# Ecological Drivers and Reproductive Consequences of Queen Cooperation in the California Harvester Ant *Pogonomyrmex Californicus*

by

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A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Approved October 2017 by the
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ARIZONA STATE UNIVERSITY

December 2017

ABSTRACT

An important component of insect social structure is the number of queens that cohabitate in a colony. Queen number is highly variable between and within species. It can begin at colony initiation when often unrelated queens form cooperative social groups, a strategy known as primary polygyny. The non-kin cooperative groups formed by primary polygyny have profound effects on the social dynamics and inclusive fitness benefits within a colony. Despite this, the evolution of non-kin queen cooperation has been relatively overlooked in considerations of the evolution of cooperative sociality. To date, studies examining the costs and benefits of primary polygyny have focused primarily on the advantages of multiple queens during colony founding and early growth, but the impact of their presence extends to colony maturity and reproduction.

In this dissertation, I evaluate the ecological drivers and fitness consequences of non-kin queen cooperation, by comparing the reproduction of mature single-queen versus polygynous harvester ant (*Pogonomyrmex californicus*) colonies in the field. I captured and quantified the total number and biomass of reproductives across multiple mating seasons, comparing between populations that vary in the proportion of single queen versus polygynous colonies, to assess the fitness outcomes of queen cooperation. Colonies in a mainly polygynous site had lower reproductive investment than those in sites with predominantly single-queen colonies. The site dominated by polygyny had higher colony density and displayed evidence of resource limitation, pressures that may drive the evolution of queen cooperation.

I also used microsatellite markers to examine how polygynous queens share worker and reproductive production with nest-mate queens. The majority of queens fairly

contribute to worker production and equally share reproductive output. However, there is a low frequency of queens that under-produce workers and over-produce reproductive offspring. This suggests that cheating by reproducing queens is possible, but uncommon. Competitive pressure from neighboring colonies could reduce the success of colonies that contain cheaters and maintain a low frequency of this phenotype in the population.

ACKNOWLEDGMENTS

I am eternally grateful to my advisor, Jennifer Fewell, for encouraging my pursuit of a risky longitudinal field project that could have easily ended in disaster. Jennifer and I have spent countless hours editing and re-editing grant applications and manuscripts, and through it all she has shown limitless patience and perseverance. I credit her trust and support for every success I've enjoyed throughout my graduate career.

I also must thank Jürgen Gadau, who was so crucial to the success of this research that he was effectively my co-advisor. I came to ASU knowing next to nothing about genetic research, but Jürgen and his lab were incredibly generous with their time and endlessly patient with a blundering field biologist who hardly knew how to use a pipette. I've admired the work of Bert Hölldobler ever since I picked up a copy of The Ants as an undergraduate. I was hoping to learn more about an ant-plant mutualism I had observed, but instead my eyes were opened to the enthralling world of social insects. This started me on the path of social insect research, and I cannot express what an honor it was to have had access to Bert's advice and expertise. My dissertation probably would have never happened without Blaine Cole, whose inspired research convinced me that this project was at all possible. Ron Rutowski was an indispensable teacher and mentor who helped me understand how my research fit into the broader field of animal behavior, and instilled in me the importance of hypothesis driven research.

This project could not have happened without the dedicated assistance of countless graduate and undergraduate students, including Ioulia Bespalova, Nathan Smith, Guo Xiaohui, Evan Farrar, Luis Garcia, Alison Willis, and many others.

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# CHAPTER 1: INTRODUCTION

## Primary Polygyny in the Harvester Ant, *Pogonomyrmex californicus*

In the several decades since it was first discovered (Mintzer and Vinson 1985), cooperation between unrelated ant queens has remained a poorly understood biological phenomenon. Most research on cooperation has focused on theories of kin selection and cooperation among relatives, but there are many examples of stable social groups composed of completely unrelated individuals, which cannot be explained through models of kin selection (Cahan and Helms 2012; Clutton-Brock et al. 2000; Holldobler et al. 2011). There are several theories that have been proposed to explain the evolution of non-kin cooperation, such as mutualism and reciprocity (Mesterton-Gibbons and Dugatkin 1992; Clutton-Brock 2002; Clutton-Brock 2009; Queller 2011).

Despite the theoretical interest in this question, finding natural contexts in which the fitness costs and benefits of non-kin cooperation can be tested has proven difficult. Most of the well-documented examples of non-kin cooperation occur in longer lived taxa, that are additionally slow to reproduce. These are not conducive to the longitudinal analysis of fitness outcomes for cooperation (Dugatkin 2002). The social insects, however, provide a series of relatively unexplored examples of non-kin cooperation. These range from parasocial (communal) groups of unrelated queens, which share nest space over a season as they produce the next year's brood (Abrams and Eickwort 1981; Danforth 2002), to the surprising example of primary polygyny, in which often non-kin queens form cooperative groups to found eusocial colonies and continue to cohabitate across the colony's lifespan as a stable cooperative group.

Primary polygyny was first documented in 1981 in a leaf-cutter ant (Moser and Lewis 1981) and has since been confirmed in multiple ant species (Mintzer and Vinson 1985; Trunzer et al. 1998; DeHeer and Herbers 2004; Qian et al. 2012), indicating multiple independent evolutionary events. The list of non-kin polygynous species has expanded considerably since its first description, in large part due to the proliferation of newer techniques for maternity analysis. Although there has been a dearth of follow-up research on the selection drivers of primary polygyny, a handful of studies on several focal species provide a solid foundation for further study.

The majority of work investigating costs and benefits of this cooperation focuses on the early colony founding stage. The presumed ancestral condition, and most common strategy ants, is colony establishment by single queens (reviewed by Bernasconi and Strassmann 1999). Several studies, however, have found benefits to multi-queen cooperation during colony establishment and early growth. These include an increase in initial worker production (Bartz and Hölldobler 1982; Thorne 1984; Trunzer et al. 1998), and increased queen and colony survival (Johnson 2004; Mintzer 1987; Cahan and Julian 1999; Clark and Fewell 2014; Overson et al 2014). However, the benefits of cooperation during colony founding and early growth do not completely explain why queen cooperation persists throughout the lifespan of the colony. Indeed, in many cases in which queens found nests together (pleometrosis), they cooperate only until first worker eclosion, at which time the queens fight until a single queen inherits the colony (Bernasconi and Strassmann 1999). The lack of a culling event under primary polygyny suggests that colonies may receive additional benefits by maintaining multiple queens

past colony establishment, into at least early colony growth and potentially though maturity.

The California Harvester Ant, *Pogonomyrmex californicus*, is ideally suited for the study of primary polygyny. Its species range includes patchy but contiguous populations in southern California (San Diego County; Figure 1.1) that vary between sites in the prevalence of queen cooperation. In the more northern sites that have been genetically sampled, colonies are almost all headed by a single queen (monogynous); while most colonies in the more southern show primary polygyny, based on observations of queen founding and on genetic analysis of mature colonies (Johnson 2004; Overson et al. 2016; Chapter 3). Sites between these two points vary somewhat clinally, with fairly even mixing of the two social structures in some intermediately located areas (Figure 1.1; Chapter 4).

The entire range of this gradient in social structure covers a distance of approximately 40 linear miles. Because of this, all sites show very similar weather patterns, and colonies share the general condition of living in disturbed arid grassland; however, level of disturbance and food availability likely vary between sites. The switch in social pattern across this narrow geographical range, however, reduces the number of ecological variables that may be driving the persistence of queen cooperation in some areas but not others. The dramatic shifts in social strategy that characterize these populations provide an almost unique opportunity to explore the natural ecological conditions driving the transition from single queen to cooperative nesting strategies.

The life cycle of these colonies also provides advantages in addressing the issue of how cooperative nesting relates to fitness outcomes. Mature harvester ant colonies reproduce through synchronized mating flights where new, winged queens and males (alates) fly from their home nest to mate and initiate new colonies. The entire annual reproductive output of a colony can be captured during this period, and the large number of alates released provides ample variation between colonies for comparative purposes. Because the California populations of *P. californicus* experience similar rainfall and temperature conditions, their reproductive flights occur across the same time windows, allowing direct comparisons of reproductive strategy and output. Thus, the reproductive costs and benefits of non-kin cooperation can be more accurately and easily quantified for primary polygyny than for other common non-kin cooperation study systems.

For this dissertation, I conducted a series of field-based experiments, combined with genetic analysis, to explore the prevalence, evolution, and stability of primary polygyny. Chapter two outlines our current state of knowledge on primary polygyny in ants, with additional consideration of similar social strategies in other insect taxa. I review all species in which primary polygyny has been discovered, and the means by which the behavior was confirmed. I also note several species in which primary polygyny potentially occurs but further confirmation is required. Our current understanding of the potential similarities between species that practice primary polygyny which are discussed in the context of their information on the potential ecological drivers of non-kin cooperative evolution. These include the issues of density and between-colony competition, the potential for brood raiding, and the selection pressures imposed by harsh

environments on colony growth and survival that may favor multi-queen cooperation. The chapter concludes with an in-depth analysis of the current state of research on primary polygyny and explores some potential avenues of future research.

Chapter Three focuses on the ecological drivers of primary polygyny. A dominant hypothesis for queen cooperation is that it may be a response to harsh environmental conditions that make solitary colony founding untenable. Alternatively, the environment where primary polygyny is found may be so rich that a polygynous colony is more equipped to exploit the local resources, mutualistically increasing the fitness of all queens in the colony. In previous research, Overson et al. (2014) identified two *P. californicus* field sites, one dominated by single queen colonies (Lake Henshaw) and one dominated by primary polygyny (Pine Valley). I explored the reproductive characteristics of colonies in these sites in the context of their local environment. *P. californicus* in this area is reproductive over approximately a month long period from mid-June to mid-July, during which time winged queens and males (alates) depart from the nest in synchrony with surrounding colonies to join mating leks. I captured the reproductive investment of colonies in these sites using suspended tent traps and quantified the total reproductive investment of colonies at both sites over a three-year period. I also performed ecological surveys of temperature, precipitation, and colony density to explore potential differences between sites that may maintain queen cooperation in one area and not the other. There were indications that colonies in the polygynous site are resource limited, so I also performed a resource supplementation experiment in a fourth year to see if the reproductive investment of colonies at the polygynous site is constrained by low resource

availability. This study represents an important first step in understanding why primary polygyny is always found in discrete regions of a species' range, and the ecological drivers that could select for the evolution of non-kin cooperation.

Chapter Four explores the costs and benefits of queen cooperation in mature *P. californicus* colonies that share the same ecological conditions, to better understand the impact of social strategy on reproduction, independently of external ecological drivers. Most research on the costs and benefits of primary polygyny are lab studies that focus on the early, founding stages of the colony. These studies suggest that queens benefit from primary polygyny during colony initiation through increased survival and more efficient worker production (Trunzer et al. 1998; Clark and Fewell 2014). These benefits may extend into early colony growth, as the colony expands from a few to a few hundred workers. However, the potential benefits of primary polygyny at colony maturity have yet to be explored. Such benefits could include a larger colony size derived from multiple egg layers or more flexible worker behaviors due to higher genetic diversity, either of which could result in higher reproductive investment. However, any colony-level advantages could also be offset by conflict between polygynous queens over reproductive resources.

To explore the question of how social strategy impacts colony and individual queen reproductive success, I located a new *P. californicus* field site between the polygynous and single-queen dominated sites studied in chapter 3, that contains a relatively even mix of monogynous and polygynous colonies. I captured and quantified the reproductive output in numbers of alates, and reproductive investment in alate mass,

for a set of focal colonies over two years to compare the reproductive characteristics of primary polygyny colonies with monogynous colonies. There was no difference in colony level reproductive investment between monogynous and polygynous colonies, but when reproduction was divided by the number of queens present in polygynous colonies, the per-queen reproductive investment was significantly less for a polygynous queen than for a solitary queen. The only benefit of being polygynous in this site that may offset the fitness cost suffered by polygynous queens was a larger colony size, which may reflect a longer colony life span that could make up for shared reproductive investment though additional reproductive opportunities for queens.

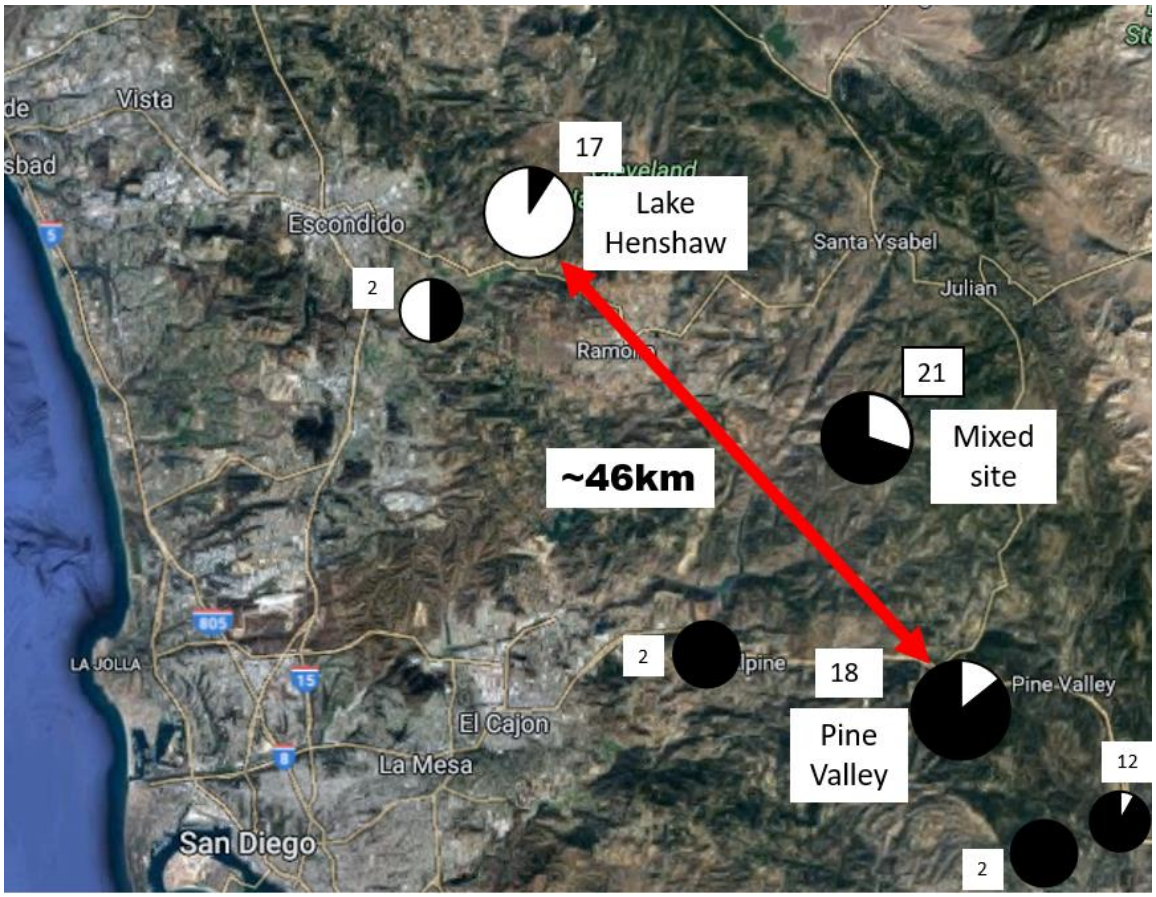
Chapter Five addresses a persistent and difficult question of primary polygyny, how cooperative queens share the demands of worker production with the benefits of alate production. The data from Chapters Three and Four offered evidence that multiple queens contribute to worker and alate production in polygynous colonies. Few studies, however, have explicitly measured the proportional contributions to alate versus worker production by cooperative queens in the context of primary polygyny (Heinze et al. 2001; Kolmer et al. 2002). We cannot confidently state that primary polygyny is a model system of non-kin cooperation until we gather further evidence that nest-mate queens receive equitable fitness outcomes in their cooperative group. Although casual observations from laboratory colonies offer no evidence of queen or worker conflict across maternal lineages, there may be subtle conflict within the colony during the reproductive season, if different queens or worker lineages nepotistically funnel resources to related alates. Queens may potentially "cheat" their cooperative group by making

lower contributions to costly worker production (which represents an individual queen contribution to group success) and produce disproportionately more alates relative to workers. If so, this would indicate the presence of queens freeloading on the work of the colony for higher personal fitness. However, I found that the overwhelming majority of queens produce worker and alates equitably, with only a small number of queens that under produced workers relative to their alate production. The spread of cheating behaviors may be kept in check by competitive pressures between colonies, which likely favor colonies that only contain cooperative queens that contribute to worker production. Regardless, the high ratio of non-cheating queens further supports the notion that primary polygyny represents a stable and fair system of non-kin cooperation.

Taken together, this dissertation provides a much needed exploration into the ecological drivers and fitness outcomes of cooperation between unrelated ant queens. The findings indicate that competitive, resource limited conditions drive the evolution and maintenance of primary polygyny. However, polygynous queens suffer fitness costs from sharing resources with other queens for reproductive investment, which may make this strategy viable only in a subset of environments where solitary colony founding is extremely challenging. Almost all polygynous queens share the benefits of alate production and the costs of worker production equally, and while potential cheating behaviors may be present in a small number of queens, this phenotype does not appear to be common or pose a threat to the cooperative system. These studies lay the groundwork for the advancement of primary polygyny as an important model system of non-kin

cooperation where lingering questions about fitness outcomes and evolutionary mechanisms can finally be directly assessed.

Figure 1.1: The study area of this project. The black area of each circle chart represents the proportion of colonies in that area that display primary polygyny, the boxed number represents the number of samples colonies. Of the sites assessed in this dissertation, 14 of 18 colonies (77.8%) surveyed in Pine Valley contained multiple queens, 14 of 21 colonies (66.7%) surveyed in the mixed site contained multiple queens, and 3 of 17 colonies (17.6%) surveyed in Lake Henshaw contained multiple queens. The small circle charts identify sites that were sampled by Overson (2011).



CHAPTER 2

# NON-KIN POLYGYNY IN ANT SOCIETIES: A REVIEW

## Introduction

In most hymenopteran social insect species, there exists a single reproductive queen in each colony, cohabiting with infertile or reproductively constrained workers (Hölldobler and Wilson 1990). It is generally accepted that the extreme cooperation and reproductive constraint of social insect workers have evolved due to inclusive fitness benefits that workers receive by increasing the reproductive success of a close relative, their mother queen (Queller and Strassmann 1998). However, some eusocial insect species display lower than expected intergroup relatedness, produced by multiple mating (polyandry; Crozier and Fjerdingstad, 2001) and/or nest sharing by multiple queens (polygyny; Hölldobler and Wilson 1977; Keller 1995). There are a handful of different mechanisms that allow polygyny to arise in a colony; queens can form groups at colony founding, or queens can be adopted by established colonies, and the relatedness of nest-mate queens varies from closely related sisters or daughters to completely unrelated foreigners. Polygyny of any type will lower relatedness within a colony, but the acceptance of unrelated queens will have a massive impact on the inclusive fitness dynamics within a colony, the implications of which are not well studied and poorly understood.

Polygyny can be divided into two major strategy types. In *secondary* polygyny, daughter queens return to their home nest after mating and become secondary queens (Hölldobler and Wilson 1977; Keller 1995). Like polyandry, secondary polygyny

generates a more diverse workforce while diluting the inclusive fitness benefits for workers. It also has the potential consequence of reducing direct fitness outcomes for individual queens due to the costs of sharing colony resources as opposed to monopolizing a colony. In turn, however, it provides a potential benefit in extending the life of the colony via replacement queens. While both polyandry and secondary polygyny reduce mean relatedness within a colony, in both social strategies workers share some relatedness above null expectations, from the population mean. Thus both strategies allow – and indeed are likely driven by – inclusive fitness benefits via kin selection.

*Primary* polygyny is a less studied and less common social structure, in which unrelated queens form social groups during the initiation of new nests. The multi-queen association persists throughout the colony lifespan and additional queens are not accepted into the group after initial colony founding (Mintzer and Vinson 1985; Johnson 2004; Overson et al. 2016). In some taxa, particularly wasps, these associations form non-randomly from sibling groups. In *Polistes*, for example, sister queens often hibernate near each other in the winter preceding nest establishment, and so are more likely than random to form kin-biased polygynous associations based on site fidelity (Spradbery 1986; Reed et al. 1988). In other taxa, however, and particularly in the ants, primary polygyny occurs directly after mating flights in which queens and males enter large population-wide mating swarms. In these cases, the relatedness of queens in polygynous associations reflects population levels, and generally approaches zero (Cahan and Helms 2012; Qian et al. 2012; Overson et al. 2016).

Oligogyny is another form of non-kin polygyny found in some ants in which unrelated queens are accepted into an already established colony, often when the resident queen is dead or dying, known as secondary oligogyny (Gadau et al. 1998). Primary oligogyny may also arise from pleometrosis during colony founding, but this is distinct from primary polygyny because as the colony grows queens become intolerant of each other and are segregated to different areas of the nest by their workers (Hölldobler and Carlin 1985). Little is known about the similarities and differences between primary polygyny and oligogyny in terms of the generated benefits or evolutionary drivers of these social structures. However, the high workforce genetic diversity, increase in egg layers, and queen redundancy that are a consequence of both structures by indicate similar evolutionary routes.

Non-kin polygyny generates a qualitatively different social structure than secondary polygyny or polyandry. Because the queens are non-relatives, colonies effectively behave as a multi-family social group, with little to no relatedness between lineages. This creates a genetic structure that is qualitatively different than polyandry or secondary polygyny, in that workers simultaneously cooperate with relatives and non-relatives. Despite this, and acknowledging a lack of studies done so far, the workers in non-kin polygynous colonies show no particular evidence of nepotism (but see Helantera et al. 2013). As with other polygynous structures, most data suggest that all queens within the colony contribute to worker production and share communal resources for individual reproductive output (Kolmer and Heinze 2000; Heinze et al. 2001; Chapter 5), indicating a stable cooperative system, but one with variable inclusive fitness benefits.

For those ant species displaying non-kin polygyny, it is generally layered onto an already existing strategy of polyandry. In *Pogonomyrmex californicus,* for example, both haplometrotic (single queen founding) and polygynous queens mate multiply, with an average of 8.22 males (Overson et al. 2016). Similarly, queens of the polygynous leafcutter ant *Acromyrmex versicolor* mate with 3 males on average (Reichardt and Wheeler 1996). Thus, mean colony relatedness in these groups is already lower than expectations for monogamous haplo-diploid populations.

Relatively little is known about non-kin polygyny, its ecological drivers, or its fitness consequences. However, as a social strategy, it provides an unrivaled opportunity to study the evolution and mechanisms of non-kin cooperation. It is found in several ant genera (Figure 1.1), indicating that the strategy has evolved independently several times. In those species displaying non-kin polygyny, it generally occurs only in a subset of populations, with other populations displaying the ancestral monogynous strategy. This allows an almost unique opportunity to directly compare and contrast the ecological conditions in which non-kin polygyny evolves, and to track its fitness consequences on colony survival and growth, and on queen reproductive success.

Here, I present a synthesis of non-kin polygyny research. Most of the species where non-kin polygyny has been identified are ants, and almost all of the experimental and theoretical work has been done on ant models, so this review focuses on ant species. I suggest ways to identify non-kin polygyny in ants, and catalogue all confirmed cases of non-kin polygyny, as well as instances where the social structure may be present but where further confirmation is needed. In addition, I discuss the ecological characteristics

that are commonly associated with non-kin polygyny in ants, and how they might drive the evolution of non-kin polygyny. To conclude, I explore the impact of non-kin polygyny on colony function and queen fitness, and suggest future research directions for this remarkable context for non-kin cooperation.

## Identifying Non-Kin Polygyny in Ants

Until recently, myrmecologists interested in social structure relied solely on physically digging up colonies, a strenuous and time consuming task. There have likely been several incidents where polygyny was accidentally overlooked because the excavation was declared a success and halted as soon as one queen was found. Monogyny is the dominant strategy in social insects, so the assumption that an excavation was complete after a single queen was found is both logical and convenient. Non-kin polygyny has only recently been focused on as a social strategy, beginning with the first discovery of a mature, multi-queen *Atta texana* colony by Moser and Lewis (1981), and continuing with the study of lab reared colonies and the genetic confirmation of unrelated queens by Mintzer and Vinson (1985), Hölldobler and Carlin (1985), and Rissing et al. (1989). It is likely that inaccuracies about the queen number of ant species are hidden in publications before this time.

As genetic techniques have become more accessible, the rate of non-kin polygyny discovery in social insect species has increased dramatically (Table 2.1). Previously, when multiple queens were found in an excavated colony it was often assumed that the queens are related and attributed to secondary polygyny (Greaves and Hughes 1974).

Secondary polygyny may be difficult to differentiate from non-kin polygyny using only genetic tools, however, especially with low allele diversity or few samples. Therefore, a combination of field-colony assessment, behavioral observation, and genetic analysis is the ideal method to accurately determine a species' social strategy. Confusing matters further, non-kin polygyny has never been found to be the only social structure used by a species; there are always other populations where queens are solitary (Helms and Helms-Cahan 2012; Overson et al. 2016). This variation in social structure between populations can generate conflicting conclusions that necessitate several surveys across a species' range to resolve.

Polygynous queens will readily tolerate unfamiliar queens in a shared lab colony, pooling brood and even dividing the labor of colony founding (Clark and Fewell 2014). The persistence of multiple queens after worker emergence in lab colonies is a good indication that primary polygyny is possible at the source population, as worker emergence is generally when cooperation breaks down if pleometrosis leads to secondary monogyny (Sommer and Hölldobler 1995). Although in at least two species it is possible to induce queen cohabitation in the lab even when it does not occur naturally (Provost and Cerdan, 1990). Considering this, the most precise way to identify non-kin polygyny is to directly examine the genetic diversity present in the workforce or alates of mature field colonies.

## The Taxonomic Distribution of Non-Kin Polygyny

*Ants-Primary Polygyny*

Primary polygyny was first described in 1981 in the Texas leaf-cutter ant *Atta texana* by Moser & Lewis (1981), after several anecdotal accounts of multiple queens inspired them to excavate a mature colony. Moser found 16 queens in an *Atta texana* field colony estimated to be 2 years old (Moser and Lewis 1981). Mintzer and Vinson showed that these cooperative queen associations were stable and beneficial to *A. texana* queen survival under laboratory conditions (Mintzer and Vinson 1985; Mintzer 1987). Subsequent evidence of primary polygyny has been reported for several leafcutter species. Primary polygyny was next identified in the temperate leaf-cutter ant species, *Acromyrmex versicolor* (Rissing et al. 1986; Rissing and Pollock 1987). Rissing et al. (1989) excavated pleometrotic starting nests, and using allozyme markers, demonstrated that the queens were non-relatives. They then reared stable multi-queen colonies in the lab as evidence of primary polygyny. Additional isoenzyme evidence indicates that at least two other South American *Acromyrmex* species, *A. striatus* and *A. heyeri*, have colonies that contain multiple unrelated queens that contribute to worker and alate production, strongly suggesting primary polygyny (Diehl et al. 2001), although supporting field colony data for these species are lacking. Primary polygyny may also occur in a Brazilian population of the fungus growing ant *Cyphomyrmex transversus* Multiple queens were found in 37.73% of colonies examined by Ramos-Lacau et al. (2012) but it is unknown if these queens were non-kin.

Several harvester ant species also practice primary polygyny in some populations. Populations of the California harvester ant, *Pogonomyrmex californicus*, display non-kin primary polygyny, as confirmed with field observation (Johnson 2004), laboratory colonies (Clark and Fewell 2014), and microsatellite analysis (Overson et al. 2014; Overson et al. 2016). Primary polygyny also occurs in a California population of the seed harvester *Veromessor pergandei*, also confirmed using microsatellites (Helms and Helms-Cahan 2012). Pleometrosis with queen culling to secondary monogyny is prevalent in adjacent California and Arizona populations (Helms & Helms-Cahan 2012). Queens of another species in a related genus, *Messor barbarus*, can be induced into stable cooperative associations in the lab, but no polygynous colonies have been found in the field (Provost & Cerdan 1990). The prevalence of primary polygyny in harvester ant species seem associated with arid environments. Two non-harvesting species that occur in arid areas also show this pattern. The honeypot ant *Myrmecosystus mimicus* practices primary polygyny in an Arizona population as confirmed by microsatellite analysis by Hölldobler et al. (2011). The mound building ant *Formica podzolica* exhibits primary polygyny in Colorado, as suggested by field excavation (Deslippe and Savolainen 1995a) and confirmed through microsatellite analysis (DeHeer and Herbers 2004).

