship between survival and the two indicators of immune potential, a generalized linear mixed model (GLMM) was applied to the data (Bates and Maechler 2009). Survival following pathogen exposure was used as the response variable. Fixed factors were fat content,

fungal inhibition, relatedness (lab only), and fresh weight (field only), while random factors were colony, and origin of parents (lab only). The model was simplified in a stepwise fashion until the AIC (Aikake Information Criteria) score could be reduced no further. All statistical analysis was performed in R (R Development Core Team 2009, v 2.9.2).

6.3 Results

Survival, percent fat loss, and CFU reduction were significantly different between colonies in both the lab (Fig 6.1, KW $p<0.001$) and the field (Fig 6.2, KW $p<0.001$). Due to the high variation within most colonies, non-parametric multiple comparisons for each test were not informative. Degree of within-colony variation was not consistent among the three measures, and could not be linked to any of the available explanatory variables. In both the laboratory and the field, colonies with low variation for one test could have higher variation for the others, but there was no consistent pattern.

No correlation was found between fat content and fungal inhibition (lab: Fig 6.3, r^2=0.15; field: Fig 6.4, r^2=0.00;). Fat content and survival were not correlated in the lab (lab: r^2=0.08; field: r^2=-0.05) nor were fungal inhibition and survival correlated (lab: r^2=-0.01; field: r^2=-0.03).

The final simplified models for both lab and field included percent fat loss but not fungal inhibition as having significant predictive power for

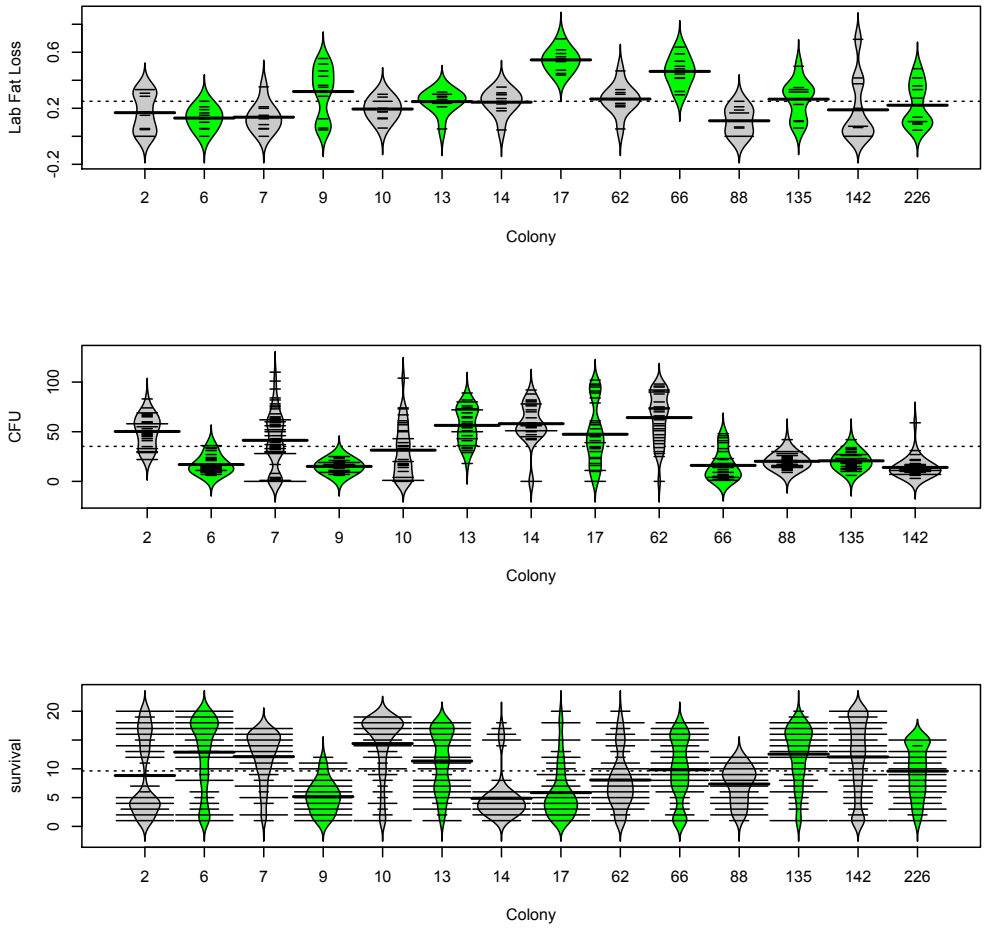


Figure 6.1 Percent body fat (top), colony forming units in the fungal inhibition assay (middle), and survival following exposure to fungal conidia (bottom) for laboratory-raised colonies. The overall mean for each beanplot is the horizontal dotted line. Category means are long solid lines. Short lines are individual observations within the density trace (shaded area).

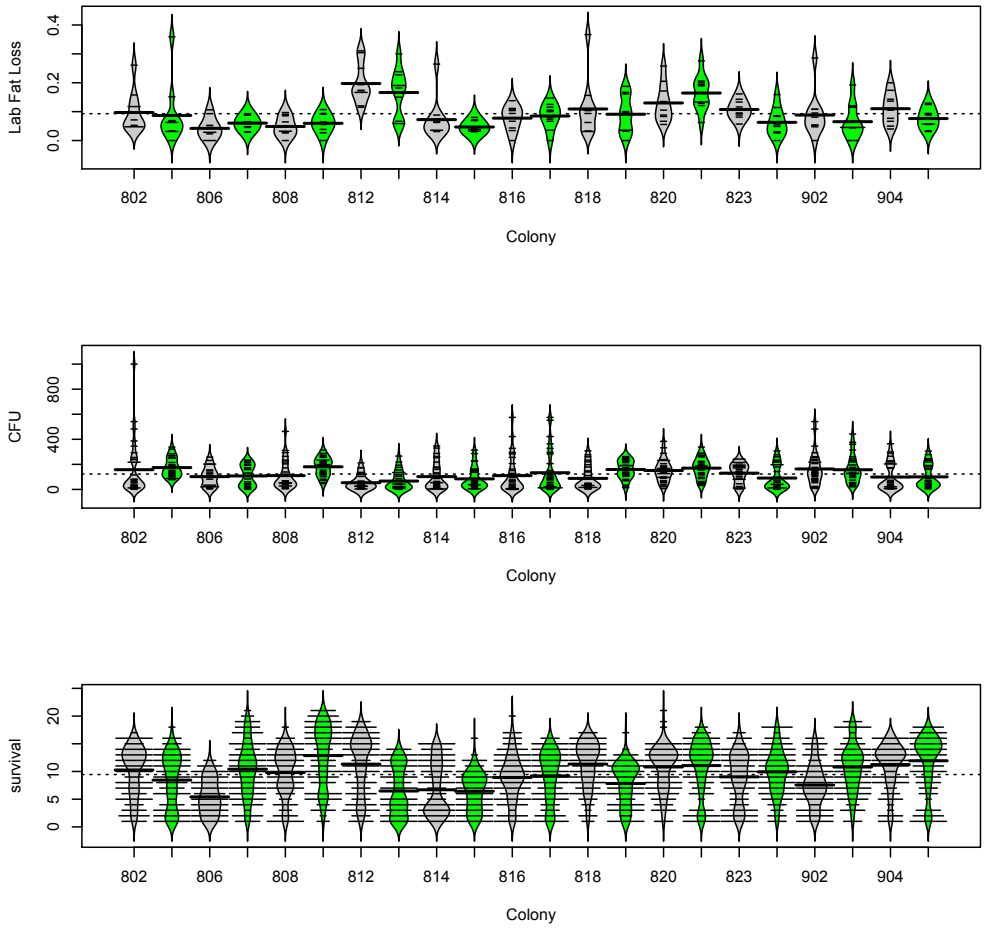


Figure 6.2 Percent body fat (top), colony forming units in the fungal inhibition assay (middle), and survival following exposure to fungal conidia (bottom) for natural colonies in Panama. The overall mean for each beanplot is the horizontal dotted line. Category means are long solid lines. Short lines are individual observations within the density trace (shaded area).

