

HOW MANY MATES IS ENOUGH? EXAMINING MULTIPLE MATING IN THE HONEY  
BEE, *APIS MELLIFERA* L.

by

KATHERINE LYNN HAGAN

(Under the Direction of Keith S. Delaplane)

ABSTRACT

Honey bee (*Apis mellifera* L.) queens are polyandrous, mating with multiple males. Polyandry results in high within-colony genetic variation; however, the adaptive drivers of this behavior are poorly understood. Most hypotheses trying to explain polyandry are variants of the “genetic variance” hypothesis, which explains that high levels of polyandry provide allelic richness to a colony, contributing to a colony’s survival and success. We investigated the mechanisms underlying queen mating patterns by utilizing artificial insemination to produce queens with a range of mating numbers and comparing their colonies for measures of fitness. We found that increasing polyandry led to lower infestation rates of *Varroa destructor* mites which supports the genetic variance hypotheses. Our findings bolster previous findings on the benefits of polyandry to colony success and support an additive relationship between polyandry and colony level phenotype.

INDEX WORDS: *Apis mellifera*, polyandry, genetic variance hypotheses, rare allele model

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## DEDICATION

This thesis is dedicated in memory of Elnora Knott, Pilar Pagés Delaplane, and Amy Janvier.

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## CHAPTER 1

### INTRODUCTION AND LITERATURE REVIEW

Across the Animal Kingdom, organisms have evolved extremely complex and strikingly diverse mating systems with which to survive and pass their genes on to the next generation [26]. Polyandry is one such mating system, in which females' mate with multiple different males and males' mate with only one female [26]. Polyandry is a fundamental behavior for informing studies in mate choice, particularly sexual selection in which individuals choose a mate based on specific attributes [88]. Darwin (1859) first proposed the concept of sexual selection in the *On the Origin of Species by Means of Natural Selection* [14]. However, it was Bateman (1948) who provided the rigorous explanation of sexual differences owing to anisogamy in fruit flies [3; 68]. The ideas of Bateman carried the understanding of female sexual behavior until the 1970s, when female birds were shown to be promiscuous, beginning the notion that females were active players of the evolutionary games [88].

With the advent of DNA fingerprinting and the seminal reviews of sperm competition in insects, increasing numbers of scientists have reported polyandry in natural litters, clutches, and broods across species, leading to the recognition of polyandry as a common and ubiquitous mating system in nature [88]. Polyandry occurs across a wide variety of taxa but has most commonly been investigated in eusocial insects. In the complex eusocial Hymenoptera, polyandry plays a super-charged role in facilitating within-nest genetic variance among the workers. The evolution of a sterile worker caste, functionally recapitulating the organismal soma [5; 78], means that hymenopteran polyandry generates in a colony the functional equivalent of

organismal heterosis. As such, polyandry has been important in the evolution of complex social behaviors [26; 73] and is considered by scientists a tool for promoting colony health in managed systems [43; 87].

### **Polyandry in Eusocial Hymenoptera**

Eusociality is defined by cooperative brood care, overlapping generations of adults, and a reproductive division of labor, with a reproductive queen and a worker caste [91]. According to Hamilton's (1964) kin selection theory, individuals are more likely to cooperate if they share genes identical by descent, which explains evolution of eusociality [29]. If relatedness amongst individuals within the group remains high, altruism is selected for as the benefits of cooperation outweigh the costs [5]. However, within eusocial Hymenoptera, polyandry poses unique consequences to kin selection as it generates intracolony genetic diversity, which depletes the high level of relatedness among individuals by reducing their potential inclusive fitness [43]. The challenges of polyandry are partially due to the haplodiploid sex determination of Hymenoptera, but also the reproductive division of labor that characterizes eusociality.