Some of the most detailed genetic and behavioral research has been performed on species of the tropical ant genus *Neoponera*. Primary polygyny has been confirmed in *Neoponera inversa* through behavioral observation in the field and lab (D'Ettorre et al. 2005) as well as with multiple microsatellite analyses (Heinz et al. 2001; Kolmer et al. 2002). In a closely related species, *Neoponera villosa*, queen cooperation has been

demonstrated in the lab (Trunzer et al. 1998) and unrelated queens have been documented in field colonies (Kellner et al. 2007), strongly suggesting primary polygyny. Microsatellites were also used to confirm primary polygyny in the Australian jumper ant *Myrmecia pilosula* (Qian et al. 2012), as well as in the red ant *Myrmicia rubra* (Pearson 1982; Pearson 1983; Seppa & Walin 1996). Finally, multiple unrelated queens have been found in mature colonies of the pleometrotic weaver ant *Oecophylla smaragdina*, strongly suggesting primary polygyny (Schluns et al. 2009).

Further confirmation is needed in several other ant species where research suggests primary polygyny may occur but is not conclusive. Multiple, unrelated queens were found in colonies of *Myrmica gallienii* in Finland using enzyme electrophoresis (Seppa 1996), but there is little discussion of colony founding or colony age. The widespread European species *Lasius neglectus* forms stable cooperative unrelated queen groups in the lab, but they have not been found in nature (Espadaler and Ray 2001). Likewise, unrelated queens of the Argentine ant *Linepithema humile* will tolerate each other when placed together in the lab (Keller 1998), suggesting a capacity for primary polygyny. Mature *Neoponera striata smith* colonies in southeastern Brazil have also been found with multiple queens, but more work is needed on queen relatedness to confirm primary polygyny (Rodrigues et al. 2011). Also in southeastern Brazil, the arboreal trap jaw ant *Odontomachus hastatus* has been found in colonies containing several queens and workers, but it is unknown if these queens are related (Oliveira et al. 2011). There are also accounts of primary polygyny in the Northeast range of *Pheidole morrisii*, but supporting data has not yet been published (Wilson 1993).

*Ants-Oligogyny*

Oligogyny was first described in the carpenter ants *Camponotus ligniperdus* and *Camponotus herculeanus* (Hölldobler 1962). It has since been confirmed in a German population of *C. ligniperdus* through microsatellite analysis (Gadau et al. 1998), and genetic analysis of *C. herculeanus* shows relatively low worker relatedness in some populations which also indicates the presence of oligogyny (Seppa and Gertsch 1996). Oligogyny is also found in the Australian meat ant, *Iridomyrmex purpureus*, through a combination of field and lab observation (Hölldobler and Carlin 1985). Unrelated queens of this species can start a colony together (primary oligogyny), or be adopted by an established colony (secondary oligogyny). Further genetic analysis confirmed that oligogynous *I. purpureus* queens are unrelated and share a workforce (Carew et al. 1997). Allozyme analysis of the subterranean ant *Lasuis flavus* also suggests oligogyny may be taking place in some colonies (Boomsma et al. 1993).

*Unicolonial ants*

Polygyny finds its extreme in the social structure of unicolonial ants (reviewed by Tsutsui and Suarez 2003). Unicoloniality is found in populations of invasive species, including the fire ant *Solenopsis invicta*, the Argentine ant *Linepithema humile*, and the crazy ant *Nylanderia fulva* (see Helanterä et al. 2009 for complete list). These social systems feature multiple, unrelated reproductive queens similar to other forms of non-kin polygyny. However, they are further characterized by a lack of typical colony territoriality; workers move freely between conspecific colonies within little to no ability

to differentiate nestmates from non-nestmates. This often makes a unicolonial territory extremely large, sometimes spanning hundreds of kilometers (Corin et al. 2007).

Evidence suggests that unicolonial colonies arose due to a genetic bottleneck when a small number of queens were introduced to a new area or continent, drastically reducing the diversity of hydrocarbon signals that can be used for nestmate identification (Suarez et al. 1999; Tsutsui et al. 2000). The reduction in genetic diversity may reduce the ants' ability to distinguish colony members from other conspecifics, leading to huge meta-colonies which share resources and workers. In contrast, primary polygyny and oligogyny have always been found within a species' natural range and colonies maintain their aggressive, territorial worker behaviors (Helms and Helms Cahan 2012; personal observation). This suggests that primary polygyny and oligogyny are evolved, adaptive cooperative strategies as opposed to the byproduct of an introduction event as seen in unicoloniality.

## Potential Ecological Drivers of Non-Kin Polygyny in Ants

To date, non-kin polygyny has only been documented in a subset of a species' range. It is the dominant social structure within discrete populations, while other areas within the species range contain only single-queen colonies (Helms and Helms-Cahan 2012; Overson 2016). This is indicative that the transition to and from polygyny is evolutionary labile, and that local ecological pressures likely drive the evolution of queen cooperation. Most studies that report non-kin polygyny give at most an anecdotal description of the environmental conditions in the area. However, there are ecological

consistencies associated with its occurrence that may offer insights into the ecological drivers for non-kin cooperation by ant queens.

*High nest density*

Non-kin polygyny is commonly reported in areas where colonies are highly clustered. High colony density decreases the survival rate for new colonies (Fowler et al. 1984), and increases competitive pressures over resources and territory for established colonies (Adams and Tschinkel 1995). The challenges that high density presents for both new and established colonies may be ameliorated through the benefits in colony size and defensive capacity generated by queen cooperation. Clustered colonies may also promote primary polygyny by forcing more contact between queens while they are searching for suitable nest sites in a limited space. High colony density relative to other populations of the species has been reported in polygynous populations of the ants *Neoponera villosa* (Trunzer et al. 1998), *Acromyrmex versicolor* (Rissing et al 1986), *Formica podzolica* (Bennett 1987), and *Pogonomyrmex californicus* (Shaffer et al. 2016). However, a lower colony density was found in a pleometrotic population of *Veromessor pergandei* (Cahan 2001).

*Brood/resource raiding*

Similar competitive pressures may also be generated by raiding events. Some ant species raid nearby colonies to capture food resources and worker brood, often destroying the target colony in the process (Hölldobler 1976; Bartz and Hölldobler 1982; Tschinkel 1992; Nonacs 1993). These raids are especially common in the period following the

mating flight, as poorly defended new colonies struggle to expand their workforce (Rissing and Pollock 1987). Raids are an extreme competitive pressure that may select for queen cooperation to increase the growth rate of the colony to better defend against attack. Raiding behavior is known in four species that also exhibit non-kin polygyny: *Acromyrmex versicolor* (Rissing et al. 1986), *Myrmecocystus mimicus* (Bartz & Hölldobler 1982), *Veromessor pergandei* (Pollock and Rissing 1985, but see Pfennig 1995).

*Temperate climate*

Most of the known ant species that display non-kin polygyny are found in temperate or desert regions, including *Formica podzolica, Acromyrmex versicolor, Atta texana, Oecophylla smaragdnia, Myrmecia pilosula, Veromessor pergandei, Myrmecocystus mimicus, Iridomyrmex purpureus,* and *Pogonomyrmex californicus*. Social insects in temperate habitats have highly synchronized mating flights that result in many queens attempting to initiate colonies simultaneously. The density of ant queens during this period may encourage the evolution of polygyny by limiting nest sites and increasing the frequency of queen contact. Tropical species generally reproduce through year-round, less synchronized mating flights that may make polygyny logistically difficult (Torres et al. 2001). However, there are a few species found in the neotropics that non-kin primary polygyny: *Neoponera villosa, Neoponera inversa, Acromyrmex striatus, Acromyrmex heyeri,* and *Odontomachus hastatus*. Resource availability is also generally lower in temperate environments than in the tropics (Fisher 1960; Leigh 1965),

a difficulty that may decrease colony success in temperate areas and make queen cooperation viable.

*Obligate foraging*

Queens of many ant species are able to nourish their first cohort of workers using only the fat and muscle reserves in her body when leaving her birth nest. These queens generally plug their nest entrance and it remains sealed until workers eclose. In other species queens do not carry enough nutrients for their first cohort and must forage after starting a new colony. Foraging is a risky endeavor for the queen that exposes her to predation, parasites, and desiccation (Fowler 1992). Interestingly, the queens of many polygynous species forage during colony initiation, including *Neoponera villosa* (Trunzer et al. 1998), *Neoponera inversa* (Kolmer and Heinze 2000), *Acromyrmex versicolor* (Rissing et al. 1989), *Acromyrmex striatus* (Diehl-Fleig and Araujo 1996), and *Pogonomyrmex californicus* (Johnson 2004). Lab studies of *P. californicus* and *A. versicolor* have shown that queen groups actually divide labor during colony founding and will specialize in foraging, nest excavation, or brood care behaviors (Rissing et al. 1989; Helms-Cahan and Fewell 2004). The pressure to forage on top of the other tasks a queen must accomplish to successfully start a colony may encourage queen cooperation through the efficiency and survival benefits generated by division of labor (Jeanson and Fewell 2008; Clark and Fewell 2014). However, queen foraging is not ubiquitous, queens of the polygynous leaf cutter *Atta texana* do not forage (Mintzer and Vinson 1985).

## State of Non-Kin Polygyny Research

Most social groups are made up of related individuals, so our understanding of social evolution is often grounded in kin selection models and the indirect benefits generated by cooperating with relatives. But there are several examples of social groups of non-kin (Clutton-Brock et al. 2000; Hacker et al. 2005; Rutte and Taborsky 2008; Hölldobler et al. 2011; Helms-Cahan and Helms 2012), indicating that direct benefits generated by cooperative systems can still select for the evolution of cooperation (Pfeiffer et al. 2005; Clutton-Brock 2009; Schino and Aureli 2010; Queller 2011). However, empirical data showing the impact of non-kin cooperation on individual fitness is lacking, largely due to the difficulty of quantifying fecundity in long lived, slow reproducing mammalian or avian social groups, which are the best studied in this context (Dugatkin 2002).

Non-kin polygyny in social insects provides a system where the relationships between social evolution, low relatedness, and individual fitness can be explored with relative ease. Cooperative queens can be cheaply raised in the lab, and all species where non-kin polygyny is found also have populations where queens are solitary which allows for fitness, behavioral, physiological, and genetic comparisons between cooperative and non-cooperative individuals of the same species. In addition, colonies are long lived but immobile, simplifying longitudinal studies of queen survival and fecundity in natural field colonies.

Most research on non-kin polygyny to date has focused on the benefits that queens receive by cooperating during the early stages of colony founding and growth. Several studies found evidence that multi-queen colonies grow more quickly than single-queen colonies, with more workers produced in a given time in *Neoponera villosa* (Trunzer et al 1998), *Atta texana* (Mintzer 1987), *Formica podzolica* (Deslippe and Savolainen 1995a), and *Iridomyrmex purpureus* (Hölldobler and Carlin 1985). There are also several studies that indicate increased survival of cooperative queens during the founding stage (Bartz and Hölldobler 1982; Mintzer 1987; Johnson 2004; Clark and Fewell 2014). These colony growth and queen survival benefits may be partially generated through division of labor between queens. Lab experiments have shown that polygynous queens will specialize in nest excavation, foraging, or brood care while they prepare for the first brood of workers to eclose (Trunzer et al. 1998; Rissing et al. 1999; Helms-Cahan and Fewell 2004). Queen division of labor should provide similar benefits to group efficiency as seen in other cooperative systems with task specialization (Wilson 1985). Sharing the metabolic demand of laying the first cohort of worker eggs likely also benefits polygynous groups (Kaib et al. 2001; Clark and Fewell 2014).

The benefits of non-kin polygyny during early colony growth closely match the well-studied benefits of pleometrosis (Bernasconi and Strassmann 1999). However, the persistence of queen cooperation past worker eclosion suggests that polygynous colonies receive benefits that extend into colony maturity and outweigh the costs of sharing colony resources for reproductive investment. The benefits of non-kin polygyny at colony maturity have never been directly investigated, though polygynous colonies likely receive

similar benefits to workforce efficiency and pathogen resistance as have been found when genetic diversity increases through polyandry (Oldroyd and Fewell 2007; Wiernasz et al. 2008). In addition, the benefits to worker production rate and worker production efficiency seen in incipient colonies may continue into colony maturity, a valuable trait in highly competitive environments (Adams and Tschinkel 1995).

The extremely low relatedness seen in non-kin polygyny may make this social structure vulnerable to queens cheating when dividing resources for reproduction, which may strain the stability of the group. Unrelated queens have no inclusive fitness benefits that discourage taking advantage of the group by hoarding resources for reproductive investment or otherwise trying to monopolize the colony's alate production. The only study that used genetic techniques to analyze the contribution of cooperative queens to reproductive investment and the workforce found little evidence of queen cheating (Heinze et al. 2001), but a higher sample size may be required for reliable support. We will never fully understand the evolution and maintenance of non-kin polygyny without more information on how these cooperative associations handle reproduction. If queens can cheat their group, it may only be a matter of time until the cheating phenotype dominates the population and the system falls apart, as is predicted by several theoretical models of non-kin cooperation (Boyd and Richerson 1988; Stevens and Hauser 2004). However, there may be mechanisms or selective forces at play that limit the growth of cheating in polygynous populations (Wade and Breden 1980). We must address these questions to determine the stability of theses cooperative groups and realize the potential of non-kin polygyny as a model system for the evolution of cooperation between non-kin.

Figure 2.1: Phylogeny of extant ant subfamilies based on Moreau et al. 2006 and Brady et al. 2006. Subfamilies containing a species where the existence of primary polygyny has been confirmed through genetic analysis and behavioral observation in the field or laboratory are indicated with a red arrow. The number of species that display primary polygyny is given in the red circles. Subfamilies containing species where the existence of oligogyny has been confirmed are indicated with a yellow arrow, with the number of species given in the yellow circle.

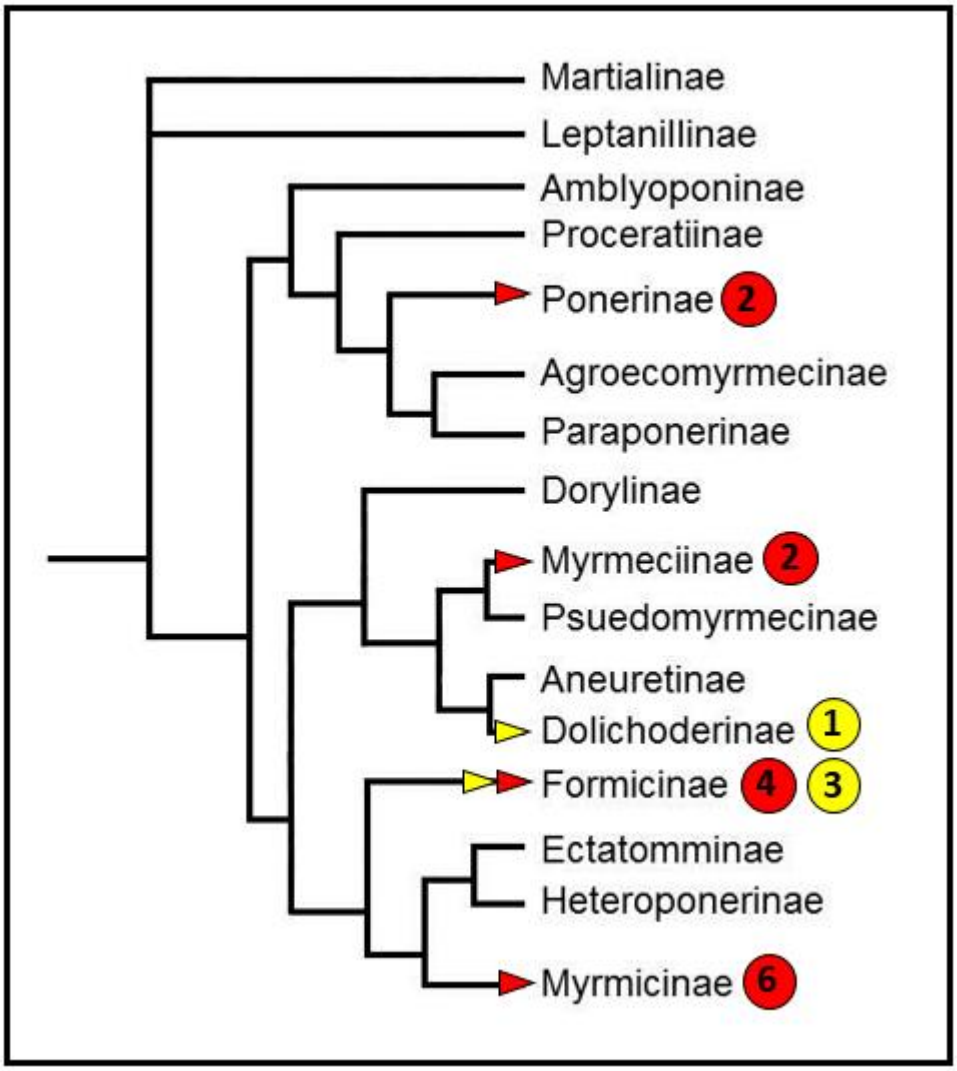


Table 2.1: Evidence of primary polygyny in ants by subfamily, genus, and species. Method indicates how data was gathered; DNA fingerprinting indicates all genetic methods including alozymes, mitochondrial DNA, microsatellites, etc., field colony observation indicates any observation of behaviors in the field or nest excavation, lab colony observation indicates any experiments with colonies transplanted or raised in the laboratory.

| Subfamily | Genus | Species | Reference | Method | Finding |
|---|---|---|---|---|---|
| ***Ponerinae:*** | *Neoponera* | *villosa* | Trunzer et al. 1998 | Field colony observation | Polygynous colonies produced more workers, no aggression, queen division of labor. |
| | | | D'Ettore et al. 2005 | Field colony observation | 70% of field colonies were polygynous. |
| | | | Kellner et al. 2007 | DNA fingerprinting | Polygynous queens unrelated. |
| | | *inversa* | Kolmer & Heinze 2000 | Lab colony observation | Polygynous queen groups persist to colony maturity in lab. |

| | | | | |
|---|---|---|---|---|
| | | | Heinz et al. 2001 | DNA fingerprinting | Polygynous queens unrelated, share worker and alate production. |
| | | | Kolmer et al. 2002 | DNA fingerprinting | Polygynous queens unrelated. |
| | | *striata smith* | Rodrigues et al. 2011 | Field colony observation | Some mature colonies were polygynous. |
| | *Odontomachus* | *hastatus* | Oliveira et al. 2011 | Field colony observation | Half of examined colonies were polygynous. |
| | | | Camargo & Oliveira 2012 | Field colony observation | Half of examined colonies were polygynous. Relatedness unknown. |
| ***Myrmicinae:*** | *Atta* | *texana* | Moser 1981 | Field colony observation | Dug up colony containing 16 queens. |
| | | | Mintzer & Vinson 1985 | Lab colony observation | Polygynous queen groups stable in lab colonies. |

| | | | | |
|---|---|---|---|---|
| | | Mintzer 1987 | Lab colony observation | Polygynous queens had higher survival rate, lost less weight, more workers in first brood. |
| *Acromyrmex* | *versicolor* | Rissing et al. 1989 | DNA fingerprinting | Polygynous queens not related. Foraging specialist emerges. No aggression or dominance. |
| | | Pollock et al. 2004 | Lab colony observation | If queen foraging specialist is kept from foraging, no queen takes her place and colony dies. "suicidal punishment". |
| | *echinatior* | Bekkevold et al. 1999 | DNA fingerprinting | Some colonies were polygynous and lower nestmate relatedness in these colonies may indicate |

| | | | | |
|---|---|---|---|---|
| | | | | unrelated queens. |
| | *striatus* | Diehl-Fleig & De Araujo 1996 | Lab colony observation | Queens forced together in lab did not fight. |
| | | Diehl-Fleig, Rocha 1998 | Lab colony observation | Queen associations persist for at least a couple months in lab. |
| | | Diehl et al. 2001 | DNA fingerprinting | Polygynous queens unrelated. |
| | *heyeri* | Diehl et al. 2001 | DNA fingerprinting | Polygynous queens unrelated. |
| *Cyphomyrmex* | *transversus* | Ramos-Lacau et al 2012 | Field colony observation | Multiple queens in 37.73% of field colonies. |
| *Messor* | *barbarous* | Provost & Cerdan 1990 | Lab colony observation | Polygyny induced in lab, not found in field. |
| | *pergandei* | Helms & Cahan 2012 | Field colony observation | Polygyny present in one area but not another, |

| | | | | |
|---|---|---|---|---|
| | | | | no clear differences in environment between populations. |
| | | Cahan & Helms 2012 | DNA fingerprinting | Polygynous queens unrelated. |
| *Myrmica* | *gallienii* | Seppa 1996 | DNA fingerprinting | Polygynous queens unrelated. |
| *Pheidole* | *tucsonica* | Rissing et al. 2000 | Lab colony observation & DNA fingerprinting | Mated queens start colonies together in lab, allozymes show they are not related. |
| *Pogonomyrmex* | *californicus* | Johnson 2004 | Lab colony observation | Polygynous queen groups persist in lab colonies. |
| | | Clark & Fewell 2014 | Lab colony observation | Queens from polygynous area less aggressive and divide labor. |

| | | | | | |
|---|---|---|---|---|---|
| | | | | Overson et al. 2014 | Lab colony observation | Queens from polygynous area less aggressive. |
| | | | | Overson et al. 2016 | DNA fingerprinting | Polygynous queens unrelated and polyanderous. |
| ***Formicinae:*** | *Formica* | *podzolica* | | Deslippe & Savolainen 1995a | Field colony observation | 27% of founding colonies in field had multiple queens. |
| | | | | Deslippe & Savolainen 1995b | Field colony observation | Polygynous colonies had more male biased reproductive output. |
| | | | | DeHeer & Herbers 2004 | DNA fingerprinting | 60% of colonies in area are polygynous with low relatedness, 4.5 queens on average. |
| | *Myrmecocystus* | *mimicus* | | Bartz & Hölldobler 1982 | Field colony observation | Queens form groups during nest founding. |

| | | | | |
|---|---|---|---|---|
| | | Hölldobler et al. 2011 | DNA fingerprinting | Polygynous queens unrelated, share worker production. |
| *Oecophylla* | *smaragdina* | Schluns et al. 2009 | Field colony observation and DNA fingerprinting | Multiple, unrelated queens in field colonies founded through pleometrosis. |
| *Cataglyphis* | *aenescens* | Cronin et al. 2015 | DNA fingerprinting | A few field colonies had multiple unrelated queens. |
| ***Myrmeciinae:*** *Myrmecia* | *pilosula* | Craig & Crozier 1979 | DNA fingerprinting | Polygynous queens likely unrelated. |
| | | Qian et al. 2012 | DNA fingerprinting | Polygynous queens unrelated. |
| | *rubra* | Person 1982 | DNA fingerprinting | Polygynous queens in some colonies unrelated. |
| | | Pearson 1983 | DNA fingerprinting | Workers from polygynous |

| | | | |
|---|---|---|---|
| | | | colonies unrelated to each other. |
| | Seppa & Walin 1996 | DNA fingerprinting | Relatedness of polygynous queens close to zero in 3 of 5 populations. |

# CHAPTER 3

# ECOLOGICAL DRIVERS AND REPRODUCTIVE CONSEQUENCES OF PRIMARY POLYGYNY

## Introduction

Non-kin cooperative groups present important test cases for understanding the diverse drivers of social evolution, because cooperation evolves in the absence of relatedness by descent. Recent work has demonstrated that social groups of non-relatives are more common than previously thought, and that many social groups that were assumed to be close kin have lower than expected genetic relatedness (Hacker et al. 2005; Cahan and Helms 2012; Clutton-Brock et al. 2000; Rutte and Taborsky 2008; Hölldobler et al. 2011). A critical dilemma for the evolution of cooperation among non-kin is that individuals within a group theoretically incur indirect fitness benefits that approach zero, yet they still suffer potential direct fitness costs associated with individual contributions to group function (Mesterton-Gibbons and Dugatkin 1992; Dugatkin 2002; Aviles 2002; West et al. 2009; Clutton-Brock 2009). For cooperation to persist in this context, there must be some balancing combination of direct individual or multilevel fitness gain, relative to competing individuals and/or groups. There has been considerable theoretical discussion of how these outcomes may be generated (Lehmann and Keller 2006; Nowak 2006; Okasha 2006; West et al. 2007; Connor 2010; Marshall 2011; Queller 2011; Van Cleve and Akcay 2014; Okasha 2016). However, empirical evaluation has been limited, especially in natural contexts, because there are few accurate measurements of direct fitness outcomes for non-kin groups (but see Leadbeater et al. 2011; Rehan et al. 2014).

Primary polygyny by ant queens provides an empirical context for evaluating the fitness costs and benefits of non-kin cooperation. In this social form, unrelated queens join together in cooperative associations during nest founding (pleometrosis). In some taxa, these associations persist through the life of the colony (primary polygyny). Because the queens are unrelated, they create what is essentially a multi-family eusocial group, with non-relative queens and workers living cooperatively (Hölldober and Wilson 1990; Overson et al. 2016; Heinz et al. 2001; DeHeer and Herbers 2004; Cahan and Helms 2012). Primary polygyny occurs infrequently across ant taxa, but multiple well-documented cases have been reported, particularly in arid environments (Mintzer 1987; Trunzer et al. 1998; Heinz et al. 2001; Helms and Helms-Cahan 2012; Helantera et al. 2013). When it does occur, it is generally present only in certain populations of a given species, while others retain the more common and ancestral strategy of single-queen founding (Helms and Helms-Cahan 2012; Overson et al. 2016). This pattern suggests that polygyny may be adaptive under a relatively narrow range of ecological conditions.

Nest founding by the California harvester ant, *Pogonomyrmex californicus,* fits this pattern of patchily distributed cooperation, making it a particularly useful empirical context to evaluate polygyny as a social strategy. Across its range, *P. californicus* includes multiple contiguous, patchily distributed populations that vary from containing mainly polygynous colonies to sites with primarily single queen colonies (haplometrosis). Polygynous and haplometrotic queens show distinct behavioral and gene expression differences in common garden experiments, indicating that cooperative versus single

queen nesting strategies are distinct and genetically linked, rather than purely phenotypic plasticity (Clark and Fewell 2014; Helmkampf et al. 2016).

Explorations of primary polygyny in harvester ants and other polygynous species have focused on the fitness components of queen survival and productivity during colony establishment and early growth (Mintzer 1987; Rissing and Pollock 1991; Clark and Fewell 2014; Overson et al. 2014). Queen cooperation may provide an important advantage during this phase of colony life-history, particularly in highly competitive or harsh environments (Rissing and Pollock 1991). Field studies of harvester ant colony demography show that nest founding and early colony establishment involve high queen and colony mortality rates (Wiernasz and Cole 1995; Wiernasz and Cole 2003). Consistent with this, multiple lab studies have shown survival benefits for polygynous queens and colonies during colony founding (Mintzer 1987; Clark and Fewell 2014), and potentially more efficient (Clark and Fewell 2014), or faster worker production (Deslippe and Savolainen 1995; Trunzer et al 1998).

The consequences of cooperation are likely to shift over time, however, as the colony progresses from early growth to reproduction at maturity. Reproduction marks an important point in queen and colony life-history, both because it most closely captures the fitness consequences of social living (McGraw and Caswell 1996), and also because it requires a shift from egg production for benefit to colony function (worker production), to direct individual fitness gain (new queen and male production). The direct fitness costs of sharing reproduction arise after the colony grows enough to become reproductively active, which often takes several years (Cole and Wiernasz 2000), and there is a dearth of

information on the impact of cooperative strategies on queen reproductive success. However, the fitness equation for queens in polygynous colonies likely involves a trade-off from the potential advantages in colony survival, growth, and resource acquisition, offset against the costs of sharing a single colony's resources to reproduce.

Layered onto the social costs and benefits of cooperation is the central, but often difficult to capture role that ecology plays in the evolution of non-kin cooperation. The relationships between reproductive investment, ecological context, and social strategy are complex, but a simple starting expectation is that polygyny may grant a larger worker force than monogyny, but that the reproductive gains from this strategy are dependent on environmental conditions. Under ideal conditions, in which early colony growth and survival are relatively high, polygyny could generate some colony-level advantage in growth and reproduction, but these might not outweigh the costs of dividing colony reproductive resources among queens. The potential benefits of non-kin cooperation for ant queens likely manifest, therefore under harsher ecological conditions with high mortality risk for single queens, and/or with strong inter-colony competition. (Mesterson-Gibbons and Dugatkin 1992; Dugatkin 2002; Krams et al. 2010; Riehl 2013).

