Causes and Consequences of Queen-Number Variation in the California Harvester Ant *Pogonomyrmex californicus*.

by

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ABSTRACT

Social insect colonies exhibit striking diversity in social organization. Included in this overwhelming variation in structure are differences in colony queen number. The number of queens per colony varies both intra- and interspecifically and has major impacts on the social dynamics of a colony and the fitness of its members. To understand the evolutionary transition from single to multi-queen colonies I examined a species which exhibits variation both in mode of colony founding and in the queen number of mature colonies.

The California harvester ant *Pogonomyrmex californicus* exhibits both variation in the number of queens that begin a colony (metrosis) and in the number of queens in adult colonies (gyny). Throughout most of its range, colonies begin with one queen (haplometrosis) but in some populations multiple queens cooperate to initiate colonies (pleometrosis). I present results that confirm co-foundresses are unrelated. I also map the geographic occurrence of pleometrotic populations and show that the phenomenon appears to be localized in southern California and Northern Baja California. Additionally, I provide genetic evidence that pleometrosis leads to primary polygyny (polygyny developing from pleometrosis) a phenomenon which has received little attention and is poorly understood. Phylogenetic and haplotype analyses utilizing mitochondrial markers reveal that populations of both behavioral types in California are closely related and have low mitochondrial diversity. Nuclear markers however, indicate strong barriers to gene flow between focal populations. I also show that intrinsic differences in queen behavior lead to the two types of populations observed. Even though populations exhibit strong tendencies on average toward haplo- or pleometrosis, within population variation exists among queens for behaviors relevant to metrosis and gyny. These results are important in understanding the dynamics and evolutionary history of a distinct form of cooperation among unrelated social insects. They also help to understand the dynamics of intraspecific variation and the conflicting forces of local adaptation and gene flow.

DEDICATION

To my mom Denise, who put up only the slightest resistance to unending attempts to keep and breed creatures of all shapes and sizes throughout her house for the better part of two decades.

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CHAPTER 1

INTRODUCTION TO THE LIFE HISTORY OF *POGONOMYRMEX CALIFORNICUS* AND PRIMARY POLYGYNY

Cooperation, where individuals act collectively to benefit members of a group, presents an evolutionary puzzle. Cooperative interactions stand in direct contrast to more straightforward selfish behaviors where individuals act to maximize their own fitness at the immediate expense of others. In extreme forms, such as when reproductive altruism occurs, this mystery is especially poignant. The sterile worker castes of the eusocial insects were specifically noted by Darwin as a special difficulty to his theory of natural selection (Darwin 1859). Darwin proposed a solution to the mystery which was later formalized by Hamilton (1964) as inclusive fitness theory. Hamilton's theory explained how an allele for altruism could go to fixation in a population if the benefits conferred to a recipient by an actor's help are greater than the costs to the actor multiplied by the probability of sharing of said allele (usually dictated by relatedness).

Historically, much of the research regarding intraspecific cooperation has focused on related individuals, invoking the indirect fitness benefits of cooperating with kin. (Alonso and Schuck-Paim 2002; Baglione et al. 2003; Bernasconi and Strassmann 1999; Bourke and Franks 1995; Clutton-Brock 2002; Creel and Rabenold 1994; Eberhard 1975; Griffin and West 2003; Hamilton 1964; Queller and Strassmann 1998). However increasing numbers of behavioral and molecular studies tracking relatedness have begun to emphasize cooperation among non-relatives (Aviles 2002; Balshine-Earn et al. 1998; Clutton-Brock 2002; Dugatkin 2002; Dugatkin and Mesterton-Gibbons 1996; McDonald and Potts 1994; Mesterton-Gibbons and Dugatkin 1992; Queller et al. 2000; Reyer 1980; Rood 1978; Toquenaga 2005).

In contrast to kin groups, groups of unrelated individuals have fitness costs associated with group living which cannot be offset by indirect benefits between cooperating kin. Thus each group member must receive direct benefits or have a higher probability to gain direct benefits in the future in order for these associations to be stable over evolutionary times (Bernasconi and Strassmann 1999). These benefits must counteract the costs of social living such as competition

with group members and sharing of resources (Silva, Macdonald, and Evans 1994), increased exposure to parasites and pathogens (Hughes, Eilenberg, and Boomsma 2002; Traniello, Rosengaus, and Savoie 2002) and energy expended in maintaining social standings (Goymann and Wingfield 2004). The importance of considering these costs as deterrents of social behavior is highlighted by the fact that despite the documented benefits which members of social groups receive, the vast majority of animals remain solitary. These systems present new mysteries as to why cooperative behaviors evolve in some ecological settings and certain taxa but not in others, what benefits these interactions give to their group members, and how they are resistant to exploitation by cheaters. A dramatic example of such cooperation among non-kin occurs in a few ant and termite species where unrelated queens join together with other queens despite being able to independently found and maintain a colony. This initial association matures into a stable cooperation which lasts throughout the life of the colony, perhaps over a decade.

The associations of nest-founding queens which occur in some ant species provide an interesting case to investigate cooperative interactions among non-relatives. Although ants are eusocial, during colony founding, queens of many ant species go through a solitary life history stage. In most species of ants, ant queens are not tolerant of the presence of conspecific queens and thus most ant colonies are haplometrotic and monogynous (Hölldobler and Wilson 1990). However, in a handful of species, such as the California seed-harvester ant *Pogonomyrmex californicus*, unrelated queens join together during the colony founding stage and not only initiate colonies together but remain together (primary polygyny), sharing resources and producing reproductive offspring for the life of the colony (perhaps more than 10 years). Individuals who participate in these associations incur costs inherent to social living and presumably receive benefits but those benefits are not fully understood. Interestingly, other populations of *P. californicus* do not exhibit this cooperative behavior and queens are highly aggressive toward conspecific queens if forced to found together in the laboratory. The selective forces that drive this behavior and maintain intraspecific variation for primary polygyny are poorly understood but are important to the understanding of the evolution of cooperation.

CHAPTER 2

THE GEOGRAPHIC EXTENT OF PLEOMETROSIS AND PRIMARY POLYGYNY IN POGONOMYRMEX CALIFORNICUS

Introduction

Initially, myrmecologists operated under the assumption that except in very extreme cases, ant colonies contained a single queen. Wheeler, a pioneering ant researcher, speculated that some colonies could be founded by more than one queen if queens from the same maternal nest should happen to come across one another while searching for a nest site (Wheeler 1910). This explanation of joint colony founding which was assumed to occur only between related queens demonstrates resistance to thinking about cooperation among unrelated individuals. In many cases individuals who are cooperating often have very straightforward costs while the benefits to their association may be less apparent. The idea that pleometrosis and polygyny were extremely rare began to change when a seminal paper described several ant species with multi-queen colonies and stated the importance of considering queen number in ant research (Hölldobler and Wilson 1977). Over the years, others have continued to add to the list of ant species which regularly exhibit more than one queen per colony (Keller 1995).

Metrosis and gyny

Haplometrosis (one queen per colony) and pleometrosis (multiple queens per colony) refer to the initial colony founding stage when queens dig a nest and raise their first cohort of brood (Hölldobler and Wilson 1990). In contrast, monogyny (one queen per colony) and polygyny (multiple queens per colony) refer to the state of a mature colony, i.e. a colony that produces sexual offspring. The terms monogyny and polygyny will be used as such in this dissertation and should not be confused with their general usage in behavioral ecology where they refer to the number of female partners a male acquires.

In independently-founded ant colonies (i.e. not dependent colony founding events which most often occur with wingless queens such as in army ants) monogynous colonies are produced

in two ways. One, when a haplometrotic colony persists with its initial single queen into the mature colony stage (primary monogyny) or two, when a pleometrotic colony undergoes a stage of queen reduction ending in only one living queen in a mature colony (secondary monogyny) (fig. 2.1). The majority of ant species are assumed to be monogynous or at least functionally monogynous (only one sexually reproducing queen per colony) (Hölldobler and Wilson 1990). The majority of monogynous colonies presumably begin as haplometrotic nests but secondary monogyny stemming from pleometrotic nests are also common in ants (Bartz and Holldobler 1982; Bernasconi and Strassmann 1999; Heinze 1993; Hölldobler and Wilson 1990; Rissing and Pollock 1987; Sasaki et al. 2005; Sommer and Holldobler 1995; Tschinkel and Howard 1983).

Polygynous ant colonies can also come about in two general ways: when pleometrosis occurs and multiple queens persist through the mature colony stage (primary polygyny), or when queens are accepted into an already established monogynous colony (secondary polygyny) (fig. 2.1). Primary polygyny is considered rare and is poorly understood but taxonomically widespread examples have been reported (Hölldobler and Carlin 1985; Johnson 2004; Mintzer 1987; Rissing et al. 1989; Trunzer, Heinze, and Hölldobler 1998). Its evolution is interesting from a theoretical standpoint because it involves life-long cooperation among unrelated queens and their offspring in a eusocial colony environment.

Secondary polygyny is distinct from primary polygyny in part because of the many variations of relatedness structure it introduces into a colony and the potential for adoption and dispersal of queens throughout the colony life. Secondary polygynous colonies run the gambit between high to low queen relatedness and from populations with distinct colonies (multicoloniality) or colonies which show no aggression over a large area (unicoloniality).

A growing number of studies of which only a few are cited, have addressed the complexity of secondary polygyny and the selective pressures potentially responsible for its evolution (Bekkevold, Frydenberg, and Boomsma 1999; Bourke 1994; Bourke and Franks 1995; Craig and Crozier 1979; Deslippe and Savolainen 1995; Janzen 1973; Pedersen and Boomsma 1999; Rissing and Pollock 1988; Yamauchi et al. 1991). There are two major hypotheses to explain secondary polygyny. The first involves ecological constraints on independent founding

(Bourke 1994; Bourke and Franks 1995; Herbers 1986; Hölldobler and Wilson 1990). The second invokes the benefits to colonies of acquiring multiple queens which increases colony survival (Nonacs 1988; Gadau et al. 1998). Additional queens in a secondary polygynous colony can theoretically be either nestmate queens returning from a mating flight or mated foreign queens from other nests. However, presumably most queens that are readopted are mated nest-mates (Keller 1995). Relatedness estimates of cohabitating queens between different species range from essentially 0 to 0.83 with a mean relatedness of 0.35 based on 23 species. Low relatedness values among queens in secondary polygynous systems have been shown to be consistent with the phenomena of relatedness erosion which occurs in a large family, especially because relatedness among queens is negatively correlated with queen number in a colony across different species (Keller 1995) which is not consistent with the idea that low relatedness among queens stems from the adoption of non-nestmate queens (Bourke and Franks 1995; Hölldobler and Wilson 1990).

To date, one population of *P. californicus* has been described as pleometrotic with incipient nests containing multiple queens (Rissing, Johnson, and Martin 2000). In the laboratory, queens collected from this area coexist without aggression well beyond the production of workers suggesting that this is a case of primary polygyny (Johnson 2004). The two distinct but interrelated phenomena of pleometrosis and polygyny both occur in *P. californicus*. Here we provide genetic evidence that queens in foundress associations are not related and that mature field colonies contain multiple unrelated reproductive queens which produce both worker and reproductive offspring. We also present a map of the known extent of multiple-queen colonies in *P. californicus*.

Methods

We characterize metrosis and gyny in populations of *P. californicus* in three ways. 1) by excavating and counting the number of nests with cofounding queens after mating flights (figs. 2.2 and 2.3), 2) by uniting dealate queens in forced associations in the lab to see how tolerant they are of conspecifics (also an indication of primary polygyny if queen associations persist), and 3) in mature colonies in nature where queens are usually not accessible, polygyny can be detected by genetically analyzing series of workers or reproductives to evaluate the number of matrilines

present (a measure of gyny). This latter technique allows the detecting of the number of queens which have survived to the mature colony stage and how many are producing workers and/or sexual offspring which has important implications for the evolutionary dynamics of the system. This is an important measure because it has been shown repeatedly that colonies can have more than one queen but be functionally monogynous with only one queen producing sexual offspring (Bourke and Franks 1995).

Study populations

We collected 12-20 males of *P. californicus* per colony from 10 colonies per population, from one documented pleometrotic and two documented haplometrotic populations prior to their summer mating flights while sexual offspring were still in the nest. On the 3rd-4th of July 2006 we collected from the California haplometrotic population (Lake Henshaw; 33° 13.928' N -116° 45.381' W; 824m) and the pleometrotic population (Cameron Valley; 32° 43.525'N -116° 27.892'W; 1,008m). These two populations are approximately 50 km apart in San Diego County, CA. On May 27th – June 6th 2008 we collected from a second haplometrotic population at the Salt River recreation area northeast of Phoenix, AZ (Coon Bluff; 33° 32.870'N -111° 38.617'W 409m). Nests were excavated and males and workers from each nest were immediately stored in 100% EtOH.

Excavation of incipient nests

To quantify frequency and level of metrosis, incipient nests were excavated shortly after mating flights and queens were counted. In all cases, excavated nests exhibited a single hole in the ground slightly larger than the diameter of a queen's body. This is important because queens can often be found in temporary non-nest associations under debris or around the base of plants between the time period of the mating flights and the excavation stage. Mapping pleometrosis on a wide scale is challenging because incipient nests are present for only a short time annually which also varies by population.

Microsatellite analysis of offspring from mature colonies

We genotyped males and workers (12-20 individuals per colony) from 12 colonies for each of the three focal populations to determine the number of queens present in mature field colonies. In addition, we genotyped 2 colonies per population for 3 extra populations. Individuals were genotyped at 5 microsatellite loci (Pb8, Ppro2, Pb5, PPro1, Pb6). These data were then used to calculate the minimum number of matrilines necessary to explain the allelic diversity observed among members of a colony (males and workers). Minimum queen number per colony was then compared among populations.

Individuals were removed from 100% EtOH then crushed in 150µl of 5% Chelex® 100 resin (Bio-Rad) in TE pH 8.0 and 1 µl proteinase K (5mg/mL) was added. Samples were incubated at 57° C for 1 hour and subsequently heated to 95° C for 5 min, then centrifuged at 14,000 rpm for 10 min. The supernatant containing DNA was stored at -20°C. Individuals were genotyped at 5 microsatellite loci: Pb5, Pb6, Pb8, PPro1, Ppro2 (Pol et al. 2008; Volny and Gordon 2002). PCR took place in a 12µl reaction volume containing 0.125 units of Taq polymerase, 2.5µl of 5X Go Taq Buffer, 0.5µl MgCl2 (50mM), 0.5µl dNTPs (10mM), and 6.4µl of H2O. Each locus was amplified separately using the following PCR program: an initial 4 min at 95° C, 38 cycles of 95° C for 30 sec, an annealing temperature cycle for 45 sec (Pb5, Pb6, Pb8= 56° C; Ppro1, Ppro2 =58° C) and 68° C for 1.5 min, and finally 68° C for 4 min. A Licor 4200 model sequencer was used for size determination of products.

Because of the difficulties which sometimes arise when adapting microsatellite loci for a different species than that which they were developed for, there are varying numbers of individuals and loci which amplified successfully for each colony. For this reason, and to be conservative in our estimates of queen number the results for matrilines per colony are reported as minimum number of queens necessary to explain the observed genetic diversity. This was done out of necessity as a more straightforward measure of effective queen number per colony with a measure of error or non-detection rate was difficult to impossible to carry-out because of the heterogeneity of the data set. In about 20% of cases colonies did not amplify for a given locus

even after multiple attempts. Out of the 210 colony/loci combinations in the study (42 colonies amplified for 5 loci) there were 48 cases where a given locus did not amplify for an entire colony.

Most of the individuals amplified are males. For 6 colonies where not enough males could be collected, workers were used in addition to or instead of males (appx. A). Male genotypes are particularly useful in resolving matrilines in this case as males have mothers but no fathers in Hymenoptera. Genotyping males in this species is also beneficial because queens are polyandrous (see ch. 5) and so the high level of genetic diversity present in workers and dealate queen offspring makes it particularly difficult to reconstruct pedigrees even when data from multiple loci are available. Utilizing male genotypes circumvents this problem. The one potential problem with using male genotypes to assess queen matrilines is that the presence of worker reproduction or brood raiding by adult colonies could confound the data by giving a false signal of the presence of additional queens. However, intense levels of brood raiding involving the removal of male offspring would be required to explain the consistent pattern of multiple matrilines we observed in the Cameron Valley population. Also, there is no reason to suspect that the pleometrotic population should have higher levels of brood raiding or higher worker reproduction than other two focal populations. In the case of worker reproduction, levels in this species should be very low as predicted by kin selection theory as the high levels of polyandry (ch. 5) should cause workers to carefully police any male production by workers that might occur in queen-right colonies (Bourke and Franks 1995).

Foundress relatedness

To calculate the average relatedness of cofoundresses, we genotyped all members of 16 queen associations. Queens were taken from individual nests, consisting of 2-7 queens shortly after mating flights from the Cameron Valley Population. Individuals were genotyped across 8 polymorphic microsatellite loci: Pb5, Pb6, Pb8, (see above) as well as Po03, Po07, Po08, (Wiernasz, Perroni, and Cole 2004), BJ04 (Gadau Lab, unpublished), and LXA GT1 (Bourke, Green, and Bruford 1997). Hamiltonian average relatedness (Hamilton 1971) was calculated according to Queller and Goodnight (1989) using the program FSTAT (Goudet 1995).

Behavioral assessment as an indication of metrosis and gyny

We have shown that queens from pleometrotic/polygynous and haplometrotic/monogynous populations exhibit distinct behavioral phenotypes on average (see ch. 5). This serves as an additional line of evidence for populations characterized through one of the other two methods above. Behavioral assessment may also serve as the sole means of characterizing a population for metrosis if we collected post-flight queens from a site but were unable to find incipient nests.

Results

Foundress relatedness

Genetic analysis confirmed that queen associations are composed of individuals with very low relatedness as the mean Hamiltonian relatedness of each set of cofoundresses across 16 incipient nests was 0.103, much lower than expected if queens were assorting with full or half sibling nest-mate queens after mating flights. The 95% confidence interval for this value based on 15,000 bootstraps overlapped with a relatedness of zero (-0.023 – 0.182).

Excavation of incipient nests

In addition to data collected by Rissing et al. (2000) and Tate Holbrook (unpublished data) We have discovered one additional pleometrotic site at Colonia Nueva Indú Baja California, Mexico with a high frequency of pleometrotic founding (figs. 2.3 and table 2.1).

Microsatellite analysis of offspring from mature colonies

The majority of the colonies from Salt River and Lake Henshaw had offspring genotypes consistent with a single queen (9 of 12 and 10 of 12 respectively; figs. 2.4 and 2.5). Contrarily, only one of the colonies from Cameron Valley did not contain evidence of multiple-queen reproduction. The number of polygynous colonies per population varied significantly between populations (Kruskal–Wallis: H=16.712, D.F.=2, p=.0002). Multiple comparisons for Kruskal-Wallis tests using Statistica 7.1 (StatSoft Inc, Tulsa, OK) revealed that the Cameron Valley

population differs statistically from both Salt River (p=.01098) and Lake Henshaw (p=.00182) but that Lake Henshaw and Salt River were not different (p>.1). Three additional populations surrounding Cameron Valley were censused but only two colonies from each population were genotyped. Although the sample size is not large enough to make comparative conclusions with the three focal populations, all of the three additional populations contained multi-queen colonies (fig. 2.4, table 1). Assuming the populations had the same frequency of polygynous nests as Lake Henshaw (2 out of 12 or 0.1667) then the chances of sampling two polygynous colonies as our first choices at Shockey Truck Trail and Alpine would have been only 2.8% ($0.1667^{\wedge 2}$). This suggests that these populations are similar in their frequency of polygyny to Cameron Valley. At Lake Sutherland which is in between Lake Henshaw and Cameron Valley one of the nests sampled was polygynous and the other was not.

Behavioral assessment as indication of metrosis and gyny

In addition to the behavioral data for the three focal populations detailed in ch. 4, we have characterized the Lyons Valley Road sight as pleometrotic and primary polygynous after 11 forced associations each containing 3 post flight queens exhibited only a single case of a death due to aggression in the laboratory for over three months.

Discussion

Our findings demonstrate that multiple foundresses of *P. californicus* indeed persist to the mature colony stage and cooperate with other unrelated queens and their progeny in a highly cooperative eusocial society. More specifically, we show that these foundresses contribute to both the sterile work force (a form of colony maintenance) and jointly produce sexual offspring which has important implications for the evolutionary dynamics of this system. We provide data demonstrating that the frequency of polygynous colonies differs markedly between populations of *P. californicus* with one population (Cameron Valley) exhibiting a much higher frequency of polygyny than the other two focal populations. Interestingly however, the Salt River and Lake Henshaw populations which had been characterized as haplometrotic and monogynous (Rissing,

Johnson, and Martin 2000) also exhibit polygyny, albeit at a much lower frequency (figs. 2.4 and 2.5). These lower levels of polygyny indicate that although there is pronounced population-level variation for gyny in this species, intra-population variation for queen number in mature colonies exists in every population tested. This mirrors the patterns observed in behavioral differences in populations (see ch. 4) as well as the pattern of variation seen in levels of metrosis (fig. 2.4).