Within a haplodiploid sex determination system, the sex of the individual is based on the number of chromosomes an individual receives [5]. Males receive a single set of unpaired chromosomes from their mother and are haploid, while females receive a set of chromosomes from each of their mother and father and are thus diploid [5; 31]. As males are unfertilized, they share 100% of their genes with their mother as no genetic recombination takes place [5]. In a monandrous system where the queen mates with a single male, female siblings are related by 75% and are predicted to act altruistically to increase their inclusive fitness [5; 29]. Because the level of relatedness between siblings in this scenario is 75%, daughters are predicted to prefer rearing siblings ( $r=0.75$ ) even in preference to their own offspring ( $r=0.5$ ) [5].

However, polyandry works against the inclusive fitness of workers as the level of relatedness drops with a queen's additional mates. Specifically, the average relatedness among individuals in a colony declines by the function  $0.25 + (0.5/m_e)$  where  $m_e$  is the effective mating number of the queen [86]. However, workers that share the same father, known as supersisters, are still related by 75% and are known as one subfamily or patriline [5; 31].

Due to the drop in relatedness, kin selection theory predicts that polyandry is likely to encourage conflict among colony members [38]. However, conflict in a eusocial system is uncommon, which may be because polyandry is derived and has only evolved in lineages which have lost the ability to reproduce (reproductive totipotency) due to a division of labor [18; 32; 38]. Additionally, polyandry and lower relatedness can lead to a reduction of conflict potential due to behaviors such as policing, where workers consume the eggs of other workers to prevent selfish reproduction [38].

Within Hymenoptera, polyandry weakens the level of relatedness with an increased number of mates and thereby reduces the level of inclusive fitness among colony members. Despite these apparent handicaps of polyandry within Hymenoptera, polyandry independently evolved 8 to 11 times in eusocial ants, bees, and wasps [89]. Therefore, polyandry provides an adaptive advantage, but only at the level of the group. Many hypotheses have been proposed to explain the potential costs and benefits of polyandrous behavior in the highly social insects [17; 24; 63]; however, circumstances favoring the behavior are still not completely understood.

### **Hypotheses for the Evolution of Polyandry**

Most empirical and theoretical studies focus on variants of one overarching model – the genetic variance (GV) hypotheses [34; 50]. Defined by Keller and Reeve (1994), the GV hypotheses propose that queen and colony fitness is increased by intracolony genetic variance

that results from polyandry [34; 65]. Increasing mating number causes an increase in the number of subfamilies within the colony [86]. With a higher number of subfamilies, genes for different phenotypes become varied among colony members, which enables individuals to adapt in varied ecological conditions [86].

Although the basic premise is the same among GV hypotheses, each attributes the adaptive advantage of polyandry to different levels of selection [86]. Some versions of the GV hypotheses focus on the consequences of polyandry at the colony level, while others focus on the queen as the main target of selection [38]. Due to differences in the selective forces, the GV hypotheses are typically divided into two categories.

#### *The first subset of GV hypotheses*

The first category concerns the haplodiploid sex determination system and relates the effects of haplodiploidy on brood viability [62; 63; 72] and conflict between workers and queens over preferred sex ratios [4; 48; 65]. These subsets of hypotheses suggest: (1) queens with low brood viability due to diploid male production have low fitness, and polyandry has evolved because it reduces variance in the production of diploid males among colonies [12; 54; 63; 72; 80], and (2) polyandry reduces conflict between queens and workers over preferred sex ratios [47; 54; 67; 70; 73].

Within the first category, polyandry is hypothesized to have evolved to reduce the production of diploid males, which are inviable in a haplodiploid sex determination system [60; 63]. Diploid males are homozygous at the sex locus [65; 95], and result from inbreeding. If a queen mates with a drone carrying a sex allele identical to her own, a large percentage of her eggs are inviable posing an energetic cost, as well as reduced brood viability and lower fitness

[65]. By mating with multiple males, a queen reduces the possibility of inviable drones and an unsuccessful colony [65].