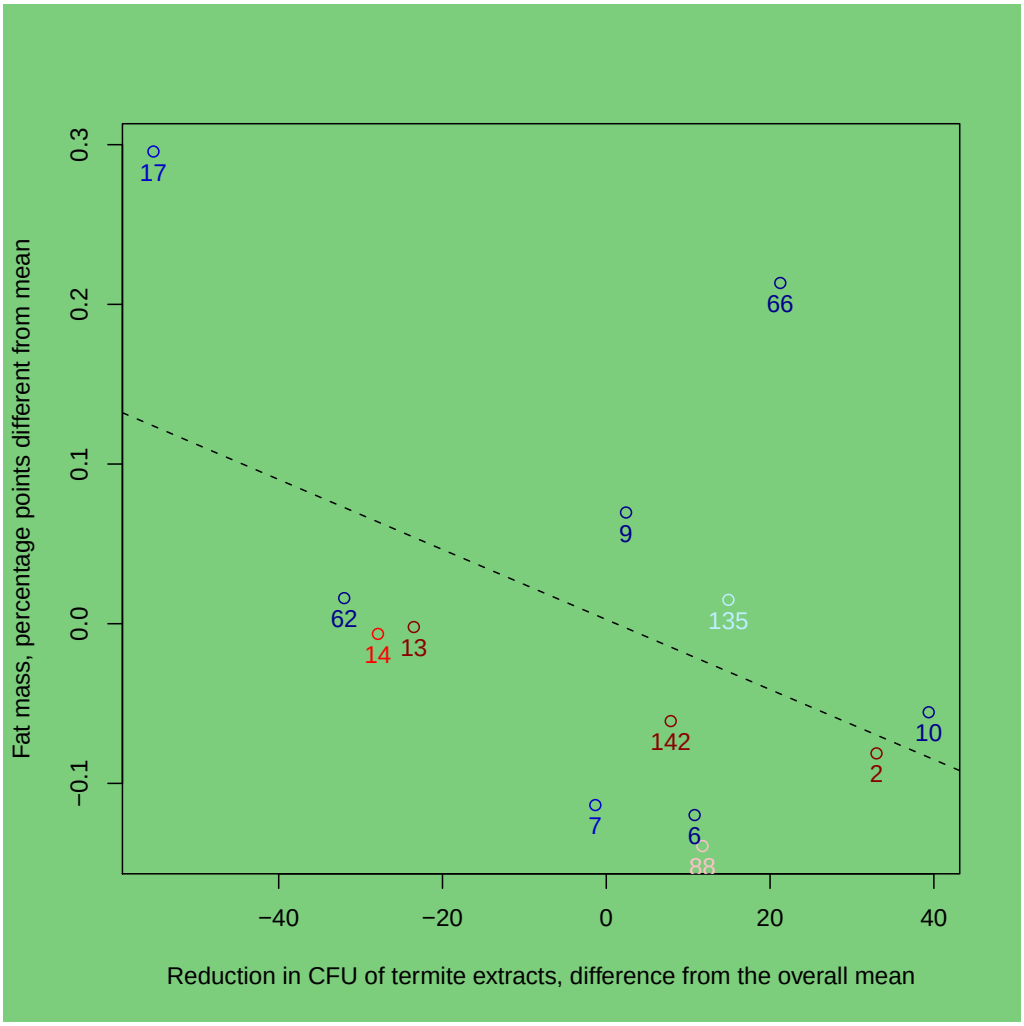


Figure 6.3 Plot of fat mass to fungal inhibition as a function of survival. Percent fat mass is indexed as the difference of the colony mean from the mean of the experiment as a whole. Fungal inhibition, measured as CFUs resulting from incubation of fungal conidia with termite extract minus control lacking termite extract, is also indexed as the difference of the colony mean from the mean of the experiment as a whole. Colonies with red symbols survived longer than average, while those in blue survived less than average. Darker colors indicate greater distance from the mean while paler colors are more similar to the mean. $r^2 = 0.15$

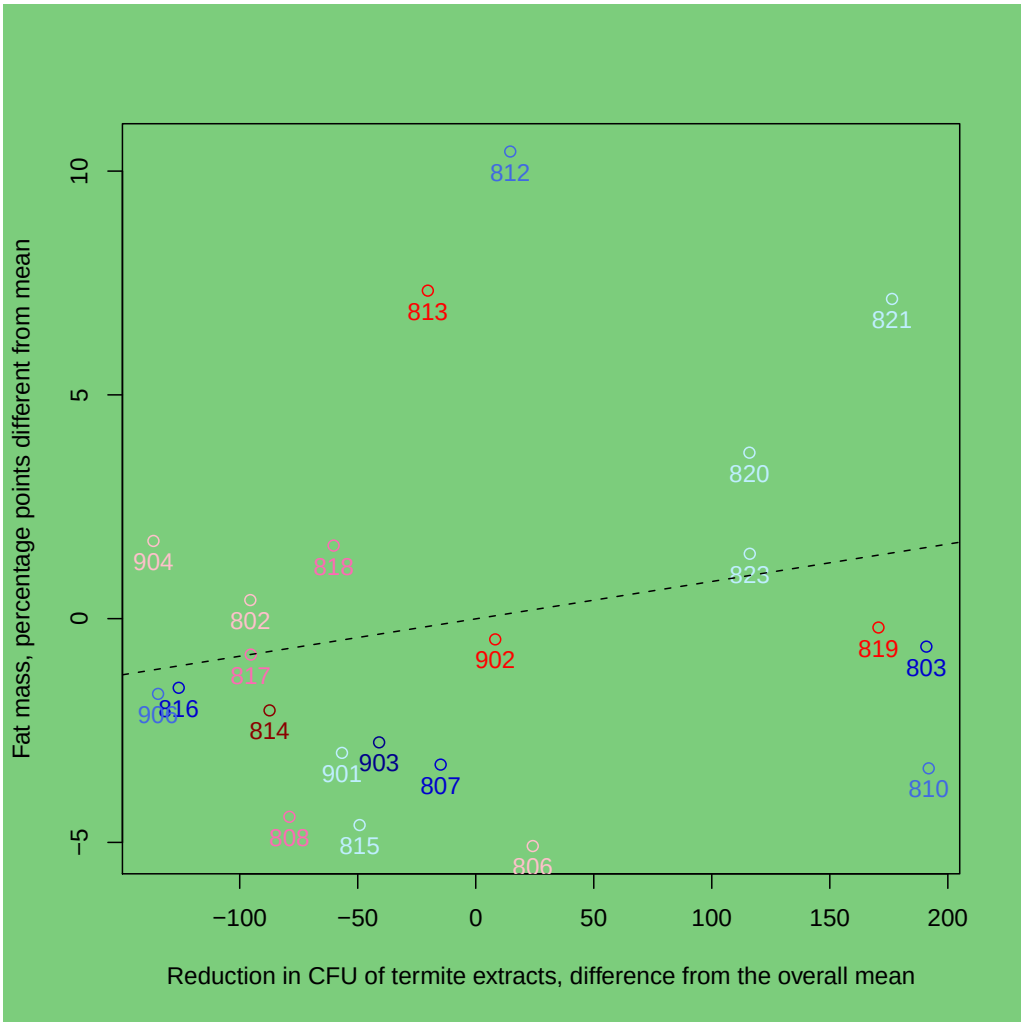


Figure 6.4 Plot of fat mass to fungal inhibition as a function of survival. Percent fat mass is indexed as the difference of the colony mean from mean of the experiment as a whole. Fungal inhibition, measured as CFUs resulting from incubation of fungal conidia with termite extract minus control lacking termite extract, is also indexed as difference of the colony mean from the mean of the experiment as a whole. Colonies with red symbols survived longer than average, while those in blue survived less than average. Darker colors indicate greater distance from the mean while paler colors are more similar to the mean. $r^2 = 0.00$

survival following pathogen exposure. Survival in laboratory colonies was best explained by percent body fat, relatedness, and colony of origin (AIC 609; model including fungal inhibition: AIC 616). Survival in field colonies was best explained by percent fat loss and colony of origin (AIC 1241; model including fungal inhibition: AIC 1253).

6.4 Discussion

These results must be interpreted with caution, as they are comparing, at the colony level, traits that seem to have great variability between nestmates, even within more genetically uniform (nestmate-founded) colonies. Unfortunately, the number of non nestmate-founded colonies included in this study was too small to test patterns based on genetic diversity, which has been shown to be important to disease resistance in ants (Hughes and Boomsma 2004; Reber et al. 2008; Seeley and Tarpy 2006).

Significant differences were found between colonies in their average fat content, degree of fungal inhibition, and survival after pathogen exposure, but there were no correlations between these measures. Therefore, the role that body fat content, either in the mass of the fat body or as stored reserves, plays in immune potential and resistance to fungal pathenogenesis in *N. corniger* remains inconclusive. While a final "best" minimal model was determined, changes to the fit of the model were minimal when the most significant factors (fat loss, relatedness, and colony of origin) were replaced

with others that had been removed from the model. A particular difficulty in interpretation of these results is the inability of the fat dissolution assay to differentiate between body condition and mass of the fat body. It is unclear whether the importance of fat is due to nutritional state (i.e. survival is condition dependent) or to some anti-microbial activity of the fat body, as the assay does not differentiate between fat body mass and bodily reserves stored as fat apart from the fat body.

One or more factors not included in this study may also be important in predicting survival after pathogen exposure. Possible missing factors include differences in diet or pathogen exposure history of the colony, or the energetic or nutritional state of the individual. The efficacy of anti-fungal compounds produced by *Nasutitermes* soldiers (Rosengaus et al. 2000*a*) may also vary between colonies and between individuals. Once these terpenes are spread on the insect cuticle they may be removed using an organic solvent (Haverty et al. 1992), however, the sodium hypochlorite rinses used in this experiment may not effectively remove them. Additionally, behavioral mechanisms, known to be highly important in protecting social insects from pathogen attack (Cremer et al. 2007), were not measured during the survival assay.