Another important implication of our matriline counts is that although high numbers of queens are regularly observed in pleometrotic nests (Rissing, Johnson, and Martin 2000) there is no genetic evidence that any more than 5 queens are contributing to a given mature colony's offspring (fig. 2.6). This suggests that queen mortality, natural or due to queen interactions, is occurring sometime between initial colony founding and the mature colony stage or that some queens present in the colony do not reproduce. It is possible that queen culling is occurring in pleometrotic associations, as low levels of mortality due to aggression have been observed in forced associations with queens from Cameron Valley (ch. 4). Two large natural associations from the Nueva Indú site with more than 20 queens each suffered high levels of queen mortality in lab nests, resulting in failed colonies with no observed aggression. In contrast, associations of 2-7 queens from the same site suffered no such mortality, for unknown reason.

Theoretical considerations of pleometrosis

Theoretical frameworks to understand the evolution of pleometrotic founding in ants have been published repeatedly (Bernasconi and Keller 1996; Bernasconi and Strassmann 1999; Heinze 1993; Tschinkel and Howard 1983; Bartz and Holldobler 1982). In *P. californicus* pleometrosis has been shown to increase queen survival during the colony founding stage (Johnson 2004). Most of the proposed benefits to pleometrotic founding, however do not explain why cooperating foundress queens would remain together permanently, which presents a challenge to understanding primary polygyny. Proposed benefits of pleometrosis include increased survival and offspring output which increases fitness for an incipient colony in competition against other incipient and mature colonies (Bartz and Holldobler 1982; Herbers 1986; Hölldobler, Grillenberger, and Gadau; Rissing and Pollock 1987; Tschinkel 1992, 1998). According to

theoretical frameworks put forth to understand pleometrosis however, the association becomes unstable after workers eclose and begin foraging because the colony shifts to an open resource system and benefits to pleometrosis no longer hold (Bernasconi and Strassmann 1999). At this point queens are expected to fight to the death over the fitness payoff of monopolizing the lifetime resources of the colony for their own reproductive output. This makes the few but taxonomically scattered cases of primary polygyny in ants where the pleometrotic relationship persists throughout colony life (Heinze et al. 2001; Johnson 2004; Kolmer, Hölldobler, and Heinze 2002; Mintzer 1987; Rissing et al. 1989; Trunzer, Heinze, and Hölldobler 1998) especially intriguing because they do not fit into this framework and the benefits which would outweigh the costs of life-long sharing of the colony's resources are unclear (Bartz and Holldobler 1982; Hölldobler, Grillenberger, and Gadau).

Primary polygyny: novel cooperation among non-relatives

Only a handful of documented cases of primary polygyny in ants exist: *Atta texana* (Mintzer 1987), *Pachycondyla sp.*(Trunzer, Heinze, and Hölldobler 1998), *Pogonomyrmex californicus* (Johnson 2004), *Acromyrmex versicolor* (Rissing et al. 1989). Cases of oligogyny also occur where mutually intolerant queens occupy the same nest but separate spatially (Gadau et al. 1998; Hölldobler and Carlin 1985; Carew, Tay, and Crozier 1997). Cases of primary polygyny have also been documented in the independently eusocial termites (Hacker et al. 2005). The phenomenon is extremely understudied despite the fact that it represents a distinct form of cooperation among nonrelatives in the social insects. This is perhaps for two reasons. One, as stated above the phenomenon does not appear to occur in a large number of species. Two, by definition primary polygynous associations come from pleometrotic foundings, and the framework constructed to understand pleometrosis does not help to explain primary polygyny; rather it predicts queen culling by queens or workers once a colony moves beyond the founding stage (Bernasconi and Strassmann 1999).

Our lack of understanding about primary polygyny is evident from several reviews and books addressing multi-queen colonies. In a review article entitled *Social life: the paradox of*

multiple queen-colonies, the only allusion to primary polygyny reads, "I will not consider associations of queens during the period of colony foundation because such associations are generally transient and do not lead to long-term polygyny" (Keller 1995). A review article entitled *Cooperation among unrelated individuals: the ant foundress case*, in reference to foundress associations, states "However, these associations are unstable because the **advantage of having multiple foundresses ends** with the emergence of adult workers" (Bernasconi and Strassmann 1999) (emphasis added). Also a prominent and important book in the field of myrmecology, *Social Evolution in Ants* makes only one statement with reference to an explanation for primary polygyny: "These species therefore represent rare cases of primary polygyny, although why they fail to revert to monogyny is unclear" (Bourke and Franks 1995).

All pleometrotic associations, and hence all cases of primary polygyny, are assumed to be composed of unrelated queens for several reasons. First, the swarm-mating behavior of ants and the subsequent dispersal of queens searching for suitable nest sites, all of which takes place over a short period of time, make it very unlikely that foundresses assort by relatedness in associations (Bernasconi and Strassmann 1999). Second, evidence from lab experiments with pleometrotic associations of *Messor pergandei*, *Acromymrex versicolor*, *Pheidole tucsonica*, *P. californicus*, and *Myrmecocystus mimicus* show queens have no preference for microsympatrically occurring queens which should be more closely related on average (Rissing, Johnson, and Martin 2000; Rissing and Pollock 1986). In contrast, similar experiments showed that the wasp *Polistes fuscatus* does indeed sort into associations by kinship (Noonan 1981). Third, evidence from allozyme markers in *Solenopsis invicta*, *M. pergandei*, and *A. versicolor* has also confirmed that ant foundresses with mating flights do not assort with kin (Hagen, Smith, and Rissing 1988; Ross and Fletcher 1985). Genetic evidence demonstrating that cooperating queens of the primary polygynous ant *Pachycondyla cf. inversa* are non-relatives is also available (Heinze et al. 2001; Kolmer, Hölldobler, and Heinze 2002).

Messor pergandei is a seed harvester ant with a similar habitat and life history to *P. californicus*, which also exhibits variation for queen number (Rissing, Johnson, and Martin 2000). Research on this ant has focused on variation for metrosis between populations. Interestingly, *M.*

pergandei exhibits a generally opposite pattern to *P. californicus* in its geographic pattern of pleometrosis. Areas with pleometrosis in *M. pergandei* occur in the harsher Mohave and Sonoran deserts and single queen colonies are much more frequent in the more productive chaparral grasslands of southern California. Higher frequencies of pleometrosis in this species have been shown to be associated with lower levels of precipitation, lower vegetation biomass and lower mature colony density (Cahan 2001). The transition between these two behavioral syndromes across this geographic cline is abrupt, suggesting that it is maintained by selection (Cahan, Helms, and Rissing 1998).

Primary polygyny may exist in *P. californicus* for several reasons. One consideration is that the phenomenon may not be adaptive but rather due to low genetic relatedness causing an inability to distinguish non-kin (ch. 3). Similarly, a founder effect may have resulted in low aggression if the initial founding individual(s) of a population were by chance more tolerant (ch. 4). If either of these explanations is correct, over time aggression may be selected for if genetic diversity were to increase in the polygynous population.

Alternatively, primary polygyny in this species may be a derived adaptive trait selected for by the environment in certain populations. Presumably then, geographically adjacent populations which vary for metrosis and gyny would also vary for the environmental conditions which select for these alternative behaviors. Evidence that these behaviors are genetic and not just plastic responses to the environment or social context are available from common garden experiments (ch. 4).

One explanation is that primary polygyny may produce and maintain larger colonies and that this may be selected for in a more competitive fitness landscape (either intra or interspecific competition). If the same benefits of larger colony size which have been documented in initial colony founding stage (Bernasconi and Strassmann 1999) were to persist into the mature colony stage this would provide an ultimate explanation for the evolution of primary polygyny. However, large mature colonies which contain qualitatively similar amounts of virgin queens and males exist at both the Cameron Valley population and at Lake Henshaw. All of the colonies we sampled at Lake Henshaw in which we later detected single matrilines were qualitatively of a similar size to

those at polygynous sites (ch. 3). Larger colony size, rather than being correlated with polygyny, seems to be much more a function of the more productive habitat of chaparral and sage grassland ecosystem of California as compared to the Mohave and Sonoran desert habitats which contain much smaller colonies of *P. californicus* with fewer sexuals. It may still be however, that generally speaking, this more productive ecosystem and the resulting higher foundress and mature colony density are driving primary polygyny through habitat saturation and increased competition. If this is the case the Lake Henshaw population may be an outlier from the general pattern and may have weaker selection for polygyny due to differences in foundress and mature colony density and hence does no exhibit the levels of primary polygyny seen in other areas of California chaparral grasslands.

Gaining a complete picture of the geographic patterns of variation in metrosis and gyny in *P. californicus* is challenging for several reasons. First, the mating season takes place for a relatively short window of time which varies across the species range. Second, in contrast to studies with *M. pergandei*, exploring the size and structure of the transition zone between the two behavioral syndromes is difficult because of habitat patchiness. Since *P. californicus* nests primarily in valleys of sandy soils with lower vegetation cover, suitable habitat can extend for some distance in Californian grasslands and then change dramatically giving way to habitats that are devoid of colonies (personal observation). For this reason, populations of *P. californicus* in this area might be thought of as islands with occasional migration events and restricted gene flow. Often these habitats exist along riverbeds or in areas where anthropogenic change has occurred such as in cleared lots or along roads. These areas are often surrounded on all sides by unsuitable habitat. As these habitats become suddenly available they may be quickly colonized by a small number of initial founders and may be strongly separated from other populations by geographic barriers. This may explain how distinct population-level behavioral differences are maintained in this species (see ch. 4).

Our results demonstrate conclusively for the first time that primary polygyny is occurring in natural colonies of *P. californicus*, a phenomenon wherein unrelated foundresses co-exist and share resources throughout the life of the colony. We also show that areas within the species range

vary dramatically in frequencies of pleometrosis and polygyny. Documenting and mapping the geographic distribution of pleometrosis and primary polygyny is a necessary first step in understanding the ultimate causes of the evolution of this cooperative phenotype

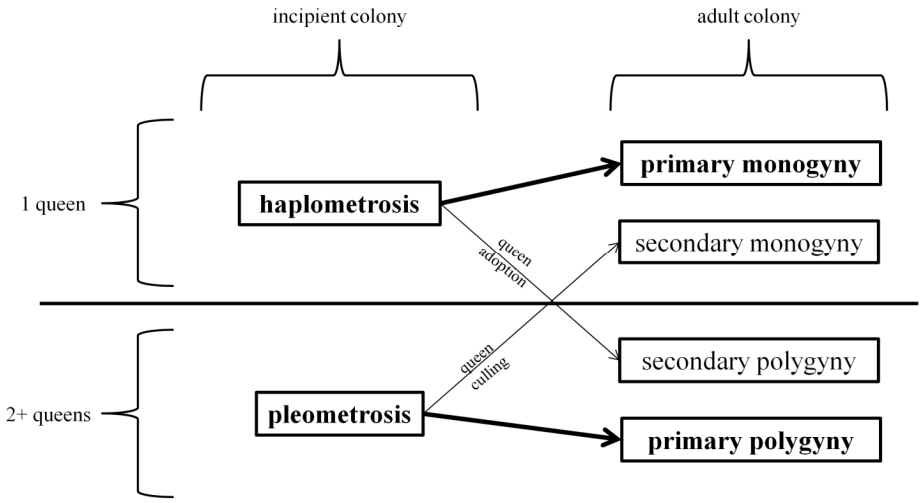

Figure 2.1. Possible routes to different forms of gyny in a mature colony from either a haplo- or pleometrotic incipient colony. Bolded terms and arrows show routes that are known to occur in *P. californicus.* Only modes of independent colony foundation (without workers) are shown. However, dependent colony founding events where queens found with workers from their natal nest also occur.

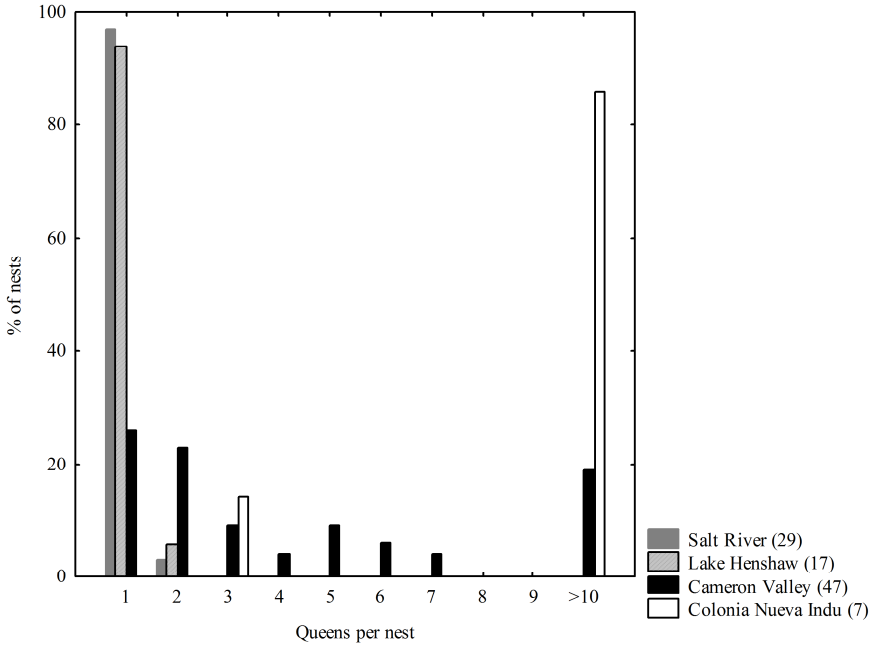

Figure 2.2. Levels of metrosis across populations. Numbers of queens per nest shown as a percentage of total founding nests excavated by population. Nests were excavated shortly after mating flights. Sample sizes per population are shown in parentheses. Salt River and Cameron Valley data are from Rissing and Johnson 2000. Lake Henshaw data are from C. Tate Holbrook (unpublished). See figure 2.3 for geographic patterns of this data.

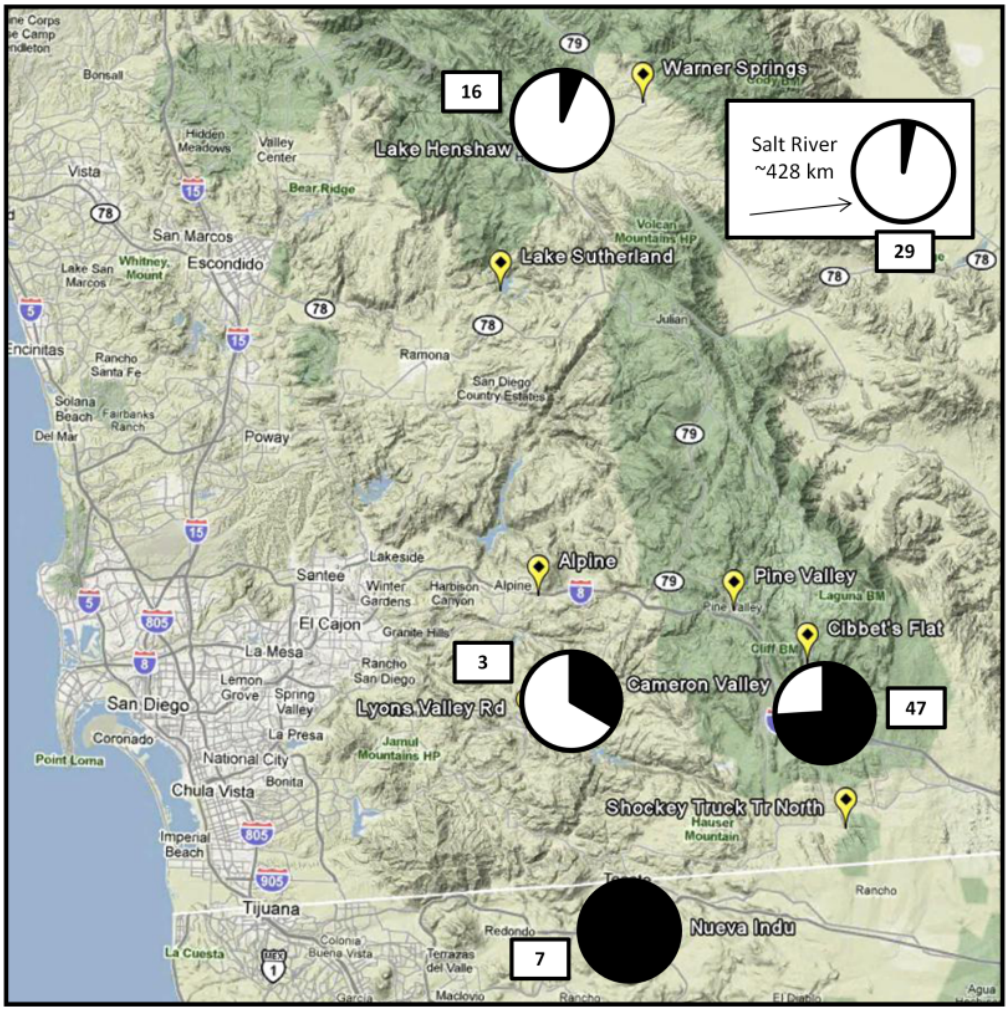

Figure 2.3. Geographic distribution of pleometrosis in sampled populations. Pleometrotic nests are shown as black representing proportion of total nests excavated. Locations with no data available which are relevant for other studies are designated as markers with diamonds. Although no formal data are available the sight at Warner Springs has been observed as haplometrotic (Robert A. Johnson personal communication; see Johnson 2004). The Salt River and Cameron Valley data are from Rissing and Johnson 2000. Lake Henshaw data is from C. Tate Holbrook (unpublished). Number of nests excavated are shown in boxes. Note that these differences are conservative in that they show only the proportion of pleometrotic nests and not the number of queens per nest which can in some cases be upwards of 60 queens in pleometrotic populations.

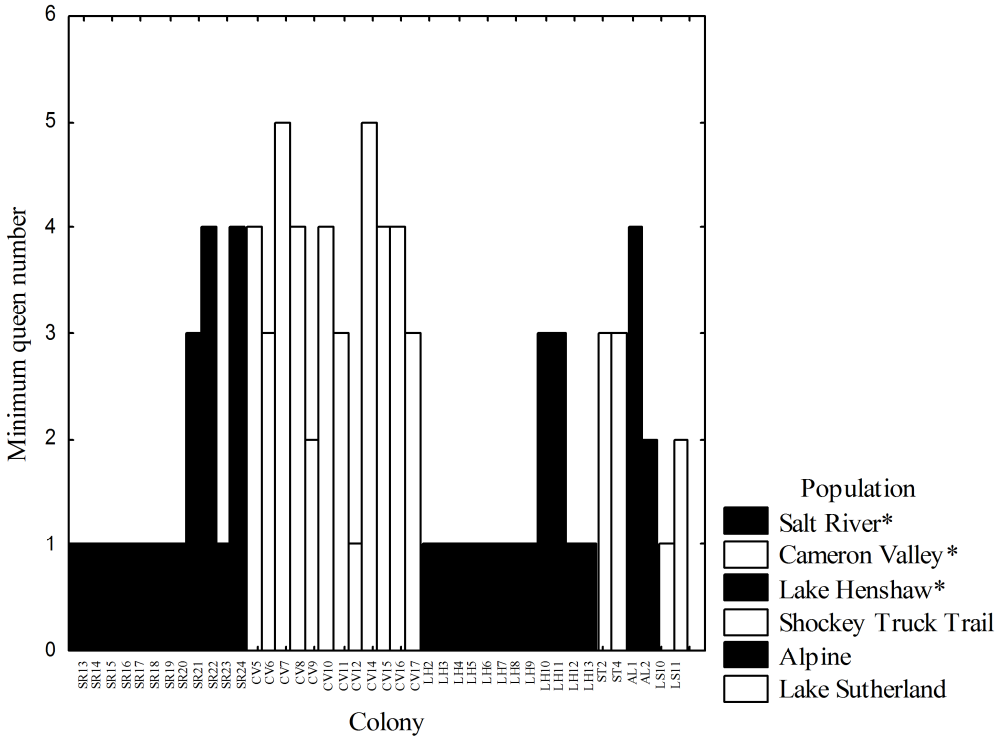

Figure 2.4. Frequency of monogyny and polygyny in mature colonies of *P. californicus*. Bars represent the minimum queen number per colony based on the allelic diversity of males and workers across 5 microsatellite loci. Major focal populations are abbreviated as follows: SR = Salt River, AZ; CV = Cameron Valley, CA; LH = Lake Henshaw, CA; ST = Shockey Truck Trail, CA; AL = Alpine, CA; LS = Lake Sutherland, CA. Black and white colored bars distinguish populations. Colonies with asterisks are focal populations with 12 colonies per populations, the remaining are additional populations where only two colonies were sampled. Note that although zero is displayed for aesthetic reasons it is not possible for a colony to have a count lower than one. See figure 2.5 for geographic patterns of this data.