California harvester ants live in arid environments where they face a diversity of potential ecological pressures, including high inter-nest and inter-specific competition, patchy nest site availability, and possible resource constraints all of which could make single queen founding difficult (Bourke and Heinze 1994; Macom and Porter 1996; Johnson 2002; Wiernasz and Cole 2003). In this study, I evaluate the reproductive consequences of queen cooperation at colony maturity, by comparing reproductive (alate)

production in single- versus multi-queen field colonies in two populations, one primarily comprised of single-queen colonies, and the other predominantly containing colonies with primary polygyny. I find no indication that polygyny generates a general reproductive advantage. My results instead indicate constraints on reproduction in the population dominated by polygyny, consistent with the expectations of the harsh environment hypothesis. I further evaluate the relationships between nest distribution, food limitation, and reproductive output, as potential indicators that polygynous colonies are indeed surviving in harsher environmental conditions. The study provides the first direct assessment of the reproductive consequences of primary polygyny as a behavioral and ecological strategy, revealing an important counter-balance to the potential advantages of queen cooperation during early colony life-history.

## Methods

*Colony reproductive output and genetic analysis of polygyny*

I quantified the reproductive output of *P. californicus* colonies at two field sites, one dominated by primary polgynous (multi-queen: MQ) colonies, and the other by single-queen (SQ) colonies. Data were collected across three annual mating seasons, spanning from 2012-2014. The primarily SQ field site is located at Lake Henshaw (LH) San Diego Cty, CA (33°14’3.96”N, 116°45’48.04”W); the MQ field site is in the town of Pine Valley (PV), San Diego Cty, CA (32°49’21.38”N, 116°31’40.24”W), approximately 40 miles away. These sites make up part of a patchily contiguous range of *P. californicus* populations, ranging from primarily single-queen colonies (Lake Henshaw), through sites

intermediate in proportion of SQ and MQ colonies, to primarily polygynous (Pine Valley; Johnson 2004; Overson et al. 2016). In a separate study, microsatellites analyses showed that 75% of colonies at Lake Henshaw contain only a single queen, while at Pine Valley 92% of colonies contain multiple queens (Overson et al. 2016).

The annual reproductive flight for *P. californicus* takes place over a 3-4 week period from mid-June to mid-July. I trapped and counted all reproductive males and females (alates) flying from a set of colonies across this timespan, bracketing before and after flights began and ended to ensure complete sampling (exact dates given in supplemental materials). In the pilot year of the study, 2012, a set of 15 colonies were randomly selected for trapping; however several colonies did not reproduce. In 2013, approximately half of the traps were selectively placed on colonies that reproduced in the previous year, and additional traps were placed on randomly selected colonies. In 2014, traps were only placed on colonies where alates were seen coming to the surface in the days preceding the mating flights, indicating reproductive participation. In 2014, I also monitored all *P. californicus* colonies at both field sites ($N_{PV} = 45$; $N_{LH} = 36$) for evidence of reproductive activity by checking colony entrances at least twice per week to determine whether alates were present. Overall, I captured alates from 10 colonies in 2012 ($N_{PV} = 4$ $N_{LH} = 6$), 24 colonies in 2013 ($N_{PV} = 11$; $N_{LH} = 13$), and 27 colonies in 2014 ($N_{PV} = 13$ $N_{LH} = 14$).

Alates were captured using suspended tent traps following the basic designs of Cole and Wiernasz (2000) and McInnes and Tschinkel (1995). The trap consisted of a circular metal base approximately 1 meter in diameter connected to a suspended

triangular tent of netting that led into a collection box. A high entrance to the collection box allowed alates to fly or crawl up and enter the box, but prevented them from leaving (image in supplemental materials). In 2012, traps were placed over colonies every morning by 7AM, and remained in place until 7PM; traps were checked every hour. Alates never flew after 1PM, so traps at Pine Valley were placed over nests from 7AM – 1PM in 2013 and 2014, to avoid vandalism. Traps were left up at all times in Lake Henshaw for all three years but no flights ever occurred after 1PM.

I removed all alates from collection boxes multiple times each day, and counted the number of males and queens. I then retained at least 10 males and 10 queens per day for genetic sampling. If the total number of trapped males or queens from a colony exceeded 100, I retained 10% of the total. All remaining alates were released undamaged, to continue on to mating swarms. Alates collected in 2012 were immediately placed in 100% ethanol for genotypic analysis; however, this technique does not permit accurate assessment of body weight. In 2013 and 2014, therefore, alates were immediately frozen after capture, and wet weights were obtained within 24 hours of capture. After weighing, samples were stored in 100% ethanol for genotypic analysis. The daily average wet weights of 2013 and 2014 samples were multiplied by the total number of males and queens released that day to estimate colony daily reproductive investment, which was then summed across days to determine total colony reproductive investment.

I determined if one or multiple queens were present in a colony by analyzing two microsatellite loci (PB6 & PB5) of 24 males from each colony (PCR protocol in Chapter 5). If only two alleles were present at each locus from the males of a colony, it was

designated as single queen colony. If more than two alleles were present, the colony was assigned as polygynous. Because male ants are products of unfertilized eggs, they have no paternal genetic information. Thus, genetic analysis of male offspring allows for direct reconstruction of the matrilines within a colony, even when queens mate multiply. Additional microsatellite analyses of queens and workers from each colony match with queen genotypes generated from male offspring and confirm that queens contribute to all casts (Chapter 5).

*Colony activity, distribution and mortality surveys*

Actual colony sizes would require destructive sampling, but I estimated proxies for colony activity for trapped colonies in 2013 and 2014, using two methodologies: by assays of the number of workers seen above ground during peak activity times, and by measuring nest surface area. *P. californicus* colonies do not always have a mound at their colony entrance, but there is always a noticeable area of cleared vegetation around the nest. To assess worker above-ground activity, I counted the number of workers entering and exiting each colony entrance for five minutes between 9:00AM and 11:00AM and/or between 4:00PM to 6:00PM. Activity measures were performed only when the temperature was between $24^{o}$ and $29.5^{o}$C. Colony above-ground activity was counted at least three times in the week after mating flights concluded, and the mean used for colony size ranking.

Both the MQ and SQ populations occur in disturbed grassland habitats with similar rainfall levels and climate. In 2013 and 2014, I measured ambient temperatures and precipitation levels at the two sites across the field season. Ambient temperature was

measured using digital thermometers hung in the shade approximately 2 m above the ground, and was recorded on the hour from 8AM to 1PM for every day that traps were set out. Annual precipitation data at each site was collected from the NOAA National Center for Environmental Information database (https://www.ncei.noaa.gov/), station USC00043914 for Lake Henshaw data and US1CASD0054 for Pine Valley.

I also measured colony densities within each site in 2013 and 2014 by walking 2 m transects and marking all active *P. californicus* colonies with an Etrex 10 GPS. I calculated colony mortality for each population by comparing all colonies that were present in 2013 but had disappeared from a 10 m radius around their GPS coordinate in 2014, by the colonies that persisted from 2013 and 2014. I calculated colony density and degree of clustering using the PASSaGE package (Rosenberg and Anderson 2011); Ripley's K and Maximum Absolute Deviations were calculated using the spatstat package (Baddeley and Turner 2005), both in R version 3.0.1 (R Core Team 2013).

*Resource supplementation*

After the mating flights concluded in 2015, I supplemented the food of a subset of colonies at the Pine Valley site, and assessed the relative impact on reproductive success relative to unfed comparison colonies during the next mating season. Supplementation was done on September 11$^{th}$, 12$^{th}$, October 10$^{th}$ and in 2016 I visited April 16$^{th}$, May 4$^{th}$, and 13$^{th}$ to provide sufficient time for the colonies to utilize the resources for alate investment in the 2016 mating flights. At each visit I provided approximately 158ml of mixed consumable resources for a set of 14 colonies that were randomly selected from a list of all colonies that had been reproductive in previous years and contained multiple

queens. I visually confirmed that the resources were collected by each target colony. The supplemented resources were composed of an equal mix of organic sesame seed, organic bulgur wheat, organic Niger seed, and organic golden flax seed. I observed all *P. californicus* colonies in the Pine Valley site for signs of reproductive activity for the duration of the 2016 mating flight and placed traps over all reproductive colonies as soon as alates were seen. I quantified the alate investment of each reproductive colony in the same method described above for the 2013 and 2014 flights.

## Results

*Reproductive output*

The timing of alate investment across the reproductive season was similar for 2013 and 2014 (when wet mass was measured) with several pulses of alates released (Figure 3.1). Of the 18 reproductive colonies trapped one or more times at Pine Valley, 2 colonies had reproduction tracked across all three years; 4 colonies were tracked for two consecutive years, and 12 for a single year. At Lake Henshaw, reproductive output was measured for 17 colonies; 3 across all three years, 9 for two consecutive years, and 5 for one year. Unless otherwise noted, data collected from the same colony over multiple years were averaged, to treat each colony as a unit of sampling; this was done for 6 of the 18 Pine Valley colonies and 12 of the 17 Lake Henshaw colonies.

I performed microsatellite analyses on males from each trapped colony to confirm whether their source colony was monogynous or polygynous. Consistent with prior reports (Overson et al. 2016), 14 of the 18 focal colonies at Pine Valley were polygynous,

while only 3 of 17 focal colonies at Lake Henshaw were polygynous. Genetic analyses were repeated every year and each colony's allele profile remained constant over time, indicating that no colonies switched from single to multi-queen or vice versa. Because some colonies of each type occurred within each site, reproductive data were analyzed by two-way Type-II ANOVA, for effects of social strategy (SQ vs MQ), and site (LH vs PV). Means and standard errors are given in text where appropriate. None of the ANOVAs had a significant interaction between study site and social structure. The full ANOVA outputs and interaction effects are given in appendix A.

Colony location had a significant effect on the probability of reproducing. In 2012, I chose focal colonies for alate trapping randomly; in that year 6 of 7 focal LH colonies, but only 4 of 10 in PV produced alates. Colonies that reproduced in 2012 were trapped again in 2013 to allow for cross-year comparisons. The 2013 focal colony data were therefore not completely randomized; however, they continued to show a consistent difference in reproduction; 13 out of 14 trapped Lake Henshaw colonies and 11 of 20 trapped Pine Valley colonies reproduced. In 2014, I surveyed all colonies at both sites for presence/absence of reproductives; 33 of 36 Lake Henshaw colonies produced alates, versus 14 of 45 Pine Valley colonies (Figure 3.2). A log linear model found that the proportion of reproductive colonies was significantly different between sites ($X^2$=5.31, df=1, p=0.02), with no effect of year ($X^2$=0.46, df=2, p=0.79).

From 2012-2014, Colonies at Lake Henshaw produced a mean of 1234.2+/-207.3(SE) alates, significantly higher than the mean of 638.6+/-119.9 for reproducing colonies in Pine Valley (2-way ANOVA: $F_{site}$=5.77, df=1, p=0.02). However,

reproductive output did not differ by social strategy (MQ: 812.4+/-126.7, SQ: 1037.0+/-216.8; 2-way ANOVA: $F_{strat}$ =0.49, df=1, p=0.49; Figure 3.3). Reproductive investment, calculated from total alate wet mass, showed a similar effect (data for 2013 and 2014 only; 2012 colonies were excluded because of storage in alcohol). Mean per-colony reproductive investment was 16514.8+/-3290.4mg at Lake Henshaw, significantly higher than the 7122.1+/-1465.1mg per-colony investment in Pine Valley (2-way ANOVA: $F_{site}$=4.59, df=1, P=0.04). Again, however, there was no difference in alate investment between single versus multi-queen colonies (MQ: 8769.5+/-1715.7mg, SQ: 14314.8+/-3287.9mg; 2-way ANOVA $F_{strat}$=0.0001, df=1, p=0.99; Figure 2.4, mean and SE of all groups found in table 3.1).

*Sex ratio and alate size*

Reproductive sex ratio was calculated for reproducing focal colonies in 2013 and 2014 (data for colonies reproducing in both years averaged across years) a total of 18 PV colonies, 16 LH colonies, 17 MQ colonies, and 17 SQ colonies. Data were arcsine transformed before analysis. Mean reproductive investment sex ratio did not differ significantly between sites. Colonies in PV allocated an average of 65.4+/-5.1% of investment into males; in LH male investment represented 52.1+/-6.35% of total (2-way ANOVA: $F_{site}$=0.001, df=1, p=0.99). Social strategy did not influence sex ratio either, with MQ colonies investing 67.9+/-3.9% into males while SQ colonies invested 49.8+/-6.1% into males (2-way ANOVA: $F_{strat}$=3.453, df=1, p=0.073; Figure 3.5). The sex ratio was also not significantly different between site or social strategy when mass was ignored and the analysis was performed on the raw number of alates released. Mating flights in

PV were on average 71.6+/-4.5% male while colonies in LH were on average 61.6+/-6.2% male (2-way ANOVA: $F_{site}$=0.014, df=1, p=0.91). MQ colonies released on average 75.3+/-3.9% males and SQ colonies released on average 59.2+/-5.9% males (2-way ANOVA: $F_{strat}$=3.164, df=1, p=0.086; Figure 3.3).

I calculated the average individual male and queen alate masses from all 2013 and 2014 flights, and compared them between sites and social structures. The average queen alate mass from all colonies was 15.7+/-0.29mg and the average male mass was 9.84+/-0.27mg. Location had a significant effect on female but not male alate size; queen alates from LH colonies were larger than from PV colonies (2-way ANOVA: $F_{site}$=8.23, df=1, p=0.0076), but there was no significant effect on male size ($F_{site}$=0.839, df=1, p=0.075). Colony social strategy had no effect on the size of male or queen reproductives ($X_{Males}$: $F_{strat}$ =0.621, df=1, p=0.44; $X_{Queens}$: $F_{strat}$=1.433, df=1, p=0.241; Figure 3.6).

*Colony activity, temperature and rainfall*

For each focal colony in 2013 and 2014, I performed above ground worker activity assays as a proxy of colony activity and size. There was no significant site effect (2-way ANOVA: $F_{site}$=3.44, df=1, p=0.073; Figure 3.7), but activity levels were generally higher in MQ than SQ colonies (2-way ANOVA: $F_{strat}$=7.613, df=1, p=0.01). I used a log-log regression between reproductive investment and above ground activity to see if a linear relationship exists between these variables. There was a significant regression when all of the colonies in this study were included (F=5.881, n=37, $r^2$=0.1194, p=0.021), and when only single-queen colonies were included (F=5.961, n=17, $r^2$=.2367, p=0.028). However, there was an insignificant linear relationship between worker activity and

reproductive investment when only multi-queen colonies were included in the regression ($F=3.657$, $n=20$, $r^2=0.1227$, $p=0.072$).

I also measured above-ground colony nest areas, within the circle cleared of vegetation around each colony. This did not vary significantly between site (2-way ANOVA: $F=0.455$, $df=1$, $p=0.51$) or social structure (2-way ANOVA: $F=0.0049$, $df=1$, $p=0.95$); however, there may not be reliable above ground architecture that correlates with colony size for this species.

Average hourly temperatures during the periods in which flights occurred were similar between the two sites. Average morning temperatures (recorded at 8 am and 12 pm) did not differ between sites in either 2013 or 2014, at 8:00AM or 12:00PM (2013: 8:00AM $t=0.351$, $df=38$, $p=0.73$; 12:00PM $t=1.21$, $df=41$, $p=0.23$; 2014: 8:00AM $t=1.45$, $df=38$, $p=0.16$; 12:00PM $t=1.35$, $df=38$, $p=0.19$, mean temperatures and SE in table 3.2). Mann-Whitney-Wilcoxon tests show that the monthly rainfall was not significantly different between sites from 2011-2012 ($W=57$, $p=0.60$), 2012-2013($W=68$, $p=0.84$), or 2013-2014 ($W=68.5$, $p=0.86$; table 3.2).

*Colony density, mortality and annual replacement*

I counted a total of 47 colonies in PV in 2013 and 55 in 2014. Between the two years, 7 colonies went absent, giving a one-year mortality rate of 14.9%. The number of colonies in LH changed from 27 in 2013 to 34 in 2014. Only 1 of the 2013 colonies died between 2013 and 2014, a mortality rate of 3.7%; mortality rates did not significantly differ between sites ($X^2=.124$, $df=1$, $p=0.12$). The colony turnover rate at Pine Valley

from 2013-2014 was 27.27% while the turnover rate was 18.75% in Lake Henshaw, because the number of colonies increased in both sites from 2013-2014 (appendix B).

The 2014 measures of colony number and nearest neighbor distance indicated that the Pine Valley site had higher density than did Lake Henshaw. Pine Valley had 55 colonies distributed unevenly across 36,995$m^2$ giving a density of 1488 colonies per square km. The average nearest neighbor distance between colonies was 16.00+/-1.62m. Lake Henshaw had 34 colonies over 40,635$m^2$ giving a density of 837 colonies per square km and an average nearest neighbor distance of 19.75+/-1.98m (Figure 3.8). Using a Ripley's (1976) K-function envelope, colony distributions at Pine Valley, but not Lake Henshaw, were above the envelope of random distribution at most distances, indicating that colonies are clustered (Figure 2.9). A Maximum Absolute Deviation test (Myllymäki et al. 2015) confirmed that Pine Valley colonies were significantly clustered (MAD=4737.4, sim=100, p=0.01), while Lake Henshaw colonies were not significantly different from random distribution (MAD=1443.2, sim=100, p=0.23). In addition, colonies in Pine Valley that did not reproduce had a significantly smaller nearest neighbor distance than colonies in Pine Valley that did reproduce (t=2.32, df=30, p=0.0273).

*Resource supplementation effects on reproduction*

I resource supplemented 14 colonies in Pine Valley. Of these, 6 (43%) survived and reproduced; 4 (29%) survived and did not reproduce; and 4 (29%) died at some point between the beginning of the supplementation regime and June of 2016. The additional 42 *P. californicus* colonies that were located in the Pine Valley site were not

supplemented during this experiment. Four (10%) of these colonies survived and reproduced; 19 (45%) survived but did not reproduce; and 19 (45%) died over the experimental period. The proportion of colonies that reproduced, survived, and died was significantly different between supplemented and non-supplemented colonies ($X^2$=7.014, df=2, p=0.030).

I also compared the mean reproductive investment of supplemented Pine Valley colonies to my 2013-14 data on reproductive investment at the PV and LH sites. Supplemented 2016 colonies had a mean reproductive investment of 16543.9+/-3270mg, which was significantly more than the 5869+/-2693.3mg average investment of non-supplemented 2016 PV colonies (t=2.52, df=9, Benjamini-Hochberg adjusted p=0.045), and which matched the reproductive investment of Lake Henshaw colonies in 2013 and 2014 ($X_{LH}$=16514.8+/-3290.4mg; t=0.006, df=15, Benjamini-Hochberg adjusted p=1). Although supplemented Pine Valley colonies had significantly more reproductive investment than non-supplemented Pine Valley colonies from 2013, 2014, and 2016 ($X_{PVsupplement}$=16543.9+/-3270mg, $X_{Pvnosupplement}$=6895.3+/-1334.7mg; t=2.732, df=7, Benjamini-Hochberg adjusted p=0.045, Figure 3.10).

## Discussion

I captured and quantified the reproductive output of *Pogonomyrmex californicus* colonies in two areas, one dominated by monogynous colonies and the other dominated by colonies that practice primary polygyny, in order to determine if ecological constraints or benefits generated through cooperation are responsible for the maintenance of primary

polygyny in some populations. One general hypothesis for queen cooperation is that it could provide a fitness advantage by accelerating colony growth, for example by producing a larger worker force to dominate the foraging landscape (Trunzer et al. 1998; Offenberg et al. 2012); the result would be to produce larger colonies with higher reproductive potential for cooperating queens. This advantage would theoretically extend across habitat types, as long as resources were available to support larger colony sizes. However, although multi-queen colonies showed higher foraging activity, the data did not indicate a clear reproductive advantage for polygynous colonies.

My results instead provide evidence consistent with the hypothesis that queen cooperation may be a response to ecological conditions that depress the success rate of colonies started by a single queen. These conditions include resource limitation and/or denser and thus more competitive populations, in which colonies are regularly subject to territorial conflict and/or brood raiding (Adams and Tschinkel 1995; Cahan 2001; Brandl et al. 2004; Hölldobler et al. 2011; Shaffer et al. 2016). The strong site-based differences in colony reproduction found consistently across all years of this study suggest that ecological conditions at Pine Valley generate stronger constraints on colony reproduction, and that colony density plays some role in this effect. Mutualistic, non-kin cooperation could potentially arise under either the accelerated growth advantage or the ecological constraint hypotheses. However, the ecological constraint hypothesis is most consistent with my observations of *P. californicus* because I found strong site differences in reproductive potential that was mediated by resource supplementation.

Evidence for polygyny as a response to adverse environmental conditions was most evident in the strong site effects on reproduction, reflected both in the low probability of colonies reproducing per year, and in the low alate investment of reproductive colonies in the polygynous site. The absence of consistent social strategy effects suggest that polygyny does not carry with it either an internal (colony limited) or intrinsic (queen physiology limited) reduction in reproductive output. Site mismatched colonies tended to reflect the investment level of other colonies in their location; polygynous colonies in Lake Henshaw had relatively high reproductive investment, while the reproductive investment of single queen colonies in Pine Valley were low relative to single-queen colonies of the other site. However, polygynous colonies in these areas contain an average of three queens (Overson 2016, unpublished data), so the per-queen fecundity of cooperative queens is substantially lower than that of monogynous queens across sites (supplementary table 3).

The influence of environmental conditions on reproductive potential was further explored with a resource supplementation experiment on Pine Valley colonies. Resource supplemented colonies were able to attain high reproductive investment levels comparable to those seen in Lake Henshaw colonies. This indicates that polygynous colonies in Pine Valley do not have inherently low reproductive potential, and the low reproductive activity I observed from Pine Valley colonies over the years of this study is likely due to environmental constraints. Precisely which environmental conditions limit the reproductive potential of Pine Valley colonies remains unclear, but low resource availability and density driven competition are likely important factors.

The lower proportion of colonies reproducing across years at the Pine Valley (primarily polygynous) site is also consistent with resource limitation, which can be further amplified by density driven intraspecific competition. In many ant species, mature colonies are flexible in their decision to participate in reproductive flights. For example, in *Pogonomyrmex occidentalis* (a monogynous species), skipping reproduction is correlated with limited resource availability in that year (Cole and Wiernasz 2000). The closer two colonies are to each other the more likely they are to compete over resources (Adams and Tschinkel 1995a; Gordon & Wagner 1997). Consistent with this, those colonies in Pine Valley that skipped reproduction were significantly closer to another *P. californicus* colony than colonies that did not skip reproduction, indicating that higher intra-colony competition reduces a colony's ability to reproduce. However, Colony age may also be responsible for this result, as harvester ant colonies generally don't reproduce until they reach a critical size (Cole and Wiernasz 2000; Smith and Tschinkel 2006), which may take several years. *P. californicus* colonies have occupied Pine Valley since at least 1997 (Johnson 2004), ample time for a matured population to develop. However, high colony turnover rate at Pine Valley may reduce the average colony age in this site and partially explain their low reproductive participation.

There is also evidence of ecological constraint within the alates themselves. Queens from Pine Valley were significantly lighter than queens from Lake Henshaw, but there is no significant difference between queen mass when the analysis performed by colony social structure. Queen mass is determined by resource provisioning by workers during larval development (Ode and Rissing 2002), so the reduced queen mass from Pine

Valley colonies suggests that they are resource limited. The lower average male mass from Pine Valley colonies, while not significant, also suggests resource limitation in that area. Although, smaller queens have been observed in other polygynous systems where resource limitation does not appear to be a factor (McInnes and Tschinkel 1995; Rüppell et al. 2001). Lower queen mass does not necessarily decrease the odds of success in the context of polygyny because queens that start colonies cooperatively or join established colonies are able to share the metabolic demands of colony founding, which reduces the amount of nutritional reserves a queen needs before leaving her home nest (Keller and Ross 1993; Rueppell and Heinze 1999). However, the larger queens produced by polygynous colonies in Lake Henshaw suggests that a polygynous colony will invest in larger queens if the resources are available.

Multiple lab studies have shown survival benefits for polygynous queens and colonies during colony founding (Mintzer 1987; Clark and Fewell 2014), as well as indicators of competitive advantage during early colony growth, including more efficient worker production (Clark and Fewell 2014), and for some species faster worker production (Deslippe and Savolainen 1995; Trunzer et al 1998). Several studies also suggest that cooperation is more likely to evolve in an adverse environment where independent colony founding success is particularly difficult (Mesterson-Gibbons and Dugatkin 1992; Dugatkin 2002; Krams et al. 2010; Riehl 2013). Like many ground-nesting ants in arid environments, harvester ant colonies are aggressively territorial (Hölldobler 1976). Young colonies face competition, predation, and brood raiding from neighboring colonies, and from other ant species; the intensity of these factors is largely

dependent on the density of colonies in the area (Adams and Tschinkel 1995a; Adams & Tschinkel 1995b).

The rarity of primary polygyny suggests that advantages during colony founding do not offset the costs of later reproduction under most environmental conditions. It is notable that these colonies are polygynous at maturity, instead of practicing pleometrosis only, in which queens cooperate until workers eclose, at which point cooperation breaks down and queens engage in battles until there is one left to inherit the colony (Bernasconi and Strassmann 1999). However, under continuous conditions of strong competition for resources and/or territory, the survival advantages of polygyny may extend to colony maturity and favor the continuation of queen cooperation. Scant ecological research has been done in the handful of other species in which primary polygyny is known, but there is anecdotal evidence that polygyny in other species is also found in relatively dense populations (Rissing et al. 1986; Bennett 1987; Trunzer et al. 1998; but see Cahan 2001).

Primary polygyny may additionally reflect a general shift in life history strategy towards more conservative reproduction in order to better survive a difficult or unpredictable environment. This strategy would damage queen fecundity in the short term, but could prove beneficial across colony life history, if it extends the colony lifespan to the point that additional mating flights recoup the short term cost (Schaffer 1974). Similar trade-offs between reproduction and survival have been observed in several species (Snell and King 1977; Partridge and Farquhar 1981; Clutton-Brock et al. 1982; Nur 1984), and the effect can be driven by environmental conditions (Callow 1973; Giesel 1976). Polygynous colonies had significantly higher worker activity counts than

single queen colonies regardless of site, indicating a larger or more active workforce which requires a resource investment at the potential cost of reproductive output. The worker activity assays I performed measured the vigorousness of foragers, the size of the foraging workforce, or a combination of these two traits. Either way the assay provides a measure of energy expended on foraging by each colony. The positive linear relationship between worker activity assays and reproductive investment further suggests that this measure reflects colony size and reproductive potential.

It is possible that Pine Valley is resource limited to the point that colonies located there have designated a higher proportion of their workforce to foraging, however if this were true we would expect monogynous colonies in this area to also display high foraging activity, which was not the case. The significantly higher activity counts by polygynous colonies across sites was driven by monogynous colonies in Pine Valley having relatively low worker activity and polygynous colonies in Lake Henshaw having relatively high worker activity. This suggests that polygynous colonies may preferentially invest resources into foraging efforts, rather than diverting them to reproductive investment in a given year.

My conclusions on the potential conservatism of polygynous colonies are limited because forager dynamics are notoriously complex and variable (Tschinkel 1999; Gordon et al. 2011), so I took several precautions to improve the reliability of the worker activity assay. The proportion of the total workforce that leave the nest for foraging or other outside activity is influenced by several factors including season (Kwapich and Tschinkel 2013) and reproductive investment (Smith and Tschinkel 2006). Temperate ant species

tend to reduce the proportion of foragers in their workforce in the winter and increase the proportion of foragers in the summer (Kwapich and Tschinkel 2013), which can make forager counts unreliable as a proxy for colony size, especially if counts are done at different times of the year. All of the activity counts were completed within a two week window in late summer which should minimize seasonal differences in the proportion of foragers between colonies. Reproductive investment can also impact the size of the workforce because colony resources are delegated to the development of alate brood instead of worker brood in preparation for a reproductive flight (Ode and Rissing 2002). Activity counts were only performed on colonies that participated in a mating flight that season, also reducing the difference between workforce characteristics of analyzed colonies.