Given the large variability of all assays within a colony, it seems likely that individual differences may also be extremely important. Unfortunately, given the destructive nature of the assays, it is not possible to test either measure of immune potential on the same individual undergoing the sur-

vival assay, further undermining the ability of these assays to pinpoint the relationship between body condition, fat body mass, circulating immune factors, and survival following pathogen exposure. If most acute infections are indeed handled by the cellular immune system (Haine et al. 2008), constitutive measures of immunocompetence such as hemocyte counts, phenoloxidase content and encapsulation rates might be more predictive of resistance to fungal infection.

Based on the superior and consistent explanatory power of fat mass compared to fungal inhibition by whole body extracts in the model, it appears that fat mass has more impact on survival than circulating factors such as AMPs. While this seems to be in contradiction with the observation that termite AMPs are not produced by the fat body (Bulmer and Crozier 2004; Lamberty et al. 2001) and thus should not depend on mass of that organ in the way observed in holometablous insects (Doums et al. 2002; Ellers 1995; Lamberty et al. 2001; Wilson-Rich et al. 2008), it is consistent with condition-dependent survival of pathogen exposure. Termites with greater body fat may have sufficient internal reserves to mount a sustained immune response. While not measured in this study, it is possible that mass of the fat body itself is not very different between individuals, and that the observed differences in fat mass are due to individual differences in the amount of bodily reserves stored as fat.

Chapter 7

Hybridization of Two Sympatric *Nasutitermes* species

Abstract

The sympatric Neotropical termites *Nasutitermes corniger* and *N. ephratae* are clearly distinguishable based on morphology, nest architecture, and defensive secretion composition. Previous molecular and morphological phylogenetic analyses have also found them to be genetically distinct. Throughout their range, they share habitat, including nesting and foraging sites. Alate production and release are synchronous. Given the extensive ecological, geographical, and behavioral overlap of these closely-related species, the potential for

interbreeding may exist. To explore this possibility, heterospecific pairs were formed experimentally to examine courtship and colony-establishment behaviors, and reproductive potential. Courtship and nest construction behavior occurred in heterospecific pairs in a similar manner to that of conspecific pairs. *N. ephratae* females paired with *N. corniger* males produced more offspring than conspecific *N. ephratae* or *N. corniger* pairs. *N. corniger* females mated to *N. ephratae* males produced significantly fewer offspring than the reciprocal cross or conspecific pairs. This was also the only pairing in which any aggression was observed. Overall, species mismatch tolerance is high, as is hybrid offspring viability, indicating a potential benefit to occasional natural hybridization. The present data, together with previous evidence from defensive secretions and isozyme analysis, suggests that hybridization occurs in the field, and that reproductive barriers between these two species may be incomplete. Genetic evidence is needed to determine the extent to which introgression occurs. Hybridization could provide a rare but important source of genetic diversity, and may ensure mating opportunities for the more abundant sex of alates in each species.

7.1 Introduction

The Neotropical termite species *Nasutitermes corniger* (Motchulsky) and *N. ephratae* (Holmgren) (Isoptera, Termitidae) are consistently recognized

as distinct on the basis of multiple criteria, including nest architecture (Thorne 1980), worker, soldier and imago (winged reproductive) morphology (Nickle and Collins 1992), defensive secretion composition (Gush et al. 1985; Howard et al. 1988), and COI and 16S rRNA genes (Miura et al. 2000; Scheffrahn et al. 2005*a*). It has also been found that *N. corniger* produces alarm pheromones, while *N. ephratae* does not (Pasteels and Bordereau 1998).

Throughout their ranges (Mexico to Bolivia), *N. corniger* and *N. ephratae* share habitat, including nesting and foraging sites. Along the Panama Canal, mature colonies of the two species can be found within several meters of each other. Both species produce winged reproductives (alates) in overlapping dispersal periods at the beginning of the rainy season, and both are known to develop nests with multiple queens and kings, an unusual trait among termites (Thorne 1985*b*).

Previous research has hinted that gene flow may occur between these species. Analysis of the defensive secretions of soldiers indicate that the diterpenes of Panamanian populations of *N. corniger* and *N. ephratae* are more similar to each other than Panamanian *N. corniger* diterpenes are to those of Costa Rican *N. corniger* (Prestwich 1983). Further, isozyme analysis of *N. corniger* and *N. ephratae* populations in southern Brazil found greater similarity between island populations of *N. corniger* and *N. ephratae* than between *N. corniger* populations on the island and the mainland (Collet and Ruvolo-Takasusuki 2003). While these results and the ecological

overlap between the two species suggest a distinct possibility of hybridization (Prestwich 1983), the maintenance of morphological and genetic differences indicate that there must also be strong barriers to inter-breeding which prevent genetic mixing.

This study explores the boundaries of reproductive isolation between these species. To test genetic or developmental constraints on hybridization, the reproductive output of heterospecific pairs was compared to that of conspecific pairs. Because these species are facultatively polygamous (*N. corniger*: e.g., Atkinson and Adams 1997; *N. ephratae*: Becker 1961), additional mate choice experiments quantified preferences for species and founding group size. Courtship and colony-establishment behavior was observed in both experiments to assess pre-zygotic barriers to hybridization, and reproductive milestones were recorded to investigate post-zygotic barriers and offspring viability.

7.2 Materials and Methods

Termite Collection

Unflown *N. corniger* and *N. ephratae* alates used in this study were collected in Gamboa, Panamá, from three separate parent colonies per species, between April and June 2008, and in April 2009. Alates were sorted by sex and colony of origin, and maintained in Petri dishes lined with moistened Whatman #1 filter paper. Individuals were placed in experiments within

24 hours of collection. All alates in this experiment were fully pigmented and shed their wings within 24 hours of pairing, indicating their readiness to mate. Wood supplied to the incipient colonies was cut from pieces of fallen mango branches on which *N. corniger* workers and soldiers had been foraging, carefully avoiding areas that showed previous termite chewing or trails which could have had pheromonal residues. All replicates were maintained in stacks in open boxes under ambient (Panamá) conditions.

Reproductive Output of Heterospecific Crosses

Conspecific pairs of both species and the reciprocal heterospecific crosses (Table 7.1) were established by placing one male and one female in filter paper-lined 60 mm Petri dishes with a small (1cm x 1cm x 5.5cm) piece of wood (Fig 7.1). A total of 24 replicates/treatment were established in 2008. To control for the possibility of parthenogenetic offspring production, unknown in these species but found in more basal termites (Matsuura et al. 2004), 12 female-female colonies were established for each species.

Incipient colonies were observed for aggression and courtship behavior for 30 minutes post-establishment. Colonies were checked for survival and nest construction activity at 24 hours and 48 hours, then examined weekly thereafter for offspring production. Complete censuses were conducted at 30 and 60 days post-establishment.

Table 7.1 Experimental treatments, hybridization

Hybridization treatments and abbreviations

	N. corniger		*N. ephratae*	
	♀	♂	♀	♂
N. corniger ♀	Ncor♀♀	Ncor♀♂		Ncor♀*x*Neph♂
N. ephratae ♀		Neph♀*x*Ncor♂	Neph♀♀	Neph♀♂

Mate choice treatments by initial alate density

	low (6)		int (10)		high(14)	
	♀	♂	♀	♂	♀	♂
N. corniger	1	2	2	3	3	4
N. ephratae	1	2	2	3	3	4

Figure 7.1 Establishment of heterospecific pairs in filter paper-lined 60 mm Petri dishes. *Nasutitermes corniger* alates are black, *N. ephratae* alates are light reddish-brown.

Mate Choice and Colony Establishment

To further explore the results of the previous experiment, pairing preferences in the presence of heterospecifics were tested in 2009 using a multi-chamber, filter paper-lined arena (Fig 7.2). Two 35mm Petri dishes with entrances cut into one side were secured in a 100mm Petri dish. Four wood-chip nesting sites, 2.5cm x 2.5cm x 0.5cm, were placed in the arena (one in each small Petri dish, two outside of the small dishes) prior to the introduction of alates.

Equal numbers of each species were placed in each arena to create low (1♀, 2♂ per species), intermediate (2♀, 3♂ per species), and high (3♀, 4♂ per species) density treatments (Table 7.1). Five replicates were established per treatment. Treatments were male biased to reflect the natural sex ratios of *N. corniger* alates collected. *N. ephratae* alate sex ratios were equal.

Each arena was observed continuously during the first 30 minutes after placement of the termites, and incidences of aggression and stereotypical courtship behavior were recorded. Arenas were monitored through 80 days post-establishment for progress in nest construction, offspring production, and survival of founding reproductives. Complete censuses were performed at 30 and 60 days post-establishment.