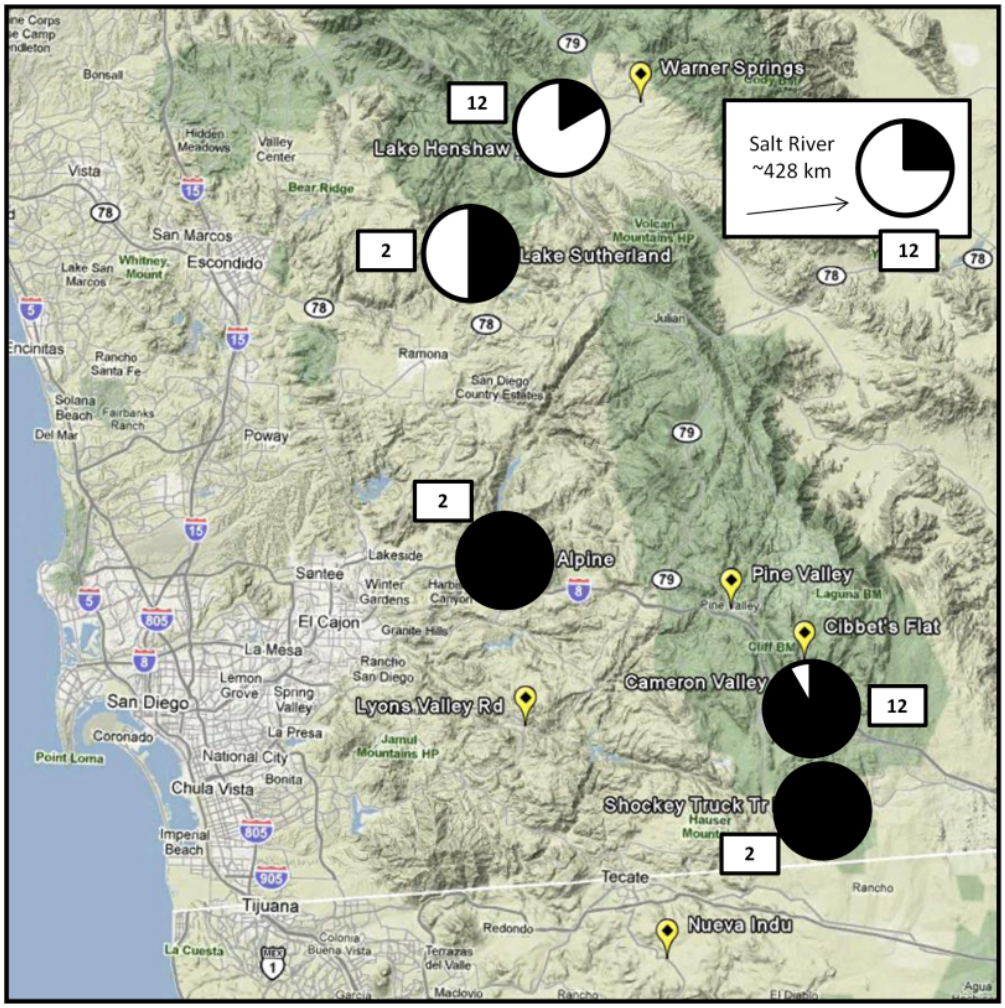

Figure 2.5. Geographic distribution of polygyny in sampled populations. Polygynous nests are shown as black representing proportion of total nests genotyped. Locations with no data available but which are relevant for other studies are designated as markers with diamonds. The number of nests genotyped is shown in boxes.

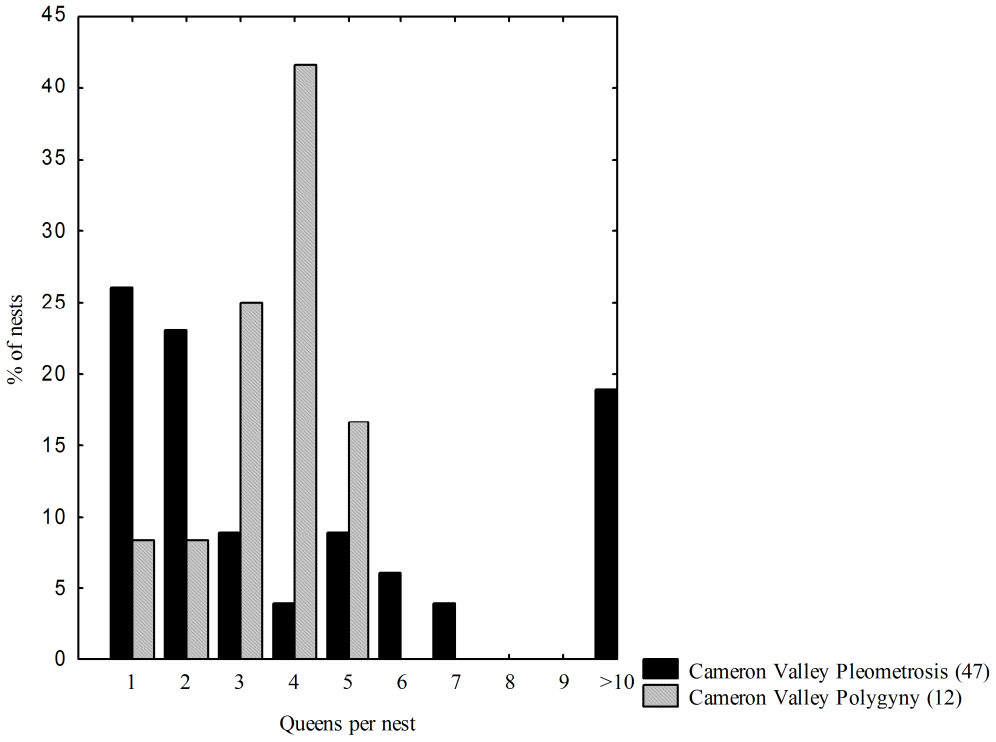

Figure 2.6. Co-occurrence of pleometrosis and polygyny at Cameron Valley. Numbers of queens per nest are shown as a percentage of total nests. Sample sizes per population are shown in parentheses. Pleometrosis data is from nests excavated shortly after mating flights. Cameron Valley pleometrosis data are from Rissing and Johnson (2000). Polygyny data is based on male and worker genotype series from mature colonies. In addition to behavioral data (ch. 4) the correspondence of these two data sources suggests that pleometrosis leads to primary polygyny in nature in *P. californicus*. Higher numbers of queens are found in incipient nests than are detected later in adult colonies.

Table 2.1. Summary of all data on metrosis and gyny in select populations of *P. californicus*

Population	Pleometrotic	Polygynous	Behavior
Alpine	n/a	2/2	tolerant
Cameron Valley	21/47*	11/12	tolerant
Lake Henshaw	1/16**	2 /12	aggressive
Lake Sutherland	n/a	1/2	n/a
Lyons Valley Road	n/a	n/a	tolerant
Nueva Indú	7/7	n/a	tolerant
Pine Valley	pleometrotic	n/a	tolerant
Shockey Truck Trail	n/a	2/2	n/a
Salt River	1/29*	3/12	aggressive
Warner Springs	haplometrotic	n/a	n/a

*(Johnson 2004); ** (Holbrook CT unpublished data)

Proportion of both incipient pleometrotic nests and genotyped mature polygynous nests for each population are shown. Also shown is evidence from behavioral data based on a summary of laboratory observations of queen associations (See ch. 4). Haplometrotic designation for the Warner Springs and pleometrotic for Pine Valley populations are from observations over several field seasons by Robert A. Johnson (personal communication), where no formal data are available. See figures 2.3 and 2.5 for geographical patterns of this data.

CHAPTER 3

THE EVOLUTIONARY HISTORY OF MULTIPLE-QUEEN COLONIES IN POGONOMYRMEX CALIFORNICUS

Introduction

Understanding how cooperation among individuals originates and is maintained over evolutionary time is of central importance in biology. Most research regarding cooperation has focused on related individuals, using kin selection as a model to explain how cooperative behavior emerges in closely related societies of organisms, with the costs of cooperation offset through indirect benefits. (Alonso and Schuck-Paim 2002; Baglione et al. 2003; Bernasconi and Strassmann 1999; Bourke and Franks 1995; Clutton-Brock 2002; Creel and Rabenold 1994; Eberhard 1975; Griffin and West 2003; Hamilton 1964; Queller and Strassmann 1998).

More recently however, in large part due to the availability of molecular tools for assessing relatedness (Hughes 1998) and careful research by behavioral ecologists, there has been increased focus on cooperation between unrelated individuals where direct benefits are required to explain its evolution (Aviles 2002; Balshine-Earn et al. 1998; Clutton-Brock 2002; Dugatkin 2002; Dugatkin and Mesterton-Gibbons 1996; McDonald and Potts 1994; Mesterton-Gibbons and Dugatkin 1992; Queller et al. 2000; Reyer 1980; Rood 1978; Toquenaga 2005).

A dramatic example of such cooperation among non-kin occurs in some ant and termite species where unrelated sexual individuals form cooperative associations despite being able to independently found and maintain a colony. This initial association matures into a stable cooperation of queens (queens and kings in termites) and their respective offspring which lasts throughout the life of the colony, perhaps in some cases over a decade (Hölldobler and Carlin 1985; Johnson 2004; Mintzer 1987; Rissing et al. 1989; Trunzer, Heinze, and Hölldobler 1998; Hacker et al. 2005). Individuals who participate in these associations incur costs inherent to social living and presumably receive benefits but those benefits are not well understood. Understanding the evolution of these phenomena is of importance to behavioral ecology and the understanding of cooperation in general.

Colony founding and colony structure in Pogonomyrmex californicus

Ants are eusocial, and members of the genus *Pogonomyrmex* are no exception with strong morphological and physiological differences between reproductive and worker castes. In the initial colony founding stage, however, independently founding queens go through a solitary life stage. This is most likely the ancestral state in the genus *Pogonomyrmex* and in *P. californicus* (Johnson 2004). During annual mating flights occurring over about one month (Robert A. Johnson personal communication and personal observations), both winged males and queens of *P. californicus* fly from their natal nests and mate. Males die shortly after and queens search for suitable nest sites. During this window of time queens may remain solitary or join with other unrelated conspecifics in a life-long cooperative venture of shared resources and presumably added fitness benefits (primary polygyny see ch.2).

Throughout most of their explored range, which extends from southern Oregon to Mazatlán, Mexico and from coastal California (including peninsular California) to western Texas, queens of *P. californicus* found nests solitarily, and nests with multiple foundresses are encountered only at very low frequencies (Rissing, Johnson, and Martin 2000). In certain populations in the region of southern California, USA however, foundress associations are common. Within this area, the majority of natal and adult nests contain multiple unrelated queens (ch. 2). The evolution of this transition and its implications for the social structure of colonies and the evolutionary dynamics which govern this novel phenomenon are decidedly important but poorly understood.

Hence, in colonies of *P. californicus* with multiple foundresses, two fundamental types of cooperation are taking place. The first is cooperation between workers and their mother queen described by kin selection, where workers sacrifice reproduction in order to increase their fitness indirectly (Eberhard 1975; Hamilton 1964; Queller and Strassmann 1998). The second distinct type of cooperation is between the unrelated co-foundresses. With the growth of the colony, this coexistence expands from involving just queens to including their respective non-reproducing and sexual offspring. A mature *P. californicus* colony of this type can thus be characterized as consisting of multiple, cooperating, unrelated, families. The evolution of this transition from a

single queen and her offspring, to multiple unrelated families in a single colony has important implications for the community structure and dynamics of the colony. It is also an independent evolution of cooperation among nonrelatives analogous to cooperative brood care in birds and cooperative interactions in other organisms (Cahan et al. 2002; Keller 1995). The proximate and ultimate causes of this colony-level trait however, are not well understood in ants (Bourke and Franks 1995; Keller 1995).

Population structure and gene flow

One of the major goals of this study was to understand how such strong population-level differences in an important colony-level phenotype are conserved within a species. Gene flow between populations of a species will erode local differentiation (Slatkin 1987). Barriers to gene flow which allow genetic differentiation between groups can come about in two ways: through assortative mating or through geographic barriers. We test for the latter by examining if there are significant genetic differences between populations and by estimating gene flow between focal populations of this species.

One possibility is that the pleometrotic/polygynous population is in fact a cryptic species; either a sister species to *P. californicus* or a more distantly related species within the genus. In this case no gene flow would occur and each species would have been free to travel its own evolutionary route. Analysis using molecular markers has detected cryptic species in a myriad of taxa (Blouin 2002; Hebert et al. 2004; Narang et al. 1993; Scheffer and Lewis 2001). This is particularly important to test in *P. californicus* because cryptic species with very similar morphology have been detected with the help of mtDNA markers in the *P. californicus* species group (Johnson and Overson).

Alternatively these populations may belong to the same species (i.e. gene flow still occurs as per the biological species concept) but barriers to gene flow may produce enough population substructure to allow for local divergence or adaptation. Similarly, complete genetic isolation may have just recently occurred, with a corresponding incipient speciation event. In both of these cases, traits may be diverging due to local adaptation and/or drift.

We also examined whether the high levels of pleometrosis and primary polygyny observed at several sites in southern California have evolved only once and subsequently spread to other locations or whether they convergently evolved in multiple areas due to similar local pressures. Understanding whether pleometrosis/polygyny has one or multiple origins in *P. californicus* is essential to understand its evolution.

Methods

Phylogeographic and mtDNA haplotype analysis

We amplified a sequence of approximately 650 bases of cytochrome c oxidase I *cox-1* for individuals scattered throughout the range of *P. californicus* (appx. B). In addition, we sequenced multiple individuals from the same population for the three focal populations (Cameron Valley, Lake Henshaw, and Salt River) to better understand the phylogenetic positions and genetic composition of these populations and their relationship to one another and their overall position in the *P. californicus* group. Upon collection, individuals were stored in 100% EtOH. DNA was extracted using a standard Chelex extraction protocol (ch. 2). We amplified partial mitochondrial cytochrome oxidase I sequences using the LCO/HCO primers in a 25µl reaction volume containing 0.01 units of Taq polymerase, 5µl of 5X Go Taq Buffer, 1µl $MgCl_2$ (50mM), 1µl dNTPs (10mM), and 13.9µl of H2O. The locus was amplified using the following PCR program: an initial 4 min at 95° C, 38 cycles of the following: 95° C for 30 sec, 45° C for 45 sec, and 68° C for 1.5 min; and finally 68° C for 4 min. PCR product was purified using Exonuclease I and Shrimp Alkaline Phosphatase (ExoSAP-IT, USB Corporation, Cleveland, Ohio, USA) for digestion of single-stranded DNA (primers) and dNTPs. Samples were sent to the School of Life Sciences core DNA laboratory at Arizona State University and sequenced using an Applied Biosystems 3730 capillary sequencer resulting in a final data set of 653 bases for each individual.

Sequences were aligned using the auto-alignment function in the program Sequencher version 4.6. Sequence trimming resulted in a final smaller dataset containing 639 nucleic acid positions. A neighbor joining tree was constructed using MEGA4 (Tamura et al. 2007). Nodal

support values were obtained with bootstraps (1000 replicates). *Pogonomyrmex maricopa* was used as the outgroup for the analysis.

Gene flow

We also examined faster evolving nuclear microsatellite markers to quantify gene flow between focal populations using 20 workers from each population (only one worker per colony). These data also allowed us to obtain unbiased heterozygosity rates by population for each locus. Individuals were amplified for 7 microsatellite loci: PO03, PO07, PO08, PB5, PB6, PB8, and BJ04 (see ch. 2 methods). F_{st} and R_{st} statistics were calculated using the program FSTAT (Goudet 1995; Wright 1950). The R_{st} statistic is similar to F_{st} but incorporates idiosyncrasies of microsatellite markers and thus may be more valid in our study (Slatkin 1995). We also measured pairwise F_{st} values and confidence intervals between each population. Additionally we analyzed these data with the program Structure v. 2.3.3 (Pritchard, Stephens, and Donnelly 2000) which infers the presence of distinct populations by calculating the probability that a given individual belongs to each population based on the individual's genotype and the genotype frequencies of each population respectively. In our analysis we assumed admixture (i.e. individuals may have ancestors from more than one population) and treated all individuals as if their real population source was unknown in order to test the extent to which only an individuals' genotype would predict its source population.

Results

Phylogeographic and mtDNA haplotype analysis

Our mtDNA phylogeny and haplotype analysis revealed close relatedness of the adjacent polygynous and monogynous populations (fig. 3.1). The entire San Diego County area across all populations sampled showed low haplotype diversity with only 2 haplotypes at our amplified region of *cox-1* in each population. We define a haplotype as any difference in nucleotide composition in our amplified sequence. At Cameron Valley 95% of 19 individuals belonged to a single haplotype (haplotype A). At Cibbets flat 92% of 25 individuals tested belonged to this same haplotype. Additionally, at Lake Henshaw 90% of 10 individuals belonged to a single haplotype

(haplotype B) which differs from haplotype A by only one base of the 639 bases of *cox-1* (appx. C). In contrast individuals from the Salt River exhibit 4 different haplotypes in only 9 individuals sequenced with the most common at 56% frequency (fig. 3.2; appx. B).

F_{st}/R_{st} analysis

In contrast to mtDNA markers, nuclear markers reveal evidence of strong genetic isolation between the three focal populations (overall test for population substructure: R_{st}=0.275; F_{st}=0.107, 99% F_{st} CI: 0.048-0.192). Intriguingly, both of the California populations are genetically differentiated to the same extent as either of them are to the Salt River population despite the fact that they are much closer to one another geographically (fig. 3.3). Pair-wise F_{st} values between focal populations reveal a consistent pattern of population substructure as follows: Cameron Valley and Lake Henshaw, 0.142 (95% CI: 0.064-0.249); Lake Henshaw and Salt River, 0.091 (95% CI: 0.039-0.156); and between Cameron Valley and Salt River, 0.094 (95% CI: 0.056-0.135). Analysis of population structure also revealed strong substructure between all three focal populations (see fig. 3.3) as most individuals were assigned a high probability of being from the population they were sampled from based on their genotype and the overall gene frequencies of each of the 3 populations in the analysis.

Discussion

mtDNA phylogeny

The geographically adjacent populations which exhibit very distinct behavioral phenotypes in *P. californicus* are phylogenetically closely related based on our partial mtDNA *cox-1* sequence data. Our results do not support the hypothesis that individuals from the polygynous population are a sister species of *P. californicus* since they are nested inside the species phylogeny (fig. 3.1). Phylogenetic analysis of 84 individuals throughout the full range of *P. californicus* based on partial *cox-1* sequence demonstrate that queens from the Lake Henshaw and Pine Valley locales are closely related despite their distinct behavioral differences (fig. 3.1). This suggests that if the polygynous population has experienced a complete genetic separation from the surrounding populations it is a comparatively recent one as only a single nucleotide

change in our amplified 639 bases of *cox-1* is distinct between individuals from the polygynous population and the nearby Lake Henshaw (appx. C). It may be however that the polygynous population has completely separated but has not had enough time since its separation from the surrounding monogynous population to accumulate enough genetic differences.

Another question is whether one origin of the pleometrosis/polygyny occurred and spread to other sites where it has been detected or whether polygyny has evolved at multiple sites independently in Pacific coastal habitat due to local selective pressures and/or genetic drift. The similar haplotype that almost all individuals from highly polygynous collecting sites share suggests that the phenomena may have evolved once and spread rather than evolving convergently in separate, preexisting populations due to similar local selection.

Although strong support values exist in the phylogeny for clades corresponding to certain geographic regions the values across the tree for most of the major clades are weak. This is most likely in part because the phylogeny is intraspecific in nature and the nodes on the tree do not represent species which have bifurcated in the past but rather are representatives from populations which are still exchanging genetic information.

F_{st} and R_{st} analysis

F_{st} and R_{st} analyses and model-based clustering methods all utilizing nuclear microsatellite markers corroborated the division seen in mtDNA and revealed a strong separation between the distinct but adjacent populations in California (fig. 3.3). The overall measures of F_{st} and R_{st} values for the populations suggest strong population substructure. Pairwise comparison reveals that all three populations have a similar level of genetic distance from one another despite the fact that the two California populations are much closer together than either is to the Salt River (~50km apart vs. ~460km).