Primary polygyny in social insects is a poorly understood social structure with the potential to become a key model for the study of cooperation between non-kin, because queens make the decision at colony founding to share effort and consequent resources with unrelated queens and the impact of this decision varies across the life history of the colony they form together. The data presented here offer a rare empirical look into the potential consequences of polygyny on queen reproduction, and thus fitness. The differences in probability of reproducing, reproductive investment, and alate size are all consistent with the suggestion that polygyny appears as a solution to environmental conditions that severely constrain colony reproduction. The harsh environment was further demonstrated when a resource supplementation regime equalized the reproductive investment levels of colonies in the polygynous population with those of colonies in the

monogynous population. Colony density appears to play roles in the evolution of cooperation in this system, potentially by increasing the frequency of intraspecific conflict and selects for multiple queen colonies in order to augment worker production; although resource availability and other ecological pressures likely also drive selection for primary polygyny in this and other systems. Polygynous ant colonies are simultaneously exemplars of simple non-kin cooperation, while also being long-lived individuals with complex life history strategies. As indicated by this study, dissecting the relationships between cooperation, survival, and reproduction requires the dissection of the balancing fitness costs across the entire life history of the colony.

Figure 3.1: Average of daily alate investment (in milligrams) from all trapped colonies across the mating season, from mid-June through early July in 2013 & 2014. Black solid line shows daily average investment of colonies in Pine Valley (PV), the mostly polygynous site; Grey broken line shows daily average investment of colonies in Lake Henshaw (LH), the mostly monogynous site. In 2013 data were collected from 11 colonies in PV and 13 colonies in LH and in 2014 data were collected from 13 colonies in PV and 14 colonies in LH.

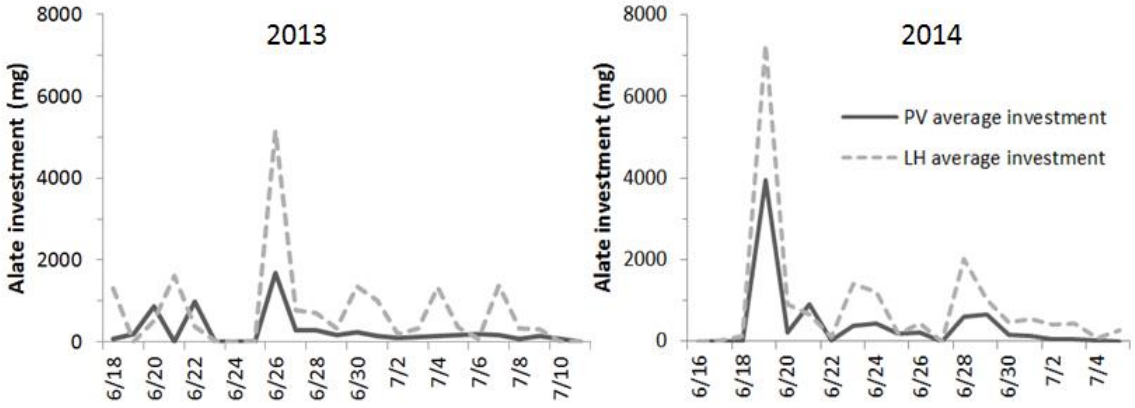


Figure 3.2: Participation in reproductive flight by site, presented as the proportion of total surveyed colonies that produced 62eproductive. In 2012 and 2013, reproductive participation was assessed only for focal colonies; in 2014, all colonies present at each site were assessed for reproductive activity, determined by whether a colony had alates present at the nest surface at any time during the mating season. A log linear model shows that the proportion of reproductive colonies is significantly different between sites ($X^2$=5.314, df=1, p=0.0212) and there was no effect of year ($X^2$=0.463, df=2, p=0.793).

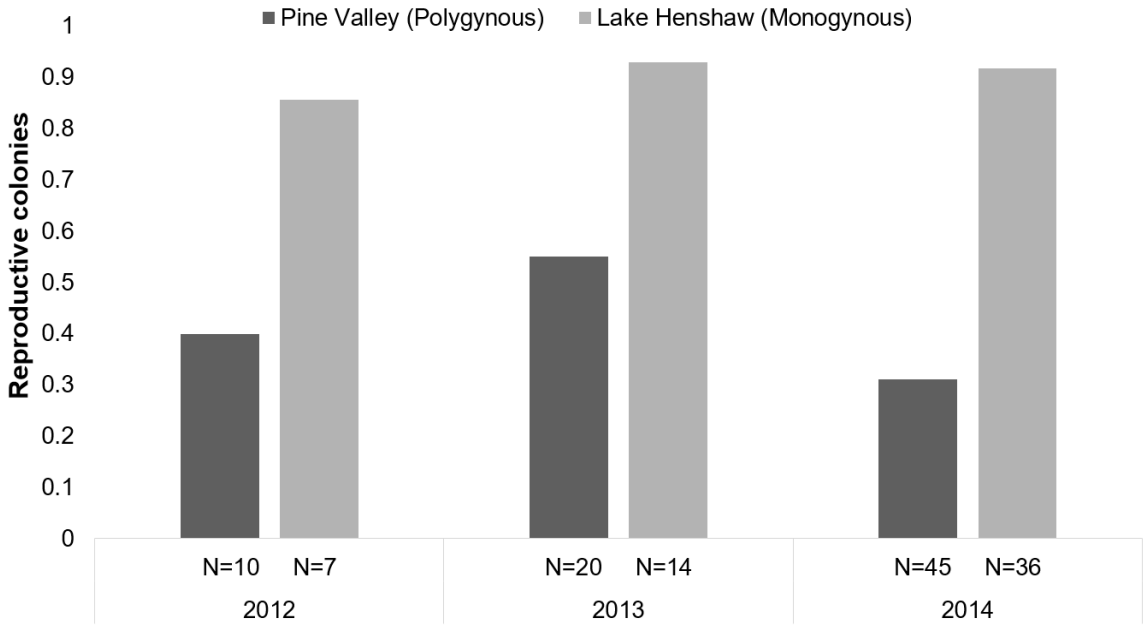


Figure 3.3: Reproductive output in number of alates captured from colonies in 2012, 2013, and 2014 mating flights, with replicated colonies averaged. A total of 18 PV and 17 LH colonies were trapped at least one year and included in the analysis. Three of the LH colonies had multiple queens and 4 of the PV colonies contained a single queen making 17 MQ colonies and 18 SQ colonies. The number of queens released is represented by the black bars, and the number of males by the grey bars. Data compare differences in output between sites (PV vs LH), and between social strategies (MQ vs SQ). Colonies in the Lake Henshaw area released significantly more alates than colonies in the Pine Valley area (A: $F_{site}$=5.77, df=1, p=0.022); however, there was no difference in alate output by colony social strategy (B: $F_{strat}$ =0.485, df=1, p=0.49). There was no significant difference between the sex ratio output by area ($F_{site}$=0.014, df=1, p=0.91) or by social strategy ($F_{strat}$=3.16, df=1, p=0.089).

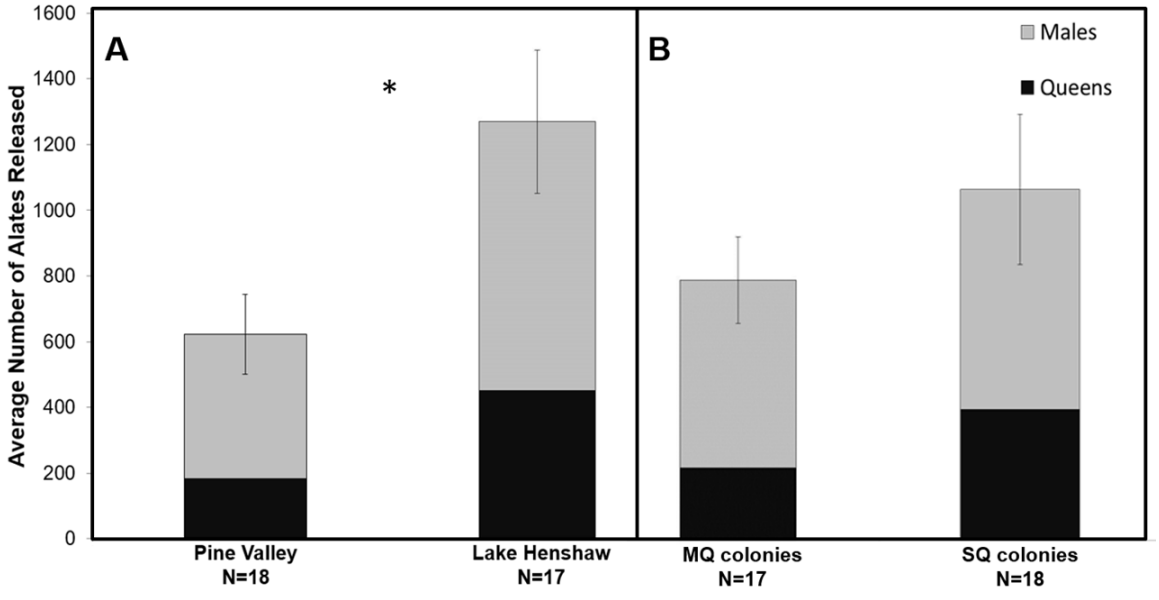


Figure 3.4: Reproductive investment in total wet weight (mg) of alates released in the 2013 & 2014 mating flights, with replicated colonies averaged. A total of 18 PV and 16 LH colonies were trapped at least one year and included in the analysis. Three of the LH colonies had multiple queens and 4 of the PV colonies contained a single queen. Lake Henshaw colonies had significantly more alate investment than Pine Valley colonies (A: $F_{site}$=4.59, df=1, P=0.041); there was no difference in alate investment by colony structure (B: $F_{strat}$=0.0001, df=1, p=0.99).

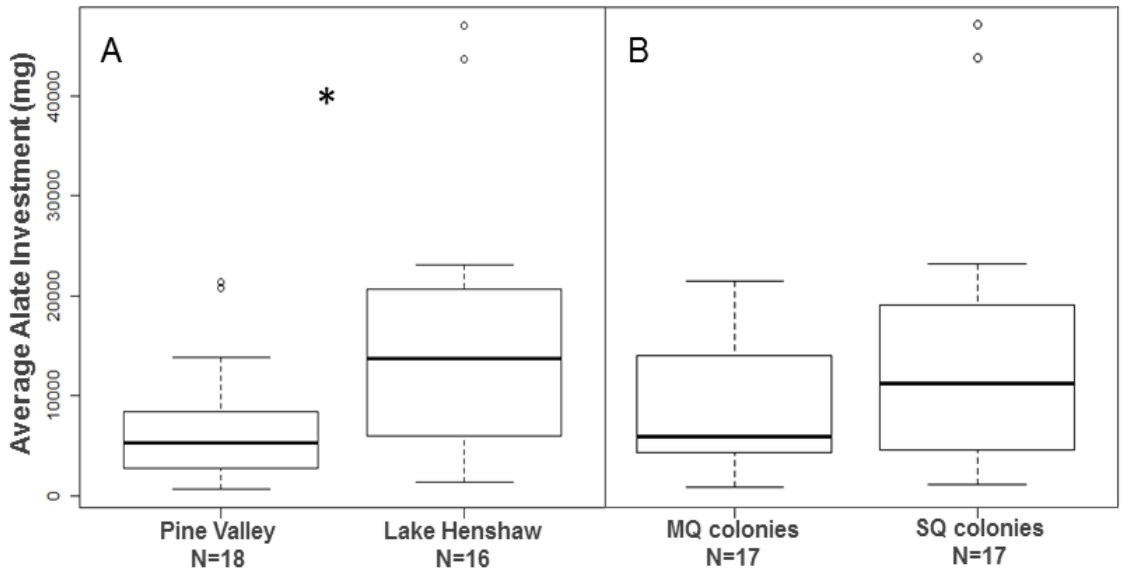


Figure 3.5: Proportional reproductive investment in males from 2013 & 2014 mating flights. Sex ratio investment was calculated using average male and queen mass per colony per day. PV and LH colonies had a similar sex ratio (A: PV male bias 65.4+/-5.1%, LH male bias 52.1+/-6.35%; $F_{site}$=0.001, df=1, p=0.987); as did MQ and SQ colonies (B: MQ male bias 67.9+/-3.9%, SQ male bias 49.8+/-6.1%; $F_{strat}$=3.453, df=1, p=0.073).

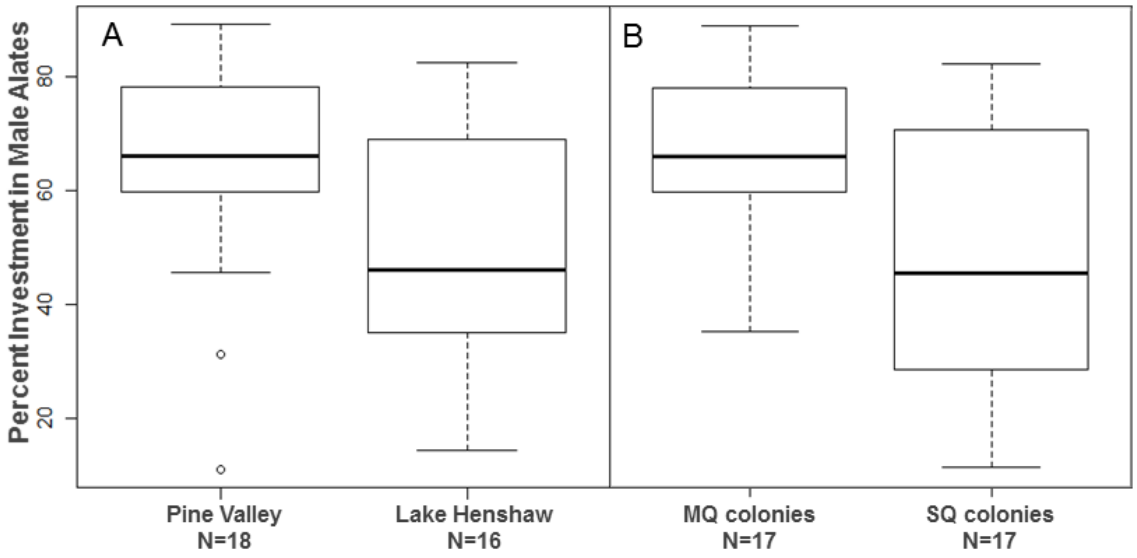


Figure 3.6: Average queen and male alate mass from the 2013 and 2014 mating flights, with data from replicated colonies combined. Plot 1 shows that queens from Lake Henshaw colonies were significantly heavier than queens from Pine Valley colonies (A: $PV_{queenmass}$=14.72+/-0.38mg, $LH_{queenmass}$=16.86+/-0.20mg; $F_{site}$=8.23, df=1, p=0.0076) but there was no difference in queen mass between MQ and SQ colonies (B: $MQ_{queenmass}$=14.78+/-0.44mg, $SQ_{queenmass}$=16.55+/-0.24mg; $F_{qnum}$=1.433, df=1, p=0.241). Plot 2 shows that males were not significantly different in mass by either location (A: $PV_{malemass}$=9.40+/-0.32mg; $LH_{malemass}$=10.38+/-0.44mg; $F_{site}$=0.839, df=1, p=0.0748), or by social structure (B: $MQ_{malemass}$=9.35+/-0.33mg, $SQ_{malemass}$=10.30+/-0.41mg; $F_{qnum}$ =0.621, df=1, p=0.437).

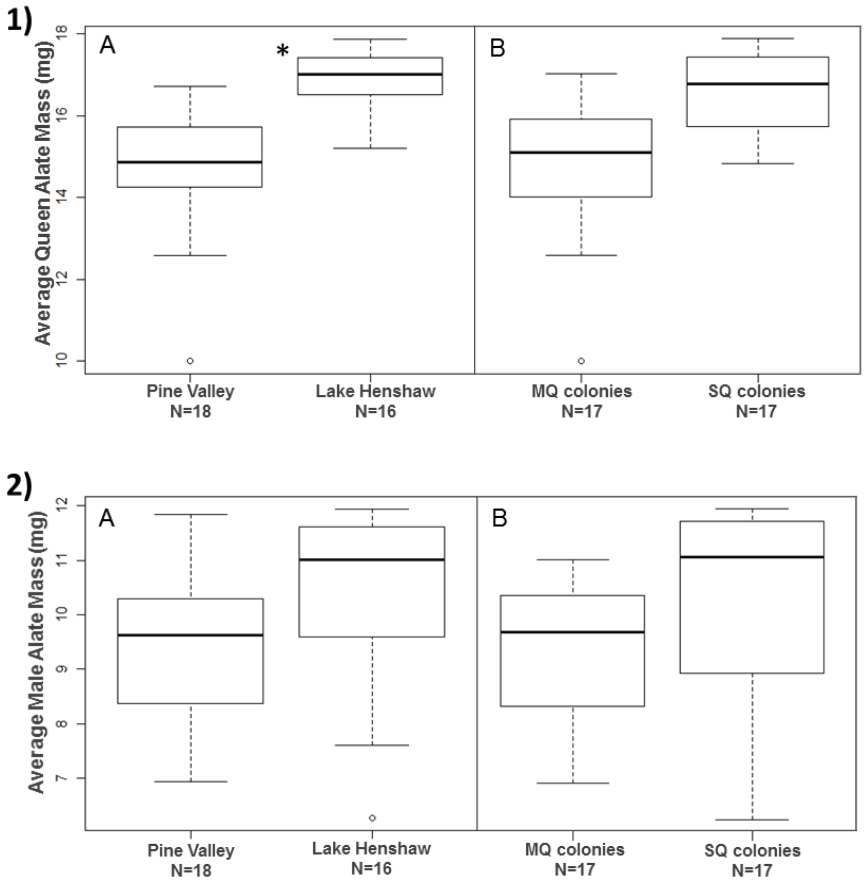


Figure 3.7: Colony size estimates by worker activity counts from all reproductive colonies in 2013 and 2014, with replicated colonies averaged. There was no significant difference in worker activity between Pine Valley and Lake Henshaw colonies (A: $PV_{activity}$=598.2+/-101.1, $LH_{activity}$=674.3+/-146.8; $F_{site}$=3.44, df=1, p=0.073), but MQ colonies had significantly higher worker activity counts than SQ colonies (B: $MQ_{activity}$=819.0+/-156.6, $SQ_{activity}$=457.5+/-52.2; $F_{qnum}$=7.613, df=1, p=0.0098).

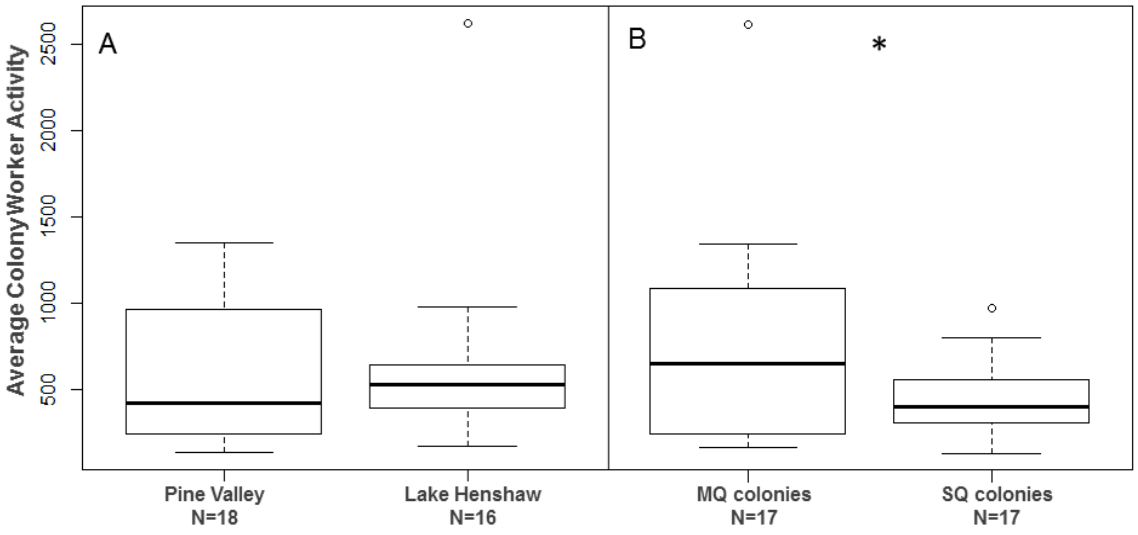


Figure 3.8: Reproductive survey of all *P.calfornicus* colonies at Pine Valley and Lake Henshaw sites in 2014. Pine Valley contained 44 colonies over .037km$^2$ and Lake Henshaw contained 34 colonies over .041km$^2$. Circles represent colonies where alates were seen at the colony entrance during the mating season, indicating that these colonies reproduced. Triangles represent colonies where no alates were seen for the extent of the reproductive season, suggesting they did not reproduce that year. Pine Valley colonies are significantly clustered (MAD=4737.4, sim=100, p=0.01). Lake Henshaw colonies are not significantly different from a random distribution (MAD=1443.2, sim=100, p=0.23).

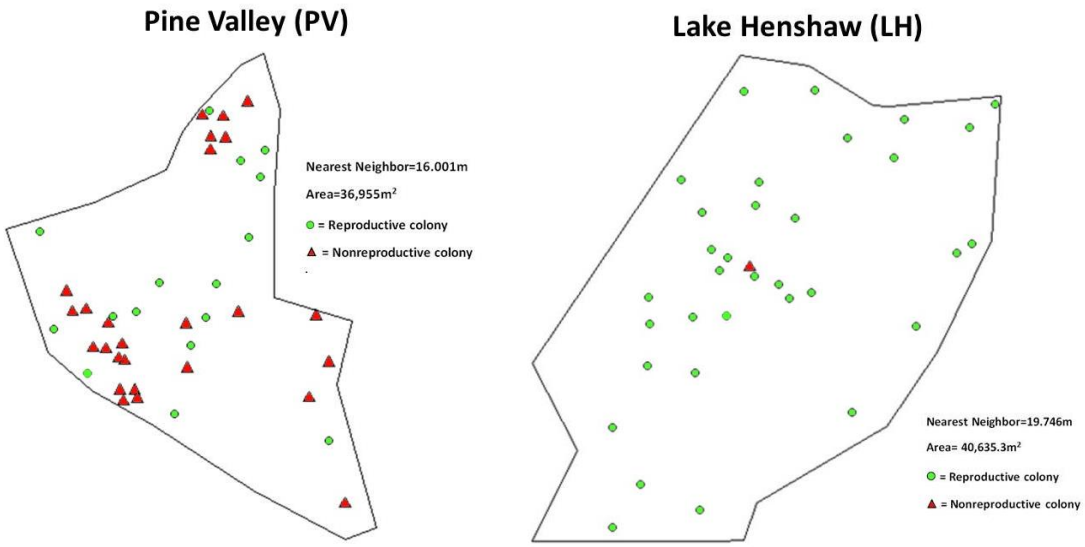


Figure 3.9: Ripley's K envelopes for Pine Valley and Lake Henshaw. The dotted line represents complete spatial randomness of colony distribution and they grey area, contained in $K_{hi}(r)$ and $K_{lo}(r)$, is the confidence envelope. The solid line represents the observed spatial pattern of colonies at each site. The solid line above the confidence envelope in Pine Valley indicates significant clustering at this site while the solid line within the confidence envelope at Lake Henshaw indicates that these colonies are randomly distributed.

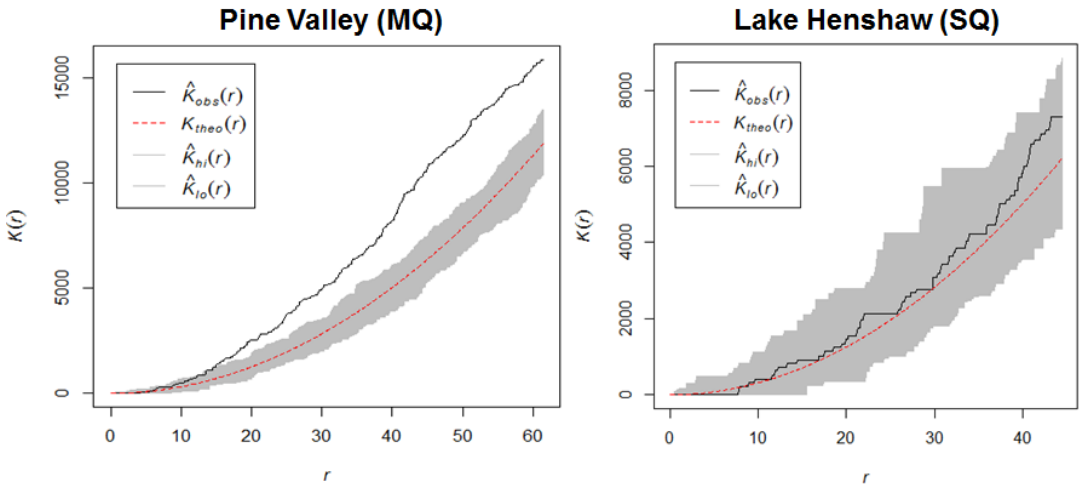


Figure 3.10: The average alate investment of Lake Henshaw colonies, unsupplemented Pine Valley colonies, and resource supplemented Pine Valley colonies. Supplemented Pine Valley colonies had significantly more reproductive investment than non-supplemented Pine Valley colonies from 2013, 2014, and 2016 ($X_{Pvsupplement}$=16543.9+/-3270mg, $X_{Pvnosupplement}$=6895.3+/-1334.7mg; t=2.732, df=7, Benjamini-Hochberg adjusted p=0.045). There was no difference in the reproductive investment between Lake Henshaw colonies and supplemented Pine Valley colonies ($X_{LH}$=16514.8+/-3290.4mg; t=0.006, df=15, Benjamini-Hochberg adjusted p=1).