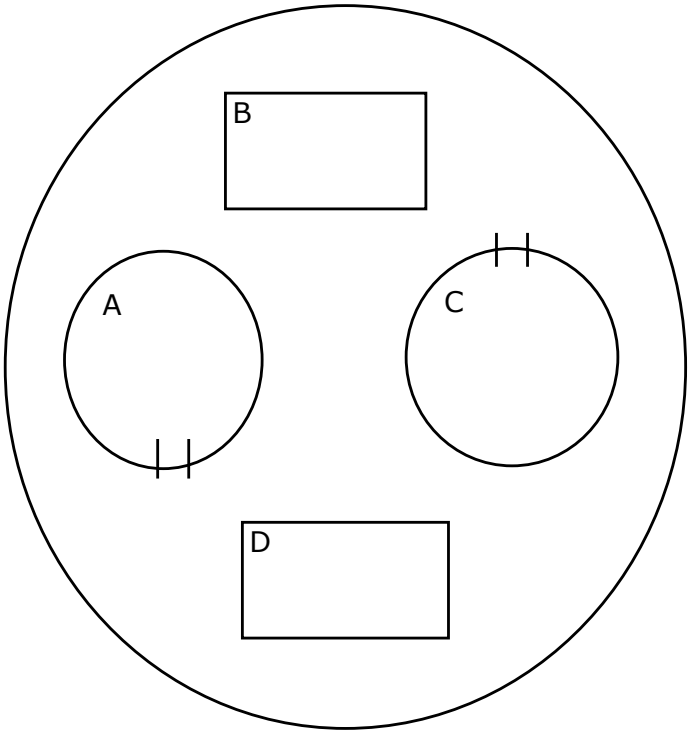


Figure 7.2 Schematic of group choice microcosm (100 mm diameter). Wood chips were placed within 30 mm petri dishes A and C, which termites could access through a door cut into the side wall. B and D are additional wood chip nesting sites, equal in size to the wood chips inside dishes A and C. Drawing is not to scale.

Statistical Analysis

Differences in colony survival and reproductive output was analyzed using Kruskal-Wallis (KW) tests, followed by non-parametric multiple comparisons when indicated (Giraudoux 2009). All statistical analysis was performed in R (R Development Core Team 2009, v 2.9.2).

7.3 Results

Survival

Heterospecific pairs formed readily in laboratory microcosms, successfully undertaking courtship, nest construction, reproduction, and parental care (Fig 7.3). Survival was significantly different between treatments at both 30 days ($p = 0.003$) and 60 days ($p = 0.001$) post-establishment. At 30 days, survival of pairs containing *N. ephratae* females (Neph♀xNcor♂, Neph♀♂) was equivalent and significantly higher than the other pairs. Survival of the two crosses was not significantly different (Fig 7.4), however both were significantly different from the two-female colonies (Ncor♀♀, Ncor♀♀) and the conspecific *N. corniger* pair (Ncor♀♂). At 60 days post-establishment, the pairs with *N. ephratae* females (Neph♀xNcor♂, Neph♀♂) were again statistically equivalent and survived significantly better than all other pairs.

Overall, pairs consisting of an *N. ephratae* female with an *N. corniger* male survived as well as *N. ephratae* pairs, while survival of the reciprocal

Figure 7.3 *Nasutitermes corniger* male (top right) and *N. ephratae* female (lower left) caring for their offspring at 30 days post-pairing.

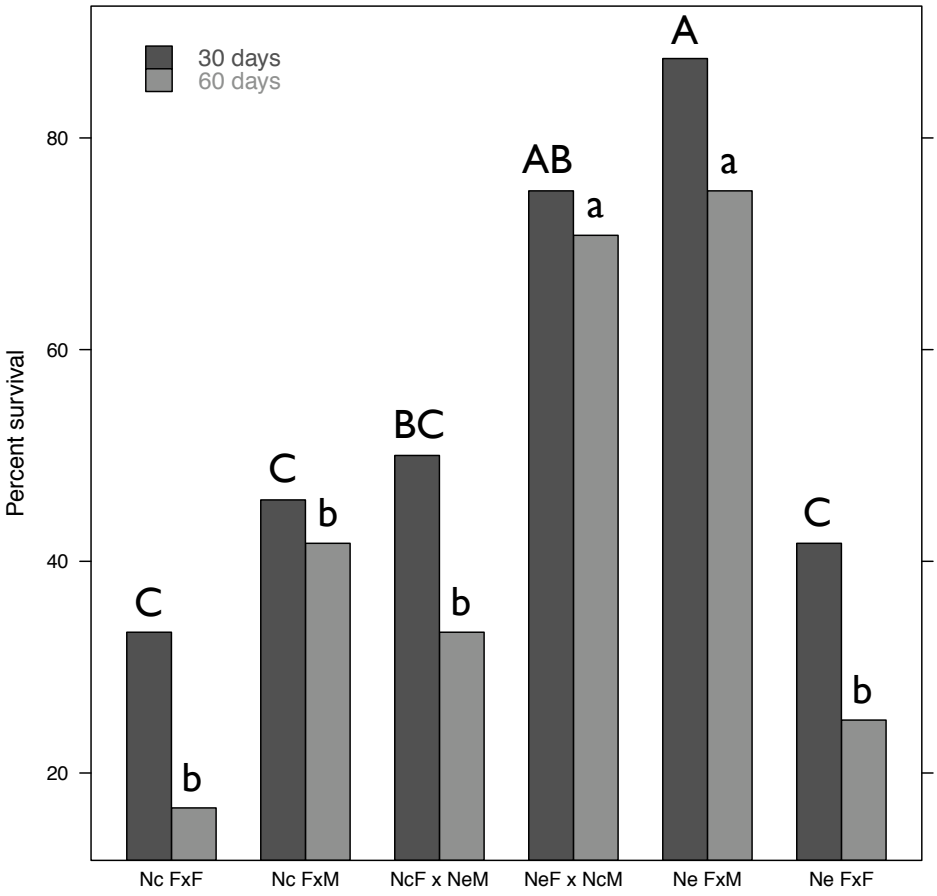


Figure 7.4 Percent survival of heterospecific and conspecific pairs at 30 and 60 days post-establishment. At 30 days, KW p = 0.003; 60 days, KW p = 0.001. Groups with different letters were found to be significantly different by non-parametric multiple comparisons.

cross was not significantly different from that of *N. corniger* pairs. Survival was low and equivalent in the two-female treatments of both species. No aggression was observed between females.

Reproductive output

Two-female colonies in this experiment produced eggs that did not hatch, and they were therefore disregarded in the statistical analysis. Total number of offspring at both 30 and 60 days post-establishment were significantly different between treatments (KW, p = 0.02; Fig 7.5). However heterogeneous subgroups could only be detected at 60 days, when pairs containing *N. corniger* females (Ncor♀xNepho♂) produced significantly fewer total offspring than any other combination, including the conspecific *N. corniger* pairs. Offspring production in Ncor♀xNepho♂ colonies was no different from that of conspecific pairs, and higher than that of the reciprocal cross.

Significant differences were indicated for number of eggs at 30 days (Fig 7.6, p = 0.02), but no differences in number of larvae (Fig 7.7, p = 0.11). No differences in egg number were detected in the pairwise multiple comparisons. KW tests of the individual offspring classes at 60 days were not significant for the eggs (p = 0.11), but were significant for larvae (p = 0.02), workers (p = 0.001), and soldiers (p = 0.001). The cross containing an *N. corniger* female (Ncor♀xNepho♂) produced fewer larvae than any other pairing. Neph♀xNcor♂ pairs produced significantly more workers than the Ncor♀xNepho♂ pairings, although neither was significantly different from