The inter-population isolation evident in these genetic analyses may be a consequence of habitat islands. In southern California, *P. californicus* occurs in open valleys of grasslands and the sagebrush *Artemisia tridentate* (Beauchamp 1986; Johnson 2004) especially in sandy soils without dense vegetation cover. In many places *P. californicus* exist only in cleared lots or along riverbeds or dirt roads in otherwise uninhabited areas. For this reason areas of suitable habitat can extend for

some distance and then end dramatically giving way to areas devoid of the species. This is the case between Lake Henshaw and Cameron Valley where areas of pine forest exist which are unsuitable for *P. californicus*. The patchiness of *P. californicus* populations in this area may be driving the strong pattern of population substructure we observe and may explain how two populations which are approximately 50km apart show similar signatures of differentiation as they each do to a population around 460km in Arizona (fig. 3.3), especially since there may be more consistent gene flow across the range of *P. californicus* in the Sonoran and Mohave desert. This barrier to gene flow may be the mechanism allowing the polygynous population to exhibit the population-level differences we observe. A follow-up test to further confirm this explanation would be to examine the F_{st} values between the Salt River and a population 50km away. The expectation would be that these two populations would have a much lower F_{st} value if they are indeed not as geographically isolated as Cameron Valley and Lake Henshaw. This would demonstrate whether the observed genetic distance between Cameron Valley and Lake Henshaw is aberrant for this species or whether it is a more general characteristic of the patchy nature of the habitat of *P. californicus* throughout its range.

Non-adaptive explanations

One possibility is that pleometrosis and primary polygyny in *P. californicus* may not be adaptive but rather due to the fixation of different neutral characters in isolated populations (Mayr, Eldredge, and Gould 1982). For instance, a newly available habitat could by chance be quickly colonized by the descendents of an unaggressive founding queen. Assuming that primary polygyny is not the optimal strategy for that environment this may be a transitory state where this population would evolve more aggressive individuals over time. This is theoretically possible because both tolerant and aggressive individuals are present in all populations (ch. 4) and mtDNA genetic signatures suggest that a founder effect and relatively fast regional expansion have occurred in southern California. However, this explanation still leaves the question as to why these tolerant individuals are present in populations in the first place.

Additionally, high genetic similarity due to a founder effect and rapid colonization of an area may act as a mechanism leading to pleometrosis and polygyny if the ability to distinguish

non-relative conspecifics is lost. This phenomenon has been observed in *Linepithema humile* where low relatedness due to founder effects has caused low aggression and unicoloniality in invasive ranges (Suarez et al. 1999; Tsutsui and Case 2001). In *P. californicus* the lack of mtDNA diversity in San Diego County supports this explanation as more than 90% of individuals from both Cameron Valley and Cibbets Flat have the same haplotype and mtDNA diversity is lower when compared to individuals from the Salt River population. The Lake Henshaw population also has low mtDNA diversity however, and foundresses exhibit aggressive behavior towards one another regularly in this population which would argue against this explanation (ch. 4). Additionally, in contrast to mtDNA markers, nuclear markers contain considerable diversity (table 2) and do not support the hypothesis that the lack of aggression in polygynous populations is correlated with genetic similarity. Also, workers in both population types have no difficulties in distinguishing foreign queens and workers and respond aggressively towards them (personal observation). For these reasons lack of genetic diversity does not seem a likely explanation as the driving force behind pleometrosis and primary polygyny in *P. californicus*.

Adaptive explanations

The other possibility is that the observed differences in *P. californicus* are an adaptive response to local selective pressure within the species range. Queens of *P. californicus* have lower mortality when in groups during the colony founding stage (Johnson 2004). This may be because of resistance to desiccation or food shortage but it is unclear why these selective pressures would be stronger in the wetter more productive habitat of grassland chaparral of San Diego County as compared to the Mohave and Sonoran desert. Additionally the Lake Henshaw population which remains largely haplometrotic and monogynous but contains habitat which is more similar to Cameron Valley than to the habitat of the Sonoran and Mohave desert populations of *P. californicus*. Intriguingly however, there are suggestions that queens from Lake Henshaw may be intermediate in certain key behaviors between Salt River and Cameron Valley (ch. 4). This presents the possibility that the more productive Californian habitat is indeed driving the evolution of primary polygyny and that the Lake Henshaw population exhibits an intermediate behavioral

phenotype. It may be that this population is in transition toward pleometrosis and primary polygyny or that the selective pressures to becoming pleometrotic/polygynous are not as strong as they are at Cameron Valley.

Although benefits to pleometrosis are more straightforward, those resulting from primary polygyny are more elusive. The polygynous colonies we measured have between 2-5 foundresses all producing sexual offspring; hence there is a life-long cost to an individual queen's reproductive output as polygynous colonies are qualitatively not 5 times larger than monogynous ones (ch. 2). For this reason, theory on the evolution of pleometrosis predicts that queen reduction should occur before the mature colony stage as queens compete to monopolize the life-long rewards of the colony (Bernasconi and Strassmann 1999). One possibility is that selection for primary polygyny is driven by interspecific competition from the sympatric *Messor andrei* (Johnson 2004). This ant species which fills a similar ecological niche has been observed engaged in fierce battles with *P. californicus* (personal observation). If these battles led to enough worker death, presumably primary polygyny could be selected for if multi-queen colonies were better able to maintain large colony sizes throughout colony life in the face of this attrition. It is important to note however, that *M. andrei* co-occurs with *P. californicus* at both monogynous and polygynous sites.

In the desert ant *Messor pergandei*, evidence suggests that variation in metrosis across a geographic cline is mediated by positive selection because the intermediate behavioral transition zone between the two phenotypes is narrow compared to that predicted from estimated dispersal rates across the contact zone (Cahan, Helms, and Rissing 1998). This provides evidence that positive selection is maintaining the divergently adapted behaviors of each region as well as a tight contact zone between them in the face of the eroding effects of gene flow. The challenge to approaching this question in *P. californicus* with similar methodology is that the explored populations straddling the transition zones do not contact one another geographically. Hence, it is difficult to estimate dispersal rates between populations because we cannot make the same simplistic assumptions about dispersal rates based on foundress flight distances that we could if the two zones were bridged by a continuous, connecting hybrid zone. Because of this, it is also difficult to know with certainty whether gene flow is still occurring. The observed F_{st} signatures

could arise from two populations with low levels of current gene flow or reflect historically connected populations which are now isolated. If gene flow is indeed occurring at the present time at any appreciable rate between Lake Henshaw and Cameron Valley this would be good evidence for positive selection for the phenotypic variation seen, as the phenotypic change between these two areas is quite abrupt over a scale of approximately 50km at maximum.

Another possibility is that frequency dependent selection affects the relative fitness values of different queen strategies. Frequency dependent selection could be driven by the social context of other queens participating in a given association and by the state of other colonies in the vicinity. The variable landscape of different social contexts may create variation in payoffs for possessing a tolerant or aggressive strategy (ch. 4) which would produce and maintain the observed variation in queen strategy. This idea is supported by studies where differing queen behavioral types in *P. californicus* (Clark, unpublished data) and *M. pergandei* (Cahan 2001) are placed in associations demonstrating that success of a given strategy is highly dependent on the social context of a queen's founding group. The complexity of this social landscape could be the sole cause of the variation seen in *P. californicus* or perhaps more likely is an additional factor along with other ecological factors which maintain the observed variation.

Our results demonstrate that queens from populations with high levels of primary polygyny in *P. californicus* are not a cryptic species sister species. Rather the populations around Cameron Valley are closely related to surrounding populations and nested inside the *P. californicus* phylogeny.

MtDNA haplotype analysis reveals either a recent selective sweep or founder effect in the area of southern California for both population types (fig. 3.2). Nuclear markers however, show a surprising amount of population subdivision between Cameron Valley and Lake Henshaw, similar to that found between southern California and the Salt River which is approximately 460km away (fig. 3.3). This suggests that barriers to gene flow exist between the behaviorally distinct populations which is corroborated by the patchy nature of the range of *P. californicus* in southern California. This genetic separation is predicted by theory, as strong gene flow would erode local differentiation whether it be due to adaptation or differences due to founder effects and/or drift (Savolainen, Pyhäjärvi, and Knürr 2007; Slatkin 1987; Storfer and Sih 1998; Wright 1990).

It is unknown whether the observed patterns of pleometrosis and primary polygyny in *P. californicus* are due to drift, an adaptive response to particular abiotic factors, or to variability in social environments. The data for *M. pergandei* support the idea that harsh desert conditions (e.g. less food and water) select for pleometrosis. *Pogonomyrmex californicus* follows the opposite pattern where pleometrosis and primary polygyny are restricted to chaparral grassland areas of southern California where colonies are larger and more productive (Johnson 2004). The Lake Henshaw site is an exception to this pattern though as it exhibits high levels of monogyny in mature colonies and also occurs in chaparral grassland. It may be that the Lake Henshaw population is in transition or that it represents an intermediate step toward primary polygyny as there is some evidence that queens occupy a behaviorally intermediate state in this area (ch. 4).

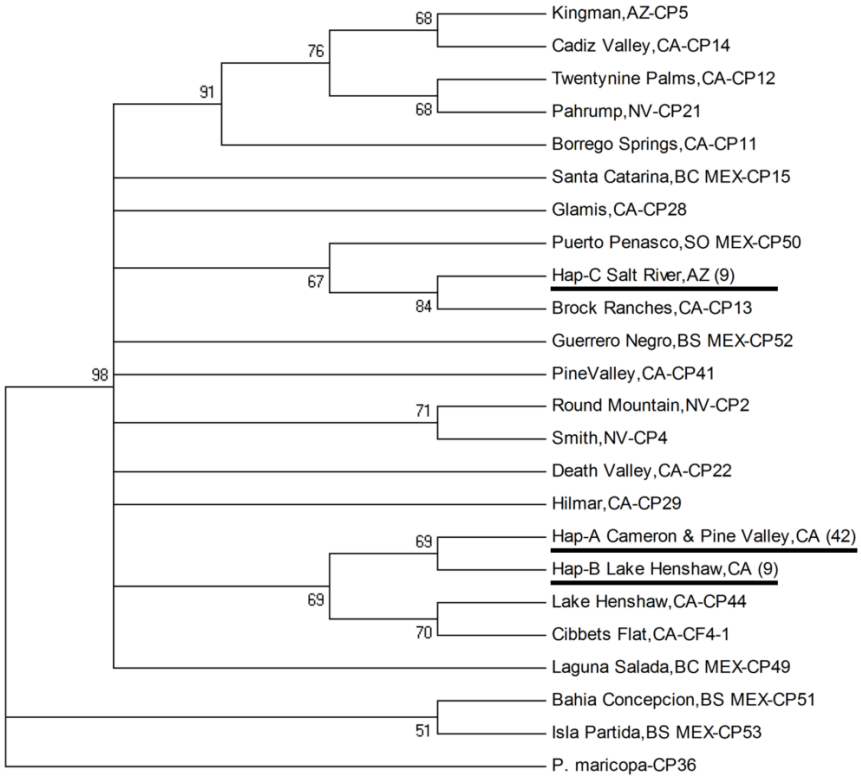

Figure 3.1. Neighbor joining tree based on 639 bases of *cox-1*. Support values are from 1,000 bootstrap replicates. Individuals from pleometrotic/polygynous populations (Cameron Valley and Pine Valley) are nested within the *P. californicus* tree, and are closely related to neighboring populations including the monogynous population Lake Henshaw. The majority haplotype of each of the focal populations is designated with Hap-A,-B, and –C respectively (underlined) and numbers in parentheses represent the number of individuals with that haplotype. For list of all sequenced individuals and their locations and haplotypes see Appendix B.

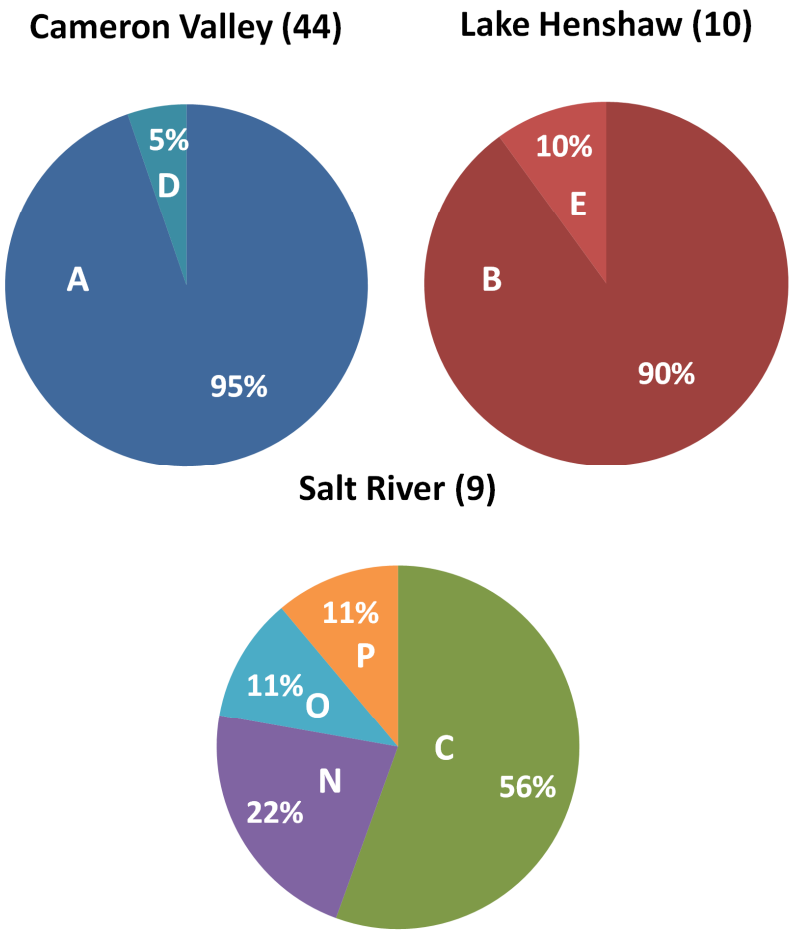

Figure 3.2. mtDNA haplotype diversity by population. Letters designate same haplotype. Number of individuals sequenced per population in parentheses. See Appendix B for details concerning individual haplotypes.

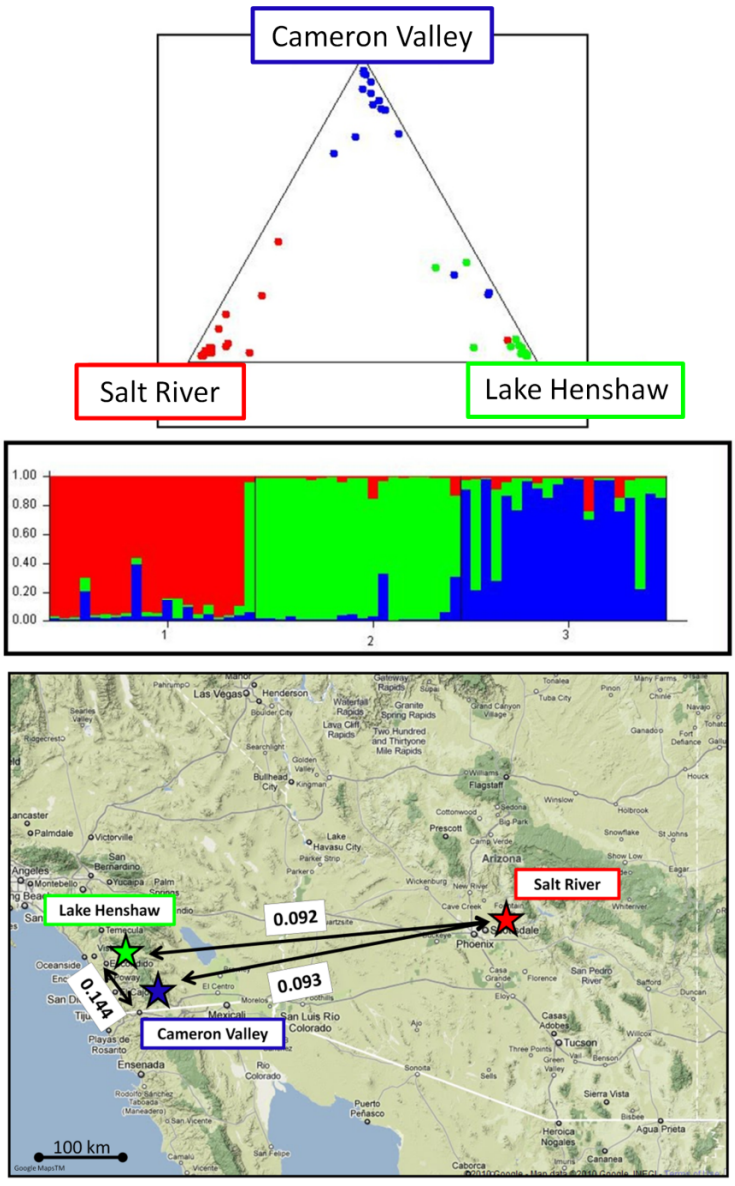

Figure 3.3. Model-based clustering analysis (using Structure 2.3.X) and inter-population F_{st} values. Top: three-way probabilities of individuals originating from a given populations. Colors denote population of origin. Proximity of a given dot to each of the three corners is that individual's probability of coming from each respective population. The same data is shown in bar graph form below with probability from zero to one shown on the Y-axis. Each vertical bar is an individual and the respective three colors represent the probabilities of that individual coming from each population. Map shows pairwise F_{st} values between focal populations.

Table 3.1. Allele numbers for each locus by population and in total.

Locus	Salt River	Cameron Valley	Lake Henshaw	Total
Pb5	11	10	12	20
Pb6	13	11	14	25
Pb8	5	2	5	7
Po03	11	12	11	16
Po7	7	4	4	10
Po8	9	4	7	14
BJ04	15	3	6	15

CHAPTER 4

FROM CONFLICT TO COOPERATION: MECHANISMS OF COLONY FOUNDING BY UNRELATED ANT QUEENS

Abstract: Although much research has explored the costs and benefits of cooperation, there has been less focus on the specific behavioral changes that must accompany the switch from a solitary to a cooperative life history. The California harvester ant *Pogonomyrmex californicus* is ideal for studying the behavioral mechanisms involved in this transition, because it demonstrates striking population-level variation in colony queen number over a close geographic range. Populations either consist of single-queen or multi-queen colonies. We compared the behavioral differences between queens from populations with large biases in single and multi-queen colonies in a laboratory experiment in which we presented queens with the alternatives of nesting together or separately among a series of nest tubes or in an open tray of dirt. Our goal was to determine which behaviors relevant to group founding exist prior to the transition to cooperative sociality and which behaviors evolve with it. As expected, queens from the pleometrotic population readily clustered around a common brood pile. Surprisingly, clustering was similar and equally common among all population types in our nest-tube choice experiment, suggesting that innate attraction to conspecifics or brood may serve as a facilitator in the transition to social aggregation. Our experiment in dirt trays revealed that the two single-queen biased populations exhibited previously undetected behavioral syndromes from one another, either exhibiting or lacking avoidance. Regardless, queens from these populations were much less likely than pleometrotic queens to tolerate one another during initial colony development, showing much higher levels of aggression. Our results suggest that behavioral mechanisms facilitating aggregation in *P. californicus* may already be in place prior to the evolution of communal sociality, while the switch from aggression to tolerance of conspecifics is a critical behavioral transition in the evolution to stable cooperative associations in this system.

Introduction

The evolutionary transition from solitary to social living is one of a series of fundamental shifts in biological scale and complexity (Hölldobler and Wilson 2009; Szathmary and Smith 1995; Wilson 1975). This transition necessarily requires associated mechanistic behavioral changes. Stable, cooperative systems cannot evolve until individuals aggregate and tolerate one another, regardless of the fitness benefits they may ultimately receive through being social. Research on the evolution of sociality has primarily focused on fitness costs and benefits associated with the transition to a cooperative system (Aviles 2002; Baker et al. 1998; Bono and Crespi 2006; Costa and Ross 2003; Cowan 1987; Jakob 1991; Macdonald 1983; Rosengaus et al. 1998; Sachs et al. 2004; Uetz and Hieber 1997), but there has been little detailed investigation of the behaviors driving and/or accompanying group formation (Jeanson et al. 2005; Tschinkel 1998).

One way to explore these mechanistic changes experimentally is to compare the interactions of individuals that form cooperative associations with those of others who are normally solitary. The behavior of newly mated ant queens during nest establishment offers the opportunity to make such a comparison. During the life cycle phase of nest establishment and initial brood production, ant queens are generally solitary (haplometrosis) (Hölldobler and Wilson 1977, 1990). However, in a subset of species, unrelated queens form communal associations (pleometrosis) in which they cooperatively construct a nest, rear brood, and in some cases forage (Bernasconi and Strassmann 1999; Bourke and Franks 1995; Cahan and Fewell 2004; Dolezal et al. 2009; Keller 1995). Because queens of both types ultimately live in eusocial colonies with worker offspring, they clearly already have the capacity to behave as social entities, but most ant queens do not form pleometrotic associations and most that do, reduce their number to only one queen shortly after worker eclosion (Bernasconi and Strassmann 1999). The behavior of solitarily founding queens has also been shown to differ from those that establish foundress associations (Cahan and Fewell 2004; Jeanson and Fewell 2008). This behavioral variation indicates that selection operates on behavioral phenotypes within the context of solitary versus cooperative living in ant foundresses. In fact, selection on behavioral phenotypes during this phase is likely to

be strong, because levels of mortality during nest establishment and early colony growth are extremely high (Cole 2009).