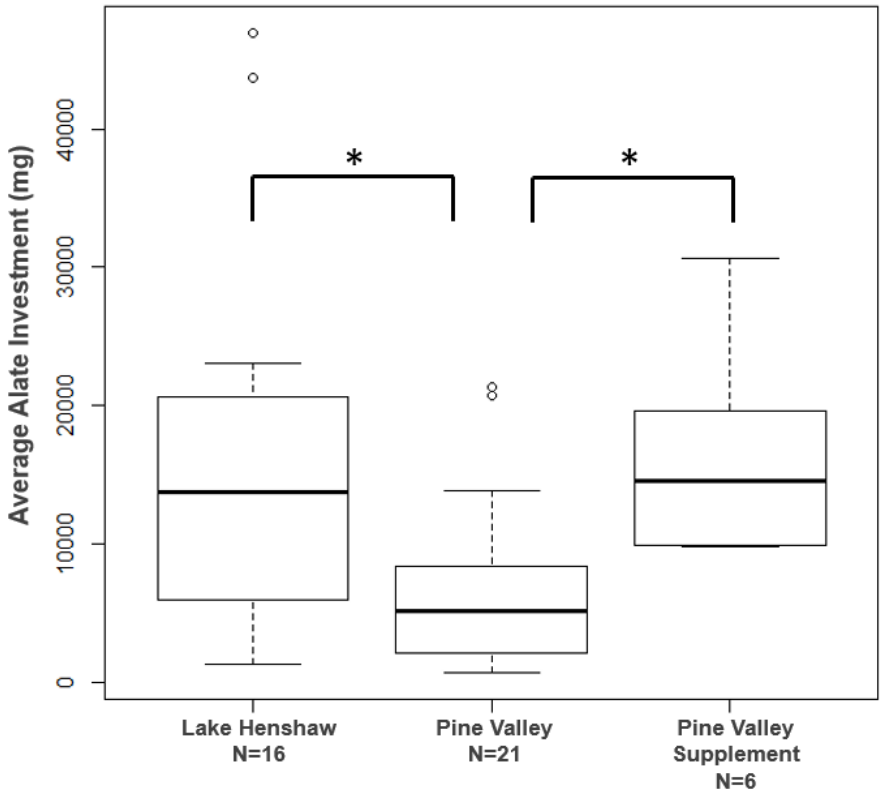


Table 3.1: Mean alate output from 2012, 2013, and 2014 in number of alates released, mean reproductive investment, sex ratios, alate mass from 2013 and 2014 calculated using daily average alate mass, as well as worker activity and above ground colony area from 2013 and 2014 surveys. Colonies replicated over years were averaged. Bold p values indicate significance.

| | *Pine Valley* | *Lake Henshaw* | *P* | *MQ colonies* | *SQ colonies* | *P* |
|---|---|---|---|---|---|---|
| *Number alates released* | 638.6+/-119.9 alates n=18 | 1234.2+/-207.3 alates n=17 | **0.022** | 812.4+/-126.7 alates n=17 | 1037.0+/-216.8 alates n=18 | 0.491 |
| *Reproductive investment* | 7122.1+/-1465.1mg n=18 | 16514.8+/-3290.4mg n=16 | **0.041** | 8769.5+/-1715.7mg n=17 | 14314.8+/-3287.9mg n=17 | 0.994 |
| *Male investment sex ratio* | 64.0+/-4.7% n=18 | 52.0+/-6.3% n=16 | 0.987 | 67.9+/-3.9% n=17 | 49.7+/-6.1% n=17 | 0.073 |
| *Queen investment sex ratio* | 36.1+/-4.7% n=18 | 47.9+/-6.3% n=16 | 0.987 | 32.1+/-3.9% n=17 | 50.2+/-6.1% n=17 | 0.073 |
| *Male output sex ratio* | 71.6+/-4.5% n=18 | 61.6+/-6.2% n=16 | 0.907 | 75.3+/-3.9% n=17 | 59.2+/-5.9% n=17 | 0.089 |
| *Queen output sex ratio* | 28.4+/-4.5% n=18 | 38.4+/-6.2% n=16 | 0.907 | 24.6+/3.8% n=17 | 40.8+/-5.9% n=17 | 0.089 |
| *Queen mass* | 14.72+/-0.38mg n=18 | 16.86+/-0.20mg n=16 | **0.0076** | 14.78+/-0.44mg n=17 | 16.55+/-0.41mg n=17 | 0.241 |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Male mass* | 9.40+/-0.32mg n=18 | 10.38+/-0.44mg n=16 | 0.367 | 9.35+/-0.33mg n=17 | 10.30+/-0.41mg n=17 | 0.437 |
| *Worker activity* | 598.2+/-101.1 n=18 | 674.3+/-146.8 n=15 | 0.073 | 819.0+/-156.6 n=16 | 457.5+/-52.2 n=17 | **0.0098** |
| *Above ground colony area* | 9228.9+/-2156.5$cm^2$ n=14 | 12861.8+/-3425.4$cm^2$ n=14 | 0.506 | 9847.3+/-2054.6$cm^2$ n=14 | 12243.4+/-3528.4$cm^2$ n=14 | 0.945 |

Table 3.2**:** A) Average daily temperature by site at 8:00AM and 12:00PM during the reproductive flights of 2013 and 2014. B) Dates that alate traps were deployed in 2011-2014 and total annual rainfall at each site.

| *A* | *Pine Valley* | *Lake Henshaw* | |
|---|---|---|---|
| *2013 8:00AM* | 21.15+/-0.47°C | 20.83+/-0.77°C | |
| *2013 12:00PM* | 30.79+/-0.80°C | 32.39+/-1.1°C | |
| *2014 8:00AM* | 20.28+/-0.70°C | 18.89+/-0.66°C | |
| *2014 12:00PM* | 29.0+/-0.76°C | 30.44+/-0.75°C | |
| *B* | Dates of alate trapping | Pine Valley rainfall | Lake Henshaw rainfall |
| *2011-2012* | 6/29 to 7/16 | 16.53 inches | 19.96 inches |
| *2012-2013* | 6/11 to 7/11 | 10.74 inches | 12.77 inches |
| *2013-2014* | 6/16 to 7/4 | 14.00 inches | 12.74 inches |

# CHAPTER 4

# REPRODUCTIVE STRATEGIES OF POLYGYNOUS AND MONOGYNOUS COLONIES IN A SHARED HABITAT

## Introduction

The evolution of intraspecific cooperation is often discussed in the context of kinship, because the indirect benefits generated by relatedness can offset the costs of altruism (Hamilton 1964; Frank 2013). Most social groups have high relatedness, fitting the theoretical expectations of kin-selection, but many cooperative systems have lower relatedness than expected or are composed of completely unrelated individuals (Hölldobler and Carlin 1985; Gadau et al. 1998; Hacker et al. 2005; Cahan and Helms 2012; Clutton-Brock et al. 2000; Rutte and Taborsky 2008; Hölldobler et al. 2011). Individuals gain no indirect fitness benefits when cooperating with non-kin, but they suffer direct fitness costs by contributing to group functions (Mesterton-Gibbons and Dugatkin 1992; Dugatkin 2002; Avilés 2002; West et al. 2007; Clutton-Brock 2009). Thus, in order for cooperation to evolve and persist between non-kin, there must be direct individual and/or shared group benefits generated via cooperation. Forces that potentially generate these benefits, such as reciprocity, mutualism, and group selection, have received considerable attention in theoretical models (Lehmann and Keller 2006; Nowak 2006; Okasha 2006; Connor 2010; Marshall 2011; Queller 2011; Van Cleve and Akcay 2014; Okasha 2016). Direct evaluation of these effects has proven difficult because most species where non-kin cooperation has been explored are long lived and reproduce

slowly, making the quantification of fitness a challenge (Seyfarth and Cheney 1988; Moller et al. 2001; Silk 2005).

An assessment of the relative fitness effects of non-kin cooperation requires accurate measurement of the survival and reproductive outcomes for individuals that cooperate, as compared to individuals outside of that social context. Primary polygyny by ant queens provides a unique opportunity to test the direct fitness outcomes of cooperation within a species and in a common environment, because both cooperative and solitary strategies are often present. In primary polygyny, several unrelated queens cohabitate and cooperate for the lifespan of their colony. Queens randomly associate into small groups after their mating flight and work together to build a nest and raise worker brood, forming what is essentially a multi-family eusocial group (Mintzer 1987; Trunzer et al. 1998; Heinz et al. 2001; Helms and Helms Cahan 2012; Helanterä et al. 2013).

The reproductive consequences of social strategies, such as polygyny, are heavily influenced by ecological factors, including weather, levels of inter-and intraspecific competition, and resource availability (Gordon and Wagner 1997; Gadau et al. 1998; Ode and Rissing 2002). Thus, comparisons of the fitness outcomes of single queen nesting versus multi-queen cooperation should be performed in a shared environment to control for ecological variation. Conditions allowing direct comparisons of reproductive strategy in a single species are generally rare, but primarily polygyny allows an almost unique opportunity to do so. Several ant species practice primary polygyny in discrete regions of their species range, but no species has been found that exclusively performs this behavior (Helms and Helms Cahan 2012; Overson et al. 2016). There are always areas of the

species range that are dominated by solitary queens, which is likely the ancestral state (Overson 2016). There can also exist transitional zones between multi-queen and single-queen populations where both colony types share a common environment.

I present one of few known cases in which both social and solitary strategies exist within a common natural environment, allowing direct empirical tests of the consequences at reproduction of choosing a social over a solitary strategy. The California harvester ant, *Pogonomyrmex californicus*, occupies contiguous but patchy populations in Southern California that vary in the proportion of colonies that practice primary polygyny (Overson et al. 2016, Chapter 3). Previous tests of genetic diversity in mature polygynous *P. californicus* field colonies show that nest-mate queens are unrelated (Overson et al. 2016), consistent with findings for other taxa that display primary polygyny (Heinz et al. 2001; DeHeer and Herbers 2004; Cahan and Helms 2012). In addition, the non-aggressive cooperation of polygynous ant queens has been observed to persist for years in lab colonies (Mintzer 1987; Kolmer and Heinze 2000; Holbrook et al. 2011; Overson et al. 2014).

I have discovered a previously unstudied site that contains a fairly even mix of single and multi-queen *P. californicus* colonies. Quantification of the reproductive characteristics of colonies in this shared environment could elucidate the costs and benefits of primary polygyny in natural field conditions, a crucial step to determine the mechanisms by which this example of non-kin cooperation has evolved. Recent work on two populations of *P. californicus*, one in an area dominated by primary polygyny and one in an area dominated by solitary queens, found that resource limited, competitive

conditions at the polygynous site constrain reproductive potential and likely drive queen cooperation (Chapter 3). That study was performed on two separate field sites (approximately 40 miles apart), and so the direct effects of primary polygyny on colony and queen fitness remain unclear.

It is important to note that the costs and benefits of cooperation shift over the lifespan of the colony, from the extremely tenuous colony founding stage (Wiernasz and Cole 2003), through early colony growth, and eventually to colony maturity and reproduction. Several studies have demonstrated benefits from queen cooperation at colony initiation and early growth, when the colony can gain a competitive advantage through faster or more efficient worker production (Deslippe and Savolainen 1995; Trunzer et al 1998; Clark and Fewell 2014). Although most research on primary polygyny has focused on early colony growth, worker production benefits may extend past early colony life. For example, higher genetic diversity in polygynous colonies may increase the workforce's task efficiency and pathogen resistance (Shykoff and Schmid-Hempel 1991; Cole and Wiernasz 1999; Oldroyd and Fewell 2007; Wiernasz et al. 2008). Queens of *P. californicus* mate multiply (Overson et al. 2016), as do many polygynous species (Kellner et al. 2007; Qian et al. 2012), suggesting that increased variation in the worker force is beneficial; however, the increased benefits of polygyny beyond those already given by polyandry are unlikely to provide a complete answer to the question of why queens remain polygynous past colony founding.

Despite the multiple studies suggesting a benefit early in the colony life history, primary polygyny is rare. Cooperative queens likely suffer intense individual costs at

colony maturity, when resources are shared by nest-mate queens for personal reproductive investment. These costs will only arise after the colony grows enough to become reproductively active, which often takes several years (Cole and Wiernasz 2000), and we lack empirical studies that directly assess how cooperative benefits balance with reproductive costs to generate a fitness outcome for polygynous queens. If the benefits of queen cooperation to colony efficiency and growth are ample, polygynous colonies may be able to dominate their territories to the extent that all queens in the colony can increase their reproductive investment to the point that the per-queen fitness is higher than that of a solitary queen. However, the benefits of polygyny may be skewed towards early colony life when the colony is vulnerable to attack and exploitation, a tactic that helps the colony get established but carries future costs at the reproductive stage. In this case, the per-queen reproductive potential would be lower for multi-queen colonies than single-queen colonies due to resource sharing between nest-mate queens. The outcome of competition between single and multi-queen phenotypes then depends on the strength of selection at the different time points of colony development. For example, if no single queen foundress in a monogynous colony will survive to reproduction, the potential delayed benefit of monogyny does not come into play and the population should become fixed for pleometrosis and polygyny.

An accurate measurement of cooperative and non-cooperative queen fitness in a common environment is crucial step towards understanding the evolutionary pathway of cooperation in this and other systems (Oli 2003; Van Horn et al. 2004; West et al. 2007). In this study, I examine how primary polygyny effects queen fecundity by capturing and

quantifying the reproductive output of multi and single-queen colonies over two annual mating flights. I use microsatellite markers to estimate queen number and assess the relationship between social structure and queen fitness. I test the hypothesis that in a common environment, primary polygyny may provide benefits that allow higher colony-level reproductive output than single-queen colonies, but may not reach levels that allow individual queen reproduction to be the primary driver of cooperation. To do so, I compare mature field polygynous and monogynous field colonies interspersed within the same field site, to control for ecological variables, including as colony density and resource availability, that have been found in my previous work to influence colony reproductive output. This study represents the first within-site comparison of the fitness outcomes for colonies that practice primary polygyny versus monogynous colonies.

## Methods

*Field site and mating flights*

To answer the question of how social strategy contributes to reproductive outcomes, I measured the reproductive output for polygynous and singe-queen colonies of *P. californicus* colonies at a site with a mix of both social strategies in Cuyamaca Rancho State Park, Southern California. The field site, off West Mesa Trail, San Diego Cty, CA (32°56’19.27”N, 116°33’46.00”W), lies between areas dominated by the two colony founding types, with populations containing primarily single queen colonies approximately 46km north of a site composed of primarily polygynous colonies (Overson

et al. 2016; Chapter 3). This makes the population extremely useful for comparative analyses.

Colonies of this species reproduce by releasing alates (winged and reproductively capable males and females) into mating leks. Alate release occurs daily across an approximately 3-4 week period from mid-June to mid-July. To determine colony social strategy and associated reproductive output, I first identified and GPS tagged all colonies in the site. After population surveys, I monitored colonies daily before the mating flights began to determine whether there were alates visible at the entrance, an indicator that the colony will reproduce. I then trapped, counted, and weighed male and female alates released from colonies for the extent of the mating flight to measure reproductive investment. I used microsatellite analyses to determine social strategy, and further to assess number of queens present in polygynous colonies (Chapter 3).

*Quantification of reproductive output and investment*

Data were collected over two annual mating seasons in the summers of 2015 and 2016. In both years, all colonies with alates coming to the surface in days preceding the mating flights were trapped to measure reproductive output. Traps were placed daily on colonies starting before mating flights began, and trapping continued until all flights were finished. Alate sampling occurred from June 20$^{th}$-July 9$^{th}$ in 2015, and June 14$^{th}$ to July 4$^{th}$ in 2016. I captured the reproductive output of a total of 21 colonies in 2015, and 13 colonies in 2016. If a colony reproduced in both years, data from that colony were

averaged across the two seasons for analyses to treat each colony as a single sampling unit.

Alates were captured by placing suspended mesh flight traps above colonies each morning (Chapter 3; design based on Cole and Wiernasz 2000). Each trap consisted of a metal cylinder surrounding the colony, with a tent-like structure above, leading into a collecting chamber. Alates flying from the colony land on the mesh and crawl up into the chamber. The traps allow exhaustive sampling of alates, without harming them. Traps were placed over all colony entrances of each colony every morning by 7AM, remained in place until 1PM, and were monitored hourly. Previous work has shown that *P. californicus* mating flights finish daily well before 1PM in this area (Chapter 3). I retained at least 10 males and 10 queens per colony per day for genetic sampling and weighing. Remaining alates were counted and released to continue flights. If the total number of trapped males or queens from a colony exceeded 100, I retained 10% of the total.

The total number of male and queen alates captured daily across the extent of the mating flight was summed and designated as each colony's reproductive output. To assess colony reproductive investment, retained alates were immediately frozen after capture, and their wet weights obtained within 24 hours. After weighing, all samples were stored in 100% ethanol at -20°C for microsatellite analysis. The daily average wet mass of males and queens were multiplied by the total number of males and queens, respectively, trapped that day (including released alates) to calculate daily colony reproductive investment. These values were summed across all days to determine total

colony reproductive investment. This methodology does not provide a direct assessment of energy investment, because wet weights also include water content. However, because these are desert species, for which water and energy content are both valuable, wet weights do represent an ecologically relevant assessment of colony investment into alates.

*Genetic determination of social strategy and queen number*

I determined the social strategy of each colony by analyzing four microsatellites (PB6, PB5, E10, E20) for 12-40 males from each reproductive colony (Chapter 5 for DNA extraction and PCR protocol). Maternal queen genotypes were reconstructed by entering the allele profiles of the males from each colony into the program COLONY v2.0 (Jones and Wang 2010), with allelic dropout and error rates set to 0.05. Male alates were used to identify queens, because they are products of unfertilized eggs, which reduces the maternal uncertainty caused from polyandry. This number of samples provides a conservative estimate of queen number, and were used in this analysis to calculate per-queen reproductive investment in polygynous colonies. A higher sample size, more microsatellites, confirmation of maternal genotypes in worker and queen alates, and a longitudinal study of queen number over years were used in a deeper genetic analysis of colony social structure that is described in Chapter 5.

*Colony Size and distribution*

Colony size cannot be directly measured non-destructively, but I made two indirect assessments of colony activity as a proxy for robustness, by measuring colony

above-ground area, and by performing counts of worker above-ground activity rates after the end of the reproductive season. To assess relative activity levels, I counted the number of workers entering and exiting each colony entrance for five minutes between 9:00AM and 11:00AM and/or between 4:00PM to 6:00PM; early trials showed that these represent peak activity times for this population. Activity measures were performed only when the temperature was between 24$^{o}$ and 29.5$^{o}$C, and colony order was randomized. Worker counts were performed at least three times for each colony, with the mean used for the final activity ranking. The above ground area that each colony had cleared of vegetation was also recorded as an additional estimate of colony size, to determine whether there was a correlation between above-ground nest area, activity rates, and reproductive output.

In 2015 I recorded colony locations within the site by walking 2m transects of the area and marking all visible *P. californicus* colonies with an Etrex 10 GPS. Colony mapping was performed before the onset of the reproductive season. Colonies at this time were at least one year old; by this age they have cleared vegetation from around the nest in a ring, making them distinctively visible. I used colony mapping data to calculate colony density and nearest neighbor distances, using the spatstat package (Baddeley and Turner 2005). I generated the associated graphs using ggplot2 package (Wickham 2009), and performed ANOVA statistics using the car package (Fox and Weisberg 2011) in R version 3.0.1 (R Core Team 2013). The mean and standard error are provided in the results when possible.

## Results

*Nearest neighbor and colony size*

Above ground colony area was significantly predicted by worker activity assays in a type-2 ANOVA model that also included nearest neighbor distance and social strategy (F=10.1, df=1, p=0.007; table 4.2). Colony area trended larger for multi-queen colonies (13707.3+/-2598.9cm$^2$) than for single-queen colonies (5342.64+/-1470.9cm$^2$), but there was no significant difference by social strategy in a model with colony activity level (F=0.001, df=1, p=0.977) or with colony activity removed (F=1.70, df=1, p=0.209). However, in a type-2 ANOVA model including worker activity, nearest neighbor distance, and social strategy, social strategy varied significantly with colony activity level. Polygynous colonies had approximately twice the activity levels (953.5+/-139.6 workers entering and exiting per 5 min) of single-queen colonies (471.8+/-53.0 workers er 5 min; F=4.96, df=1, p=0.040, table 4.2). The average nearest neighbor distance for all colonies in the site was 11.43+/-0.98 m, and did not differ between social strategies (Polygynous colonies: 11.64+/-1.36 m; monogynous colonies: 11.02+/-1.24 m). A type-2 ANOVA model that included social strategy, worker activity, and reproductive investment found that nearest neighbor distance did not vary with any of these factors (Appendix C).

*Colony reproduction and queen number*

In the mapping surveys, I identified 34 *P. californicus* colonies in 2015, and 32 in 2016. Across the site, five colonies died between 2015 and 2016, as indicated by colony

absence at the GPS coordinates, and 3 new colonies became established, giving a year mortality rate of 14.7% and a turnover rate of -0.063%. A total of 21 of the 34 colonies identified in 2015 (61.8%) reproduced. In 2016, 13 of the 32 identified colonies (40.6%) reproduced. All reproducing colonies in 2016 had also reproduced in 2015. Microsatellite analysis indicated that 14 of the 21 reproductive colonies in 2015 contained multiple queens, and 7 contained a single queen, giving a 66.7% occurrence of primary polygyny in reproducing colonies. Repeated microsatellite profiles of colonies reproducing in 2015 and 2016 indicated that no colonies changed their queen number between years. Similarly to 2015, microsatellite assays indicated that 8 of the reproductive colonies in 2016 were polygynous (61.5%), and the other 5 had a single queen. Polygynous colonies contained an average of 2.57+/-0.29 queens with a range of 2 to 6 queens.

Polygynous colonies released an average of 992.4+/-159.1 alates in a reproductive season, while single queen colonies released an average of 696.7+/-100.9. Reproductive investment, as assessed by mean wet weight of alates released per colony, was 12501.1+/-2022.3mg for polygynous and 9436.3+/-1279.7mg for single queen colonies. I used a multi-factorial type-2 ANOVA model to determine whether variation in per-colony reproductive investment or alate number was significantly influenced by colony activity, nearest neighbor distance, and/or social strategy. Colony activity level was the only significant predictor of alate investment ($F_{inv}$=30.6, df=1, $p<0.001$; Table 4.1A). Social strategy did not significantly predict alate investment in any version of the model. Similarly, in a multi-factorial type 2 ANOVA that included activity, nearest neighbor distance, and social strategy, alate number was predicted only by colony activity level

($F_{num}$=12.0, df=1, p=0.004; Table 4.1B). There was one significant interaction between all three variables in the model of alate number (F=5.9, df=1, p=0.03; Table 4.1B).

*Per-queen reproductive investment*

I divided the reproductive investment of the colonies by the number of queens present in each respective colony to approximate the annual reproductive gains of cooperative and solitary queens as a component of fitness. A one-way ANOVA was then performed to compare the per-queen reproductive investment in polygynous colonies versus monogynous colonies. Cooperative queens had a mean alate investment of 5375.4+/-1060.8 mg per season, significantly less than the average 9436.3+/-1279.7mg investment of a solitary queen (F=5.345, df=1, p=0.0322, Fig 4.1). The raw number of alates released per-queen also trended lower for polygynous queens (426.1+/-83.3) than for monogynous queens (609.9+/-123.2), but this difference was not significant (F=3.841, df=1, p=0.0649).

*Alate mass and sex ratio*

I examined whether a colony's area, worker activity, social structure, or reproductive investment predicted the average male or queen alate mass. When analyzed in a multi-factorial type-2 ANOVA model, neither average queen nor male mass were significantly predicted by these factors (Appendix C). As with reproductive investment and total alate number, individual male and female alate masses did not vary between social strategies (Polgynous colonies: males, 10.83+/-0.18mg, females 15.40+/-0.20mg; Monogynous colonies: males, 10.62+/-0.36mg; females 15.34+/-0.40mg).

I also used a multi-factorial type-2 ANOVA to test whether sex ratio, or the proportion of males relative to total alates, varied significantly by social structure and colony activity. significantly influence colony sex ratio, the proportion of males or queens that a colony released during their mating flight. The sex ratio of each colony was arcsin transformed before all analyses, to correct for potential skew effects of the proportional data. Alate sex ratios did not vary significantly in a model that included social structure and activity (Appendix C). Multi-queen colony reproductive output was on average 50.5+/-4.2% male, similar to single-queen colony reproductive output which was on average 50.2+/-8% male. The sex ratio of reproductive investment similarly was not significantly influenced by social structure or activity (Appendix C). Investment sex ratio was slightly less male biased than reproductive output because the lighter weight of males was taken into account; multi-queen colonies invested 42.5+/-3.8% of their mating flight into males and single-queen colonies invested 43.6+/-7.7% of their mating flight into males.

*Regressions of worker activity and alate investment*

I explored the relationship between worker activity and alate investment by performing linear regressions of these two factors. When all colonies in this study were analyzed, I found a significant positive relationship between worker activity and reproductive investment ($F=35.7$, $n=21$, $r^2=0.6527$, $p=9.47E-6$; Figure 4.2). When colonies were separated by social structure, single-queen colonies alone did not show a significant relationship between activity and investment ($F=0.0186$, $n=7$, $r^2=0.0037$, $p=0.8969$; Figure 4.2), however multi-queen colonies alone still have a significant

positive relationship between worker activity and reproductive investment ($F=35.81$, $n=14$, $r^2=0.749$, $p=6.39E-5$; Figure 4.2).

## Discussion

Social groups must balance the survival, growth, and/or competitive benefits that may emerge from cooperation (Connor 1995; Möller et al. 2001; Kokko et al. 2001; Clutton-Brock 2002), with the potential costs that can arise through sharing group resources (Clutton-Brock and Parker 1995; Bshary and Grutter 2005; Raihani et al. 2012). Groups of non-kin are particularly susceptible to the costs of cooperation because there are no balancing indirect fitness benefits from kin. Previous work strongly suggests that the polygynous strategy of some *P. californicus* queens is genetically linked (Helmkampf et al. 2016; Overson et al. 2016), and may benefit queens in highly competitive or resource limited environments, where the early costs of being solitary likely outweigh the costs of sharing reproductive resources with other queens. Indeed, the mean reproductive investment of colonies in the mixed population (11479+/-1428mg) is in between the mean levels of the highly polygynous site (7122+/-1465mg) and highly monogynous site (16515+/-3290mg) discussed in Chapter 3, further supporting the link between ecological stress and frequency of primary polygyny. However, my analysis of reproductive investment for colonies in this shared field sited provide little evidence to support the hypothesis that the benefits generated by queen cooperation compensate for the reproductive costs of individual alate production in an environment where both single and multi-queen colonies survive to reproduction.

*The costs of polygyny in per-queen reproduction*

Polygynous and single queen colonies were similarly successful at producing reproductives, even across the two years of higher and then lower total output. However, when reproductive investment is divided by the mean number of queens present in a colony, solitary queens gain a significantly higher individual reproductive investment than do cooperative queens. In other data, I demonstrate that queens in polygynous colonies do, indeed, share reproductive investment (Chapter 5). Thus, the polygynous colony does not represent a case of extreme within-colony reproductive skew, as is seen in some polygynous wasps, in which a dominant queen gains all of the colony's resources (Queller et al. 1997; Seppä et al. 2002).

For a long-lived organism, such as an ant colony, however, fitness represents a complex interaction in which queen and colony survival probability, early growth rate and colony stability, as well as competitive ability and reproductive capacity all play roles. My results indicate that, at least in this environment, polygynous colonies may gain some advantage at the group level in colony size and/or robustness, as indicated by colony activity assays. The capacity to reach a larger colony size and other shifts in life history strategy could reflect the principal benefits to bringing colonies into maturity in this system.

*Polygyny effects on colony life history strategy*

This study found significantly higher worker activity levels in polygynous colonies, which may reflect a key benefit of queen cooperation under primary polygyny.

Larger or more active colonies are better able to dominate their competitive environment, which may provide the survival and resource benefits needed to maintain queen cooperation into colony maturity. Higher worker activity counts could be generated by two, non-exclusive emergent traits: 1) multiple queens have a higher egg laying rate than a single queen which allows the colony to reach and maintain a larger workforce and/or 2) multiple queens produce a workforce with higher genetic diversity which increases the range of conditions under which workers will forage (Wiernasz et al. 2008; Mattila and Seeley 2011). Either of these explanations could cause the higher worker activity levels I found in polygynous colonies, but more detailed dissection of colony growth and workforce dynamics are needed to determine the relative importance of these traits during the evolution of queen cooperation.

While it may seem logical that an ant colony will increase its reproductive investment as it becomes larger in size, this is not always the case. There is only one other *Pogonomyrmex* species in which the relationship between colony size and reproductive investment has been explored. Cole and Wiernasz (2000) found that there was no relationship between colony size and reproductive investment in *Pogonomyrmex occidentalis*, a species in which queens are always solitary. Similarly, the monogynous *P. californicus* colonies in this study show no relationship between colony size and reproductive investment. However, the strong positive correlation between size and reproduction in polygynous *P. californicus* colonies may reflect that a novel adaptation has coevolved with queen cooperation in this system (Figure 4.2).

Previous work has shown that primary polygyny evolves in areas of high environmental pressure that may make solitary colony founding and growth prohibitively difficult (Chapter 3). In addition to queen cooperation, a harsh evolutionary environment may also select for more conservative reproductive decisions in order to improve survival. The linear reproductive trajectory I observed in polygynous colonies could be generated if they have developed a stricter target for resource investment into reproduction, which is determined by colony size, and any additional resources are invested into the workforce. If polygynous colonies have similar reproductive targets, they would align to a similar reproductive trajectory and likely achieve a larger workforce as was also seen in this and other studies (Chapter 3). Alternatively, queen cohabitation may intrinsically produce a more predictable reproductive trajectory. If each queen has their own resource target for reproductive investment, the target of a group of queens may become more predictable as conciliation mediates the range of queen phenotypes and generates a common reproductive trajectory for polygynous colonies. A similar phenomenon is found in foraging ants where the nutrient target of a group of ants is more predictable than that of a single ant (Dussutour and Simpson 2008).

*Polygyny and colony sex ratio*

It is difficult to frame these results in a broader context, as this is the first detailed analysis of the reproductive characteristics for colonies that practice primary polygyny. However, the reproductive consequences of secondary polygyny, the re-acceptance of related daughter queens into the colony, provide some context for my findings. Several studies suggest that as the relatedness within a colony decreases as queens are added in

secondary polygyny, the colony tends to have a more male biased reproductive investment (Chan and Bourke 1994; Evans 1995; Chapuis and Keller 1999; Kümmerli et al. 2005). This may be caused by workers preferentially caring for male brood to create a higher male bias as colony relatedness decreases and workers become less related to female alates (Boomsma and Grafen 1990; Pamilo 1991).