The second hypothesis within the first category proposes that polyandry evolved to reduce the conflict between castes, the queen and her workers, over preferred sex ratios [47; 54; 73]. As the queen passes on 50% of her genes with every egg she lays, she is expected to favor a 1:1 ratio of male to female within the colony [16]. Workers however face a different dilemma due to polyandry. Workers that share the same father are related by 75%, while workers that have different fathers only have a probability of a 25% relatedness [5]. As drones are haploid, workers are also related to their brothers by 25%. Due to this asymmetry in relatedness between the siblings, workers are expected to prefer a 3:1 ratio of rearing sisters over brothers [16]. By practicing polyandry, a mother decreases the average relatedness amongst her daughters to ~33%, while daughter/brother relatedness remains 25%. By reducing the magnitude of this differential, polyandry very nearly restores the workers' interest in a 1:1 sex ratio in agreement with their mother [90].

#### *The second subset of GV hypotheses*

The second category of hypotheses refers to ecological fitness benefits that stem from the genetic diversity within the worker population [54; 62; 63; 65; 72]. These hypotheses propose that: (1) genetic variance allows an increased expression of caste- [13; 54] or task-specialization [7; 8; 20; 23; 54-58; 61], (2) genetic variance increases the range of environments a colony can tolerate [13; 52; 54; 57; 59; 60], and (3) genetic variance increases colonial resistance to parasites and pathogens [81-83].

According to the first hypothesis, GV promotes an increased expression of behavioral polymorphism [54]. With an increase in the number of subfamilies due to polyandry, there is a



higher level of behavioral diversity allowing for an efficient division of labor through task specialization by individual workers [50]. With a higher level of behavioral diversity, workers are predicted to increase colony level efficiency and fitness through specializing in specific tasks [17]. Such tasks include acquiring food through foraging, general nest maintenance like guarding and undertaking, and nursing the brood or the queen [42]. Due to the increase in colony efficiency, task specialization could be a selective force in the evolution of polyandry [65].

The second hypothesis is that GV increases the range of environmental conditions a colony can tolerate [13; 54; 57; 60]. Like the first hypothesis, the behavioral variation due to multiple mating, may help buffer colonies against fluctuations in the environment [9; 13; 64; 65].

The final hypothesis connected to worker diversity because of polyandry is the parasite and pathogens hypothesis. Under this hypothesis, genetically diverse colonies are more resistant to parasites and pathogens [54; 81-83], and polyandry evolved as an adaptive response to high numbers of parasites and pathogens [50]. A key assumption of this hypothesis is that susceptibility or resistance to pathogens varies between different patriline in the colony [36; 81]. Of the second category of hypotheses, the parasite and pathogens hypothesis has garnered the most empirical support. Genetically diverse colonies of honey bees have demonstrated lower incidence of diseases when compared to genetically uniform colonies [39].

Outside of the GV hypotheses, other hypotheses have been proposed, but based on a review by Crozier and Page (1985), most have been deemed improbable [13]. For example, intra-spermathecal sperm competition was initially proposed [41; 54]. However, this hypothesis was rejected by Crozier and Page (1985) because it depends on the successful males yielding high quality offspring, and there is no reason to expect a correlation between sperm competitiveness and offspring quality [13]. A second proposed hypothesis is based on the

volume of sperm a queen requires to lay successfully during her life span [11]. A queen typically lives 2 to 3 years, and continuously lays eggs throughout her lifetime [92]. However, this hypothesis also is implausible as males do not transfer their sperm directly into the spermatheca, but ejaculate into the female's sexual tract, the bursa copulatrix [2]. Due to this action, much of the spermatozoa is discarded that the queens receive during mating [13].

Although there are many different interpretations of the genetic variance hypotheses, it is likely that a variety of forces selected for the evolution of polyandry in the eusocial Hymenoptera. Multiple hypotheses, and the interpretations of those hypotheses, in concert with life history traits shaped the selection for the evolution of polyandry, and these together maintain the high rate of polyandry within *Apis*. Despite the undue focus on the numerous hypotheses seeking to explain polyandry, the effect of polyandry at the colony level remains overwhelmingly beneficial. For example, field studies indicated that polyandrous honey bee colonies demonstrated population growth, weight gain, and survival when compared to their monandrous counterparts [16].