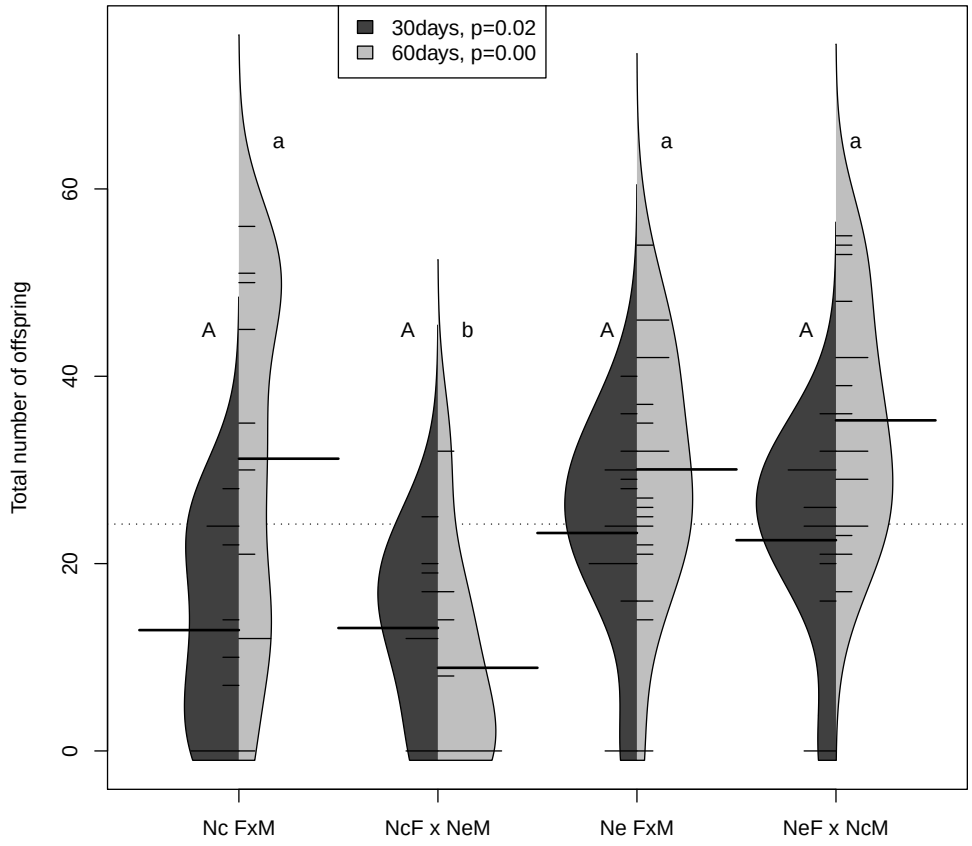


Figure 7.5 Total offspring produced by heterospecific and conspecific pairs at 30 days (KW p = 0.02) and 60 (KW p = 0.11) days post-establishment. The overall mean for the beanplot is the horizontal dotted line. Category means are long solid lines, and the short lines are individual observations within the density trace (shaded area). Individual beans are split by census date. Heterogenous subgroups could not be distinguished for 30 days, although the Kruskal-Wallis test was significant. Heterogeneous subgroups for 60 days post-establishment are indicated next to the corresponding half of each bean.

the conspecific pairs. Both *N. ephratae*-female containing pairings produced significantly more soldiers than the Ncor♀xNepho♂, but were not different from the conspecific *N. corniger* pairings.

Courtship

In the original no-choice experiments, aggression was observed in one-third of the Ncor♀xNepho♂ replicates during the first five minutes. In each case, the female snapped at or bit the male as he initiated contact. In one pair, aggression extended to a face-off in which the female and male tried to bite each other for several seconds. Pairs in which aggression was initially observed were constructing nests together at 24 and 48 hours post-pairing. Most Ncor♀xNepho♂ pairs initially showed some agitation (running rapidly around the dish, separately), but then settled down to explore the arena within five minutes.

In contrast, no aggression or agitation was observed in the reciprocal heterospecific pairs (Neph♀xNcor♂). In two such replicates the female and male avoided interacting for the first five minutes, however in two other replicates, tandem running was initiated immediately. No unusual agitation was observed within the initial 30-minute observation period for the Neph♀xNcor♂ replicates; during that time the heterospecific alates were observed resting together, tandem running, engaging in mutual grooming, and exploring the wood. No aggression was observed in any of the other treatments or at any other time point. Agonism was apparantly transient

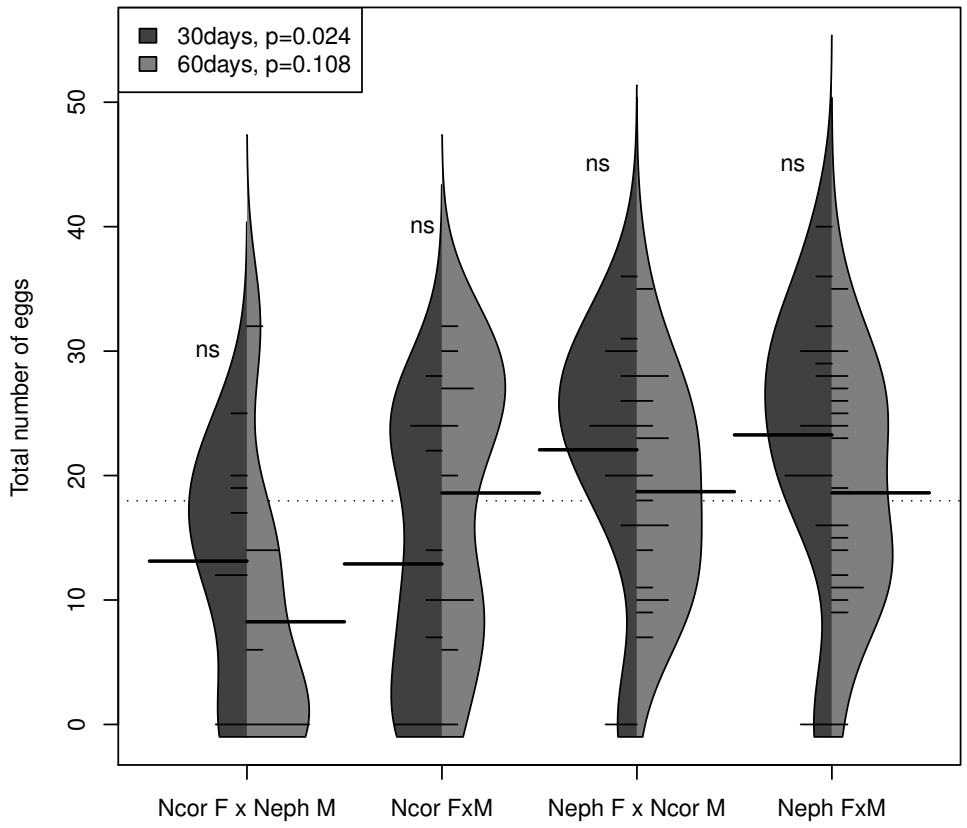


Figure 7.6 Number of eggs produced by heterospecific and conspecific pairs at 30 days (KW p = 0.11) and 60 days (KW p = 0.02) post-establishment. The overall mean for the beanplot is the horizontal dotted line. Category means are long solid lines, and the short lines are individual observations within the density trace (shaded area). Individual beans are split by census date. Heterogenous subgroups could not be distinguished for 30 days, although the Kruskal-Wallis test was significant; heterogeneous subgroups were not determined for 60 days, because the Kruskal-Wallis test was not significant.

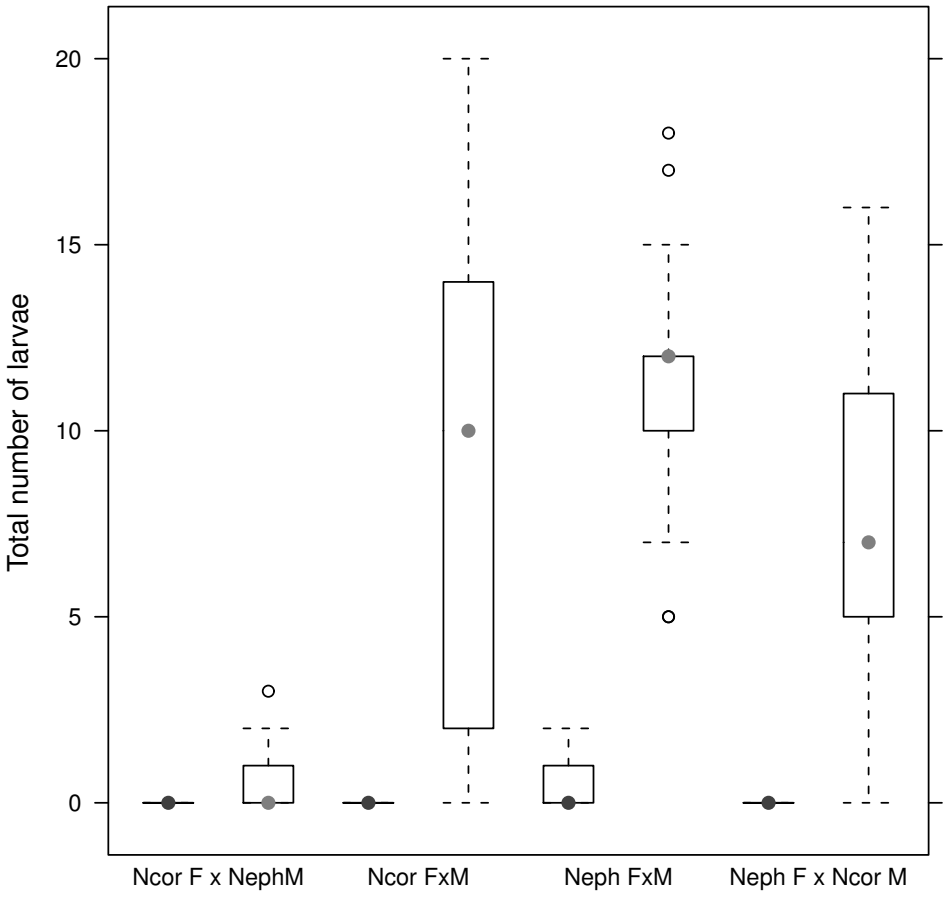


Figure 7.7 Number of larvae produced by heterospecific and conspecific pairs at 30 days(KW p = 0.11) and 60 days (KW p = 0.02) post-establishment.