Contrary to what has been observed under secondary polygyny, there were no significant changes in reproductive characteristics under primary polygyny. Quantification of the reproductive flights yielded no colony-level differences in output, investment, sex ratio, or alate mass between social structures. Although intracolony relatedness is reduced by primary polygyny, likely more so than under secondary polygyny, it does not appear that the workforce or queens alter their sex ratio. Shifts in reproductive characteristics that accompany other forms of cooperation are often generated by conflict within the group (Ratnieks and Reeve 1992; Keller and Reeve 1994; Herbers et al. 2001). The lack of significant reproductive alteration in the context of primary polygyny may indicate that little to no conflict exists between these cooperative queens or their workforce. Indeed, low levels of conflict are often a theoretical requirement for the stable and enduring non-kin cooperation observed under primary polygyny (Kokko et al. 2001; Clutton-Brock 2009; Frederickson et al. 2011).

*Conclusion*

This study represents an important first step in our understanding of the evolutionary mechanisms of primary polygyny. As with most early explorations into a

biological phenomenon, we have generated as many new questions as we have answered. This study suggests that a central benefit of primary polygyny is a more active workforce, which could be the result of a larger workforce capacity or a more genetically diverse workforce. However, this benefit does not bolster the annual per-queen reproductive investment of a polygynous colony to a higher level than that of a solitary queen. How queen cooperation is maintained in the face of this fitness cost remains an open question. Polygynous colonies may have a longer lifespan that makes up for the annual reproductive cost, or the survival benefits during early colony growth may be strong enough to cement queen cooperation for the colony's lifespan regardless of the future costs. The latter explanation may only be a viable strategy in certain environments where ecological conditions are especially harsh, which could explain why this behavior has never been found to spread throughout a species' range. Theoretical models of non-kin cooperation have struggled for decades with questions about evolutionary mechanisms, group member cheating, multi-level selection, and ecological drivers, among others. Primary polygyny represents a unique system where we can finally begin to answer the elusive questions of non-kin cooperation with empirical data from natural field conditions, which could finally put to rest some of the oldest and most fundamental problems of social evolution.

Figure 4.1: The per-queen reproductive investment of colonies that practice primary polygyny versus monogynous colonies. Solitary queens, who do not share their reproductive output, release significantly more alate biomass than cooperative queens in an annual mating flight. Significant difference ($P<0.05$) is noted by asterisk.

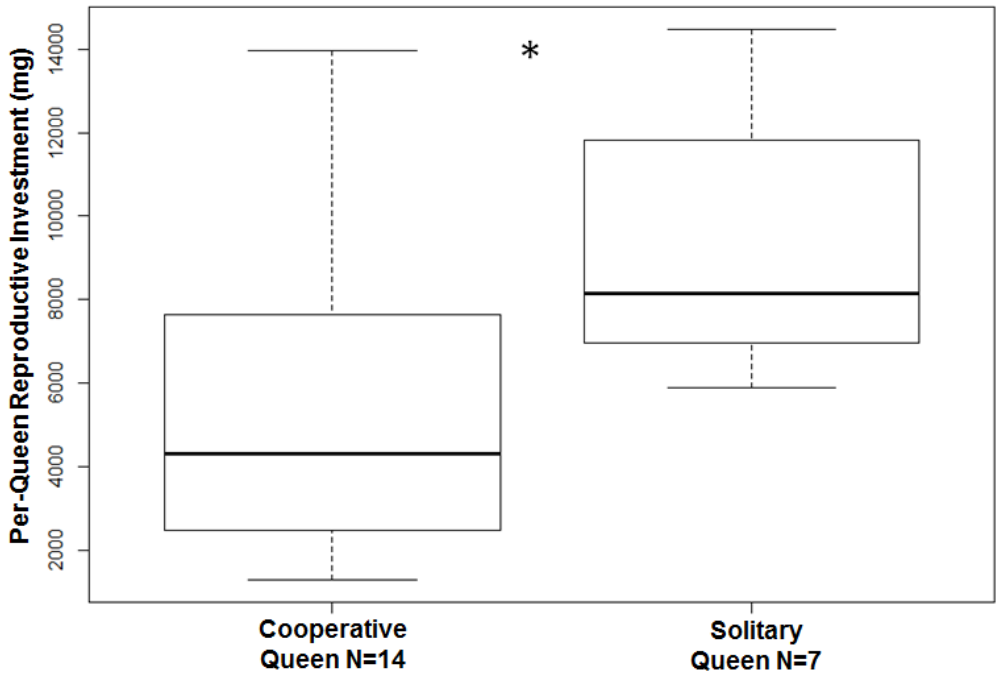


Figure 4.2: The relationship between worker activity, as a proxy of colony size, and reproductive investment. All colonies in the study show a significant regression of activity and investment (F=35.7, n=21, $r^2$=0.6527, p=9.47E-6), the regression line has an intercept at 2872.7, a slope of 10.855, and an $R^2$ of 0.6527. Single-queen colonies do not show a significant relationship between worker activity and investment (F=0.0186, n=7, $r^2$=0.0037, p=0.8969), the regression has an intercept of 8743.2, a slope of 1.4691, and an $R^2$ of 0.0037. The multi-queen colonies retain the significant regression (F=35.81, n=14, $r^2$=0.749, p=6.39E-5) with an intercept of 545.45, a slope of 12.539, and an $R^2$ of 0.749.

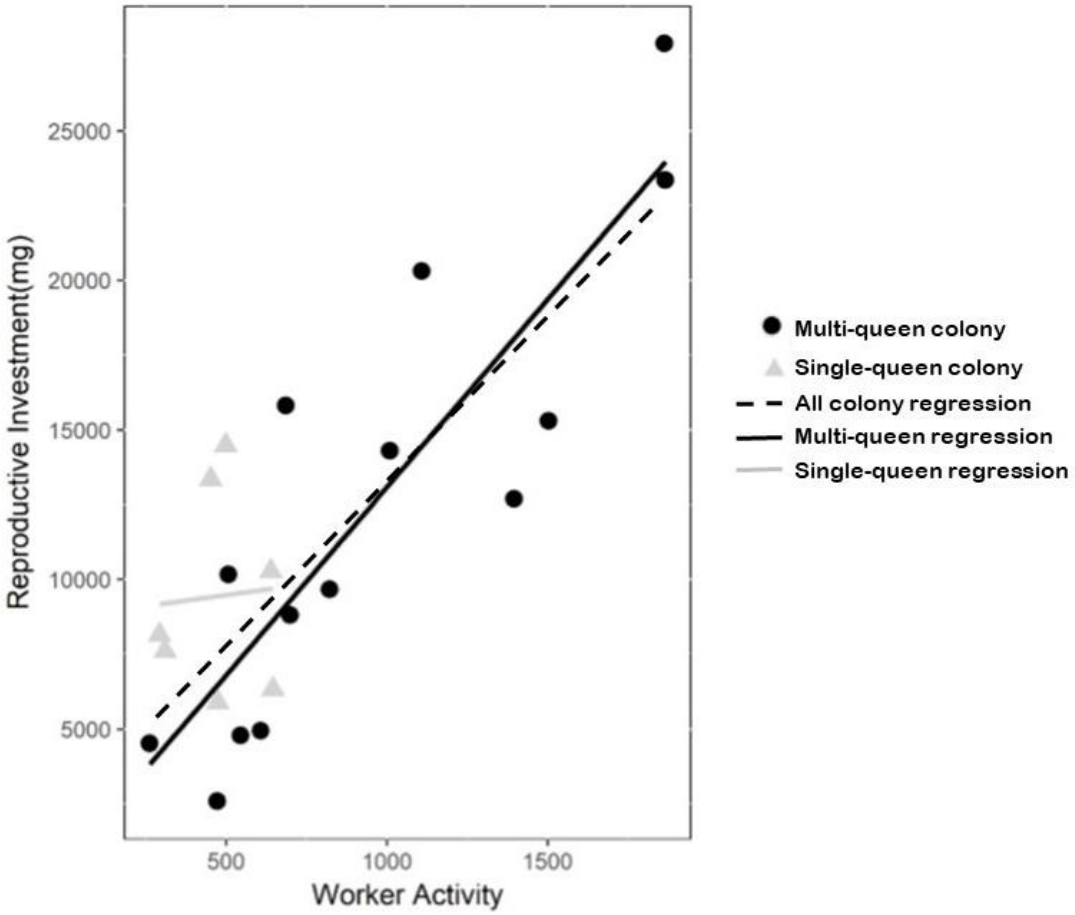


Table 4.1: Results of type II ANOVA models of worker activity levels, above ground colony area, colony social strategy, and all interactions for A: amount of alate investment in mg, and B: total number of alates released. Bold P values indicate significance ($P<0.05$).

| | *A) Alate investment, n=21* | | | | *B) Alate number, n=21* | | | |
|---|---|---|---|---|---|---|---|---|
| | Sum sq | df | F | P | Sum sq | df | F | P |
| *Worker Activity* | 537006251 | 1 | 30.635 | **0.00009** | 3008554 | 1 | 34.475 | **0.00005** |
| *Nearest Neighbor Dist.* | 13724546 | 1 | 0.7830 | 0.3923 | 156979 | 1 | 1.7988 | 0.203 |
| *Social Strategy* | 22563083 | 1 | 1.2872 | 0.2771 | 110975 | 1 | 1.2716 | 0.279 |
| *Activity:NNdist* | 12432766 | 1 | 0.7093 | 0.4149 | 157288 | 1 | 1.8023 | 0.202 |
| *Activity:Strategy* | 12590975 | 1 | 0.7183 | 0.4120 | 136060 | 1 | 1.5591 | 0.234 |
| *NNdist:Strategy* | 1275627 | 1 | 0.0728 | 0.7916 | 73402 | 1 | 0.8411 | 0.376 |
| *Activity:NNdist:Strategy* | 1176279 | 1 | 0.0671 | 0.7997 | 515182 | 1 | 5.9034 | **0.030** |

Table 4.2: Results of type II ANOVA models of A: colony above ground area by worker activity, nearest neighbor distance, and social strategy, and B: worker activity by nearest neighbor distance and social strategy. Bold P values indicate significance (P<0.05).

***A) Colony Area, n=21***

| | Sum sq | df | F | P |
|---|---|---|---|---|
| *Worker Activity* | 601310647 | 1 | 10.091 | **0.0073** |
| *Nearest Neighbor Dist.* | 1270446 | 1 | 0.0213 | 0.8861 |
| *Social strategy* | 50743 | 1 | 0.0009 | 0.9771 |
| *Activity:NNdist* | 22140742 | 1 | 0.3716 | 0.5527 |
| *Activity:strategy* | 2599478 | 1 | 0.0436 | 0.8378 |
| *NNdist:Strategy* | 4075696 | 1 | 0.0684 | 0.7978 |
| *Activity:NNdist:Strat* | 89483761 | 1 | 1.5017 | 0.2422 |
| | **B) Worker Activity, n=21** | | | |
| | Sum sq | df | F | P |
| *Nearest Neighbor Dist.* | 13387 | 1 | 0.0626 | 0.8055 |
| *Social Strategy* | 1062305 | 1 | 4.9643 | **0.0397** |
| *NNdist:Strategy* | 12723 | 1 | 0.0595 | 0.8103 |

# CHAPTER 5

# DIVISION OF WORKER AND REPRODUCTIVE OFFSPRING BY COHABITING QUEENS: DO COOPERATIVE QUEENS CHEAT?

## Introduction

Social groups are defined by their cooperative behaviors, but there is often conflict within social groups because the reproductive interest of all group members rarely aligns perfectly. Individuals can benefit by exploiting the work of group members for personal fitness gains, a social phenomenon known as cheating (Trivers 1971). Theory predicts that cheating phenotypes will emerge in social groups as relatedness within the group decreases (Trivers 1985; Keller 1999), which has recently received strong support from the study of social microbes (Gilbert et al. 2007; Sandoz et al. 2007; Kuzdzal-Fick et al. 2011; Pollak et al. 2016; Inglis et al. 2017). Social groups composed of unrelated individuals are especially vulnerable to cheating behaviors because cheaters do not lose indirect fitness benefits by taking advantage of group members they do not share genes with. Cheaters gain fitness benefits by exploiting their group, so if left unchecked they will theoretically outcompete non-cheaters and spread through a population, potentially leading to the collapse of the cooperative system (Trivers 1971; Axelrod and Hamilton 1981; Bull and Rice 1991). However, there are several examples of stable non-kin cooperation that somehow keep the frequency of cheating phenotypes in check (Mesterton-Gibbons and Dugatkin 1992; Dugatkin 2002; Clutton-Brock 2009), but it has been difficult to observe the emergence and control of cheating phenotypes outside

of the social microbes. Unrelated social groups could resist cheating phenotypes in several ways including: 1) the social benefits generated by cooperating in a group outweigh the potential benefits of cheating (Clutton-Brock et al. 1999; Kokko et al. 2001), 2) the groups has means of controlling the spread of cheating behaviors through punishment and policing (Clutton-Brock and Parker 1995), and 3) competitive interactions with other groups select for more functional or efficient groups that contain a higher proportion of cooperative individuals (Maynard Smith 1964; Shaffer et al. 2016). Further study of stable non-kin social groups are needed to better understand how the spread of cheating is curtailed and non-kin cooperation can be maintained, particularly in multicellular organisms.

Primary polygyny in social insects is an ideal system to study the stability of non-kin groups and the dynamics of cheating. In this rare social structure, primarily found in ants, multiple queens form a cooperative association at colony founding that persists through the life of the colony (Hölldobler and Wilson 1977; Mintzer and Vinson 1985; Trunzer et al. 1998). These queens are completely unrelated (Kolmer et al. 2002; DeHeer and Herbers 2004; Cahan and Helms 2012; Overson et al. 2016), but do not develop any apparent hierarchy and all assist in nest initiation and worker production (Rissing et al. 1989; Heinze et al. 2001; Clark and Fewell 2014). Primary polygyny has been documented in several ant species (Mintzer 1987; DeHeer and Herbers 2004; Johnson 2004; Kellner et al. 2007; Hölldobler et al. 2011; Qian et al. 2012), and the stability of cooperative queen groups have been observed in lab (Mintzer 1987; Kolmer and Heinze 2000; Johnson 2004; Clark and Fewell 2014) and field conditions (Helms and Cahan

2012; Chapter 3). Most research on primary polygyny has focused on colony initiation, where queen cooperation generates survival and worker production benefits (Trunzer et al. 1998, Deslippe and Salvolainen 1995; Clark & Fewell 2014). There is some opportunity for queens to cheat early in colony life by avoiding metabolically costly worker production or dangerous tasks such as foraging, but queens do not appear to exploit the group at this stage (Heinze et al. 2001; Hölldobler et al. 2011). However, once the colony becomes reproductively mature, queens have an opportunity to unfairly exploit colony resources for personal reproductive output. Little is known about how cooperative queens share resources for reproductive investment (but see Heinze et al. 2001 and Kolmer et al. 2002), but this period is highly vulnerable to cheating due to the direct relationship with queen fitness.

Ant colonies reproduce through winged queens and males (alates) that fly from the nest in sync with conspecific colonies to mating leks (Hölldobler and Wilson 1990). Mated queens then leave the lek to found a new colony. Reproductive decisions are controlled at multiple levels. Queens lay eggs at a rate that varies between queens (Tschinkel 1988; Kwapich et al. 2017), and regulate if a laid egg is fertilized or unfertilized (Passera et al. 2001; de Menten et al. 2005). Being haplodiploid, a fertilized egg can become either a sterile worker or a new queen while an unfertilized egg can only become a reproductive male. In many species, workers then control the resource provisioning for developing larvae and determine if the females will become workers or queens, and if the males are allowed to develop at all (Hammond et al. 2002; Mehdiabadi et al. 2003). Cooperative queens are probably not able to cheat by directly provisioning

their larvae with more resources than the larvae of other queens because it would be logistically difficult and metabolically demanding. It is also unlikely that workers preferentially provision related brood because it does not appear that workers can identify their lineage from others in a polygynous colony (Snyder 1993; DeHeer and Ross 1997; Holzer et al. 2006; but see Helentara et al. 2013).

However, there are strategies that queens may utilize to take advantage of their social group during reproduction. Any strategy that reduces a queen's contribution to the workforce while increasing her contribution to reproductive output would be considered cheating because worker production is metabolically costly and uses stored sperm, but worker production is crucial for the function and survival of the colony as a whole. A queen could cheat by regulating the timing of egg production to increase her proportion of the brood during the reproductive season. She could also consume the eggs of other queens to reduce resource competition for her brood. Queens may also be able to cheat their cooperative group by overproducing unfertilized male eggs. This would provide a way to circumvent worker control, because unfertilized eggs can only develop into reproductive males, unlike fertilized eggs that can become sterile workers instead of queens. This strategy may be especially harmful to colony survival because every queen that preferentially produces unfertilized eggs will decrease the worker production rate of a colony at every stage of the colony's lifecycle.

I explored the possibility of queen cheating in a population of the harvester ant *Pogonomyrmex californicus* that practices primary polygyny. I captured male and queen alates as they flew from colonies over several successive annual mating flights and

concurrently collected workers from each colony. I used genotyped (microsatellites) queens, males, and workers from each colony to reconstruct queen genotypes which were used to assign maternity to alates and workers. These data allow us to see if cooperative queens fairly divide the metabolic cost of worker production with the fitness benefits of reproductive investment, and if the proportions are consistent over years. My evidence suggests that a low frequency of a novel cheating phenotype exists in this polygynous population. However, queen cooperation has been stable in this population for at least 20 years (Johnson 2004), suggesting that internal colony dynamics or competitive interactions may limit the spread of this cheating phenotype in the population.

## Methods

*Sample collection*

I captured alates and workers from *P. californicus* colonies from a disturbed grassland field site in the town on Pine Valley, San Diego Cty, CA (32°49'21.38"N, 116°31'40.24"W). Previous work has shown that this area is dominated by primary polygyny (Johnson 2004; Overson 2016; Chapter 3). The mating flight in this area takes place over a 3 to 4 week period from mid-June to mid-July. I trapped alates as they flew from colonies over the extent of the mating flight every year from 2012-2015 using suspended tent traps described in Chapter 3. Every day, I retained 10 males and 10 queens from each colony for genetic analysis. If more than 100 males or queens flew from a colony on that day, I saved 10% of the total. All remaining alates were released

undamaged to preserve the condition of the population. After the mating flights had ended, I collected at least 60 workers from the nest entrances of all sampled colonies. Alates and workers were immediately frozen at -20°C and stored in 100% ethanol within 24 hours of capture before being transported back to Arizona State University for genetic analysis.

I captured alates and performed genetic analysis on a total of 11 colonies, seven of which I captured for multiple annual mating flights. Some colonies did not reliably reproduce every mating season, and some died over the course of the experiment, but I was able to capture one mating flight for four colonies, two mating flights for four colonies, three mating flights for two colonies, and four mating flights for one colony.

*DNA extraction*

I extracted DNA using a Chelex extraction protocol (Gadau 2009). Ants were removed from the ethanol and their heads were cut from their bodies. The heads were individually crushed in 50µl of 5% Chelex® 100 resin (Bio-Rad) suspended in 1X TE buffer pH 8.0 before adding 1µl Proteinase K (5 mg/ml) to each sample. I then incubated the samples at 57°C for at least 1 hour, heated them to 95°C for 5 minutes, and centrifuged at 14,000rpm for 10 minutes. I pipetted the resulting supernatant into sterile tubes to be used as the DNA template and stored them at -20°C.

*PCR and microsatellite analysis*

Alates and workers were genotyped by at least 5 of the following microsatellite loci, with additional used when more clarification was required: Pb5, Pb6 (Volny and

Gordon 2002); Po03 (Wiernasz et al. 2004); E9, E10, E19, E20, and B16 (unpublished; sequences for unpublished primers given in Appendix D). PCR reactions took place in 12 µl reaction volumes containing 6 µl $dH_20$, 2.4 µl of 5x GoTaq buffer (Promega), 1 µl $MgCl_2$ (50mM), 0.5 µl dNTPs (10mM), 0.5 µl forward and reverse primer, and 0.1 µl Taq polymerase. PB6, E9, E10, and B16 were tagged with a 700nm fluorescent label while PB5, E19, E20, and Po03 were tagged with an 800nm fluorescent label so primers from these two groups were often run together using the following PCR program. An initial 5 minute at 94°C, 37 cycles of the following three stages: (1) 20 seconds at 94°C, (2) a 30 second annealing stage at 56°C, and (3) 30 seconds at 72°C, with a final 5 minute stage at 72°C. A Licor 4300 model sequencer was used to measure size of the PCR products. All gels were scored by the same individual (BH) and reference samples from previous runs were always included to maintain standard allele identities.

*Genotype reconstruction*

Maternal queen genotypes were reconstructed by entering the allele profiles of all males from a colony into the program COLONY v2.0 (Jones and Wang 2010), with allelic dropout and error rates set to 0.05. I was only interested in the maternity of the samples so haploid males were used to reconstruct the maternal genotypes and avoid the added complexity of paternity. The resulting queen genotypes were checked for redundancy and combined when possible. All males, alate queens, and workers of the colony were then assigned to the maternal line that fit their allele profile. Overlapping alleles occasionally prevented the assignment of an individual to a single mother; these samples were not included in further analyses.

Chi square tests were used to determine if the overall proportions of worker and alate production deviated from the expected fair proportions within each polygynous colony. A standardized residual post-hoc was then used to determine which queen deviated from her expected production of which caste. Chi square values were generated with JMP 13.0 and ANOVAs were run using the car package (Fox and Weisberg 2011) in R version 3.0.1 (R Core Team 2013).

## Results

Of the eleven colonies analyzed, three colonies contained two queens, two colonies contained three queens, three colonies contained four queens, one colony contained six queens, one colony contained seven queens, and one colony contained eight queens. Seven of the eleven colonies were analyzed over multiple years, and there was a single instance of a queen's alleles disappearing from the colony's genetic pool. This queen contributed to three consecutive mating flights of her colony before disappearing in the fourth and final sample year. There were no instances of novel alleles appearing in a colony's gene pool after the first analyzed mating flight.

Ten of the 11 colonies showed an overall difference in the observed caste investment by nest-mate queens in at least one year. A total of 22 annual mating flights were captured and analyzed from the 11 colonies, 17 of these flights had a significant difference in the observed caste investment by nest-mate queens (Appendix E). A standardized residual post-hoc analysis was used to find the specific caste and queen that

significantly deviated from the expected investment. Overall there were three instances of a queen overproducing males, one instance of worker underproduction, one instance of worker overproduction, and one instance of male underproduction. Two queens deviated from their colony's expected investment into workers, queens, and males over a single mating flight (Appendix E).

I designated a queen as a cheater if she produced a significantly higher proportion of reproductive male and/or queen alates or under produced workers relative to her nest mate queens. Of the 22 mating flights analyzed in this study, a single cheater queen was identified in 3 flights. However, two of these flights were from the same colony in different years that contained the same cheater queen, so a cheater queen was identified in 2 of the 11 colonies for at least one year. In three flights the cheater significantly overproduced males ($_{queenA}$Residual=3.93, crit.residual=2.86; $_{queenB}$Residual=3.39, crit.residual=2.86; $_{queenC}$Residual=3.31, crit.residual=2.86), and in one she significantly under-produced workers ($_{queenD}$Residual=4.16, crit.residual=2.86).

I calculated the ratio of alate to worker production for every queen that participated in each mating flight to compare the values of cheaters versus non-cheaters. Any zero values in the alate or worker production of a queen were replaced with one for the calculation of ratios. Cheaters had an average alate to worker ratio of 24.67+/-12.67, significantly higher than the non-cheater ratio of 2.98+/-0.359 (ANOVA: $F=62.278$, $df=1$, $p<0.0001$). Most of the inequality between alate and worker production for cheaters came from male production. The average male to worker production ratio for cheater queens was 23.33+/-11.84, significantly higher than the non-cheater ratio of 1.85+/-

0.0.226 (ANOVA: F=95.34, df=1, p<0.0001). However, the average queen to worker production ratio from cheater queens was 1.67+/-0.667, not significantly different than the queen to worker production ratio of non-cheater queens at 1.28+/-0.162 (ANOVA: F=0.2024, df=1, p=0.654).

Both of the two colonies where a cheater queen was identified through post-hoc testing had their mating flights captured and analyzed for more than one year. Only one queen, who overproduced male alates, was identified who cheated consistently over two mating flights. However, differences in overall investment levels and sample sizes between years may have obstructed the detection of some queens with biased caste investment. I combined the maternity results for all queens in the six colonies captured over multiple years and reran the standardized residual post-hoc tests to further gauge consistency in caste investment by cheaters over years.

One of the six colonies contained a cheater queen when all mating flights were combined. This was the same colony that contained a queen that consistently overproduced males in two separate mating flights. When the three mating flights of this colony were combined, this cheater queen (Queen A) was found to significantly overproduce male alates (Residual=5.19, crit.residual=2.86), and significantly under-produce workers (Residual=4.40, crit.residual=2.86). There was another queen (Queen D) in this colony that significantly overproduced workers (Residual=3.92, crit.residual=2.86) and under-produced males (Residual=4.60, crit.residual=2.86; Figure 5.1). The post-hoc test did not identify a consistent cheater queen in any of the other colonies (Table 5.1).

## Discussion

When individuals cooperate there exists the potential for one or more group members to garner additional benefits by abstaining from costly activities or by unfairly utilizing shared resources. If these selfish behaviors emerge they could become fixed into a cheating phenotype that spreads rapidly throughout the population (Axelrod and Hamilton 1981; Velicer et al. 2000; Doebeli et al. 2004; Nowak 2006). Cooperative groups of unrelated individuals are particularly at risk because there are no kinship benefits gained by helping group members that may offset the benefits of cheating (Hamilton 1964; Van Dyken et al. 2011). I explored the prospect of cheating within cooperative groups of unrelated ant queens during reproductive investment. Primary polygyny is relatively rare in ants and may only persist in the absence of cheating behaviors, in which case we would expect cooperative queens to share reproductive investment equally. However, if a cheating phenotype is detected in these colonies it would provide a rare example of a non-kin cooperation that can support the presence of cheaters.

The results indicate that almost all polygynous *P. californicus* queens fairly divide worker and reproductive production. This result further verifies that primary polygyny is a solid example of stable, fair non-kin cooperation that persists in a natural context. However, it appears that a low frequency of cheating does exist in polygynous *P. californicus* colonies. I identified two queens that produce more alates and fewer workers than the other queens of her colony, and found that these reproductive patterns are consistent over multiple years and mating flights. Interestingly, these queens primarily

overproduce male alates, which may represent a strategy to bypass worker influence. Although cheater queens should have higher fitness than cooperative queens, cheating individuals are quite rare, with most colonies composed completely of cooperative queens.

Cheating was only identified in 2 out of the 45 queens (4.4%) analyzed in this study. However, almost all colonies significantly varied from the expected proportions of alates and workers produced by each queen. Few of the differences between queens were extreme enough to achieve significance in a post-hoc test, which quickly loses power as queen number increases. While there is natural variation in the proportion workers and alates produced by a queen that surely contributed to the significant tests at the colony level, the post-hoc analysis likely missed some cheater queens due to low power, especially in colonies with a high number of queens. However, even if potential cheater queens are included using a less conservative adjusted Bonferroni approach (Keppel 1991), the prevalence of queen cheating remains low (15.6%).

For cheating to be considered an adaptive, distinct phenotype, the caste production ratio of cheater queens must be consistent over years. Otherwise skewed production ratios may simply be caused by random factors such as variation in egg development and/or sampling skew. One of the two cheater queens identified in this study reduced the overall amount of investment between two mating flights two years apart, from twelve alates and zero workers produced in the first year to two alates and two workers in the second year, which may indicate declining health (Table 5.1). The other cheater queen identified in this study displayed consistency in alate and worker

production ratio over three separate mating flights. This suggests that cheaters are able to maintain their unfair caste production ratio over multiple years, effectively freeriding off the worker production of other queens by mainly producing procreative offspring.

The overinvestment of alates by cheating queens tended to be highly male biased, while most cooperative queens had fairly equal investment into male and queen alates (Table 5.1). This may represent a strategy that cheater queens have adopted in order to circumvent worker or nest-mate queen influence on the cheater's reproductive output (Trivers 1974). Ants are haplodiploid, meaning that a fertilized diploid egg can develop in either a worker or an alate queen while an unfertilized haploid egg can only develop into a male. In most species workers can influence whether diploid eggs will become new workers or queens by controlling the amount and quality of food fed to the larvae while they grow (Sundstrom et al. 1996; Hammond et al. 2002; Beekman and Ratnieks 2003). Thus, a cheater queen may not be able to ensure that the diploid eggs she lays will result in reproductive progeny. However, a haploid egg can only develop into a reproductive male, so highly male biased reproduction may be a strategy adopted by cheater queens to more tightly control their fitness outcome.