Pogonomyrmex californicus exhibits striking variation in solitary versus group living among queens. Throughout most of their explored range in the deserts of western North America, queens of the species primarily found nests solitarily. However, one population in San Diego County, California exhibits pleometrosis, with 2 to over 20 queens per nest (Rissing, Johnson, and Martin 2000). Genetic sampling from mature colonies has corroborated that this is a case of primary polygyny where multiple queens remain together beyond worker emergence and produce sexual offspring (ch. 2). This is in contrast to most incidences of pleometrosis in ants where queens found nests together but initiate mortal fighting after the founding stage until only one queen remains (Bernasconi and Strassmann 1999). Because pleometrosis is rare in the genus *Pogonomyrmex* and occurs within a limited area of the species distribution, it is presumed to be a derived character for this species (Johnson 2004). This variation in colony founding behavior between populations provides a particularly useful and straightforward context within a single species to compare behavioral changes occurring with the transition to group living.

A starting point in exploring the transition from solitary to group behavior is to consider what behavioral attributes are already present in the context of solitary living. Here, we compare social interactions and survival in three populations of *P. californicus*, in two of which queens initiate nests as solitary individuals (haplometrosis), while queens of the third population form communal associations (pleometrosis). We explore key behaviors which are likely necessary for social group formation, including spatial clustering and tolerance. We ask whether elements of these key social phenotypes are already present in the haplometrotic populations, and what changes are associated with the transition to communality.

In order to cooperate, individuals must first aggregate. Organisms aggregate actively in two ways, when they come together via attraction to external factors such as patches of resources, or based on mutual attraction to conspecifics, the latter of which has been termed congregating (Fletcher 2006; Parrish and Hamner 1997; Stamps 1988). To be a true social group, individuals must congregate independently of external conditions; however, the transition to congregation

could be mediated through factors that induce aggregation (Beauchamp 1986; Jeanson and Deneubourg 2007; Jeanson et al. 2005; Muller 1998; Seeley and Morse 1978; Visscher, Morse, and Seeley 1985). In the case of *P. californicus*, excavated nests are an attractive resource for foundresses, and could serve as a catalyst for aggregation. Once in the same nest, queens could also aggregate around another resource, brood. The presence of brood functions as a key mechanism in transitioning from a solitary to a eusocial existence in wasps, by serving as a cue for adult offspring to remain on the nest (James Hunt, personal communication). In this case a behavioral response (maternal attraction to brood) already present in the solitary condition, functions to facilitate the transition from a solitary life history to eusociality. We ask whether similar cues, including conspecifics, brood and nest chambers, serve as attractants for aggregation in our haplometrotic populations, and whether they can provide a pre-condition for the transition to the communal social systems of these ant foundress associations.

We also examined the occurrence of avoidance behaviors and aggression as potential barriers to cooperative sociality. Ant colonies form territories and exhibit avoidance behavior, and aggression between ants of different colonies is widespread (Hölldobler and Wilson 1990). Although not always a negative social phenotype (for example in cases of social regulation), aggression that produces significant fitness costs could be considered as a barrier which must be modified in the transition to cooperative sociality(Cahan, Helms, and Rissing 1998). Thus a general expectation is that unrelated foundresses initially introduced into a social context with non-relatives should exhibit avoidance behavior and/or aggression and that the evolution of cooperation involves a transition from avoidance to attraction and from aggression to tolerance of conspecifics. Aggression should not, however, be automatically assumed for solitary species; some naturally haplometrotic ants are tolerant when forced together in laboratory conditions (Fewell and Page Jr 1999).

In this study, we bring groups of queens from either predominantly haplometrotic or pleometrotic populations into the laboratory, and allow them to cluster with one another or to segregate among a series of nest tubes in Experiment-A, or in an open tray of dirt in Experiment-B. We also compare tolerance versus aggression within groups, and monitor their effects on

individual survival. These explorations provide insight into the assumptions made by current models of the evolution of cooperation and conflict, by identifying which behavioral attributes may be assumed present prior to the transition to sociality and which evolve as novel behaviors or innovations. Through this approach, we hope to understand the targets that natural selection acts upon during the transition to sociality.

Methods: Experiment-A

Study populations and collection methods

We collected mated foundresses of the California harvester ant, *P. californicus*, from one pleometrotic and two haplometrotic populations during their respective summer mating flights (see Cahan and Fewell 2004 and Rissing, Johnson, and Martin 2000 for population information). On June 6-9, 2006 we collected 267 foundresses from the Arizona haplometrotic population (H-A) at the Salt River recreation area northeast of Phoenix, AZ (33° 32.870'N, 111° 38.617'W; 409m). On July 2-4, 2006 we collected queens from the California haplometrotic population (H-C) and the pleometrotic population (P-C), which are approximately 50 km apart in San Diego county, CA. Ninety foundresses were collected from H-C at Lake Henshaw (33° 13.928' N, 116° 45.381' W; 824m), and another 90 foundresses were collected from two P-C sites 9 km apart: Pine Valley, CA (32° 49.420'N 116° 31.680'W 1,133m) and Cibbet Flat Campground (32° 46.614'N, 116° 26.819'W; 1,265m).

At each collection site we gathered newly-mated foundresses from the ground shortly after their nuptial flights and placed them into individual plastic tubes with a moist piece of paper towel. Queens were brought into the lab within 1-3 days, and were weighed and individually marked before being placed as groups of 3 into nest boxes. These groups were composed of haphazardly chosen queens from the same population; each trio of queens was placed simultaneously into the foraging area of the nest box. We set up 89 nest boxes for H-A, 30 for H-C, and 30 for P-C.

Each nest box consisted of a transparent plastic container (11cm x 11cm x 3.5cm) with four glass test tubes (7.5cm x 1cm) fused to each wall. The tubes served as independent nest sites

and the central chamber as a common foraging area. Each test tube contained water behind a cotton plug and was covered with aluminum foil to provide darkness. Kentucky bluegrass seed was provided in the center of the container as food ad libitum throughout the experiment.

Experimental design and behavioral observations

Initial observations indicated that queens required a 24-hour 'settling-in' period after placement into the nest box, during which they explored the foraging area, frequently antennated one another, and occasionally entered and explored the nest tubes. To capture behavior after the initial exploration period, we made observations twice daily for the first week (morning and afternoon) and then once daily over a total of 89 days. During each observation we recorded queen mortality, the location of each queen, noting whether she was in one of the four nest tubes or the foraging area and also presence/absence and location of brood. We also recorded any observed incidents of overt aggression and, whenever possible, noted the identity of both the aggressor and the target of aggression. An aggressive incident was defined as either one queen grasping another with her mandibles or a live queen exhibiting damaged or missing body parts. Damage serves as strong indirect evidence of aggression. In multiple previous experiments under similar laboratory conditions, this type of damage to a live queen has never been observed outside the context of aggression, while observed aggression almost always corresponds to damaged or missing body parts.

Analysis of survival and aggression

Queen survivorship was compared across the 3 focal populations using a log rank multiple comparison survival analysis. To test for an effect of queen weight on survival we ranked the 3 queens within a nest box as heaviest, middle-weight, and lightest. We then tested for overall differences between the 3 weight classes for each population separately with the survival analysis. To measure aggression, nest boxes were divided into two categories at the end of the experiment: those in which aggression had been observed at any time during the experiment and those in

which no evidence of aggression was seen. A χ^2 contingency table was used to detect differences among populations in the frequency of nest boxes with aggression.

We also analyzed mortality linked to aggression. A queen's death was considered to co-occur with aggression if she died within 48 hours of being observed as the target of aggression and/or her body was severed in some way. Nest boxes were divided into two categories: those in which deaths corresponding with aggression had been observed and those in which no such deaths were observed. A χ^2 contingency table was used to detect differences among populations in the number of nest boxes in which aggression-related deaths occurred.

Analysis of clustering

We scored each nest box daily over the first 40 days of the study for presence of clustering behavior. Clustering was defined as occurring when either two or three queens simultaneously occupied a single nest tube, rather than being in different tubes. A forty day cutoff was chosen for the clustering analysis; this corresponded to the approximate half-way point of the experiment and beyond this time the cumulative mortality of queens in both haplometrotic treatments made statistical comparisons across populations difficult. During the first week, when two observations were made per day, the analysis included only one of the two observations, chosen at random. Mortality occurred throughout the study, and when nest boxes no longer contained at least two living queens they were removed from the clustering analysis.

To determine whether queens actively clustered together, we compared the number of nest box observations where an aggregation was observed with that expected if queens were to choose a nest tube without regard to the presence of other queens. We generated the latter expectation by simulating a data set with the same number of nest boxes and observation days as the observed data. Each simulated nest box had the same number of live queens in tubes per day as the corresponding real nest box. Queens located in the foraging area, not in a nest tube, were not counted for that observation period in both the real and simulated data sets (occurred for only 6.7% of all queen observations). For each day, the presence of a cluster was determined from the probability that two or more queens would be in the same tube if each chose a tube independently.

The full data set was simulated 1,000 times, using MatLab (The MathWorks, Inc. Natick, MA, U.S.A.). The average proportion of nest boxes with clusters per day was then calculated across the 1000 simulations. To estimate the 95% confidence interval of the mean, we sorted the 1,000 simulated proportions and excluded the highest and lowest 2.5% of values.

We next tested for differences in clustering between populations by using Matlab to generate three separate 1,000 bootstrapped versions of the observed data, one for each population. Each bootstrapped version was produced by resampling the real data with replacement to create a new data set of the same size. The sampling unit was the entire series of observations for an individual nest box. For each day of each bootstrapped data set, we calculated the proportion of nest boxes with clusters, as described above for the real data. We then determined the mean and 84% confidence interval of this proportion on each day, across the 1,000 bootstrapped samples. We used 84% confidence intervals, because these are expected to yield a true significance level of 5% for this test (Payton, Greenstone, and Schenker 2003). These values were determined separately for each population; we judged two populations to differ in degree of clustering whenever their confidence intervals did not overlap. Comparisons were made on each of the first 40 days of the experiment, except for a few days on which no observations took place. Less than 40 pairwise comparisons were available between two given populations because of some days in which observations did not take place. The number of missing days varied among population pairs, such that 32 daily comparisons were made between H-A and P-C and between H-C and P-C, but 30 were made between H-A and H-C. Copies of both MatLab programs are available from the authors upon request.

Methods: Experiment-B

Study populations and collection methods

As a follow-up to Experiment A, we ran a similar experiment in open containers of dirt rather than the enclosed plastic choice-boxes. Although in this setup we lacked the ability to observe what nesting queens were doing below ground, we were able to observe how queen behaviors relevant to pleometrosis and primary polygyny might change with a more natural setting

with more space and the ability for queens to excavate their own hole rather than only choosing a nest tube. Foundresses were collected during the mating flights of 2010 from the same sites as before except for that due to lack of availability of queens at Cameron Valley, queens from a site at Pine Vally, CA (13 km away) were used instead (32° 49.420'N, 116° 31.680'W; 1132m). This site has been monitored annually for over a decade (unpublished data Johnson, RA, and Jennifer Fewell Lab) and is a confirmed pleometrotic site (Johnson 2004) and strong evidence suggests that it is also polygynous (ch. 2-3).

Within 36 hours, queens were brought into the lab, weighed, and individually marked before being placed as groups of 3 into separate 36x43x17cm (LxWxH) plastic trays filled with 10cm of dirt. There were 20 replicate trays observed for each population. At the start of the experiment, randomly chosen queens from the same population were placed simultaneously into the center of each tray starting at 7:00 am. Over the next 8 hours, scanning observations took place approximately every 6 minutes for each tray (75 total observations per tray and 1500 tray-observations per population). Beyond this, starting on the second day most queens had settled underground and observations were made twice daily for one week and then once daily for the following week at which point observations ended. Kentucky bluegrass seed was provided as food ad libitum throughout the experiment by sprinkling it lightly throughout the tray.

Analysis of behavior

During scanning observations we recorded any instance of aggression (i.e. biting with mandibles) and excavating behavior (i.e. digging a hole in a series of dirt removal bouts with mandibles). When an excavated hole was large enough for a queen to fit fully inside it, we assigned it a unique identifying number and recorded the queen or queens that were associated with its initial excavation. A “queen-carrying” behavior which had not been seen in Experiment-A was also observed, presumably because of the new context of nest excavation or the more natural larger surface for queens to interact. This entailed one queen physically picking up another from the surface of the ground or from inside an excavated hole and physically relocating her some distance away. The carried queen assumed a pupal position with all her legs tucked near her body

without struggling. Often the carried queen would wait between 10 seconds to a minute after being set down to begin moving again.

Three weeks later at the end of the experiment, all trays were carefully excavated and censused and individual queen survival and location were assessed as well as the presence of brood. This allowed us to compare the last known nest hole that a queen was observed in to where she finally chose to reside and clear up ambiguities as to location of some queens whose nest decisions were missed with the scanning observations. Due to desiccation which occurred in the Salt River population almost all individuals had deceased at the 3 week mark when censusing took place and hence no final data for mortality was collected for this population. Because the experiment for the Salt River population was run earlier we altered our watering protocol which avoided this problem for the California populations.

For our analysis of the three populations we compared aggression, queen survivorship at the end of the experiment, occurrence of queen-carrying, and of cohabitation (more than one queen sharing a nest). Differences in the mean number of surviving queens per tray across populations were tested with an ANOVA. A χ^2 contingency table was used to detect differences between populations for all other comparisons. In the case of aggression and queen-carrying behavior we compared the number of trays per population that either did or did not exhibit the focal behavior at any time during the experiment. This was done to avoid problems of pseudo-replication caused by queens of a given tray repeatedly exhibiting a behavior. For comparison of cohabitation, we compared both the number of trays per population that had a joint founding event after 24 hours (when the majority of queens had settled underground) and the number of trays which contained at least one instance of co-habitating queens at the end of the experiment. Because Experiment-B was a follow-up experiment similar to Experiment-A, and we had *a priori* expectations of both the predicted differences and their directionality, we did not correct for alpha inflation.

Results: Experiment-A

Survival

Queen survival differed significantly among the three populations (Overall log-rank: χ^2 = 42.66, P < 0.0001; fig. 4.1) with P-C queens exhibiting higher survival than queens from either the H-A (Log-rank: $\chi 2$ = 4.98, P < 0.0001) or H-C (Log-rank: $\chi 2$ = 5.95, P < 0.0001, Bonferroni corrected α=0.0167) populations. Differences in queen survival between the two haplometrotic populations were not significant (Log-rank: χ^2 = 1.952, P = 0.0509, Bonferroni corrected α=0.0167). Relative queen weight within nest boxes was not significantly associated with mortality in any of the three populations: P-C (Overall log-rank: χ^2 = 0.105, P = 0.949), H-C (Overall log-rank: χ^2 = 2.83, P= 0.243), (Overall log-rank: χ^2 = 4.425, P = 0.109).

Aggression

The three populations varied in the number of nest boxes in which aggression was observed (Chi-square test: χ^2 = 6.58, P < 0.0001; table 4.1). Queens of both haplometrotic populations exhibited much more aggression than the pleometrotic queens. (Chi-square test: χ^2 = 11.30, P < 0.0001; table 4.1). When the pleometrotic population was removed from the analysis, the two haplometrotic populations did not differ significantly in aggression, (Chi-square test: χ^2 = 0.57, P = 0.4498) or in aggression-linked mortality (Chi-square test: χ^2 = 0.08, P = 0.7720).

All 3 populations contain both tolerant and aggressive behavioral types, at least at low frequencies. Three of the 117 nest boxes (2.6%) from the two haplometrotic populations remained pleometrotic throughout the duration of the experiment (90 days), with all three queens residing in the same nest tube beyond the time of the emergence of workers, demonstrating that more tolerant individuals are present even in haplometrotic populations. Meanwhile, in the pleometrotic population six out of 30 nest boxes failed to retain all three foundresses throughout the experiment (20%) due to mortality caused by aggression (removal of head or gaster).

Clustering behavior

Queens in all populations had a strong tendency to cluster within a common nest tube, as indicated by the significantly higher occurrence of clustering in the observed data compared to simulations in which queens chose nest tubes independently of one another (fig. 4.2). Surprisingly, there was little difference between haplometrotic and pleometrotic populations in the occurrence of clustering (fig. 4.3). Queens from the H-A population were never significantly less clustered than those from the P-C population, and even exceeded them on 7 of the 32 days for which data were available to make a comparison. H-C did not significantly differ from P-C queens in clustering on any of the 32 days. The H-C queens were significantly less clustered than H-A queens for 6 of the 30 days available for comparison.

In the majority of all observations for all queen types, living queens grouped together in a single nest tube. The foundresses usually gathered around a single brood pile (> 98% of nest boxes had clusters with the presence of brood). When individual foundresses were not observed in a cluster with other queens they were often foraging for seeds or were injured and were located in the common foraging area. Across 17,525 total queen observations for the duration of the entire experiment (84 days), queens were observed in the foraging area only 1,176 times (6.7% of all queen observations). Out of 7,056 nest box observations across populations, queens were observed in separate nest tubes only 366 times or 5.19% of observations. The majority of occurrences where queens were located in different nest tubes within the same nest box were for one or two days at which times queens were again found clustered together. There were only 6 occurrences during the experiment where queens were observed in separate nest tubes for 3 or more consecutive observation days.

Results: Experiment-B

Aggression and survival

Queen survival differed significantly between the Lake Henshaw and Pine Valley populations (Student T-test: T=-5.20, D.F. 38, $p<0.00001$; fig. 4.4) presumably due largely to aggression as was the case among clustered foundresses from Lake Henshaw in Experiment-A.

Because of high mortality due to desiccation in our nest boxes the survival data from the Salt River population was not used in this comparison. However, only 2 out of 20 nest boxes from the Salt River ever had a joint-founding event (more than 1 queen nesting together) so in most cases mortality would not have stemmed from aggression as queens were not presumably not together but we cannot rule this out entirely as tunnels may have connected underground. Observed aggression was also significant between the three populations (Chi-square test: $\chi^2 = 17.89$, P = 0.0001) with no aggressive interactions observed in the Pine Valley population and 16 and 20 such interactions observed in the Lake Henshaw and Salt River populations respectively. When the Pine Valley population was removed from the experiment the differences in aggression were no longer significant (Chi-square test: $\chi^2 = 5.01$, P = 0.082).

Carrying behavior and joint-founding

When queens came into contact with one another they either ignored one another, antennated one another peacefully, had an aggressive interaction, or sometimes engaged in queen-carrying behavior. Carrying behavior occurred when one queen picked up another and relocated it some distance away. Queen-carrying behavior also occurred in the context of nest construction, when a queen entered a hole that another queen was excavating. Instead of returning with a ball of dirt on her next pass, the excavating queen would emerge carrying the other queen in her mandibles and thereafter deposit her on the ground some distance from the nest. The frequency of observed queen-carrying behavior was significant between populations. Queen-carrying behavior was observed only once in the Pine Valley population and was observed 10 (6 of 20 trays) and 15 times (11/20 trays) in the Lake Henshaw and Salt River populations respectively (Chi-square test: $\chi^2 = 11.91$, P = 0.003). When the Pine Valley population was removed from the analysis no significant difference remained (Chi-square test: $\chi^2 = 2.56$, P = 0.278). Queen carrying in the context of removal from an excavated nest was observed in 5 trays and only the Salt River population (Chi-square test: $\chi^2 = 10.9$, P =0.004).

The frequency of joint founding events also differed significantly between populations. After the 24 hour settling-in period when queens had excavated and\or chosen their underground

nest sites 11/20 trays had a joint-founding event in the Pine Valley populations, 15/20 in the Lake Henshaw population, and only 2/20 in the Salt River population (Chi-square test: $\chi^2 = 17.81$, P =0.00014). When the Salt River population was removed from the contingency table the difference was no longer significant (Chi-square test: $\chi^2 = 1.76$, P =0.415). However at the end of the experiment the Pine Valley population had 20/20 trays with a joint-founding event whereas Lake Henshaw had only 3/20 (Chi-square test: $\chi^2 = 29.56$, P <0.000001).