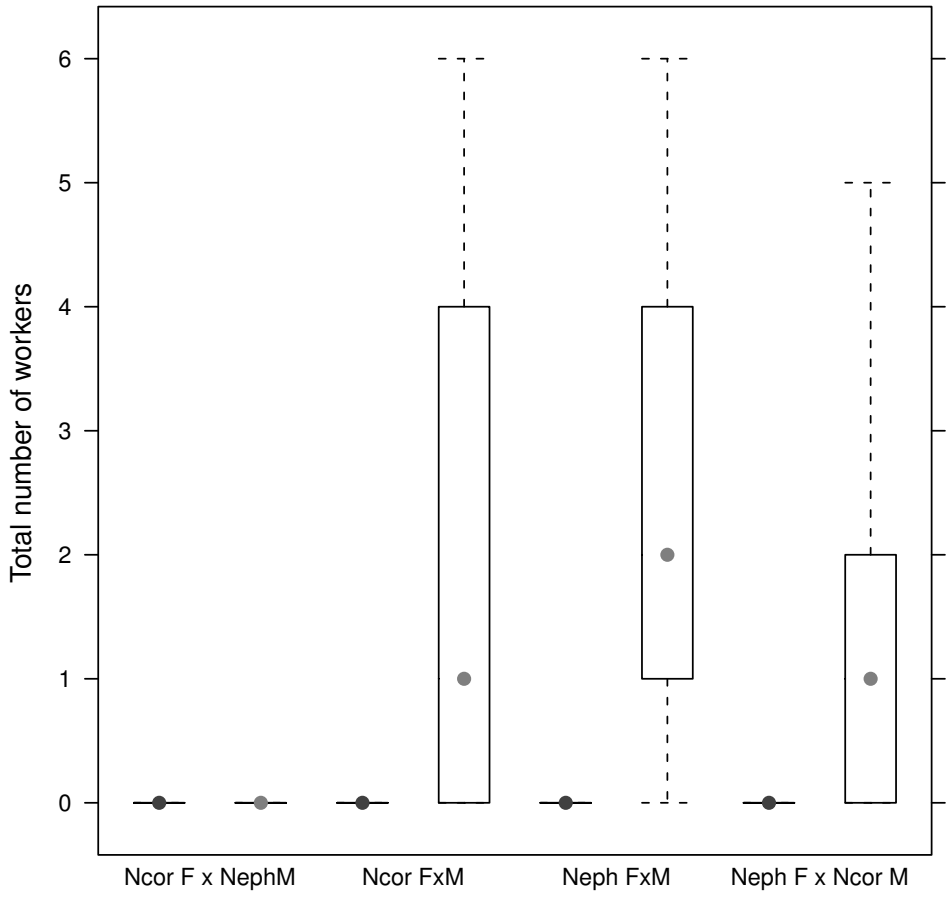


Figure 7.8 Number of workers produced by heterospecific and conspecific pairs at 60 days post-establishment (KW p = 0.001).

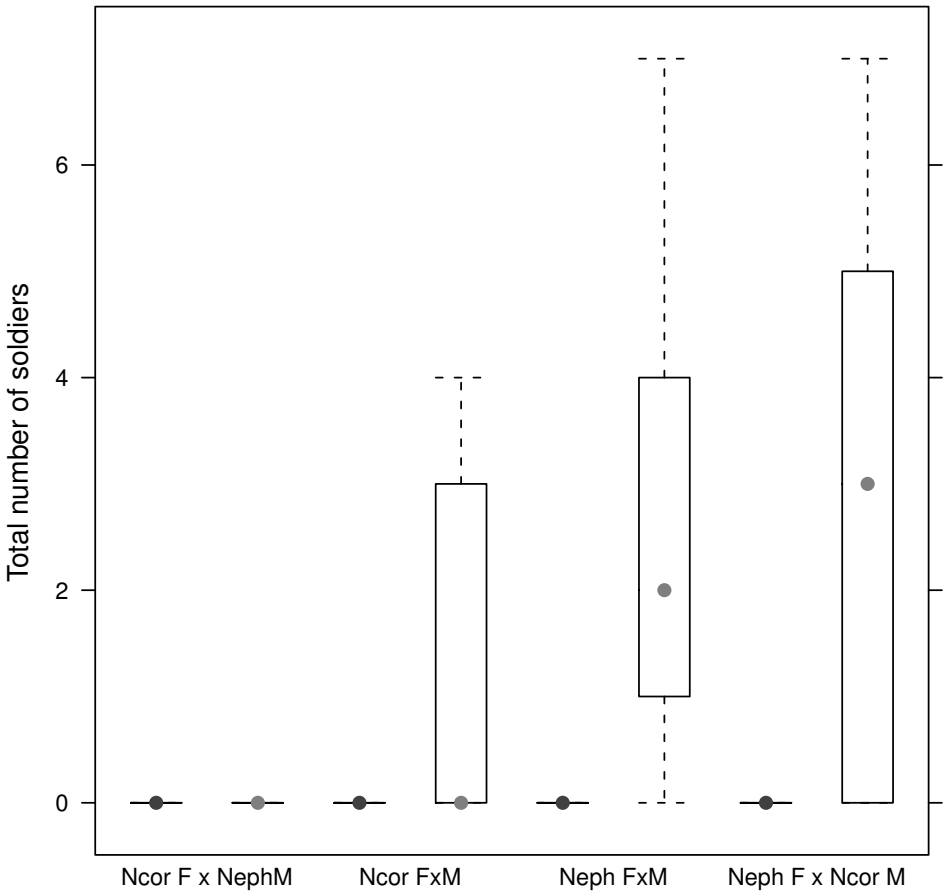


Figure 7.9 Number of soldiers produced by heterospecific and conspecific pairs at 60 days post-establishment (KW p = 0.001).

and non-lethal as survival rates did not differ from those of conspecific pairs of the female's species.

Mate choice and colony establishment

Heterospecific pairs as well as conspecific pairs formed in the mate choice arenas, and engaged in the typical courtship behavior of tandem running (male following female, antennating her abdomen, while female searches for a nest site). Heterospecific pairs were maintained through numerous encounters with other alates and opportunities to re-form into conspecific pairs. No overt aggression was observed in this experiment.

In spite of the availability of nesting sites, more mixed-species partnerships were formed than conspecific partnerships at both high and low alate densities, and typical group size was 3 or 4 (Table 7.2). Nearly two-thirds (19 out of 29) of the groups observed contained members of both species. At the intermediate densities, founding groups were more often conspecific or dominated by one species. Group membership, excluding deaths, was generally stable after 24–48 hours, although occasionally an individual would change groups following the death of one or more group members.

Table 7.2 Chosen group size and composition as a function of initial alate density

	Initial density			
	6	10	14	overall
mean group size +/- SD	3.1 +/- 1.07	3.8 +/- 1.39	4.3 +/- 2.42	3.8 +/- 1.85
median group size	3	4	3	3
mode group size	3	2,3,4,5	3	3
con:heterospecific groups	3:4	5:4	2:11	10:19
most common group size/type	4 conspecifics	3 conspecifics	3 heterospecifics	4 conspecifics

7.4 Discussion

Survival and reproductive success of heterospecific pairs in this study depended on the species of the female partner, and Neph♀xNcor♂ pairs often exceeded conspecific pairs in both survival and offspring production. The significantly lower reproductive output of Ncor♀xNeph♂ pairs indicates that postmating isolating do barriers exist. Although founder survival was not significantly different from that of conspecific *N. corniger* pairs, reproductive success was significantly lower. Development and early stage viability of hybrid offspring appear to be compromised in heterospecific pairings with *N. corniger* females.

While this study does not test whether this effect is entirely genetic or may be mediated by *Wolbachia* or other cytoplasmic incompatibilities (Bordenstein and Rosengaus 2005), the data are consistent with theories of genetic isolation (Dobzhansky 1951). These theories predict that the cross involving the rarer sex of each species, in this case *N. corniger* females and *N. ephratae* males, will be more strongly selected against than the cross between the more abundant sexes. The interaction of multiple isolating factors may also place stronger selection pressure on one cross than on the reciprocal (Matsubayashi and Katakura 2009), which could result in differences between crosses as seen here. Males are the more abundant sex of *N. corniger* alates (Thorne 1982*b*, and pers. obs.), while *N. ephratae* sex ratios are equal or slightly female-biased (pers. obs.).

Interestingly, when given the opportunity to select a mate from a pool of con- and hetero-specifics, the majority of chosen founding groups observed contained members of both species. Pre-reproductive isolating mechanisms would have predicted nearly complete segregation by species (Arbuthnott 2009; Pfennig 2003). Identifying pheromones, indicating both species and sex, are likely highly conserved within the genus *Nasutitermes* (Buděšínský et al. 2005), and tend to differ in relative concentrations of compounds rather than in absolute composition (Pasteels and Bordereau 1998). Individual variation in expression could perhaps further blur distinctions between prospective mates.

The rarer females of *N. corniger* should in theory be choosier than *N. ephratae* females or *N. corniger* males (Dobzhansky 1951), and indeed only *N. corniger* females were observed initiating aggression toward heterospecifics. However, those isolated instances of aggression did not ultimately inhibit courtship or nest construction in heterospecific pairs. The low levels of aggression and the prevalence of courtship behavior, ultimately resulting in nest establishment between hetrospecifics, suggests that courtship behavior is similar. Although courtship behavior is known to be important in reproductive isolation of many species (Coyne and Orr 2004), it is a weak or insufficient barrier between these species.