The production of diploid eggs is also costly for a queen because it requires sperm from their limited stored supply. The only time an ant queen receives sperm is when she first leaves her home nest and mates during the nuptial flight. A queen never mates again after this period, all the sperm used to fertilize worker and queen eggs come from the single mating event. Stored sperm is a limiting resource for queens, and in some species the limiting factor for the longevity and size of a colony is the number of sperm a queen

can store (Tschinkel 1987; Fjerdingstad and Boomsma 1998). Once a queen's sperm is depleted, she can no longer produce workers and the colony is doomed to dwindle and die. Cheater *P. californicus* queens still produce queens and some workers, suggesting that their male biased caste investment is not a result of sperm depletion. Instead, the overproduction of males may represent an additional cheating strategy in which queens attempt to conserve their stored sperm while nest-mate queens deplete their reserves on worker and alate queen production. Cheaters may gain a substantial fitness boon if they are able to switch their reproductive sex ratio to alate queen production once their nest-mates are low on sperm, because there would be less resource competition with the larvae of other lineages during development. This strategy could potentially increase the proportion of the cheater queen's sperm that develop into reproductive queen alates instead of workers.

A cheater queen produces a higher proportion of reproductive alates than other queens, so this strategy likely provides a higher lifetime fitness than cooperative queens. In isolation, individual selection would favor the benefits of being a cheater and we would expect the phenotype to proliferate in a population. However, this phenotype may not spread widely throughout the population if colonies that contain a cheater queen are less likely to survive to reproductive maturity. Cheaters in this study increased their personal alate investment at the cost of worker production. Even if a cheater still produces her share of workers, the resources she uses to overproduce alates might otherwise be used to produce workers by nest-mate queens. Thus, a cheater queen likely reduces the worker production rate of a colony. Primary polygyny in *P. californicus*

occurs in areas with higher colony density, which likely increases the competitive pressure exerted on the colonies (Chapter 3). In a highly competitive environment the ability of a colony to produce workers is crucial to survive territorial interactions. A colony's workforce can be rapidly depleted from losses during fights over resources, reducing the ability of the colony to forage and defend itself from future attacks (Wiernasz and Cole 1995). This may be especially important during early colony growth, when small colonies can be completely wiped out by neighboring mature colonies if they do not become large and established quickly (Hölldobler 1976; Bartz and Hölldobler 1982; Adams and Tschinkel 1995b; Sanders and Gordon 2004).

In an uncommon circumstance such as this, when competitive pressure is high and cooperative groups are permanent, selection acting at the colony level may override selection on individual queens (Wilson 1975; Wilson 1987). Selection on the traits of the group, in this case high worker production, may limit the spread of a cheating phenotype regardless of its fitness benefits to the individual. If future work confirms that the low frequency of the cheating phenotype is stable over time, this would provide a unique model system that demonstrates an observable evolutionary influence by multi-level selection (Wilson 1989; Wilson and Sober 1994; Aviles 2002; Hölldobler and Wilson 2009). The finding that the proliferation of an uncooperative phenotype is restricted by group selection would be a valuable addition to other recent advances in our understanding of the mechanisms and prevalence of multi-level selection in nature (Boza and Szamado 2010; Pruitt and Goodnight 2014; Okasha 2016; Schaffer et al. 2016; Pruitt et al. 2017).

*Conclusion*

Maternity tests of males, queens, and workers from 11 colonies that are headed by multiple, unrelated queens have revealed that there is a low frequency of a cheater phenotype present in this cooperative system. Cheater queens overproduce reproductive alates, specifically males, often at the expense of worker production. Although cheating provides additional fitness benefits to queens, these behaviors are not widespread in the population, potentially because colonies that contain cheaters are at a competitive disadvantage in their highly dense, harsh environment. This provides a rare example of a trait that is selected for at the individual level being overridden by the selective pressures on the cooperative group. The dynamics of cheating in the context of primary polygyny may represent a long sought after model system for the evolution and maintenance of stable altruism between non-kin.

Figure 5.1: The proportion of males, queens, and workers produced by the queens of two polygynous colonies, with all years where reproduction was captured combined. (A) Colony 1 contains one cheater queen, Queen A, who over-produced males ($_{male}$residual=5.19, crit.residual=2.86) and under-produced workers ($_{worker}$residual=4.40, crit.residual=2.86). (B) Queens equally divide the alate and worker production in colony 5.

Table 5.1: The males, queens, and workers produced by each queen in the eleven polygynous colonies surveyed for this study. If a colony was analyzed over multiple years, maternity results were combined for all years. Colony ID, years reproduction was analyzed, and the critical standardized residual value for each colony are given in the leftmost column. Queens deviate significantly ($p<0.05$) from their expected output when a cell's standardized residual value is greater than the critical value.

| | Queen ID | Males produced | Male Standardized Residual | Queens produced | Queen Standardized Residual | Workers produced | Worker Standardized Residual |
|---|---|---|---|---|---|---|---|
| **Colony 1** | A | 77 (89.5%) | **5.18** | 5 (5.8%) | 2.79 | 4 (4.7%) | **4.40** |
| 2012, 2015, 2016 | B | 44 (46.3%) | 0.51 | 22 (23.16%) | 0.95 | 29 (30.5%) | 0.10 |
| Critical residual value=2.86 | C | 33 (40.7%) | 1.18 | 15 (18.5%) | 0.081 | 33 (40.7%) | 1.56 |
| | D | 2 (4%) | **4.6** | 17 (34%) | 2.45 | 31 (62%) | **3.92** |
| **Colony 2** | A | 28 (45.9%) | 0.25 | 6 (9.8%) | 0.66 | 27 (44.3%) | 0.72 |
| 2013, 2014, 2015, 2016 | B | 49 (43.0%) | 0.58 | 20 (17.5%) | 0.41 | 45 (39.5%) | 0.37 |
| Critical residual value=2.86 | C | 52 (66.7%) | 2.59 | 12 (15.4%) | 0.14 | 14 (18.0%) | 2.80 |
| | D | 46 (38.3%) | 1.34 | 20 (16.7%) | 0.182 | 54 (45.0%) | 1.37 |
| **Colony 3** | A | 14 (63.6%) | 1.75 | 8 (36.4%) | 0.375 | 0 (0%) | 2.49 |
| 2013 | B | 15 (37.5%) | 0.25 | 13 (32.5%) | 0.073 | 12 (30.0%) | 0.221 |
| Critical residual value=2.93 | C | 5 (45.5%) | 0.286 | 6 (54.5%) | 1.33 | 0 (0%) | 1.76 |
| | D | 8 (30.8%) | 0.74 | 4 (15.4%) | 1.49 | 14 (53.8%) | 2.47 |
| | E | 12 (33.3%) | 0.632 | 12 (33.3%) | 0.157 | 12 (33.3%) | 0.586 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Colony 4** | | | | | | | |
| 2013, 2014, 2015 | A | 99 (33.7%) | 1.35 | 90 (30.1%) | 1.41 | 105 (35.7%) | 0.197 |
| Critical residual value=2.63 | B | 75 (47.8%) | 1.85 | 29 (18.5%) | 1.93 | 53 (33.8%) | 0.27 |
| **Colony 5** | | | | | | | |
| 2014, 2015 | A | 44 (39.6%) | 0.0566 | 30 (27.0%) | 0.905 | 37 (33.3%) | 0.886 |
| Critical residual value=2.63 | B | 46 (39.0%) | 0.0548 | 43 (36.4%) | 0.878 | 29 (24.6%) | 0.859 |
| **Colony 6** | A | 12 (40%) | 0.215 | 15 (50%) | 1.67 | 3 (10%) | 1.99 |
| 2014, 2015 | B | 7 (31.8%) | 0.441 | 6 (27.3%) | 0.439 | 9 (40.9%) | 0.956 |
| Critical residual value=3.08 | C | 6 (54.5%) | 0.917 | 1 (9.1%) | 1.37 | 4 (36.4%) | 0.399 |
| | D | 7 (50%) | 0.757 | 2 (14.3%) | 1.20 | 5 (35.7%) | 0.406 |
| | E | 6 (60%) | 1.16 | 4 (40%) | 0.408 | 0 (0%) | 1.73 |
| | F | 5 (27.8%) | 0.679 | 4 (22.2%) | 0.773 | 9 (50%) | 1.57 |
| | G | 8 (80%) | 2.19 | 0 (0%) | 1.81 | 2 (20%) | 0.567 |
| | H | 2 (7.69%) | 2.47 | 14 (53.8%) | 1.89 | 10 (38.4%) | 0.810 |
| **Colony 7** | A | 11 (47.8%) | 0.88 | 7 (30.4%) | 0.103 | 5 (21.7%) | 0.845 |
| 2015 | B | 9 (47.4%) | 0.767 | 4 (21.1%) | 0.821 | 6 (31.6%) | 0.00 |
| Critical residual value=2.76 | C | 9 (24.3%) | 1.24 | 14 (37.8%) | 0.670 | 14 (37.8%) | 0.670 |
| **Colony 8** | | | | | | | |
| 2015 | A | 5 (17.9%) | 1.76 | 13 (46.4%) | 1.65 | 10 (35.7%) | 0.342 |
| Critical residual value=2.63 | B | 25 (50.0%) | 1.32 | 10 (20.0%) | 1.24 | 15 (30.0%) | 0.256 |
| **Colony 9** | A | 26 (59.1%) | 2.19 | 8 (18.2%) | 1.36 | 10 (22.7%) | 1.10 |
| 2015 | B | 17 (36.2%) | 0.265 | 16 (34.0%) | 0.603 | 14 (29.8%) | 0.285 |
| Critical residual value=2.76 | C | 11 (22.4%) | 1.82 | 17 (34.7%) | 0.699 | 21 (42.9%) | 1.32 |
| **Colony 10** | A | 18 (31.6%) | 0.702 | 18 (31.6%) | 0.312 | 21 (36.8%) | 1.14 |
| 2014, 2016 | B | 11 (68.8%) | 2.06 | 3 (18.8%) | 1.05 | 2 (12.5%) | 1.21 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Critical residual value=2.86 | C | 9 (42.9%) | 0.421 | 7 (33.3%) | 0.051 | 5 (23.8%) | 0.423 |
| | D | 19 (32.2%) | 0.636 | 24 (40.7%) | 0.882 | 16 (27.1%) | 0.235 |
| **Colony 11** | A | 10 (28.6%) | 1.21 | 14 (40.0%) | 1.29 | 11 (31.4%) | 0.178 |
| 2015, 2016 | B | 5 (41.7%) | 0.01 | 2 (16.7%) | 0.761 | 5 (41.7%) | 0.754 |
| Critical residual value=3.04 | C | 9 (34.6%) | 0.570 | 11 (42.3%) | 1.33 | 6 (23.1%) | 0.627 |
| | D | 13 (50.0%) | 0.643 | 7 (26.9%) | 0.139 | 6 (23.1%) | 0.627 |
| | E | 8 (66.7%) | 1.33 | 1 (8.3%) | 1.30 | 3 (25.0%) | 0.304 |
| | F | 3 (60.0%) | 0.628 | 2 (40.0% | 0.488 | 0 (0%) | 1.22 |
| | G | 11 (44.0%) | 0.167 | 3 (12.0%) | 1.54 | 11 (44.0%) | 1.30 |

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# APPENDIX A

## TWO-WAY TYPE II ANOVA FULL RESULTS WITH INTERACTIONS OF PINE VALLEY AND LAKE HENSHAW COLONIES, 2012-2014

| | df | Sum Sq | Mean Sq | F | P |
|---|---|---|---|---|---|
| **Alate output by site** | 1 | 2904390 | 2904390 | 5.7696 | 0.02249 |
| **Alate output by social structure** | 1 | 244235 | 244235 | 0.4852 | 0.49128 |
| **Interaction** | 1 | 243735 | 243725 | 0.4842 | 0.49173 |
| | df | Sum Sq | Mean Sq | F | P |
| **Alate investment by site** | 1 | 485925561 | 485925561 | 4.5867 | 0.04046 |
| **Alate investment by social structure** | 1 | 5320 | 5320 | 0.0001 | 0.99439 |
| **Interaction** | 1 | 76906135 | 76906135 | 0.7259 | 0.40096 |
| | df | Sum Sq | Mean Sq | F | P |
| **Alate investment sex ratio by site** | 1 | 0.0001 | 0.0001 | 0.001 | 0.987 |
| **Alate investment sex ratio by social structure** | 1 | 0.1931 | 0.1931 | 3.453 | 0.0733 |
| **Interaction** | 1 | 0.0355 | 0.0355 | 0.635 | 0.4321 |
| | df | Sum Sq | Mean Sq | F | P |

| | | | | | |
|---|---|---|---|---|---|
| **Alate output sex ratio by site** | 1 | 0.0007 | 0.0007 | 0.014 | 0.9073 |
| **Alate output sex ratio by social structure** | 1 | 0.1676 | 0.1676 | 3.164 | 0.0858 |
| **Interaction** | 1 | 0.0873 | 0.0873 | 1.648 | 0.2094 |
| | df | Sum Sq | Mean Sq | F | P |
| **Worker activity by site** | 1 | 718285 | 718285 | 3.4403 | 0.07348 |
| **Worker activity by social structure** | 1 | 1589526 | 1589526 | 7.613 | 0.00978 |
| **Interaction** | 1 | 43277 | 43277 | 0.2073 | 0.65218 |

# APPENDIX B

## OVERVIEW OF REPRODUCTIVE AND ECOLOGICAL DATA, PINE VALLEY AND LAKE HENSHAW 2012-2014

| | 2012 | 2013 | 2014 |
|---|---|---|---|
| PV colonies with traps | 10 | 20 | 14 |
| LH colonies with traps | 7 | 14 | 14 |
| PV Mating flights trapped | 4 | 11 | 13 |
| LH Mating flights trapped | 6 | 13 | 14 |
| PV first mating flights trapped (new colony) | 4 | 6 | 5 |
| LH first mating flights trapped | 5 | 9 | 3 |
| PV haplometrotic colonies | 0 | 3 | 3 |
| LH pleometrotic colonies | 2 | 2 | 2 |
| PV percent haplo | no data | 27.30% | 23.10% |
| LH percent pleo | no data | 15.40% | 14.30% |
| PV days trapped | 18 | 31 | 20 |
| LH days trapped | 10 | 24 | 20 |
| PV total alates released (all colonies) | 1737 | 7243 | 8732 |
| LH total alates released (all colonies) | 5321 | 17573 | 15744 |
| PV average alates released by colony | 289.5 | 637.9 | 671.69 |
| LH average alates released by colony | 886.8 | 1367.8 | 1326.4 |
| PV alate investment total (mg) | 5963.91 (dry) | 74699.1 | 103052.17 |
| LH alate investment total | 32869.2 (dry) | 227395.9 | 246250.3 |
| PV alate investment colony average | 1987.97 (dry) | 6790.8 | 7927.1 |
| LH alate investment colony average | 6573.84 (dry) | 17492 | 17589.3 |
| percent investment difference | 331% | 257.5837898 | 221.8882063 |
| PV male investment total | 4654.24 (dry) | 53640.8 | 57620.6 |
| LH male investment total | 12063.5 (dry) | 110875.7 | 138585 |
| PV male investment colony average | 1551.41 (dry) | 4876.4 | 4432.6 |
| LH male investment colony average | 2412.71 (dry) | 8528.9 | 9898.93 |
| PV percent male investment average | 84 | 71.8 | 61.7 |
| LH percent male investment average | 36.8 | 48.8 | 59.1 |
| PV survey area | no data | 36955$m^2$ | 36955$m^2$ |
| LH survey area | no data | 26509.9$m^2$ | 40635.3$m^2$ |
| PV colony number total | no data | 47 | 55 |
| LH colony number total | no data | 27 | 34 |

| | | | |
|---|---|---|---|
| PV colony density | no data | 1190 per km² | 1488 per km$^2$ |
| LH colony density | no data | 905 per km² | 837 per km² |
| PV nearest neighbor | no data | 15.47 m | 16 m |
| LH nearest neighbor | no data | 17.33 m | 19.75m |
| PV percent non repro trapped colonies | 60% | 45% | 7.10% |
| LH percent non repro trapped colonies | 14.30% | 7.10% | 0% |
| PV percent non reproductive all colonies | no data | no data | 72.30% |
| LH percent non reproductive all colonies | no data | no data | 2.94% |

# APPENDIX C

## ANOVA FULL RESULTS WITH INTERACTIONS FOR COLONIES IN MIXED POPULATION, 2015 & 2016

Multifactorial Type II ANOVA results for nearest neighbor distance

| **Nearest Neighbor** | Sum of Squares | Df | F | P |
|---|---|---|---|---|
| Worker activity | 5.915 | 1 | 0.2965 | 0.5953 |
| Social strategy | 3.288 | 1 | 0.1649 | 0.6913 |
| Reproductive investment | 20.774 | 1 | 1.0415 | 0.3261 |
| Activity:strategy | 34.053 | 1 | 1.7073 | 0.2140 |
| Activity:investment | 59.062 | 1 | 2.9611 | 0.1090 |
| Strategy:investment | 0.264 | 1 | 0.0132 | 0.9101 |
| Activity:strat:investment | 44.464 | 1 | 2.2292 | 0.1593 |

Multifactorial Type II ANOVA results for queen and male mass

| **Male mass** | Sum of Squares | Df | F | P |
|---|---|---|---|---|
| Worker activity | 0.01274 | 1 | 0.0383 | 0.8514 |
| Area | 0.00015 | 1 | 0.0004 | 0.9839 |
| Social strategy | 0.85673 | 2 | 1.2871 | 0.3427 |
| Reproductive investment | 0.01189 | 1 | 0.0357 | 0.8563 |
| Activity:area | 1.74492 | 1 | 5.2430 | 0.0619 |
| Activity:strategy | 0.07916 | 1 | 0.2378 | 0.6431 |
| Area:strategy | 0.85514 | 1 | 2.5695 | 0.1601 |
| Activity:investment | 0.41317 | 1 | 1.2415 | 0.3078 |
| Area:investment | 0.00160 | 1 | 0.0048 | 0.9470 |
| Strategy:investment | 0.23335 | 1 | 0.7012 | 0.4345 |
| Activity:area:strategy | 0.10973 | 1 | 0.3297 | 0.5867 |

| | | | | |
|---|---|---|---|---|
| Activity:area:investment | 0.09514 | 1 | 0.2859 | 0.6121 |
| Activity:strategy:investment | 0.02740 | 1 | 0.0823 | 0.7838 |
| Area:structure:investment | 1.05214 | 1 | 3.1614 | 0.1257 |
| **Queen mass** | Sum of Squares | Df | F | P |
| Worker activity | 0.0463 | 1 | 0.0783 | 0.7890 |
| Area | 0.8686 | 1 | 1.4706 | 0.2708 |
| Social strategy | 1.6845 | 2 | 1.4259 | 0.3114 |
| Reproductive investment | 0.3739 | 1 | 0.6331 | 0.4565 |
| Activity:area | 1.0521 | 1 | 1.7813 | 0.2304 |
| Activity: strategy | 0.0230 | 1 | 0.0389 | 0.8501 |
| Area: strategy | 0.7510 | 1 | 1.2714 | 0.3026 |
| Activity:investment | 0.0563 | 1 | 0.0953 | 0.7680 |
| Area:investment | 0.1497 | 1 | 0.2534 | 0.6326 |
| Structure:investment | 0.7638 | 1 | 1.2932 | 0.2988 |
| Activity:area: strategy | 0.0487 | 1 | 0.0825 | 0.7836 |
| Activity:area:investment | 0.9890 | 1 | 1.6743 | 0.2433 |
| Activity: strategy:investment | 0.1587 | 1 | 0.2687 | 0.6228 |
| Area:strategy:investment | 0.0915 | 1 | 0.1549 | 0.7075 |

Multifactorial Type II ANOVA results for sex ratios of alate output and investment

| **Male output sex ratio** | Sum of Squares | Df | F | P |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| Worker activity | 0.0881 | 1 | 2.6354 | 0.1229 |
| Social strategy | 0.0204 | 1 | 0.6089 | 0.4459 |
| Activity:strategy | 0.0016 | 1 | 0.0466 | 0.8316 |
| **Male investment sex ratio** | Sum of Squares | Df | F | P |
| Worker activity | 0.0557 | 1 | 1.7518 | 0.2032 |
| Social strategy | 0.0086 | 1 | 0.2688 | 0.6108 |
| Activity:strategy | 0.0025 | 1 | 0.0786 | 0.7826 |

# APPENDIX D

## SEQUENCES FOR UNPUBLISHED PRIMERS

Forward and reverse sequences for unpublished primers used in this study:

| | |
|---|---|
| E9-F | TTTTAGACCATTTTAACG |
| E9-R | AAGATTTTCATCGCGAAACG |
| E10-F | TGATTCAACGAGGTGAGC |
| E10-R | TTTGTTTCCTTGCGTTAGGG |
| E19-F | AGAGTCACCTTGGCGCTTC |
| E19-R | GAATCATCTGCCTCCGGTAA |
| E20-F | CTTTCGCGTACTCATCGTCA |
| E20-R | ATCTGCGATCTTTGGGAGAA |
| B16-F | AATGAACCGATCATGTTG |
| B16-R | CGAATTTAACGCAATTTGGAA |

# APPENDIX E

## CASTE RATIO INVESTMENT BY POLYGYNOUS QUEENS, DIVIDED BY YEAR

Worker and reproductive partitioning by polygynous queens, divided by mating flights. Highlighted cells indicate significant deviation from expected output.

| **Colony 1** | | | | | |
|---|---|---|---|---|---|
| **2012** | males | queens | workers | total | |
| **Q1** | 47 | 3 | 0 | 50 | |
| **% Q1** | 0.94 | 0.06 | 0 | | |
| **% total** | 0.66197183 | 0.1875 | 0 | | a=.00426 |
| **cell chi2** | 15.4514 | 1.5133 | 17.2932 | | critical value = 2.86 |
| **residual** | 3.93082689 | 1.2301626 | 4.15850935 | | |
| **Q2** | 18 | 10 | 18 | 46 | |
| **% Q2** | 0.39130435 | 0.2173913 | 0.39130435 | | |
| **% total** | 0.25352113 | 0.625 | 0.39130435 | | |
| **cell chi2** | 1.7505 | 3.6045 | 0.2746 | | |
| **residual** | 1.32306462 | 1.8985521 | 0.5240229 | | |
| **Q3** | 6 | 0 | 13 | 19 | |
| **% Q3** | 0.31578947 | 0 | 0.68421053 | | |
| **% total** | 0.08450704 | 0 | 0.2826087 | | |
| **cell chi2** | 1.6922 | 2.2857 | 6.2888 | | |
| **residual** | 1.30084588 | 1.5118532 | 2.50774799 | | |
| **Q4** | 0 | 3 | 15 | 18 | |
| **% Q4** | 0 | 0.1666667 | 0.83333333 | | |
| **% total** | 0 | 0.1875 | 0.32608696 | | |
| **cell chi2** | 9.609 | 0.3217 | 12.3669 | | |
| **residual** | 3.09983871 | 0.567186 | 3.51666035 | | |
| **total** | 71 | 16 | 46 | | |
| | | | | | |
| **2015** | males | queens | workers | total | |
| **Q1** | 12 | 0 | 0 | 12 | |
| **% Q1** | 1 | 0 | 0 | | |
| **% total** | 0.42857143 | 0 | 0 | | |
| **cell chi2** | 11.5238 | 3.33 | 4 | | |
| **residual** | 3.3946723 | 1.8248288 | 2 | | |
| **Q2** | 8 | 3 | 5 | 16 | |
| **% Q2** | 0.5 | 0.1875 | 0.3125 | | |

| | | | | | |
|---|---|---|---|---|---|
| **% total** | 0.28571429 | 0.15 | 0.20833333 | | |
| **cell chi2** | 0.508 | 0.4694 | 0.0208 | | |
| **residual** | 0.71274119 | 0.6851277 | 0.14422205 | | |
| **Q3** | 6 | 10 | 9 | 25 | |
| **% Q3** | 0.24 | 0.4 | 0.36 | | |
| **% total** | 0.21428571 | 0.5 | 0.375 | | |
| **cell chi2** | 1.4251 | 1.344 | 0.0533 | | |
| **residual** | 1.19377552 | 1.1593101 | 0.23086793 | | |
| **Q4** | 2 | 7 | 10 | 19 | |
| **% Q4** | 0.10526316 | 0.3684211 | 0.52631579 | | |
| **% total** | 0.07142857 | 0.35 | 0.41666667 | | |
| **cell chi2** | 3.9302 | 0.562 | 2.1228 | | |
| **residual** | 1.9824732 | 0.7496666 | 1.45698318 | | |
| **total** | 28 | 20 | 24 | | |
| | | | | | |
| **2016** | males | queens | workers | total | |
| **Q1** | 18 | 2 | 4 | 24 | |
| **% Q1** | 0.75 | 0.0833333 | 0.16666667 | | |
| **% total** | 0.31578947 | 0.0869565 | 0.14814815 | | |
| **cell chi2** | 2.0268 | 1.8937 | 0.6667 | | |
| **residual** | 1.42365726 | 1.3761177 | 0.81651699 | | |
| **Q2** | 18 | 9 | 6 | 33 | |
| **% Q2** | 0.54545455 | 0.2727273 | 0.18181818 | | |
| **% total** | 0.31578947 | 0.3913043 | 0.22222222 | | |
| **cell chi2** | 0.0301 | 0.4274 | 0.7353 | | |
| **residual** | 0.17349352 | 0.6537584 | 0.85749636 | | |
| **Q3** | 21 | 5 | 11 | 37 | |
| **% Q3** | 0.56756757 | 0.1351351 | 0.2972973 | | |
| **% total** | 0.36842105 | 0.2173913 | 0.40740741 | | |
| **cell chi2** | 0.0642 | 1.0524 | 0.3311 | | |
| **residual** | 0.25337719 | 1.0258655 | 0.5754129 | | |
| **Q4** | 0 | 7 | 6 | 13 | |
| **% Q4** | 0 | 0.5384615 | 0.46153846 | | |
| **% total** | 0 | 0.3043478 | 0.22222222 | | |

| | | | | | |
|---|---|---|---|---|---|
| **cell chi2** | 6.9815 | 6.4675 | 2.3269 | | |
| **residual** | 2.64225283 | 2.543128 | 1.52541798 | | |
| **total** | 57 | 23 | 27 | | |

| **Colony 2** | | | | | |
|---|---|---|---|---|---|
| **2013** | males | no queens flew | workers | total | |
| **Q1** | 8 | 0 | 8 | 16 | |
| **% Q1** | 0.5 | 0 | 0.5 | | |
| **% total** | 0.112676 | | 0.177778 | | a=.00426 |
| **cell chi2** | 0.3283 | | 0.518 | | critical value = 2.86 |
| **residual** | 0.572975 | 0 | 0.719722 | | |
| **Q2** | 18 | 0 | 13 | 31 | |
| **% Q2** | 0.580645 | 0 | 0.419355 | | |
| **% total** | 0.253521 | | 0.288889 | | |
| **cell chi2** | 0.05 | | 0.0789 | | |
| **residual** | 0.223607 | 0 | 0.280891 | | |
| **Q3** | 32 | 0 | 7 | 39 | |
| **% Q3** | 0.820513 | 0 | 0.179487 | | |
| **% total** | 0.450704 | | 0.155556 | | |
| **cell chi2** | 2.7685 | | 4.3681 | | |
| **residual** | 1.663881 | 0 | 2.09 | | |
| **Q4** | 13 | 0 | 17 | 30 | |
| **% Q4** | 0.433333 | 0 | 0.566667 | | |
| **% total** | 0.183099 | | 0.377778 | | |
| **cell chi2** | 1.5658 | | 2.4705 | | |
| **residual** | 1.251319 | 0 | 1.571782 | | |
| **total** | 71 | 0 | 45 | | |
| | | | | | |
| **2014** | males | queens | workers | total | |
| **Q1** | 18 | 3 | 11 | 32 | |
| **% Q1** | 0.5625 | 0.09375 | 0.34375 | | |
| **% total** | 0.327273 | 0.15 | 0.22 | | |