### **Consequences and Modern Effects of Polyandry**

Polyandry is clearly an advantageous behavior, and the benefits appear to outweigh the costs. But addressing the costs and benefits for eusocial Hymenoptera requires considering both the individual and colony levels. For honey bees, the individuals considered are the queen and her workers (males, or drones, die upon mating and only incur cost to the colony for rearing them). Evolutionarily, the costs typically occur at the individual level, while the benefits occur at the level of the superorganism.

As the queen is the sole reproducer in the colony, polyandrous behavior has potential to be extremely costly for both the individual and the colony. In the early days of her life, a queen

flies to drone congregation areas where she mates with multiple males on the wing. The mating flight poses a significant energetic cost, but also incurs risk from predation, harsh weather, and exposure to disease infection [60; 75]. In other non-social species, polyandry has also been demonstrated to reduce lifespan [1], which may be an additional cost to the queen. Energetically, queens that return to the colony with an insufficient number of mates are forced to embark on additional mating flights, increasing the costs with each additional trip [38; 76].

For the other caste in the colony, the workers, polyandry is costly to the level of relatedness amongst individuals. Workers that share the same father (supersisters) are related by 75%, while workers with different fathers (subsisters) only have a probability of sharing 25% of their genes [5]. The average relatedness among workers with a polyandrous mother can be explained by the equation  $sisters = 0.25 + (0.5/k)$ , where  $k$  is the mating number of the mother. This value asymptotes to 0.3 relatedness at  $m=6$ , which means this is the average level of relatedness amongst workers is roughly 30% [5]. When considering the level of relatedness amongst the workers, it is also important to consider the cost of the worker with their reduced relatedness to the next sister who becomes a queen. Workers would prefer to rear a supersister (75%), but the vast majority tend to raise a sister with  $r=0.3$  relatedness [90].

The benefits of polyandry result from an increased level of fitness, which for the colony is measured in terms of the lifetime number of surviving swarms and drones produced [50]. GV hypotheses are closely connected to the benefits of polyandry, as the level of fitness for a colony increases by intracolony genetic variance [65]. Empirical support for the benefits of polyandry due to genetic variance has been demonstrated by stable colony homeothermy [25; 33; 43; 52; 53], reduced risk of brood pathogens [66; 79; 85], and heterozygosity maintenance at the sex locus [63; 86]. In honey bees, differences amongst patriline were also shown to influence the

type of forage [57], distance travelled to forage [58], foraging time of day [37], scouting [19; 45], and dance communication [21; 44]. Additionally, newly founded genetically diverse colonies were found to build comb faster and store greater amounts of food when compared to their genetically uniform counterparts, when controlling for the number of workers and environmental conditions [43].

Although emerging mechanisms leading to the evolution of extreme polyandry remain conjectural, the consequences of polyandry are clearly beneficial at the colony level. While the consequences of polyandry are important for understanding the maintenance of the behavior in eusocial Hymenoptera, the benefits do not explain the extreme levels of polyandry seen within the *Apis* genus.

### **Problem with Extreme Polyandry**

Queens within all *Apis* species are hyperpolyandrous because they mate more than six to ten times [54]. According to theory, a queen captures 90% of the alleles from the breeding population in her first six mates. Beyond the sixth mate, there are vanishingly marginal increases in intracolony genetic heterogeneity [5; 54; 62] [51; 54] or variation in the distribution of diploid males [62; 63]. Therefore, the current explanations for the evolution of polyandry cannot explain the extreme levels of polyandry shown by *Apis* [48; 54; 60]. Although the GV hypotheses are the most common explanations for the evolution of polyandrous behavior, they are limited. The problem with most GV hypotheses is that they assume linear increase in fitness due to genetic diversity, and do not include non-linear fitness benefits or the steep fitness increase because of extreme polyandry [38]. Additionally, GV hypotheses are problematic as the benefits of GV are a function of selection acting on the colonies after mating has taken place [86], which explains the maintenance of polyandry, but not the evolution of the behavior.