Mate choice criteria, such as body mass, can interfere with species recognition (Pfennig 1998). Individuals in this study were not weighed, however choice experiments with *N. corniger* suggest that mate choice and choice

of founding group size are weakly correlated with female mass (Chapter 4). Hybrid mating has been found to increase fitness under challenging ecological conditions in other species (Pfennig 2007), with lower-quality females (lower mass relative to body size) more likely to switch to a heterospecific mating strategy, presumably perceiving both their own phenotype and the environmental conditions. Thus, mass or some other indicator of mate quality may trump species recognition cues.

Divergence times between these species are not known (R. Scheffrahn and M. Engel, pers. comm.), but based on the results of the current study, reproductive isolation between the species is apparantly not complete. It is unclear whether heterospecific mate choice is a recognition error or an adaptive choice, however, heterospecific pairing does not decrease fitness, at least for the *N. ephratae* female x *N. corniger* male combination. Rather than simply making the best of a bad job (Reyer 2008), the most abundant sex from each species may paradoxically increase its fitness by choosing the "wrong" mate.

Hybridization between these two termite species may be an important source of genetic diversity in isolated populations, such as the island populations (Collet and Ruvolo-Takasusuki 2003) or recently disturbed areas (Prestwich 1983) sampled in the previous studies. Field observations of intermediate colony morphology in mainland areas of well-developed forest (pers. obs.), combined with the present results, indicate that heterospecific pairings may be a rare but ubiquitous phenomenon. The fertility of F1

(hybrid) alates is not directly known given that several years are required for a new colony to produce alate brood, however the defensive secretion analysis of Prestwich (1983) and the isozyme evidence of Collet and Ruvolo-Takasusuki (2003) indicates that hybrid alates are likely to be fertile and backcross with the parent species when they do occur. Genetic analysis of a large number of colonies in the population may further delineate divergence times and amount of introgression between these species.

Chapter 8

Summary and Conclusions

This dissertation presents the first detailed observations of incipient colony development in *Nasutitermes corniger*, and the first systematic evaluation of hypotheses concerning pleometrosis in termites. First I will summarize the contributions of this study to our knowledge of termite colony foundation. Next, I present evidence from this study relevant to each of the hypotheses for the evolution of pleometrosis. In closing, I will discuss the major contributions of these experiments to the study of pleometrotic colony foundation and termite mating systems, and the likely ontogeny of polygamous mature colonies of *N. corniger*.

8.1 Incipient colony development in *N. corniger*

Egg laying commences soon after flight and mate/nest site selection, as suggested by a previous study in the closely-related *Nasutitermes ephratae* (Becker 1961). In contrast to both that study and a recent examination of colony foundation in *Trinervitermes trinervoides* (Adam and Mitchell 2009), oviposition was continuous. In both the laboratory and in the field, *N. corniger* queens did not wait for the first clutch of eggs to mature into workers before laying additional eggs, with the result that there were offspring at various developmental stages at any given time.

The first helper in incipient colonies of *N. corniger* is most often a worker, or a worker and a soldier may mature on the same day. This is similar to the other studies in derived termites (Adam and Mitchell 2009; Becker 1961). Some colonies in these studies were observed to produce only workers and no soldiers in the first several broods, unlike the more basal termites in which a soldier always differentiates in the first brood (Thorne 1997).

The highest mortality was seen early in colony development, with about one-half of laboratory colonies failing in the first 30 days post-establishment. Mortality was 90% within the first 90 days. This is may be an over-estimate of an incipient colony's chances for success in the field, as predators and competitors such as ants were carefully excluded from the experiments. On

the other hand, the open environment of a petri dish and the constant disturbance through handling and censuses may impose more severe stresses on the reproductives than the cloistered "nuptial chamber", as well as exposing the young colonies to more pathogens and perhaps more suitable conditions for pathogen growth.

Pleometrosis was rare in the field, with only 5% of recovered colonies containing more than one king. No colonies were found containing multiple queens, in spite of the consistent recovery of polygynous mature colonies in this species (Table 1.1 and references therein). Under laboratory conditions, persistance of pleometrotic groups was very low (Chapter 2) but somewhat higher when alates were allowed to choose their own co-founders (Chapter 4).

8.2 Evaluation of hypotheses on the evolution of pleometrosis

Hypotheses for the evolution of pleometrosis were initially presented in Chapter 1 (Table 1.2). Each hypothesis will be briefly reviewed, followed by a discussion of relevant evidence from this study. A summary of these arguments can be found in Table 8.1.

Previous research has found a distinct survival benefit to group colony foundation in the social Hymenoptera (Adams and Tschinkel 1995; Bartz and Hölldobler 1982), with some exceptions (Deslippe and Savolainen 1995).

Table 8.1 Hypotheses on the evolution of non-monogamy in termites.

Hypothesis	Evidence from this study
Polygamy reduces the risk of colony extinction due to founder death.	Individuals in larger groups did not survive longer. Colonies founded by groups generally died sooner than those founded by pairs.
Dispersal is risky. Predation or competition for nest sites severely limit chances for success.	No evidence for "hotspots" of pleometrosis in the field. No effect of nest site availability on founding group sizes. Predation not tested in these experiments, but thought to impact pairs and pleometrotic groups similarly.
Benefits of increased genetic diversity: behavioral polymorphisms lead to greater colony efficiency.	Behavioral observations during the first 90 days were inconclusive.
Benefits of increased genetic diversity: greater resistance to parasites and pathogens.	Following immune challenge, mortality in polygamous colonies was not relatively lower than in pairs. Large variability was found in worker immune potential, not correlated with colony characteristics.
Intraspecific parasitism.	No behavioral evidence found. Determination of differential reproductive effort awaits genetic analysis of offspring.
Increased reproductive output and colony growth.	Some groups have more offspring sooner than pairs, however in general pairs and trios exceed quintets.
Phylogenetic constraints.	Individuals from particular colonies more often choose pleometrosis. Individuals from other parent colonies choose to establish colonies with fewer co-founders.

The only previous study addressing this question in termites (Darlington 1984), did not find increased survival of pleometrotic groups compared to pairs. The hypothesis that pleometrosis reduces the risk of colony extinction due to founder death predicts that colonies should survive longer when established by more co-founders.

This hypothesis was not supported in this study, and in fact individuals in quintets consistently died more quickly than those placed in pairs and trios, which were roughly equivalent in survival. If excess founders were being eliminated to reach an optimal group size, mortality of individuals in quintets should have been higher than mortality of those in pairs and quintets, but the survival rate of colonies should have been the same. That was not found to be the case. In these experiments, colonies founded by quintets were actually more likely to go extinct than those founded by pairs or trios. Increasing group size beyond a pair or trio significantly increased the likelihood of a colony going extinct.

Pleometrosis fostered by risky dispersal, including predation pressure and nest site limitation, should result in "hotspots" of pleometrotic colony foundation in areas where these stressors are particularly acute. This was previously found to be the case for *Macrotermes michaelseni* (Brandl et al. 2001, 2004), which is more likely to found colonies pleometrotically under marginal conditions at the edge of its range. However, no such clustering of pleometrotic incipient colonies was found in the field for N. corniger. Pleometrotic incipient colonies were found in different sites in each year,

with different local conditions including moisture, heterospecific competitors, and density of mature colonies. In both the laboratory and the field, pleometrotic groups could be found isolated or near other incipient colonies, apparently without regard for availability of additional nest sites. Similarly, nest site availability had no effect on founding group sizes in microcosm and mesocosm choice experiments. Higher alate densities resulted in initially larger founding groups, but mortality during the first weeks of decreased group size below levels found in the lower-density treatments.

Predation is most severe during dispersal, although ant predation on founding pairs and incipient colonies was observed in the field. This factor was not tested in these experiments, but resilience of pleometrotic founding groups to such overwhelming attacks is not expected to differ from that of pairs.

The role of physiological and behavioral polymorphisms in colony-level resistance to disease or in increasing energetic efficiency, brought about by increased levels of within-colony genetic diversity, is still under debate (Brown and Schmid-Hempel 2003; Oldroyd and Fewell 2007; Seeley and Tarpy 2006; Wilson-Rich et al. 2009). Calleri et al. (2006*a*) suggest a behavioral, but not physiological, basis for increased survival of progeny from outbred monogamous pairs compared to those from inbred monogamous pairs in a primitive termite. Possible benefits of genetic diversity during colony foundation were tested by observing behavior of pairs and pleometrotic groups during nest construction and parental care.

Behavioral observations did not rule out efficiencies in division of labor during nest construction or parental care of offspring in polygamous colonies, but suggested that some experimental pleometrotic groups cooperated better than others. In the experiments reported here, mortality of individuals in polygamous colonies was not relatively lower than that of individuals in pairs following exposure to either a parasitic nematode or a fungal pathogen, and was generally higher for individuals in larger groups. This indicates that not only is there no pathogen protection benefit of pleometrosis, but that individuals may actually be more susceptible to pathogen attack during this time, in contradiction to the group size and group diversity benefits reported for groups of non-reproductives.