| | | | | | |
|---|---|---|---|---|---|
| **cell chi2** | 1.0914 | 0.8778 | 0.2531 | | |
| **residual** | 1.044701 | 0.93691 | 0.50309 | | |
| **Q2** | 13 | 5 | 13 | 31 | |
| **% Q2** | 0.419355 | 0.16129 | 0.419355 | | |
| **% total** | 0.236364 | 0.25 | 0.26 | | |
| **cell chi2** | 0.03 | 0.0003 | 0.029 | | |
| **residual** | 0.173205 | 0.017321 | 0.170294 | | |
| **Q3** | 6 | 4 | 5 | 15 | |
| **% Q3** | 0.4 | 0.266667 | 0.333333 | | |
| **% total** | 0.109091 | 0.2 | 0.1 | | |
| **cell chi2** | 0.0545 | 1.0667 | 0.1667 | | |
| **residual** | 0.233452 | 1.032812 | 0.408289 | | |
| **Q4** | 18 | 8 | 21 | 47 | |
| **% Q4** | 0.382979 | 0.170213 | 0.446809 | | |
| **% total** | 0.327273 | 0.4 | 0.42 | | |
| **cell chi2** | 0.3473 | 0.0306 | 0.2574 | | |
| **residual** | 0.589322 | 0.174929 | 0.507346 | | |
| **total** | 55 | 20 | 50 | | |
| | | | | | |
| **2015** | males | queens | workers | total | |
| **Q1** | 2 | 3 | 8 | 13 | |
| **% Q1** | 0.153846 | 0.230769 | 0.615385 | | |
| **% total** | 0.095238 | 0.15 | 0.347826 | | |
| **cell chi2** | 1.2034 | 0.2779 | 2.3709 | | |
| **residual** | 1.096996 | 0.527162 | 1.539773 | | |
| **Q2** | 6 | 7 | 5 | 18 | |
| **% Q2** | 0.333333 | 0.388889 | 0.277778 | | |
| **% total** | 0.285714 | 0.35 | 0.217391 | | |
| **cell chi2** | 0.0015 | 0.3361 | 0.3335 | | |
| **residual** | 0.03873 | 0.579741 | 0.577495 | | |
| **Q3** | 8 | 4 | 0 | 12 | |
| **% Q3** | 0.666667 | 0.333333 | 0 | | |
| **% total** | 0.380952 | 0.2 | 0 | | |
| **cell chi2** | 4.1915 | 0.0167 | 4.3125 | | |

| | | | | | |
|---|---|---|---|---|---|
| **residual** | 2.047315 | 0.129228 | 2.076656 | | |
| **Q4** | 5 | 6 | 10 | 21 | |
| **% Q4** | 0.238095 | 0.285714 | 0.47619 | | |
| **% total** | 0.238095 | 0.3 | 0.434783 | | |
| **cell chi2** | 0.5187 | 0.0482 | 0.7974 | | |
| **residual** | 0.720208 | 0.219545 | 0.892973 | | |
| **total** | 21 | 20 | 23 | | |
| | | | | | |
| **2016** | males | queens | workers | total | |
| **Q1** | 0 | 0 | 0 | 0 | |
| **% Q1** | | | | | |
| **% total** | | | | | |
| **cell chi2** | | | | | |
| **residual** | | | | | |
| **Q2** | 12 | 8 | 14 | 34 | |
| **% Q2** | 0.352941 | 0.235294 | 0.411765 | | |
| **% total** | 0.428571 | 0.444444 | 0.636364 | | |
| **cell chi2** | 0.2857 | 0.1111 | 0.8182 | | |
| **residual** | 0.534509 | 0.333317 | 0.904544 | | |
| **Q3** | 6 | 4 | 2 | 12 | |
| **% Q3** | 0.5 | 0.333333 | 0.166667 | | |
| **% total** | 0.214286 | 0.222222 | 0.090909 | | |
| **cell chi2** | 0.2269 | 0.2135 | 0.9127 | | |
| **residual** | 0.47634 | 0.462061 | 0.955353 | | |
| **Q4** | 10 | 6 | 6 | 22 | |
| **% Q4** | 0.454545 | 0.272727 | 0.272727 | | |
| **% total** | 0.357143 | 0.333333 | 0.272727 | | |
| **cell chi2** | 0.0978 | 0.0053 | 0.1755 | | |
| **residual** | 0.31273 | 0.072801 | 0.418927 | | |
| **total** | 28 | 18 | 22 | | |

| | | | | | |
|---|---|---|---|---|---|
| **Colony 3** | | | | | |
| **2013** | males | queens | workers | total | |

| | | | | | |
|---|---|---|---|---|---|
| **Q1** | 14 | 8 | 0 | 22 | |
| **% Q1** | 0.636364 | 0.363636 | 0 | | a=.0034 |
| **% total** | 0.259259 | 0.186047 | 0 | | critical value = 2.93 |
| **cell chi2** | 3.0727 | 0.1406 | 6.1926 | | |
| **residual** | 1.752912 | 0.374967 | 2.488494 | | |
| **Q2** | 15 | 13 | 12 | 40 | |
| **% Q2** | 0.375 | 0.325 | 0.3 | | |
| **% total** | 0.277778 | 0.302326 | 0.315789 | | |
| **cell chi2** | 0.0625 | 0.0053 | 0.0487 | | |
| **residual** | 0.25 | 0.072801 | 0.220681 | | |
| **Q3** | 5 | 6 | 0 | 11 | |
| **% Q3** | 0.454545 | 0.545455 | 0 | | |
| **% total** | 0.092593 | 0.139535 | 0 | | |
| **cell chi2** | 0.0818 | 1.7785 | 3.0963 | | |
| **residual** | 0.286007 | 1.333604 | 1.759631 | | |
| **Q4** | 8 | 4 | 14 | 26 | |
| **% Q4** | 0.307692 | 0.153846 | 0.538462 | | |
| **% total** | 0.148148 | 0.093023 | 0.368421 | | |
| **cell chi2** | 0.5538 | 2.2135 | 6.0999 | | |
| **residual** | 0.744177 | 1.487784 | 2.469798 | | |
| **Q5** | 12 | 12 | 12 | 36 | |
| **% Q5** | 0.333333 | 0.333333 | 0.333333 | | |
| **% total** | 0.222222 | 0.27907 | 0.315789 | | |
| **cell chi2** | 0.4 | 0.0248 | 0.3439 | | |
| **residual** | 0.632456 | 0.15748 | 0.58643 | | |
| **total** | 54 | 43 | 38 | | |

| **Colony 4** | | | | | |
|---|---|---|---|---|---|
| **2013** | males | queens | workers | total | |
| **Q1** | 34 | 23 | 40 | 97 | |
| **% Q1** | 0.350515 | 0.237113 | 0.412371 | | |
| **% total** | 0.62963 | 0.676471 | 0.851064 | | a=.0085 |

| | | | | | |
|---|---|---|---|---|---|
| **cell chi2** | 0.5938 | 0.0837 | 1.1492 | | critical value = 2.63 |
| **residual** | 0.770584 | 0.28931 | 1.072007 | | |
| **Q2** | 20 | 11 | 7 | 38 | |
| **% Q2** | 0.526316 | 0.289474 | 0.184211 | | |
| **% total** | 0.37037 | 0.323529 | 0.148936 | | |
| **cell chi2** | 1.5159 | 0.2136 | 2.9334 | | |
| **residual** | 1.231219 | 0.462169 | 1.712717 | | |
| **total** | 54 | 34 | 47 | | |
| | | | | | |
| **2014** | males | queens | workers | total | |
| **Q1** | 27 | 32 | 43 | 102 | |
| **% Q1** | 0.264706 | 0.313725 | 0.421569 | | |
| **% total** | 0.45 | 0.744186 | 0.754386 | | |
| **cell chi2** | 3.3088 | 0.7677 | 1.2216 | | |
| **residual** | 1.819011 | 0.876185 | 1.10526 | | |
| **Q2** | 33 | 11 | 14 | 58 | |
| **% Q2** | 0.568966 | 0.189655 | 0.241379 | | |
| **% total** | 0.55 | 0.255814 | 0.245614 | | |
| **cell chi2** | 5.819 | 1.3501 | 2.1483 | | |
| **residual** | 2.41226 | 1.161938 | 1.465708 | | |
| **total** | 60 | 43 | 57 | | |
| | | | | | |
| **2015** | males | queens | workers | total | |
| **Q1** | 38 | 35 | 22 | 95 | |
| **% Q1** | 0.4 | 0.368421 | 0.231579 | | |
| **% total** | 0.633333 | 0.833333 | 0.407407 | | |
| **cell chi2** | 0.0585 | 3.4717 | 3.6027 | | |
| **residual** | 0.241868 | 1.86325 | 1.898078 | | |
| | | | | | |
| **Q2** | 22 | 7 | 32 | 61 | |
| **% Q2** | 0.360656 | 0.114754 | 0.52459 | | |
| **% total** | 0.366667 | 0.166667 | 0.592593 | | |
| **cell chi2** | 0.091 | 5.4067 | 5.6108 | | |
| **residual** | 0.301662 | 2.325231 | 2.368713 | | |
| **total** | 60 | 42 | 54 | | |

| Colony 5 | | | | | |
|---|---|---|---|---|---|
| **2014** | males | queens | workers | total | |
| **Q1** | 30 | 23 | 14 | 67 | |
| **% Q1** | 0.447761 | 0.343284 | 0.208955 | | |
| **% total** | 0.5 | 0.425926 | 0.378378 | | a=.0085 |
| **cell chi2** | 0.4285 | 0.0385 | 0.3559 | | critical value = 2.63 |
| **residual** | 0.654599 | 0.196214 | 0.596574 | | |
| **Q2** | 30 | 31 | 23 | 84 | |
| **% Q2** | 0.357143 | 0.369048 | 0.27381 | | |
| **% total** | 0.5 | 0.574074 | 0.621622 | | |
| **cell chi2** | 0.3418 | 0.0307 | 0.2839 | | |
| **residual** | 0.584637 | 0.175214 | 0.532823 | | |
| **total** | 60 | 54 | 37 | | |
| | | | | | |
| **2015** | males | queens | workers | total | |
| **Q1** | 14 | 7 | 23 | 44 | |
| **% Q1** | 0.318182 | 0.159091 | 0.522727 | | |
| **% total** | 0.466667 | 0.368421 | 0.793103 | | |
| **cell chi2** | 0.5049 | 1.2897 | 2.696 | | |
| **residual** | 0.710563 | 1.13565 | 1.64195 | | |
| **Q2** | 16 | 12 | 6 | 34 | |
| **% Q2** | 0.470588 | 0.352941 | 0.176471 | | |
| **% total** | 0.533333 | 0.631579 | 0.206897 | | |
| **cell chi2** | 0.6534 | 1.669 | 3.4889 | | |
| **residual** | 0.808332 | 1.291898 | 1.86786 | | |
| **total** | 30 | 19 | 29 | | |

| Colony 6 | | | | | |
|---|---|---|---|---|---|
| **2014** | males | queens | workers | total | |
| **Q1** | 9 | 8 | 0 | 17 | |
| **% Q1** | 0.529412 | 0.470588 | 0 | | a=.0021 |

| | | | | | |
|---|---|---|---|---|---|
| **% total** | 0.310345 | 0.363636 | 0 | | critical value = 3.08 |
| **cell chi2** | 0.5439 | 1.3215 | 4.6143 | | |
| **residual** | 0.737496 | 1.149565 | 2.148092 | | |
| **Q2** | 3 | 2 | 3 | 8 | |
| **% Q2** | 0.375 | 0.25 | 0.375 | | |
| **% total** | 0.103448 | 0.090909 | 0.157895 | | |
| **cell chi2** | 0.0298 | 0.1052 | 0.3162 | | |
| **residual** | 0.172627 | 0.324345 | 0.562317 | | |
| **Q3** | 5 | 0 | 4 | 9 | |
| **% Q3** | 0.555556 | 0 | 0.444444 | | |
| **% total** | 0.172414 | 0 | 0.210526 | | |
| **cell chi2** | 0.4336 | 2.8286 | 0.9926 | | |
| **residual** | 0.658483 | 1.681844 | 0.996293 | | |
| **Q4** | 4 | 1 | 3 | 8 | |
| **% Q4** | 0.5 | 0.125 | 0.375 | | |
| **% total** | 0.137931 | 0.045455 | 0.157895 | | |
| **cell chi2** | 0.1419 | 0.912 | 0.3162 | | |
| **residual** | 0.376696 | 0.954987 | 0.562317 | | |
| **Q5** | 2 | 4 | 0 | 6 | |
| **% Q5** | 0.333333 | 0.666667 | 0 | | |
| **% total** | 0.068966 | 0.181818 | 0 | | |
| **cell chi2** | 0.0949 | 2.3706 | 1.6286 | | |
| **residual** | 0.308058 | 1.539675 | 1.276166 | | |
| **Q6** | 2 | 4 | 6 | 12 | |
| **% Q6** | 0.166667 | 0.333333 | 0.5 | | |
| **% total** | 0.068966 | 0.181818 | 0.315789 | | |
| **cell chi2** | 1.776 | 0.0139 | 2.3098 | | |
| **residual** | 1.332666 | 0.117898 | 1.519803 | | |
| **Q7** | 2 | 0 | 2 | 4 | |
| **% Q7** | 0.5 | 0 | 0.5 | | |
| **% total** | 0.068966 | 0 | 0.105263 | | |
| **cell chi2** | 0.0709 | 1.2571 | 0.7699 | | |
| **residual** | 0.266271 | 1.121205 | 0.877439 | | |

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|---|---|---|---|---|---|
| **Q8** | 2 | 3 | 1 | 6 | |
| **% Q8** | 0.333333 | 0.5 | 0.166667 | | |
| **% total** | 0.068966 | 0.136364 | 0.052632 | | |
| **cell chi2** | 0.0949 | 0.6584 | 0.2426 | | |
| **residual** | 0.308058 | 0.811419 | 0.492544 | | |
| **total** | 29 | 22 | 19 | | |
| | | | | | |
| | | | | | |
| **2015** | males | queens | workers | total | |
| **Q1** | 3 | 7 | 3 | 13 | |
| **% Q1** | 0.230769 | 0.538462 | 0.230769 | | |
| **% total** | 0.125 | 0.291667 | 0.130435 | | |
| **cell chi2** | 0.4424 | 1.545 | 0.3484 | | |
| **residual** | 0.665132 | 1.24298 | 0.590254 | | |
| **Q2** | 4 | 4 | 6 | 14 | |
| **% Q2** | 0.285714 | 0.285714 | 0.428571 | | |
| **% total** | 0.166667 | 0.166667 | 0.26087 | | |
| **cell chi2** | 0.1133 | 0.1133 | 0.4731 | | |
| **residual** | 0.336601 | 0.336601 | 0.687823 | | |
| **Q3** | 1 | 1 | 0 | 2 | |
| **% Q3** | 0.5 | 0.5 | 0 | | |
| **% total** | 0.041667 | 0.041667 | 0 | | |
| **cell chi2** | 0.1552 | 0.1552 | 0.6479 | | |
| **residual** | 0.393954 | 0.393954 | 0.804922 | | |
| **Q4** | 3 | 1 | 2 | 6 | |
| **% Q4** | 0.5 | 0.166667 | 0.333333 | | |
| **% total** | 0.125 | 0.041667 | 0.086957 | | |
| **cell chi2** | 0.4657 | 0.5212 | 0.0016 | | |
| **residual** | 0.682422 | 0.721942 | 0.04 | | |
| **Q5** | 4 | 0 | 0 | 4 | |
| **% Q5** | 1 | 0 | 0 | | |
| **% total** | 0.166667 | 0 | 0 | | |
| **cell chi2** | 5.1854 | 1.3521 | 1.2958 | | |
| **residual** | 2.277147 | 1.162798 | 1.138332 | | |
| **Q6** | 3 | 0 | 3 | 6 | |

| | | | | | |
|---|---|---|---|---|---|
| **% Q6** | 0.5 | 0 | 0.5 | | |
| **% total** | 0.125 | 0 | 0.130435 | | |
| **cell chi2** | 0.4657 | 2.0282 | 0.5741 | | |
| **residual** | 0.682422 | 1.424149 | 0.757694 | | |
| **Q7** | 6 | 0 | 0 | 6 | |
| **% Q7** | 1 | 0 | 0 | | |
| **% total** | 0.25 | 0 | 0 | | |
| **cell chi2** | 7.7782 | 2.0282 | 1.9437 | | |
| **residual** | 2.788942 | 1.424149 | 1.394166 | | |
| **Q8** | 0 | 11 | 9 | 20 | |
| **% Q8** | 0 | 0.55 | 0.45 | | |
| **% total** | 0 | 0.458333 | 0.391304 | | |
| **cell chi2** | 6.7606 | 2.6585 | 0.981 | | |
| **residual** | 2.600115 | 1.630491 | 0.990454 | | |
| **total** | 24 | 24 | 23 | | |

| | | | | | |
|---|---|---|---|---|---|
| **Colony 7** | | | | | |
| **2015** | males | queens | workers | total | |
| **Q1** | 11 | 7 | 5 | 23 | |
| **% Q1** | 0.478261 | 0.304348 | 0.217391 | | |
| **% total** | 0.37931 | 0.28 | 0.2 | | a=.0057 |
| **cell chi2** | 0.7744 | 0.0107 | 0.7133 | | critical value = 2.76 |
| **residual** | 0.88 | 0.103441 | 0.844571 | | |
| **Q2** | 9 | 4 | 6 | 19 | |
| **% Q2** | 0.473684 | 0.210526 | 0.315789 | | |
| **% total** | 0.310345 | 0.16 | 0.24 | | |
| **cell chi2** | 0.5881 | 0.6737 | 0 | | |
| **residual** | 0.766877 | 0.820792 | 0 | | |
| **Q3** | 9 | 14 | 14 | 37 | |
| **% Q3** | 0.243243 | 0.378378 | 0.378378 | | |
| **% total** | 0.310345 | 0.56 | 0.56 | | |

| | | | | | |
|---|---|---|---|---|---|
| **cell chi2** | 1.5459 | 0.4483 | 0.4483 | | |
| **residual** | 1.243342 | 0.669552 | 0.669552 | | |
| **total** | 29 | 25 | 25 | | |

| **Colony 8** | | | | | |
|---|---|---|---|---|---|
| **2015** | males | queens | workers | total | |
| **Q1** | 5 | 13 | 10 | 28 | |
| **% Q1** | 0.178571 | 0.464286 | 0.357143 | | a=.0085 |
| **% total** | 0.166667 | 0.565217 | 0.4 | | critical value = 2.63 |
| **cell chi2** | 3.0907 | 2.7254 | 0.1172 | | |
| **residual** | 1.758039 | 1.650879 | 0.342345 | | |
| **Q2** | 25 | 10 | 15 | 50 | |
| **% Q2** | 0.5 | 0.2 | 0.3 | | |
| **% total** | 0.833333 | 0.434783 | 0.6 | | |
| **cell chi2** | 1.7308 | 1.5262 | 0.0656 | | |
| **residual** | 1.315599 | 1.235395 | 0.256125 | | |
| **total** | 30 | 23 | 25 | | |

| **Colony 9** | | | | | |
|---|---|---|---|---|---|
| **2015** | males | queens | workers | total | |
| **Q1** | 26 | 8 | 10 | 44 | |
| **% Q1** | 0.590909 | 0.181818 | 0.227273 | | a=.0057 |
| **% total** | 0.481481 | 0.195122 | 0.222222 | | critical value = 2.76 |
| **cell chi2** | 4.8031 | 1.8525 | 1.2136 | | |
| **residual** | 2.191598 | 1.361066 | 1.101635 | | |
| **Q2** | 17 | 16 | 14 | 47 | |
| **% Q2** | 0.361702 | 0.340426 | 0.297872 | | |
| **% total** | 0.314815 | 0.390244 | 0.311111 | | |
| **cell chi2** | 0.0703 | 0.3631 | 0.0811 | | |

| | | | | | |
|---|---|---|---|---|---|
| **residual** | 0.265141 | 0.602578 | 0.284781 | | |
| **Q3** | 11 | 17 | 21 | 49 | |
| **% Q3** | 0.22449 | 0.346939 | 0.428571 | | |
| **% total** | 0.203704 | 0.414634 | 0.466667 | | |
| **cell chi2** | 3.3021 | 0.4894 | 1.75 | | |
| **residual** | 1.817168 | 0.699571 | 1.322876 | | |
| **total** | 54 | 41 | 45 | | |

| **Colony 10** | | | | | |
|---|---|---|---|---|---|
| **2014** | males | queens | workers | total | |
| **Q1** | 6 | 10 | 14 | 30 | |
| **% Q1** | 0.2 | 0.333333 | 0.466667 | | |
| **% total** | 0.214286 | 0.37037 | 0.636364 | | a=.00426 |
| **cell chi2** | 2.2091 | 0.0257 | 3.4381 | | critical value = 2.86 |
| **residual** | 1.486304 | 0.160312 | 1.854211 | | |
| **Q2** | 11 | 1 | 0 | 12 | |
| **% Q2** | 0.916667 | 0.083333 | 0 | | |
| **% total** | 0.392857 | 0.037037 | 0 | | |
| **cell chi2** | 10.928 | 2.4454 | 3.4286 | | |
| **residual** | 3.305753 | 1.563777 | 1.851648 | | |
| **Q3** | 4 | 7 | 2 | 13 | |
| **% Q3** | 0.307692 | 0.538462 | 0.153846 | | |
| **% total** | 0.142857 | 0.259259 | 0.090909 | | |
| **cell chi2** | 0.1119 | 1.3077 | 0.7912 | | |
| **residual** | 0.334515 | 1.143547 | 0.889494 | | |
| **Q4** | 7 | 9 | 6 | 22 | |
| **% Q4** | 0.318182 | 0.409091 | 0.272727 | | |
| **% total** | 0.25 | 0.333333 | 0.272727 | | |
| **cell chi2** | 0.125 | 0.2143 | 0.013 | | |
| **residual** | 0.353553 | 0.462925 | 0.114018 | | |
| **total** | 28 | 27 | 22 | | |

| | | | | | |
|---|---|---|---|---|---|
| **2016** | males | queens | workers | total | |
| **Q1** | 12 | 8 | 7 | 27 | |
| **% Q1** | 0.444444 | 0.296296 | 0.259259 | | |
| **% total** | 0.413793 | 0.32 | 0.318182 | | |
| **cell chi2** | 0.2796 | 0.0875 | 0.0851 | | |
| **residual** | 0.528772 | 0.295804 | 0.291719 | | |
| **Q2** | 0 | 2 | 2 | 4 | |
| **% Q2** | 0 | 0.5 | 0.5 | | |
| **% total** | 0 | 0.08 | 0.090909 | | |
| **cell chi2** | 1.5263 | 0.3558 | 0.6124 | | |
| **residual** | 1.235435 | 0.59649 | 0.78256 | | |
| **Q3** | 5 | 0 | 3 | 8 | |
| **% Q3** | 0.625 | 0 | 0.375 | | |
| **% total** | 0.172414 | 0 | 0.136364 | | |
| **cell chi2** | 1.2423 | 2.6316 | 0.2022 | | |
| **residual** | 1.114585 | 1.622221 | 0.449667 | | |
| **Q4** | 12 | 15 | 10 | 37 | |
| **% Q4** | 0.324324 | 0.405405 | 0.27027 | | |
| **% total** | 0.413793 | 0.6 | 0.454545 | | |
| **cell chi2** | 0.3179 | 0.6575 | 0.0471 | | |
| **residual** | 0.563826 | 0.810864 | 0.217025 | | |
| **total** | 29 | 25 | 22 | | |

| **Colony 11** | | | | | |
|---|---|---|---|---|---|
| **2015** | males | queens | workers | total | |
| **Q1** | 2 | 11 | 9 | 22 | |
| **% Q1** | 0.090909 | 0.5 | 0.409091 | | a=.0024 |
| **% total** | 0.068966 | 0.55 | 0.5 | | critical value = 3.04 |
| **cell chi2** | 5.9425 | 2.9922 | 1.615 | | |

| | | | | | |
|---|---|---|---|---|---|
| **residual** | 2.437724 | 1.729798 | 1.270827 | | |
| **Q2** | 1 | 0 | 0 | 1 | |
| **% Q2** | 1 | 0 | 0 | | |
| **% total** | 0.034483 | 0 | 0 | | |
| **cell chi2** | 0.7432 | 0.2985 | 0.2687 | | |
| **residual** | 0.86209 | 0.546352 | 0.518363 | | |
| **Q3** | 3 | 2 | 3 | 8 | |
| **% Q3** | 0.375 | 0.25 | 0.375 | | |
| **% total** | 0.103448 | 0.1 | 0.166667 | | |
| **cell chi2** | 0.0618 | 0.0631 | 0.3368 | | |
| **residual** | 0.248596 | 0.251197 | 0.580345 | | |
| **Q4** | 13 | 6 | 3 | 22 | |
| **% Q4** | 0.590909 | 0.272727 | 0.136364 | | |
| **% total** | 0.448276 | 0.3 | 0.166667 | | |
| **cell chi2** | 1.27 | 0.049 | 1.4332 | | |
| **residual** | 1.126943 | 0.221359 | 1.197163 | | |
| **Q5** | 5 | 1 | 2 | 8 | |
| **% Q5** | 0.625 | 0.125 | 0.25 | | |
| **% total** | 0.172414 | 0.05 | 0.111111 | | |
| **cell chi2** | 0.6825 | 0.8068 | 0.0104 | | |
| **residual** | 0.826136 | 0.89822 | 0.10198 | | |
| **Q6** | 2 | 0 | 0 | 2 | |
| **% Q6** | 1 | 0 | 0 | | |
| **% total** | 0.068966 | 0 | 0 | | |
| **cell chi2** | 1.4864 | 0.597 | 0.5373 | | |
| **residual** | 1.21918 | 0.772658 | 0.733008 | | |
| **Q7** | 3 | 0 | 1 | 4 | |
| **% Q7** | 0.75 | 0 | 0.25 | | |
| **% total** | 0.103448 | 0 | 0.055556 | | |
| **cell chi2** | 0.9296 | 1.194 | 0.0052 | | |
| **residual** | 0.964158 | 1.092703 | 0.072111 | | |
| **total** | 29 | 20 | 18 | | |
| | | | | | |
| **2016** | males | queens | workers | total | |
| **Q1** | 8 | 3 | 2 | 13 | |

| | | | | | |
|---|---|---|---|---|---|
| **% Q1** | 0.615385 | 0.230769 | 0.153846 | | |
| **% total** | 0.266667 | 0.15 | 0.083333 | | |
| **cell chi2** | 1.4139 | 0.0751 | 1.1649 | | |
| **residual** | 1.189075 | 0.274044 | 1.079305 | | |
| **Q2** | 4 | 2 | 5 | 11 | |
| **% Q2** | 0.363636 | 0.181818 | 0.454545 | | |
| **% total** | 0.133333 | 0.1 | 0.208333 | | |
| **cell chi2** | 0.0473 | 0.3184 | 0.5751 | | |
| **residual** | 0.217486 | 0.564269 | 0.758353 | | |
| **Q3** | 6 | 9 | 3 | 18 | |
| **% Q3** | 0.333333 | 0.5 | 0.166667 | | |
| **% total** | 0.2 | 0.45 | 0.125 | | |
| **cell chi2** | 0.2306 | 3.5149 | 1.3759 | | |
| **residual** | 0.480208 | 1.874807 | 1.172988 | | |
| **Q4** | 0 | 1 | 3 | 4 | |
| **% Q4** | 0 | 0.25 | 0.75 | | |
| **% total** | 0 | 0.05 | 0.125 | | |
| **cell chi2** | 1.6216 | 0.0061 | 2.2348 | | |
| **residual** | 1.273421 | 0.078102 | 1.494925 | | |
| **Q5** | 3 | 0 | 1 | 4 | |
| **% Q5** | 0.75 | 0 | 0.25 | | |
| **% total** | 0.1 | 0 | 0.041667 | | |
| **cell chi2** | 1.1716 | 1.0811 | 0.0681 | | |
| **residual** | 1.082405 | 1.03976 | 0.26096 | | |
| **Q6** | 1 | 2 | 0 | 3 | |
| **% Q6** | 0.333333 | 0.666667 | 0 | | |
| **% total** | 0.033333 | 0.1 | 0 | | |
| **cell chi2** | 0.0384 | 1.7441 | 0.973 | | |
| **residual** | 0.195959 | 1.320644 | 0.986408 | | |
| **Q7** | 8 | 3 | 10 | 21 | |
| **% Q7** | 0.380952 | 0.142857 | 0.47619 | | |
| **% total** | 0.266667 | 0.15 | 0.416667 | | |
| **cell chi2** | 0.031 | 1.2614 | 1.4934 | | |
| **residual** | 0.176068 | 1.123121 | 1.222047 | | |

| | | | | | |
|---|---|---|---|---|---|
| **total** | 30 | 20 | 24 | | |