Discussion

Ant foundress associations provide a useful context in which to explore both the mechanisms and consequences of cooperation among non-kin. Since, they consist of unrelated adults, cooperation is not rewarded via indirect fitness gains and individuals must receive direct benefits to make these associations stable (Clutton-Brock 2002). To understand how such cooperating groups evolve, we must also understand the behavioral mechanisms that can facilitate or impede such a transition. Organisms cannot shift from a solitary to a cooperative state unless certain mechanistic changes take place. These modifications do not necessarily have to be large; they can be the alteration of a preexisting behavior or the execution of a behavior in a new context, producing evolutionary novelty. In the ant foundress case, the simple behavioral transition of queen tolerance for conspecifics seems to facilitate a long-term cooperative association between multiple unrelated queens and their worker offspring.

Behavioral mechanisms in the transition to sociality

Because ant colonies are territorial and queens face competition from nearby queens, we might expect the solitary behavioral condition for ant queens to be one of avoidance or aggression. If so, a transition from avoidance to attraction would be requisite for the switch to communal living. Interestingly, however, we found that in Experiment-A, haplometrotic *P. californicus* queens preferentially clustered together despite being offered multiple nest tubes (fig. 4.3). Their tendency to cluster could not be separated from that of the pleometrotic queens; the majority of queens from each of our three populations clustered together in one nest tube around a single

brood pile for the duration of the experiment. This suggests that regardless of avoidance or aggression that they may exhibit in different contexts, queens of *P. californicus* have an innate attraction to conspecific queens or brood, regardless of whether they possess tolerance for the continued presence of conspecific foundresses. Worker brood could serve as a means of indirect attraction between queens; as such, the drive for parental care toward brood serves as a pre-condition for the aggregative behavior necessary for cooperative sociality. The attractive force of brood within the nest has also been suggested to provide a proximate mechanism for the transition to eusociality in wasps, as adult offspring shift from dispersing to brood care within the natal nest (James Hunt, personal communication). In both cases, proximate behaviors already present in the solitary context set the stage for the evolution of a novel social phenotype, in this case communal sociality. Within communal systems, clustering around the cue of brood presence may transition into congregating, in which queens are directly attracted by the presence of other queens.

One of the paradoxical outcomes of Experiment-A was the lack of avoidance behavior exhibited by foundresses from the Salt River population resulting in a discrepancy between the high levels of clustering observed in the lab and the low frequency of pleometrosis observed in nature at the Salt River (Johnson 2004). In all populations of *P. californicus*, queens are obligate foragers and must gather food to raise their first workers and thus do not permanently seal their nest entrances during the colony founding stage like other claustral members of the genus (Johnson 2004). Since mating flights in this species take place over several weeks rather than in a punctuated flight after heavy rain like other *Pogonomyrmex*, diffuse waves of queens searching for nest sites are continually incoming. Incoming queens are attracted to the preformed holes of other queens which are left open and will enter them upon discovery (personal observation). These holes are an important resource because they minimize the time queens are at the surface and reduce the amount of digging they must perform (Tschinkel 1998). If the initial founding queen has since died, holes may also be filled with food or brood, both valuable resources. This is especially likely in *P. californicus*, as queens must leave their nests repeatedly to forage at least in the early stages of founding (Johnson 2004).

Because of this combination of life history characteristics and the discrepancy observed regarding the Salt River population, it remained an open question as to what was precluding queens from entering and remaining in the nests of other queens in nature like they did in the laboratory. Queen-carrying, a form of avoidance behavior, provides an answer to this question. In contrast to Experiment-A where the Salt River population exhibited high levels of clustering, in Experiment B only 2 out of 20 trays ever had an observed joint-founding event during the duration of the experiment (table 4.2). Presumably this was due at least partially to the co-occuring queen carrying behavior which was seen only in Experiment B. Although queen carrying behavior was observed repeatedly in both the Lake Henshaw and Salt River populations, queens were only observed removing other queens from their nests in the Salt River population (observed in 5/20 trays). Often the invading queen would be removed repeatedly in succession as she repeatedly tried to enter. On one of the 5 occasions an incoming queen usurped a hole under construction by successfully carrying and removing the queen who had initiated excavation. This behavior of carrying conspecifics called adult transport has been examined in many ant species including *Pogonomyrmex californicus* and is most commonly utilized by ants during nest transport (Hölldobler and Wilson 1990; Hölldobler and Wilson 2009). Adult carrying in a unique context in *P. californicus* takes on new meaning as it allows queens to physically avoid queens whom they do not tolerate from their nest while both parties avoid lethal fighting. This avoidance behavior functioned upstream of the mortality that occurred in Experiment-A in the Salt River population. In Experiment-B, queens from the Salt River population were still attracted to one another and/or to excavated nests and attempted to enter them but were almost invariably removed as 18/20 trays from this populations contained no joint founding events. Although queen-carrying did occur in both the Salt River and Lake Henshaw population, it was never observed in the context of queen-removal from a nest in any population but Salt River. The Lake Henshaw population which lacked the queen-nest removal behavior had more trays containing a joint founding event after 24 hours than even Pine Valley (15/20 vs. 11/20). However, high levels of mortality occurred in the Lake Henshaw population which reduced pleometrosis to only 3/20 trays by the end of the experiment compared to 20/20 for Pine Valley. This was similar to the pattern seen in Experiment-A and

presumably was mortality driven by aggression as it was in Experiment-A. This suggests that the Lake Henshaw population may be in a transitional state between the Salt River and Pine Valley populations in that they failed to produce the avoidance behavior observed in the Salt River populations but still exhibit high levels of aggression unlike clustering individuals at Pine Valley. The fact that after the first 24 hours only 11/20 trays in the Pine Valley population contained pleometrotic foundings but by the final census all 20 trays did is strong evidence that even queens who excavated their own holes changed nests sites to cluster with conspecifics after initially excavating their own nest.

The results of our study show that on average, foundresses from pleometrotic populations exhibit much more tolerance towards conspecifics than those from haplometrotic populations. They also demonstrate that previously undetected differences exist even among populations which were classified as "haplometrotic". The results of Experiment-B where queens from Lake Henshaw still failed to separate when those from Salt River did suggests that Lake Henshaw may exhibit secondary monogyny in some cases where queens found together but eventually fight until only one remains. Our initial hypothesis, that intrinsic differences in tendency to aggregate or segregate in queens would alone explain the population level variation in frequency of pleometrosis, was not supported. In Experiment-A the tendency to cluster between populations did not differ significantly (fig. 4.3) demonstrating that queens from all population types of *P. californicus* have an innate attraction to either conspecifics and/or brood. These results indicate that a switch from a solitary to a social life history in founding queens of *P. californicus*, a colony level trait, is based on a switch to higher tolerance of conspecifics, an individual level trait.

In contrast to clustering, aggression and queen-removal behavior in the haplometrotic populations served as a barrier to social group formation. Consistent with expectations, our results indicate that a switch from a solitary to a social life history in this species is based, at least in part, on the evolution of increased tolerance for conspecifics. In those associations exhibiting aggression, what initially seemed to be tolerant behavior was interrupted when one queen grasped another in its mandibles, beginning a struggle that lasted several hours and ended with the

severing of the head or gaster of the attacked ant. This pattern of punctuated aggression was commonly observed for both haplometrotic populations. Queen removal from a nest provided a mechanism upstream from costly battles for intolerant queens to avoid another queen's presence.

Simple genetic architecture underlying variation in cooperation

The transition from solitary to cooperative nest founding poses the questions of how genetic architecture is reshaped by selection for sociality. Recently, several studies have reported simple single-gene mechanisms driving variation in aggregation and cooperation. In the fire ant *Solenopsis invicta*, the social organization of a colony has been shown to be associated with genetic variation at the Gp-9 locus. Colonies composed of workers with only the BB genotype tolerate only one BB queen resulting in monogyny, whereas colonies with workers bearing b alleles tolerate multiple Bb queens resulting in a polygynous colony form (Krieger and Ross 2002). In the nematode *Caenorhabditis elegans*, variation in whether worms aggregate with conspecifics on food sources is explained largely by a polymorphism at a single amino acid of the G protein-coupled receptor npr-1(Gloria-Soria and Azevedo 2008). In the yeast *Saccharomyces cerevisiae*, a key gene, FLO1, determines whether cells cooperate to form a protective biofilm. The gene also provides a built-in mechanism to direct cooperation toward other FLO1 carriers and protects against cheaters that would benefit from the film without producing it (Smukalla et al. 2008). These examples illustrate that relatively simple genetic changes can influence a complex social trait. In this way, a behavior such as solitary or communal breeding can be selected for quickly based on the fitness payoffs associated with each strategy.

Our research demonstrates that simple behavioral changes such as tolerance to nearby conspecifics and lack of the queen-removal behavior result in a population-level difference in colony social structure in *P. californicus*. Although aggression against other adults and the queen carrying behavior serve as a barrier to social evolution in of *P. californicus*, the level of variation in these behaviors observed both within and across population types suggests it is potentially responsive to selection. Tolerant individuals (as determined by our experiment, i.e. not engaging in aggressive behaviors or queen-removal) were present in our haplometrotic populations and

likewise, aggressive individuals were present in pleometrotic associations, albeit at low frequencies. This intrapopulation variation should allow selection to quickly alter the frequency of communal living based on the local fitness costs and benefits imposed by the environment. It suggests also that the fitness benefits between solitary and cooperative nest founding vary considerably across even the small spatial scale of these populations. Our findings also reveal that contrary to what was previously known, behavior varies even for populations previously characterized and grouped as haplometrotic. In both the choice experiment and in the dirt tray experiment, foundresses in the Lake Henshaw population still exhibited high levels of clustering and subsequent reduction (secondary monogyny). It could be argued that this was an artifact of our experimental design as even queens from the Salt River population clustered in Experiment-A. However, in the trays of Experiment-B, queens from the majority of individuals did not cluster presumably because of a queen's aggressive interactions and carrying behavior in defense of her excavated hole. The fact that queen removal from a nest was not observed in the Lake Henshaw population and high levels of clustering occurred demonstrates variation for behavioral mechanisms even between populations with colonies that eventually become monogynous.

Foundress density and aggregation

How do benefits to communal living vary across these *P. californicus* populations, such that one population has a high frequency of pleometrosis, while others, including geographically adjacent ones, do not? One suggested possibility is that pleometrosis is a proximate response to high foundress densities (Tschinkel and Howard 1983). Foundress density can be influenced by the number of foundresses after mating flights and any factor that promotes queen crowding: established colonies, microtopography, nest-site limitation, etc. Queens from populations with higher densities of foundresses may be limited for possible nest sites, with consequent negative fitness effects if queens act aggressively when they come in contact. Work on the ants *Solenopsis invicta* and *Lasius niger* has demonstrated that the frequency of cooperative founding by pleometrotic queens is related to queen density after mating flights; thus, queen number per colony is a proximate response to local conditions (Nonacs 1992; Sommer and Holldobler 1995;

Tschinkel and Howard 1983; Ward, Webster, and Hart 2006). These results, however, are for populations of already pleometrotic ants. According to the density hypothesis, local queen densities could influence selection for pleometrosis as well as contribute to the context for its expression. No formal data have been collected for post-flight foundress densities of *P. californicus*; however, anecdotal evidence from the collection of foundresses suggests that there are no major differences in initial foundress density between our focal populations. Measuring post-flight queen density in this species is challenging due to the fact that mating flights are drawn out over several weeks and most likely vary from year-to-year for a given population.

In both Experiment-A and B we started with an equal queen density, but queens still exhibited strong population-specific differences in behaviors that have relevance to cooperative founding. This suggests that behavior is the primary regulator of the observed variation in pleometrosis, rather than proximate effects of local queen density. Our experiment was, in effect, a common garden experiment where foundresses retained behavioral differences when they were removed from their respective environments and placed in the laoratory.

Our results suggest that a relatively small number of key mechanistic differences separate solitary founding queens from closely related populations in which queens behave as cooperative units with non-relatives. Certain behaviors already in place, such as attraction to conspecifics and brood, take on new meaning in a cooperative context that in this case stabilizes the group as a social unit. Because of these preexisting behaviors, the evolution of sociality in this system may be associated with a small number of key behavioral shifts, the most important being increased tolerance of conspecifics. Simple genetic and behavioral changes may produce a novel social phenotype with multiple matrilines, each consisting of groups of non-reproducing workers and sexual offspring. These matrilines co-exist and share resources and reproductive fitness within the same nest. The small mechanistic changes that lead to this cooperation in turn have dramatic downstream effects: The increasing genetic diversity introduced with each new matriline decreases average relatedness within the nest, weakening the role of kin selection. Increased colonial diversity has also been shown to affect disease resistance and division of labor and colony efficiency (Fewell and Bertram 1999; Hughes and Boomsma 2004; Liersch and Schmid-Hempel

1998; Smith et al. 2008; Tarpy 2003). The transition to multi-queen colonies also affects population structure as effective population size and potentially population-level genetic diversity increase. All of these changes have implications for the further evolution of the system and stand in contrast to adjacent populations consisting of single-queen colonies.

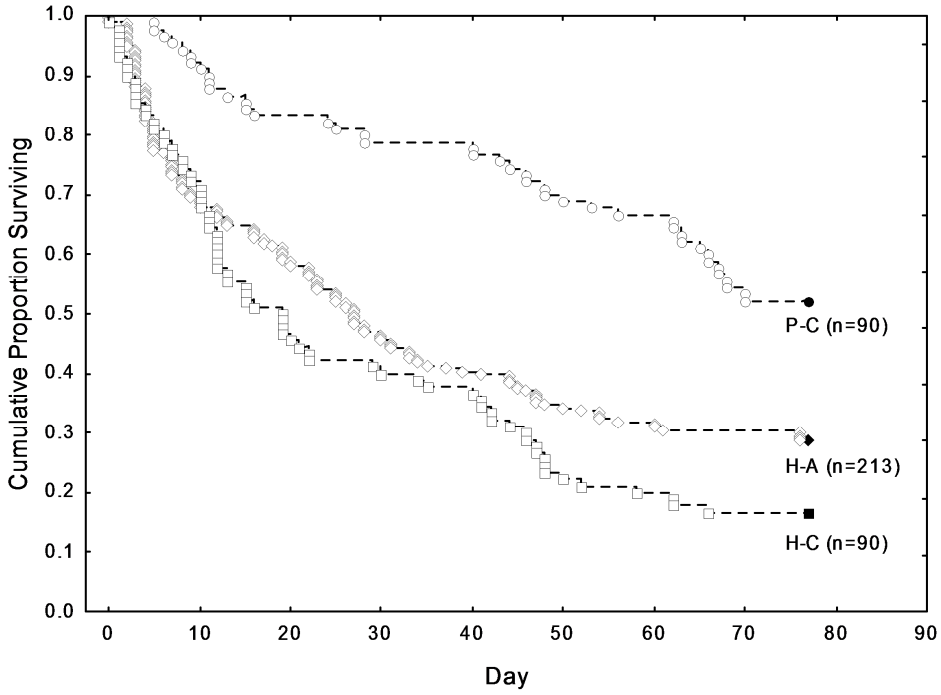

Figure 4.1. Cumulative proportions of surviving queens for each population per day over the first 77 days of the experiment (Experiment-A).

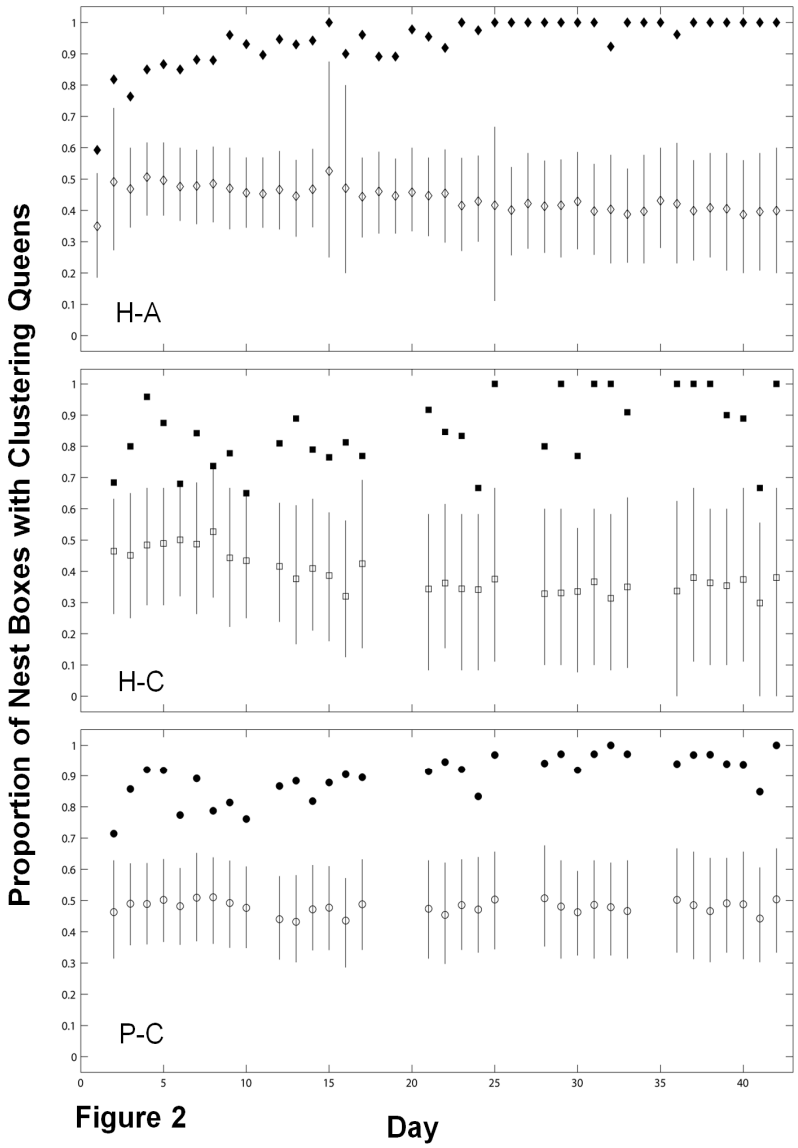

Figure 4.2. Comparison of observed queen clustering (closed symbols) with that of simulated queens (open symbols) who choose nest tubes without regard for the presence of another queen (Experiment-A). Shown are the percentages of nest boxes per day that exhibited clustering for each population. Error bars are 95% confidence intervals for simulated data (based on 1000 bootstraps). All three populations differ significantly from the simulated values indicating that all queens actively cluster. H-A N=71, H-C N=30, P-C N=30.

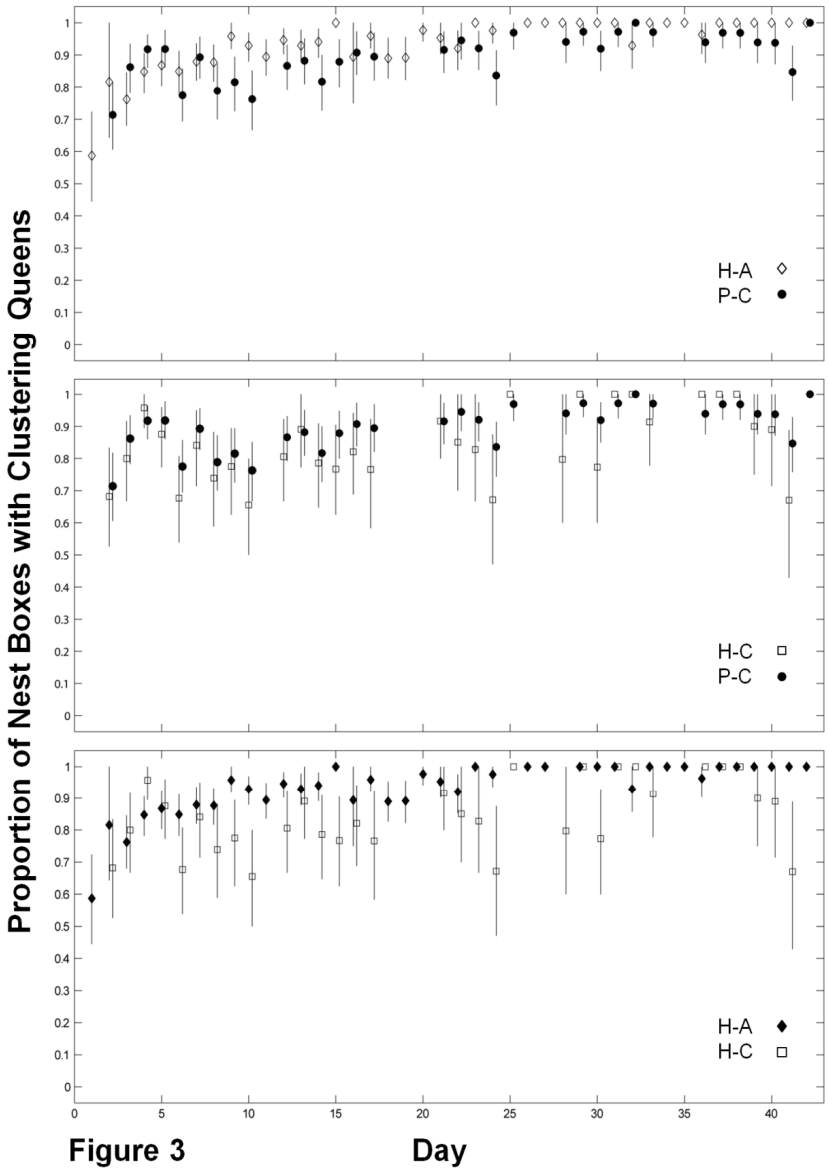

Figure 4.3. Comparison of queen clustering behavior between populations for Experiment-A. Shown are three day running means of our clustering index for 1,000 bootstrapped samples. Error bars show 84% confidence intervals. There was no significant difference in the clustering behavior of queens coming from haplometrotic or pleometrotic populations H-A N= 71, H-C N= 30, P-C N= 30.