It has been suggested that social insects have evolved unique mechanisms to combat challenges associated with group living, particularly in terms of parasites and pathogens (Hughes et al. 2002; Walker and Hughes 2009; Wilson-Rich et al. 2009). Indeed, genetic diversity within a colony has been found to improve resistance to *Metarhizium anisopliae* exposure in ants (Hughes and Boomsma 2004). The survival of workers following pathogen exposure, as well as two measures of immune potential, varied greatly within and between termite colonies in my experiments. These measures of immune potential were not correlated with colony characteristics, including genetic similarity due to nestmate rather than non-nestmate parents. Genetic similarity and heterozygosity of individuals from field colonies awaits genetic analysis.

Hypothetical intraspecific parasitism could occur when an individual minimizes their investment during the costly initial nest construction and production of the first broods, and then takes over reproductive duties in the colony after the development of helpers. Manipulation of reproductive effort in the presence of co-founders has been observed in some ant species (Bernasconi and Strassmann 1999), while no such cheating has been found in others (Aron et al. 2009). No evidence for intraspecific parasitism was found in the behavioral observations in this study. Rather, the limited observations of parental care and nest construction activities suggest a generally egalitarian division of labor. Samples have been collected to ascertain differential contributions to the offspring pool using microsatellite markers.

Faster growth of colonies founded by multiple reproductives is well-supported for certain ant species (Deslippe and Savolainen 1995; Johnson 2004; Sasaki et al. 2005), and has also been observed in the facultatively pleometrotic termite *M. michaelseni* (Kaib et al. 2001). In the experiments reported here, certain pleometrotic colonies had higher reproductive output compared to monogamous pairs, as predicted by the faster colony growth hypothesis, however in general pairs and trios exceeded quintets. Quintets often laid eggs more quickly in experimental colonies, but smaller groups had steadier growth and were more successful in hatching their eggs into larvae, and then developing those larvae into workers and soldiers. In group size choice experiments, there was no significant effect of group size on number of offspring at 60 days post-establishment. No colonies established

in the study have reached maturity and produced alates, so it remains unknown whether successful pleometrotic groups can achieve this milestone faster or more consistently than monogamous pairs.

There is some circumstantial evidence from this study to support a genetic basis for pleometrosis. The phylogenetic constraint hypothesis suggests that individuals from certain lineages should be more likely to found colonies in pleometrotic groups, or to be more successful in such founding groups, than individuals from other lineages. This was pattern was seen in the data from the mate-choice experiments. Colony of origin itself was not a significant predictor of mortality in no-choice experiments, however, it is clear from the choice experiments that composition of the group is important to success. The combinations of founding group members in no-choice experiments are probably not what the alates themselves would have chosen, potentially leading to incompatibilities between co-founders.

Alates of *N. corniger* are far more tolerant of additional founding group members than are alates of more basal termites (Nutting 1969), and young incipient colonies will merge without the fighting and reduction in the number of primary queens and kings seen in primitive species (Thorne et al. 2003). Thus, there may not be a distinct preference for group colony foundation (Gotzek and Ross 2007, in fire ants), but rather an enhanced propensity for cooperation or varying degrees of "lack of intolerance", as suggested by tests of aggression and experimental mergers between mature colonies (Adams et al. 2007).

8.3 Concluding remarks

For pleometrosis to be maintained in the population, this alternative strategy must do no worse than monogamous pairs in terms of survival and reproductive fitness. For it to be favored, pleometrotic groups must survive and/or reproduce better than monogamous pairs. On average, termites in this study placed into groups fared worse than those placed in pairs. Rare pleometrotic groups survived as well as, and produced as many or more offspring than, monogamous pairs. Termites given a choice demonstrated a preference, linked with colony of origin, for founding colonies in larger or smaller groups. Those choosing to be in pleometrotic groups survived as well or slightly less well than those that chose to be in pairs. Surviving pleometrotic groups under natural and semi-natural conditions, however, reproduced as well as pairs, and sometimes at a higher rate than pairs.

Pleometrotic groups in the field are rare, approximately 5% of incipient colonies recovered in the first 90 days post-flight. All such colonies found in this study contained two kings and one queen. In the 23% of mature *N. corniger* colonies found to contain multiple unrelated reproductives, queens nearly always outnumber kings (Table 1.1 and references therein). This indicates that the colonies I encountered post-flight are not the same ones that would eventually survive to establish permanent mature arboreal nests. Polygamous colonies of *N. corniger* are thus not pleometrotic, or in other words, are not originally established (founded) by multiple reproductives.

To achieve observed queen:king ratios in polygamous mature colonies from the monogamous pairs and king-biased trios found post-flight, there must be fusion of incipient colonies or adoption of additional alates early in colony development. Research presented in this dissertation as well as reports on the potential for fusion of mature colonies (Adams et al. 2007), support this "meet and merge" mechanism for the ontogeny of polygamous colonies. Given the high densities at which incipient colonies may sometimes be found (pers. obs.), it is inevitable that young colonies will meet. In the more primitive termites, this has disastrous consequences for the founding reproductives — a fight to the death (Thorne et al. 2003). This is understandable, given the pressure for maintaining high relatedness within the colony.

Why is this pressure insufficient to enforce monogamy in higher termites? In the more derived Termitidae, the presence of fixed sterile castes, with no reproductive potential, relaxes the requirement of high within-colony relatedness is relaxed. This may permit primary reproductives, as well as their offspring, to have a more relaxed view of accepting additional queens or kings into a merged incipient colony. While *N. corniger*, workers and soldiers from mature colonies, in general, aggressively attack non-nestmates, including alates (Adams et al. 2007; Levings and Adams 1984; Thorne 1982*a*, and pers. obs.), experimental mergers of intact laboratory-established colonies (discussed below), suggest that workers and soldiers in incipient colonies are highly tolerant of non-nestmates, including young

queens and kings. It would be interesting to know whether incipient colonies repel or accept newly-flown alates after their first year post-establishment, or if tolerance is limited to individuals that already "smell" like a queen (D'Ettorre et al. 2004; Weil et al. 2009). Clearly there is a limited window of opportunity during which colony mergers generally occur, although the results of Adams et al. (2007) suggest that some colonies remain tolerant and retain the potential for mergers during maturity.

Merging with a neighboring incipient colony could in fact be a selective advantage: by accepting an additional reproductive pair that have already passed through the initial mortality bottleneck, along with their attendant workforce, colony size and reproductive capacity double instantly. This advantage over other colonies of the same age could lead to production of alates that then disperse and pass on these "super-cooperative" genes sooner than would be possible with monogamy. Multiple kings are not necessary to provide sperm for multiple queens, so in this scenario supernumerary kings are probably eliminated through competitive interactions. This was also suggested by Thorne (1982*a*, *b*, 1984), and must certainly be the case, as the few pleometrotic groups found in the field had more kings than queens, while all mature colonies dissected in the course of this study had one or more queens but only one king. Direct evidence for king reduction has been seen in another polygamous termite, *Macrotermes herus* (Darlington 1988).

The limited evidence available at this point indicates the feasibility of this meet-and-merge hypothesis. Preliminary experiments not reported in

this dissertation indicate that founding pairs will readily accept additional reproductives during the first 60 days post-establishment, and that young colonies, up to at least 9 months post-establishment in the lab (approximately 200 workers and soldiers), and possibly beyond, will peacefully meet and merge. Developing colonies in the laboratory have occasionally been observed foraging in each others' containers. In some cases this results in aggression and mortality, while in other cases they apparently mix without conflict.

Such co-mingling would appear to be a real-world phenomenon as well, based on tests involving naturally-established mature colonies with well-developed arboreal carton nests. Some mature colonies are not aggressive toward each other and will fuse when placed adjacent to each other in the field (Adams et al. 2007). In addition, polycalic colonies have been found with (multiple) unrelated reproductives in various nests (Atkinson and Adams 1997), suggesting colony fusion after the initial construction of the arboreal carton nest.

This work refutes the idea that polygamous colonies in this species are established by pleometrosis. The ontogeny of polygamous colonies of *N. corniger* is thus different from that of *Macrotermes michaelseni*, the only other facultatively polygamous termite in which it has been examined. Clearly, then, there has been more than one elaboration of non-monogamous mating systems in termites. This may impact our view of the evolution of the derived termites, as well as challenging theories on the development of

mating systems in social insects (Boomsma 2009*a*, *b*), to this point based on knowledge of the social Hymenoptera.

The studies reported in this dissertation fill large gaps in our knowledge of the natural history of this species and of this clade of higher termites in general. Open questions still remain regarding the period of colony development between the end of the first rainy season and the establishment of a mature arboreal nest. From the data presented here, this appears to be the critical period for the development of polygamy in *N. corniger*. The possibility of a genetic component to increased tolerance of conspecifics remains to be explored, as well as its impact on the meet-and-merge hypothesis for the origin of polygamous colonies.

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