Fuchs and Moritz (1998) attempted to explain nonlinear fitness benefits that occur through polyandry with a worker ‘specialist’ model, in which specialist workers needed to be rare in the colony [24; 38]. However, there is little empirical support for this model, and often conflicting evidence that genetically diverse colonies perform better as a collective (too many cooks in the kitchen?) than less genetically diverse colonies [25; 33; 43; 50; 57; 64], as too many worker specialists, would provide additional cost at the colony level [38].

Further research is required to understand how fitness benefits accrue in *Apis* and the mechanisms of worker behavior that may explain the seemingly stochastic mating behavior in *Apis*. If polyandrous behavior were simply a matter of collecting genes, a queen would be expected to stop at around 6 mates. However, for the western honey bee, *Apis mellifera* L, a queen often doubles mates with the species average at around 12 mates [16]. But, based on microsatellites, outliers as high as 44 and 77 mates are present in the population [93]. Thus, more work is needed to understand how GV extends to colony fitness and phenotype.

Utilizing a large range of polyandrous values, the goal of this work is to address how the adaptive benefits and costs of polyandry occur. This is the first study to assess the effects of polyandry across a range of values elucidating the mechanisms encouraging honey bee queens to mate multiply. Previous studies have demonstrated that colony fitness increases with high levels of polyandry, but the mechanisms encouraging queens to mate at such high rates has yet to be elucidated. Understanding these mechanisms of multiple mating in honey bees may lead to broad changes in traditional trait-based genetic selection as well as further understanding for the evolution of polyandry in social insects more generally.

## CHAPTER 2

### HOW MANY MATES IS ENOUGH? EXAMINING MULTIPLE MATING IN THE HONEY BEE, *APIS MELLIFERA* L.<sup>1</sup>

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<sup>1</sup> Hagan, Katherine, Bartlett, Lewis J., and Delaplane, Keith S. To be submitted to the Journal of Functional Ecology

## ABSTRACT

In the first weeks of her life, a honey bee (*Apis mellifera* L) queen mates on the wing with several males, storing their sperm in her spermatheca and using it thereafter to fertilize all her female worker progeny. This behavior is known as polyandry and is a feature of all highly eusocial Hymenoptera. Most hypotheses seeking to explain polyandry are variants the genetic variance hypothesis, which suggests that higher levels of polyandry provide a diverse set of genetics to the colony contributing to colony survival and success. However, the extreme level at which honey bee queens' mate is poorly understood. Theory dictates that 90% of the population alleles is captured in a queen's first 6 matings, yet the species average is ~12 mates and outliers as high as 77 are known. In this study, we sought to investigate the mechanisms underlying honey bee queen patterns and to determine the effect of polyandry on colony fitness. We utilized artificial insemination to produce queens with a range of mating numbers – each inseminated with 1, 2, 4, 8, 16, or 32 males – and compared their colonies for measures of fitness, including colony bee populations, and colony load of the economically-damaging mite parasite *Varroa destructor*. Using a generalized linear mixed model, we found extreme polyandry led to lower infestation rates of mites through increasing levels of polyandry, which supports the hypotheses that the benefits of polyandry accrue additively. These results suggest that the relationship between polyandry and colony fitness is driven by ecologic competitiveness deriving from increased genetic variance in the colony. This could explain why honey bee queens routinely mate at double the rate theory predicts. Our findings bolster previous evidence for the benefits of polyandry to colony success and suggest that benefits of

increasing polyandry accrue additively.

## INTRODUCTION

Across the Animal Kingdom, organisms have evolved extremely complex and strikingly diverse mating systems with which to survive and pass their genes on to the next generation [26]. One such mating system is polyandry, in which females' mate with multiple different males and males' mate with only one female [26]. Polyandry has most commonly been investigated in eusocial Hymenoptera (the wasps, ants, and bees) because this behavior has evolved multiple times independently throughout the order, and many species, including the western honey bee, exhibit high mating numbers, termed extreme polyandry [38].