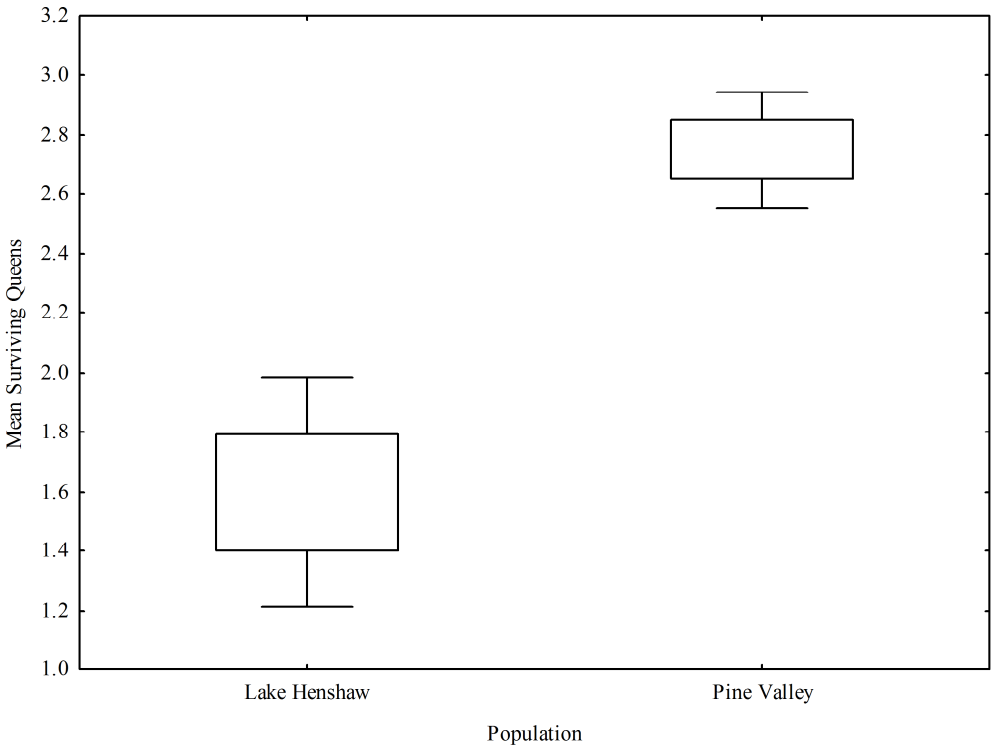

Figure 4.4. Mean queen survivorship per tray for Experiment-B across populations (initially 3 queens per tub). Whiskers represent standard error from the mean.

Table 4.1. Aggression and mortality due to aggression

Population	No. of nest boxes	Aggression	Mortality due to Aggression
H-A	89	83%	76%
H-C	30	77%	73%
P-C	30	37%	20%

The percentage of nest boxes by population in Experiment-A where an aggressive interaction occurred at any time during the experiment (Aggression). The third column shows the percentage of nest boxes with aggression-induced mortality.

Table 4.2. Summary of observed behaviors relevant to metrosis and gyny in both experiments A (choice-boxes) and B (dirt pans).

	aggression		queen removal		joint founding		tolerance	
	Exp A	Exp B	Exp A	Exp B	Exp A	Exp B	Exp A	Exp B
Pine Valley	low	none	n/a	low	high	high	high	high
Lake Henshaw	high	high	Low	low	high	high	low	low
Salt River	high	high	High	high	low	low	low	n/a

CHAPTER 5

THE EFFECTS OF PRIMARY POLYGYNY ON POLYANDRY IN *POGONOMYRMEX CALIFORNICUS*

Introduction

Although polyandry (a female mating with multiple male partners) is common in animals and more particularly insects (Arnqvist and Nilsson 2000), in the social Hymenoptera, obligate multiple mating by females is restricted to a limited number of taxonomically widespread species. Initially, measurements of polyandry in the social Hymenoptera were observational (Hölldobler and Wilson 1990) but have since been enhanced by the advent of molecular tools (Page Jr and Metcalf 1982). Molecular analyses are particularly important as it has been shown that counts of queen matings are often biased because multiple matings produce an average colony relatedness similar to monandry when one male receives a disproportionately large paternity share (Boomsma and Ratnieks 1996).

Facultative polyandry has been documented repeatedly in the social Hymenoptera, where in addition to single matings, multiple matings by females occur at varying frequency between around 1-5 times (Boomsma and Van Der Have 1998; Pamilo 1993; Paxton et al. 1999; Sundström 1994; Sundström, Chapuisat, and Keller 1996). Obligate polyandry, where queens almost invariably mate with high numbers of males however, is more rare and restricted to a handful of known genera. Obligate polygyny has been reported from honey bees (Moritz et al. 1995; Palmer and Oldroyd 2000; Tarpy, Nielsen, and Nielsen 2004), vespine wasps in the genus *Vespula* (Foster and Ratnieks 2001; Ross 1986), army ants (Denny et al. 2004; Kronauer et al. 2006; Kronauer, Johnson, and Boomsma 2007; Kronauer, Schöning, and Boomsma 2006; Kronauer et al. 2004), leaf-cutter ants (Bekkevold, Frydenberg, and Boomsma 1999; Boomsma 1996; Boomsma, Fjerdingstad, and Frydenberg 1999; Fjerdingstadt and Boomsma 2000; Murakami, Higashi, and Windsor 2000; Villesen et al. 1999), seed harvester ants (Cole and Wiernasz 2000; Cole and Wiernasz 1999), and the ant *Cataglyphis cursor* (Pearcy et al. 2004).

Within the seed harvester genus *Pogonomyrmex* several species have been measured for effective mating frequencies revealing high levels of polyandry including *P. occidentalis* (Cole

and Wiernasz 2000; Cole and Wiernasz 1999), *P. barbatus* (Volny and Gordon 2002), *P. rugosus* (Gadau et al. 2003), *P. badius* (Rheindt et al. 2004), and two South American species *P. inermis* and *P. pronotalis* (Pol et al. 2008). The discovery that *P. pima* from the sister subgenus *Ephebomyrmex* is monandrous (Johnson et al. 2007) suggest that high levels of polyandry may be derived within the *Pogonomyrmex sensu stricto* sub genera but as the mating frequency of closely related genera to *Pogonomyrmex* have not been measured this is unknown.

Costs of polyandry

The potential costs to multiply-mating females include increased risk of damage and predation (Arnqvist 1989; Parker 1979; Rowe 1994; Wing 1988), as well as infection with pathogens or parasites (Hurst et al. 1995). In most social Hymenoptera, the events surrounding mating and colony foundation take place during a dangerous time of risks resulting in high mortality (Hölldobler and Wilson 1990) and multiple mating increases the time that founding queens are exposed to these risks.

Hypotheses to explain polyandry

Many hypotheses have been presented to explain the evolution and maintenance of polyandry in the social Hymenoptera and these have been reviewed at length (Brown and Schmid Hempel 2003; Cole 1983; Crozier and Fjerdingstad 2001). The “sperm limitation” hypothesis proposes that polyandry may have evolved to allow queens to store more sperm and in turn have longer-lived and larger colonies (Boomsma and Ratnieks 1996; Cole 1983; Fjerdingstad and Boomsma 1998). However this hypothesis leaves unclear why males would not be under directional selection to provide enough sperm for a given queen (Crozier and Page 1985). Queens may also mate-multiply to avoid diploid male production (Crozier and Page 1985; Page Jr 1980; Page Jr and Metcalf 1982; Pamilo et al. 1994), or to bring their daughters’ preferred sex ratio more in line with their own (Moritz 1985; Queller 1993; Starr 1984) . The “convenience hypothesis” suggests that queens may mate multiply only to avoid conflict with ambitious males (Alcock et al. 1978).

Another set of hypotheses proposes that an increase in colonial genetic diversity leads to colony-level benefits. These benefits may come through increased pathogen and parasite resistance (Baer and Schmid-Hempel 1999; Liersch and Schmid-Hempel 1998; Schmid-Hempel 1994; Sherman, Seeley, and Reeve 1988; Shykoff and Schmid-Hempel 1991; Tarpy 2003) or through increased colony performance through the addition of morphological and behavioral variation to the colony (Jones et al. 2004; Julian and Fewell 2004; Mattila, Burke, and Seeley 2008; Mattila and Seeley 2007; Oldroyd and Fewell 2007; Page et al. 1995; Hughes et al. 2003; Rheindt, Strehl, and Gadau 2005; Smith et al. 2008; Fewell and Page 1993). This increased variation can then lead to greater task specialization by workers and in turn increased colony efficiency and success.

Increased genetic diversity as an explanation for the evolution of polyandry in *Pogonomyrmex* seems particularly likely as a correlation between colony fitness and polyandry has been discovered in *P. occidentalis*. During an 8-year study tracking 1,492 colonies Cole and Wiernasz demonstrated a strong advantage to colony growth correlated with the increased genetic diversity provided by polyandry (Cole and Wiernasz 1999; Wiernasz, Perroni, and Cole 2004). This increased growth in turn led to strong effects on colony survival and reproductive success.

Here we report the first mating frequencies based on molecular data for *P. californicus*. Unlike all other members of the *Pogonomyrmex sensu stricto* tested to date (which are all polyandrous), some colonies of *P. californicus* exhibits an additional source of intracolonial genetic diversity through primary polygyny (ch. 2). This presents the unique opportunity to test the genetic benefits hypotheses for the evolution of polyandry. In this species, unrelated queens exhibit a life-long cooperation, and both found and maintain colonies together. Additionally, populations of *P. californicus* exhibit striking differences in the frequency of primary polygyny from low to high, which causes dramatic variation in the genetic diversity and average relatedness of colony members (ch. 3). This unique combination of life history traits in *P. californicus* allows us to test the prediction that the Cameron Valley population will exhibit a lower mating frequency than other monogynous populations. This would support the hypothesis that the potential costs of

multiple mating have selected against polyandry in populations of *P. californicus* where high levels of intracolonial genetic diversity have been achieved through polygyny.

Methods

Sampling

We collected series of 20-50 workers from excavated nests of *P. californicus* from the Lake Henshaw and Salt River monandrous populations. For collection dates and locale information refer to the chapter 2 methods. A pilot experiment with worker series from 4 adult colonies from the primary polygynous population at Cameron Valley revealed much greater genetic diversity than at either of the other two populations which we presumed was due to the combined effects of multiple polyandrous females present in the nest. This high amount of diversity made statistically discerning matriline from patriline in a colony very difficult. Hence, for analysis of the Cameron Valley populations we collected worker offspring only from the same mother queen by separating queens in the lab. As low levels of polygyny have been detected in the monogynous populations (ch. 2), when data from series of males confirming monogyny were available (9 out of 11 colonies for the Lake Henshaw population) we preferentially used those colonies. When no confirmation of monogyny was available we made sure during analysis that worker genotypes were all consistent with a single queen before proceeding with the estimate of queen mating frequencies.

Microsatellite analysis

DNA was extracted using a standard Chelex extraction protocol and genotyped using PCR amplification at combinations of the following loci for each colony series: Pb5, Pb6, Pb8 (Volny and Gordon 2002); PO03, PO07, PO08 (Wiernasz, Perroni, and Cole 2004); PPro1, Ppro2 (Pol et al. 2008); BJ04 (Gadau lab, unpublished); Pr2 (Gadau et al. 2003); and LXA GT1 (Bourke, Green, and Bruford 1997). A Licor 4200 model sequencer was then used for size determination of products (see ch. 2 methods for details). In our analysis, difficulties occurred stemming from the use of primers developed for other species including high numbers of null alleles (on some occasions up to 30% of individuals from a colony) and extreme differences in product yield

between samples from the same colony which led many samples to be unreadable. In order to avoid these problems, we ran up to 8 different loci for each sample and removed poorly amplified loci from the data set. Regardless, all colonies in our analysis are amplified for 4-7 loci except for 3 from the Lake Henshaw population which are only amplified for 3 loci (appx. E).

Data analysis

To analyze our data, we followed Kronauer, et al. (2007). The program MateSoft (Moilanen, Sundstrom, and Pedersen 2004) was used to construct putative queen and male genotypes from worker offspring from the genotypes of worker offspring. Since the genotypes of workers from a single matriline are not independent from one another, we obtained unbiased allele frequencies from the putative queen and male genotypes. Initially, all worker alleles were entered at equal frequency we then used a new data set of only queen and male genotypes to estimate the allele frequencies for the populations by entering male haploid genotypes as diploid homozygotes and duplicating all of the diploid queen genotypes. This allowed us to calculate allele frequencies using the program FSTAT (Goudet 1995). We then reanalyzed the worker dataset using the revised allele frequencies to calculate mating statistics.

Using MateSoft we calculated the observed number of mates per queen (k_{obs}) as well as the sum of squared paternity contributions of each colony (Pamilo 1993; Pedersen and Boomsma 1999). We then calculated the effective mating frequencies per colony M_{e1} (Starr 1984), M_{e2} (Pamilo 1993), and M_{e3} (Nielsen, Tarpy, and Reeve 2003). For mating frequency comparison among the focal populations we calculated the observed patriline number for each colony. We used the observed number of queen mates rather than a measure of effective mating frequencies to avoid problems with using a statistical test on the output from a derived estimator of effective mate number. When multiple potential queen genotypes existed for a colony we first eliminated any genotypes with a lower than 10% probability and averaged the remaining patriline numbers for each putative queen to get a total average k_{obs} for each colony. We used k_{obs} for our statistical comparison to avoid problems with comparing a derived calculated value like effective mating frequency.

Results

P. californicus exhibited high levels of polyandry like other measured species within the genus. Across our entire dataset (875 individuals from 36 colonies) the mean k_{obs} was 8.81 males (+/- 0.52 SE) and the mean effective mating frequency (m_{e1}) was 8.22 males (+/-1.04 SE). High numbers of queen matings were measured in all populations investigated (table 5.1). Our comparison of observed queen matings showed a significant difference in mating frequency among the three populations (ANOVA: F=8.19, D.F. 2, p<0.002; fig. 5.1). Tukey post-hoc comparisons of the three populations indicate that the Salt River population had a significantly higher mating frequency than either Cameron Valley (MS=6.86, D.F. 33, p<0.002) or Lake Henshaw (p=0.01). There was no significant difference between the mating frequencies of the two California populations (p= 0.77). A more conservative comparison with the lowest possible queen number for each colony (rather than the average mate number of all putative queens per colony) produced the same trend of significance among populations.

The three estimates of effective mating frequency also revealed higher levels of polyandry at the Salt River population (table 1). The m_{e2} and m_{e3} estimators of Pamilo and Nielsen caused erroneous negative values and other suspected erroneous values of high mating frequency in some colonies (table 5.1). This occurred in colonies which contained very high numbers of detected patrilines relative to their sample sizes causing the estimators to produce extremely biased results because the algorithms do not work within a certain window of sample sizes and patriline numbers. Hence, to avoid these errors we used k_{obs} to compare the level of polyandry between populations.

Discussion

The high mating frequencies observed in *P. californicus* are similar to those reported in other species of *Pogonomyrmex* (Cole and Wiernasz 2000; Gadau et al. 2003; Pol et al. 2008; Rheindt et al. 2004; Volny and Gordon 2002). Our prediction that lower levels of polyandry would be detected in polygynous relative to monogynous populations was not the case. Rather, significantly lower levels of polyandry were measured in both the Cameron Valley and Lake

Henshaw populations as compared to the Salt River population (fig. 5.1). Although levels of polyandry were lowest in the polygynous Cameron Valley, contrary to our prediction there was no significant difference between it and the monogynous Lake Henshaw 40 km away. This suggests that variation for polyandry in *P. californicus* maps onto geographical location (California chaparral grassland vs. Sonoran desert) rather than being driven by the evolution of primary polygyny in certain populations. An alternative possibility is that polygyny does have a causal role in the evolution of the depression of polyandry. If this were the case, it would be required to think of the Lake Henshaw population as an outlier in a larger area throughout southern California and Northern Baja Mexico where both primary polygyny and depressed levels of polyandry like those measured were strongly correlated. Also it is important to note that although the mating frequency at the Salt River population is indeed significantly higher than the others, all populations of *P. californicus* would be considered obligately polyandrous as compared to the majority of ant species so far investigated.

Polygynous colonies of *P. californicus* thus have high levels of genetic diversity from several unrelated and multiply-mated foundresses in a single colony. Whether primary polygyny or polyandry evolved to increase colonial genetic diversity or whether the observed increase in genetic diversity in the polygynous *P. californicus* population is merely an epiphenomenon of these two life history characters in *P. californicus* is unknown. Assuming that colonial genetic diversity increases fitness as it does in *Pogonomyrmex occidentalis* (Cole and Wiernasz 1999; Wiernasz, Perroni, and Cole 2004), it may be that, contrary to our predictions, polyandry is still selected for despite its costs even in polygynous colonies of *P. californicus*. This seems to be the case especially since even in the polygynous populations where the majority of colonies have genetic diversity due to multiple, unrelated queens, mating frequency measurements are still roughly equivalent to other mating frequencies from other monogynous species of *Pogonomyrmex*.

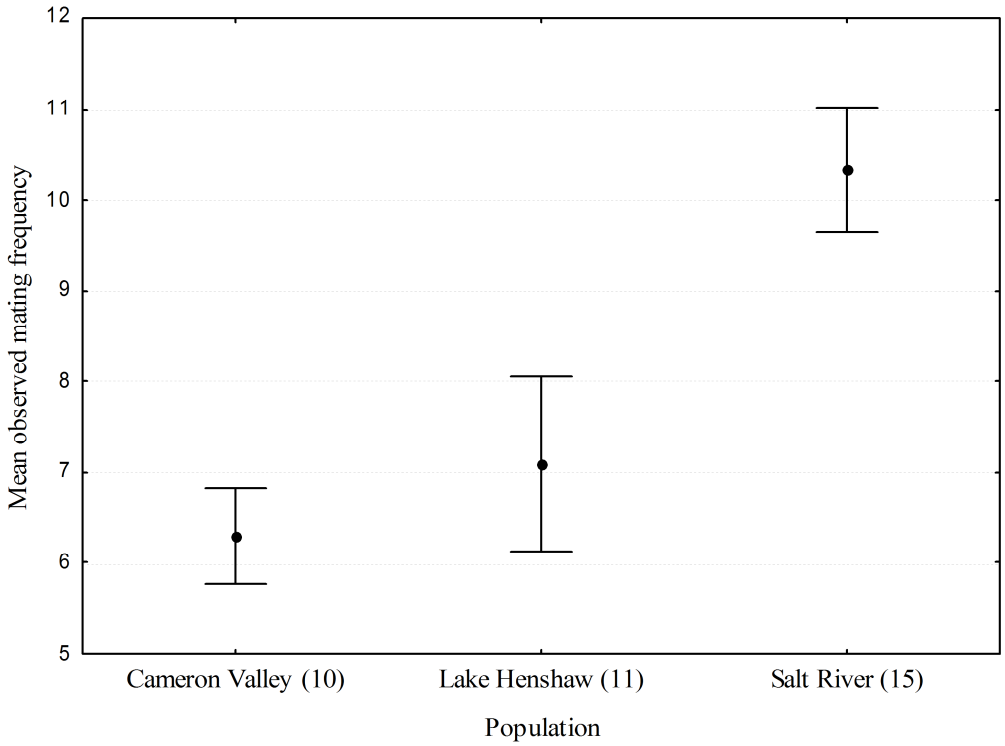

Figure 5.1. Population means and standard error of observed mating frequency for each queen (k_{obs}). Numbers in parenthesis represent colonies sampled per population.