In the western honey bee, queens' mate with multiple males in a single reproductive bout, store sperm, and use it to produce their lifetime cohort of diploid worker daughters and future queens. During her single mating flight, the queen gathers enough sperm to last her 3 to 5-year life span. Theory shows that over 90% of the breeding population's alleles is captured by a queen in her first 6 mates (Figure 1) [16; 65]. However, the species average is around  $m=12$  mates [84], and frequently higher, with values up to  $m=77$  evidenced in the literature [22; 93; 94]. One effect of polyandry is reducing average worker relatedness in the colony [74], so it is believed that polyandry cannot evolve unless its colony level benefits outweigh its costs to workers [67].

Field studies conducted by Tarpy and Pettis (87), Mattila and Seeley (43), and Delaplane et al (16) show that an increase in mating frequency is positively correlated with colony performance through reduced loads of the parasitic mite *Varroa destructor*, improved productivity of workers, and increased colony success at founding new colonies [43; 46; 87]. Many hypotheses have been put forth to explain the benefits of polyandry with the primary focus set on the umbrella of genetic variance (GV) hypotheses [34], which posit that high



levels of polyandry provide a diverse set of genetics to the colony, improving overall colony survival and fitness. Although the base presumption is the same among the different genetic variance hypotheses, considerable nuance exists between the different subsets and the hypotheses themselves. Polyandry confers benefits through: (1) reducing the production of diploid males [63], (2) reducing conflict between the queen and her workers over preferred sex ratios [4; 48; 65], (3) increasing expression of caste or task specialization [54], (4) increasing the types of environments that a colony can tolerate and (5) increasing genetic heterogeneity in colonial resistance to parasites and pathogens [81].

Alternatively, Fuchs and Moritz (1998) explain the benefits of polyandry through a rare allele model, in which a queen mates with multiple males to capture rare specialist alleles leading to an increase in colony fitness [24; 38]. However, there is very little empirical support for this model and further work must be done to investigate the mechanisms motivating a honey bee queen to mate multiply.

Previous studies demonstrate the colony-level benefits of polyandrous behavior, but the mechanisms underlying this behavior are poorly understood. For example, Delaplane et al. (16) show that queens artificially inseminated with the semen of 30 or 60 drones had increased brood rearing and lower *Varroa destructor* mite infestations, than those inseminated with 15 drones [16]. On the other hand, Oldroyd et al. (57) found that colonies at higher levels of polyandry perform less well than those with lower polyandry [52]. Other studies found no effect of polyandry on colony measures [27; 50]. However, no previous work has investigated polyandry across a range of values. To investigate the link between multiple mating and colony performance, we artificially inseminated honey bee queens with 1, 2, 4, 8, 16, or 32 drones and tracked colony fitness measures over the span of 5 months.

As a response variable, we focused on a character of high fitness returns – colony levels

of the parasitic mite *Varroa destructor*. To investigate the mechanisms underpinning the benefits of polyandrous behavior in honey bees, we used artificial insemination to provide a range of polyandrous conditions and measured respective colony mite loads. By providing a geometric range of polyandry levels, we attempt to (1) illustrate how our model fitness parameter changes across a range of polyandrous conditions unmatched in the scientific literature and (2) gain understanding of the way colony level benefits accrue. If the response variable asymptotes near the  $m=6$  mark of Palmer and Oldroyd, then it simply mirrors GV predictions. If it asymptotes significantly further than  $m=6$ , this would support a rare allele hypothesis. If a linear response continues indefinitely, this suggests either that phenotypic benefits accrue additively, apparently uncoupled from the genotypic limits suggested by Palmer and Oldroyd (2000) or else that beneficial rare alleles occur in high numbers [65]. By manipulating queen mating number and measuring colony fitness, we can inform management practices such as artificial insemination, deliver guidance in optimum mating numbers, and improve managed colony health and productivity.