Table 5.1. Summary of mating frequency data for each colony from three populations. Displayed are number of workers sampled per colony (*n*), observed queen mating frequency per colony averaged across putative queen genotypes (k_{obs}), the lowest queen mating frequency measured per colony ($min\ k_{obs}$) and effective mating frequencies estimated using m_{e1}, m_{e2}, and m_{e3} from (Starr 1984; Pamilo 1993; Nielsen, Tarpy, and Reeve 2003) respectively. Population codes are as follows: CV=Cameron Valley, LH=Lake Henshaw, SR=Salt River. Because of high patriline numbers compared to sample size in some colonies effective mating frequency estimators produced erroneous and suspect results. These values are marked with asterisks.

Table 5.1 Observed and effective mating frequencies across populations

Colony	*n*	k_{obs}	*min* k_{obs}	m_{e1}	m_{e2}	m_{e3}
CV18	30	5.75	5	5.7	6.8	6.7
CV19	26	4.00	4	2.6	2.8	2.8
CV20	26	6.00	6	5.4	6.6	6.5
CV21	31	8.00	8	3.8	4.2	4.2
CV22	30	13.00	10	12.4	20.6	19.7
CV23	30	8.50	5	6.5	8.1	7.9
CV-d20	30	6.00	6	4.4	5.0	4.9
CV-d37Q1	31	6.00	6	3.7	4.1	4.1
CV-d37Q2	31	6.00	6	3.0	3.2	3.2
CV-e31	30	7.00	7	4.0	4.4	4.4
C V Overall	295	7.0	6.3	5.2	6.6	6.4
LH1	19	3.00	2	2.3	2.5	2.5
LH2	20	2.50	2	2.0	2.1	2.1
LH3	16	8.50	8	11.8	41.8	31.2
LH4	19	12.00	11	15.3	75.4*	52.5*
LH5	20	10.00	9	9.7	18.0	16.6
LH6	20	11.00	11	9.5	17.2	15.8
LH7	20	5.50	5	4.6	5.7	5.6
LH8	20	10.50	10	11.9	27.9	24.5
LH13	20	8.00	8	9.6	17.6	16.2
LH14	20	6.67	6	5.2	6.7	6.5
LH15	20	6.00	6	5.4	7.1	6.9
LH Overall	214	7.6	7.1	8.0	20.2	16.4
SR5	17	10.67	9	14.9	110.7*	61.3*
SR6	18	10.50	10	13.7	53.6	39.9
SR7	19	14.00	14	34.6	-39.9*	-52.4*
SR8	19	12.50	12	18.5	613.8*	133.9*
SR1	19	9.00	7	8.2	13.8	12.8
SR2	20	11.00	11	1.2	1.2	1.2
SR3	18	8.00	8	8.7	15.7	14.4
SR4	19	10.00	8	13.1	40.2	32.7
SR9	18	8.00	8	10.8	25.5	22.0
SR10	18	11.00	11	15.3	95.2*	58.9*
SR11	20	8.50	7	6.8	9.9	9.4
SR-ROPC-1	39	16.00	16	13.8	20.8	20.2
SR-ROPC-2	45	11.00	11	9.9	12.4	12.3
SR-ROPC-3	37	13.00	13	9.4	12.3	12.1
SR-ROPC-4	40	10.00	10	9.6	12.4	12.2
SR Overall	366	10.9	10.3	12.6	66.5	26.1
Species Overall	875	8.81	8.22	9.10	*	*

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APPENDIX A

SAMPLE SIZES OF MALES AND WORKERS

Appendix A: Sample sizes of males and workers per colony as well as minimum queen number deduced from genetic data.

Colony	Males	Workers	Queen #
Salt River 13	12	0	1
Salt River 14	12	0	1
Salt River 15	12	0	1
Salt River 16	12	0	1
Salt River 17	12	0	1
Salt River 18	12	0	1
Salt River 19	12	0	1
Salt River 20	12	0	1
Salt River 21	12	0	3
Salt River 22	12	0	4
Salt River 23	12	0	1
Salt River 24	12	0	4
Cameron Valley 5	20	0	4
Cameron Valley 6	20	0	3
Cameron Valley 7	20	0	5
Cameron Valley 8	20	0	4
Cameron Valley 9	20	0	2
Cameron Valley 10	18	0	4
Cameron Valley 11	20	0	3
Cameron Valley 12	20	0	1
Cameron Valley 14	20	0	5
Cameron Valley 15	20	0	4
Cameron Valley 16	20	0	4
Cameron Valley 17	20	0	3
Lake Henshaw 2	12	0	1
Lake Henshaw 3	12	0	1
Lake Henshaw 4	12	0	1
Lake Henshaw 5	12	0	1
Lake Henshaw 6	10	0	1
Lake Henshaw 7	12	0	1
Lake Henshaw 8	10	0	1
Lake Henshaw 9	6	0	1
Lake Henshaw 10	12	0	3
Lake Henshaw 11	12	0	3
Lake Henshaw 12	12	0	1
Lake Henshaw 13	12	0	1
Shockey Truck Trail 2	10	10	3
Shockey Truck Trail 4	10	10	3
Alpine 1	0	20	4
Alpine 2	0	20	2
Lake Sutherland 10	10	10	1
Lake Sutherland 11	10	10	2

APPENDIX B

SAMPLE NAMES, POPULATION OF ORIGIN, AND MTDNA HAPLOTYPE INFORMATION

Appendix B: Sample names, population of origin and mtDNA haplotype designation of all individuals in phylogeography and haplotype analyses.

ID	Population	State	Country	Haplotype	Lat	Lon
CP-60	Alpine	CA	USA	A	32.83	-116.74
AV-1	Alvarez	BC	MEX	K	32.51	-116.28
CP-11	Borrego Springs	CA	USA	T	33.27	-116.40
CP-14	Cadiz Valley	CA	USA	AB	34.08	-115.35
CP54	Cameron Valley	CA	USA	A	32.73	-116.46
CP55	Cameron Valley	CA	USA	A	32.73	-116.46
CP56	Cameron Valley	CA	USA	A	32.73	-116.46
CP57	Cameron Valley	CA	USA	A	32.73	-116.46
CP58	Cameron Valley	CA	USA	A	32.73	-116.46
CV10	Cameron Valley	CA	USA	A	32.73	-116.46
CV11	Cameron Valley	CA	USA	A	32.73	-116.46
CV12	Cameron Valley	CA	USA	A	32.73	-116.46
CV13	Cameron Valley	CA	USA	A	32.73	-116.46
CV14	Cameron Valley	CA	USA	A	32.73	-116.46
CV18-1	Cameron Valley	CA	USA	A	32.73	-116.46
CV18-2	Cameron Valley	CA	USA	A	32.73	-116.46
CV18-3	Cameron Valley	CA	USA	A	32.73	-116.46
CV18-4	Cameron Valley	CA	USA	D	32.73	-116.46
CV5	Cameron Valley	CA	USA	A	32.73	-116.46
CV7	Cameron Valley	CA	USA	A	32.73	-116.46
CV8	Cameron Valley	CA	USA	A	32.73	-116.46
CV9	Cameron Valley	CA	USA	A	32.73	-116.46
2-1	Cibbets Flat	CA	USA	D	32.78	-116.45
3-1	Cibbets Flat	CA	USA	A	32.78	-116.45
3-2	Cibbets Flat	CA	USA	A	32.78	-116.45
3-3	Cibbets Flat	CA	USA	A	32.78	-116.45
3-4	Cibbets Flat	CA	USA	A	32.78	-116.45
3-5	Cibbets Flat	CA	USA	A	32.78	-116.45
3-6	Cibbets Flat	CA	USA	A	32.78	-116.45
3-7	Cibbets Flat	CA	USA	A	32.78	-116.45
4-1	Cibbets Flat	CA	USA	D	32.78	-116.45
4-2	Cibbets Flat	CA	USA	A	32.78	-116.45
4-3	Cibbets Flat	CA	USA	A	32.78	-116.45
5-1	Cibbets Flat	CA	USA	A	32.78	-116.45
5-2	Cibbets Flat	CA	USA	A	32.78	-116.45
6-1	Cibbets Flat	CA	USA	A	32.78	-116.45
6-2	Cibbets Flat	CA	USA	A	32.78	-116.45

6-3	Cibbets Flat	CA	USA	A	32.78	-116.45
7-1	Cibbets Flat	CA	USA	A	32.78	-116.45
7-2	Cibbets Flat	CA	USA	A	32.78	-116.45
7-3	Cibbets Flat	CA	USA	A	32.78	-116.45
7-4	Cibbets Flat	CA	USA	A	32.78	-116.45
CP-38	Cibbets Flat	CA	USA	A	32.78	-116.45
CP-39	Cibbets Flat	CA	USA	A	32.78	-116.45
NI-1	Colonia Nueva Indu 1	BC	MEX	G	32.50	-116.59
CP-48	Coon Bluff	AZ	USA	C	33.55	-111.64
SR-13	Coon Bluff	AZ	USA	N	33.55	-111.64
SR-14	Coon Bluff	AZ	USA	C	33.55	-111.64
SR-15	Coon Bluff	AZ	USA	O	33.55	-111.64
SR-17	Coon Bluff	AZ	USA	P	33.55	-111.64
SR-18	Coon Bluff	AZ	USA	C	33.55	-111.64
SR-19	Coon Bluff	AZ	USA	C	33.55	-111.64
SR-20	Coon Bluff	AZ	USA	C	33.55	-111.64
SR-21	Coon Bluff	AZ	USA	N	33.55	-111.64
CP-22	Death Valley 2	CA	USA	W	36.27	-116.82
CP-27	Deming 2	NM	USA	C	32.30	-107.77
CP-49	Dunas Laguna Salada	BC	MEX	Y	31.87	-115.18
CP-25	El Paso	NM	USA	C	31.83	-106.12
FV-1	Francisco Zarco	BC	MEX	L	32.09	-116.56
CP-28	Glamis	CA	USA	X	33.00	-115.07
GB-1	Golden Beach	BC	MEX	M	30.37	-115.83
CP-52	Guerrero Negro	BCS	MEX	AA	27.97	-114.04
CP-29	Hilmar	CA	USA	R	37.38	-120.85
CP-30	Hilmar 2	CA	USA	R	37.40	-120.85
CP-53	Isla Partida	BCS	MEX	AD	24.57	-110.40
JR-5	Jorge's Ranch	BC	MEX	J	31.64	-116.50
CP-5	Kingman	AZ	USA	S	35.18	-114.02
CP-43	Lake Henshaw	CA	USA	B	33.23	-116.76
CP-44	Lake Henshaw	CA	USA	E	33.23	-116.76
CP-45	Lake Henshaw	CA	USA	B	33.23	-116.76
CP-46	Lake Henshaw	CA	USA	B	33.23	-116.76
CP-47	Lake Henshaw	CA	USA	B	33.23	-116.76
LH-2	Lake Henshaw	CA	USA	B	33.23	-116.76
LH-5	Lake Henshaw	CA	USA	B	33.23	-116.76
LH-6	Lake Henshaw	CA	USA	B	33.23	-116.76
LH-7	Lake Henshaw	CA	USA	B	33.23	-116.76
LH-8	Lake Henshaw	CA	USA	B	33.23	-116.76
LM-4	Lake Morena	CA	USA	A	32.69	-116.52

CP-7	Las Cruces 1	NM	USA	C	32.32	-106.83
LV-5	Lyons Valley Rd	CA	USA	A	32.71	-116.75
CP-23	Moapa	NV	USA	C	36.67	-114.58
NU-1	Nuevo Uruapan	BC	MEX	I	30.07	-115.70
CP-40	Pine Valley	CA	USA	A	32.82	-116.53
CP-41	Pine Valley	CA	USA	G	32.82	-116.53
CP-42	Pine Valley	CA	USA	A	32.82	-116.53
CP-51	Playa Bahia Concepcion	BCS	MEX	AC	26.73	-111.91
CP-50	Puerto Penasco	SON	MEX	Z	26.88	-112.03
SV-10	San Vicente	BC	MEX	J	31.33	-116.25
CP-15	Santa Catarina	BC	MEX	V	29.73	-115.13
CP-4	Smith	NV	USA	Q	38.83	-119.33
CP-16	Smith 2	NV	USA	Q	38.83	-119.33
SC-1	Sweetwater CO Park	CA	USA	F	32.65	-117.06
CP-12	Twenty-nine Palms	CA	USA	U	34.17	-116.07
VH-1	Villa Hidalgo	BC	MEX	H	30.97	-116.15
CP-37	White Mt. 2	CA	USA	Q	37.60	-118.40

APPENDIX C

NUCLEOTIDE DIFFERENCES BETWEEN ALL MTDNA HAPLOTYPES

Appendix C: Nucleotide differences between all mtDNA haplotypes included in the phylogeny for 639 bases of cox-1 amplified by LCO/HCO. Numbers in the first row represent numerical position on our trimmed amplified fragment from 3 to 625. Haplotype A is designated as the reference sequence and all differences from this sequence are displayed with "." representing an identical base.

ID	Hap.	3	10	15	19	40	46	52	58	82	88	97	103	112	160	163	199	205	220	241	244	250	256	271	289	295	298	301	307	310	319	322	328
CP-38	A	C	C	T	C	C	C	A	T	C	A	T	G	A	A	A	C	T	A	A	T	A	A	C	A	A	T	A	T	A	C	T	T
CP-43	B	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CP44	E	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CV18-4	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CP-41	G	T	.	.	.	.	.	.	?	.	.	.	.	.	G	G	.	C	.	.	.	G	.	.	.	T	.	.	C	.	.	.	.
CP-48	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
CP4	Q	T	.	.	.	.	.	.	.	.	.	.	.	.	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CP5	S	T	.	.	.	.	.	.	.	.	.	C	.	.	G	.	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	.	C
CP11	T	T	.	.	.	.	.	.	?	.	.	C	A	.	.	.	.	.	T	.	.	G	.	.	G	.	.	G	.	G	.	.	C
CP12	U	T	.	.	.	.	.	?	?	.	.	C	.	.	G	.	.	.	T	.	.	G	.	.	G	.	.	.	.	.	.	.	C
CP14	AB	T	.	.	.	.	.	?	?	.	.	C	.	.	G	.	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	.	C
CP15	V	T	.	.	.	.	.	G	.	.	.	.	.	.	G	G	.	.	.	.	?	G	?	.	.	.	.	G	.	.	.	.	.
CP16	Q	T	.	.	.	.	.	.	.	.	.	.	.	.	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CP22	W	T	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	?	.	.	.	.	G	.	.	.	C	.
CP28	X	T	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	G	.	.	.	.	.	G	.	.	.	.	.
CP29	R	T	.	.	.	.	.	.	?	.	.	.	.	.	.	.	.	.	.	.	.	.	?	.	.	.	.	.	.	.	.	.	.
CP37	Q	T	.	.	.	.	.	.	.	.	.	.	.	.	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CP49	Y	T	.	.	.	T	.	.	.	.	.	.	A	.	G	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	.	.	.
CP50	Z	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
CP51	AC	T	T	.	.	.	.	.	.	T	G	.	A	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.
CP52	AA	T	.	.	.	.	.	.	.	.	.	C	.	.	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	T	.	.
CP53	AD	T	.	.	.	.	T	G	.	.	.	.	A	C	G	.	T	.	.	.	.	.	.	T	.	.	A	.	.	.	.	.	.

ID	Hap.	349	352	367	370	382	391	400	412	436	473	487	508	511	529	532	535	538	544	547	553	562	586	589	598	601	604	607	610	613	619	625
CP-38	A	G	C	A	C	A	C	C	T	T	A	T	A	A	G	T	A	T	A	A	T	A	A	T	G	G	G	A	G	T	T	T
CP-43	B	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CP44	E	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CV18-4	D	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CP-41	G	A	.	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	A	.	.	.
CP-48	C	.	.	.	T	.	.	.	.	C	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	A	.	.	.	C	.	.
CP4	Q	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	T	.	.	.
CP5	S	.	.	.	T	.	.	.	.	C	G	.	.	T	.	.	.	.	.	.	.	.	.	.	.	A	A	G	.	.	.	.
CP11	T	.	.	G	T	.	.	.	.	C	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	A	A	G	.	.	.	.
CP12	U	.	.	.	T	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	G	.	.	.	A	A	G	.	.	.	.
CP14	AB	.	.	.	T	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	A	G	.	.	.	.
CP15	V	.	.	.	T	.	.	.	.	C	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	A	.	.	.	.	.	.
CP16	Q	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	T	.	.	.
CP22	W	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	A	.	G	T	.	.	.
CP28	X	.	.	.	T	.	.	.	.	C	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	A	.	T	.	.	.	.
CP29	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	T	A	.	.	.
CP37	Q	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	T	.	.	.
CP49	Y	A	.	.	T	.	.	.	.	C	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	A	.	.	A	.	.	.
CP50	Z	.	.	.	T	.	.	.	.	C	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.
CP51	AC	A	.	.	T	.	.	T	C	.	.	C	.	.	A	.	.	.	.	.	.	.	T	.	A	A	A	.	A	.	.	C
CP52	AA	A	.	.	T	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.
CP53	AD	A	.	.	T	G	.	.	C	.	.	C	.	.	A	.	G	C	.	.	.	.	T	C	A	A	A	G	.	.	.	C

APPENDIX D: COMPLETE TRIMMED SEQUENCE OF THE SECTION OF *COX-1* AMPLIFIED FOR CP-38 BY THE PRIMERS BEN AND JERRY

Appendix D: Complete trimmed sequence of the section of cox-1 amplified for CP-38 by the primers Ben and Jerry which was used in our analyses.

TACTTGAGCCGGAATAATCGGATCTTCAATAAGAATGATCATTCGCCTTGAACTTGGTTCATGTAATAGTCTAATTAATAACGAACAAATCTATA
ATTCTATGGTAACAAGACATGCTTTTATTATAATTTTTTTCATAGTTATACCCTTTATAATTGGAGGATTTGGAAATTTTCTTGTCCCATTAATACT
TGGTACCCCTGATATAGCATTCCCTCGAATAAATAATATAAGATTTTGACTTCTCCCACCATCAATCTCCTTATTAATCCTAAGAAACTTTATTGG
AAGAGGAGTTGGAACCGGTTGAACTATTTACCCTCCTCTTGCATCAAATATTTTTCATAGGGGCCCTTCAGTTGATATATCCATTTTCTCTTTACA
TATTGCCGGTATATCCTCAATTCTTGGTGCAATTAATTTTATCACAACAATTTTAAATATACACCATACAAATTTCACTATAGATAAAATTCCTCT
TTTAGTTTGATCCATTCTAATTANTGCAGTACTTTTATTATTATCTCTGCCTGTACTTGCAGGAGCAATTACTATATTATTAACTGATCGAAATTTA
AATACTACATTTTTTGACCCGAGGGGGGGAGGGGATCCAATTTTATATCAACATTTATTTTG

APPENDIX E: SAMPLE SIZES AND LOCI AMPLIFIED FOR MATING FREQUENCY ANALYSIS OF FOCAL POPULATIONS

Appendix E: Sample sizes and loci amplified for mating frequency analysis of focal populations.

Colony	n	Pb5	Pb6	Pb8	Po03	Po07	Po08	Ppro1	Ppro2	BJ04	Pr2	LxAGT1	Total Loci
SR-ropc1	39	x	x	x							x	x	5
SR-ropc2	46	x	x	x							x	x	5
SR-ropc3	37	x	x	x							x	x	5
SR-ropc4	40	x		x							x	x	4
SR1	20				x	x	x	x		x			5
SR2	20				x	x	x	x		x			5
SR3	20				x	x	x	x		x			5
SR4	20				x	x	x	x		x			5
SR5	20		x	x	x			x		x			5
SR6	20		x	x	x			x		x			5
SR7	20		x	x	x			x		x			5
SR8	20		x	x	x		x	x		x			6
SR9	20				x	x	x	x		x			5
SR10	20				x	x	x	x		x			5
SR11	20				x	x	x	x		x			5
CV18	30	x	x	x		x	x		x				6
CV19	26	x	x	x		x	x		x				6
CV20	30	x	x	x		x	x						5
CV21	31	x	x	x	x	x	x		x				7
CV22	30	x	x	x	x	x	x		x				7
CV23	30	x	x	x	x	x	x		x				7
CV-d20	30				x	x	x	x		x			5
CV-d37a	30				x	x	x	x		x			5

Colony	n	Pb5	Pb6	Pb8	Po03	Po07	Po08	Ppro1	Ppro2	BJ04	Pr2	LxAGT1	Total Loci
CV-e21	30				x	x	x	x		x			5
LH1	20					x	x			x			3
LH2	20					x	x			x			3
LH3	20					x	x			x			3
LH4	20	x	x	x	x	x	x						6
LH5	20	x	x	x	x					x			5
LH6	20	x	x	x	x					x			5
LH7	20	x	x	x	x					x			5
LH8	20	x	x	x	x	x	x						6
LH13	20	x	x	x	x	x	x						6
LH14	20	x	x	x	x	x	x						6
LH15	20	x	x	x	x	x	x						6