## MATERIALS AND METHODS

### *Study Area and Experimental Design*

The experiment was conducted at the University of Georgia Horticultural Farm (33.884783, -83.421114) in Watkinsville, Georgia during the summer of 2019 and into the spring of 2020. Colonies were each started between March 20-21, 2019 with a standard package containing one queen and 3 lb. (1.4 kg) workers housed in a single deep 10 frame super (Figure 2). Forty virgin queens were reared and artificially inseminated beginning April 15, 2019. There were 6 to 8 colonies representative of our different insemination levels.

### *Artificial Insemination*

Queens were each instrumentally inseminated with standard methods [10; 30]. Each

virgin queen was instrumentally inseminated with a ~1.5 to 2.0  $\mu$ L mixture comprised of physiological saline 15% of volume) and the semen of 1 drone, 2 drones, 4 drones, 8 drones, 16 drones, or 32 drones. Batches representing multiple drones were each expressed into an Eppendorf tube, manually mixed to approach equal representation per drone, and redrawn into the same syringe tip subsequently used for inseminating a queen. As our independent variable was polyandry *per se* and not drone source, we randomly selected 10 colonies to serve as a source for drones. Our different treatments, excluding our single inseminated queens, were each a mixture of semen from a random sampling of drones from the 10 colonies. Each queen received a semen mixture volume of ~4  $\mu$ L, thereby controlling for any effects of volume on insemination [69]. Following insemination, each queen was marked with a unique number and housed in one of the previously installed package colonies. Over the next successive two days, each queen was treated with CO<sub>2</sub> for 10 minutes to encourage egg laying [40].

#### *Colony Management and Sample Collection*

Queens were released after 1-3 days and colonies managed according to standard practices. Colonies were fed supplemental sugar solution, and additional supers were provided as required to permit colony growth. After all queens were released, all colonies were maintained for 5 months to ensure adequate queen egg laying performance and ensure that the progeny of the experimental queens were the workers we monitored. Following this initial period of attrition, the beginning numbers of queens/colonies for each insemination group were  $m=1$  ( $N=6$  colonies),  $m=2$  (7 colonies),  $m=4$  (6 colonies),  $m=8$  (7 colonies),  $m=16$  (8 colonies), and  $m=32$  (6 colonies).

#### *Dependent Variables*

The relative numbers of Varroa destructor mites were determined using sticky screens, which were placed in the screened bottom board the day of assessment and removed three

days later. The mites were then counted on the screens.

Ten colonies were selected to randomly provide drones for artificial insemination. Depending on the level of artificial insemination, drone source colonies were annotated for each queen to investigate the role of specific drone sources in colony health measures. Specific drone colonies sources were analyzed using a generalized linear mixed model to determine if patriline was driving our results.

### *Statistical Analysis*

All analyses were carried out using R [71]. The rate of colony survival during the experiment (over the course of 5 months) and the rate of colony survival post experiment (to Spring 2020) was assessed using a generalized linear model with a binomial error structure. Our other colony health metrics including total number of bees, total number of mites per colony, and mites per bee were analyzed using a generalized linear mixed model. Because it was a generalized linear mixed model, type III ANOVA and chi-square statistics were used. Using a generalized linear mixed model allowed us to account for multiple time points per colony and nesting of colonies within each polyandrous treatment. Our fixed effects were timepoint and polyandry. Only the colonies that survived the duration of the experiment were analyzed for the level of total mites. The effect of time was included as mite populations grew over the course of the experiment. Mites per bee were calculated by dividing the number of mites by adult bee population. Mites per bee were 9<sup>th</sup> root transformed to meet the assumptions of the generalized linear mixed model. Shapiro Wilcox test was used to test for normality.

## RESULTS

There was a significant effect of polyandry ( $\chi^2_{1,3} = 5.68$ ;  $p=0.017$ ) on number of mites per colony. Using a Poisson distribution, polyandry was shown to correlate negatively with

the total number of mites. Therefore, increasing levels of polyandry reduced total number of mites. To account for time between our sampling periods, timepoint was included as a categorical variable in our analysis. We found that there was a significant effect of timepoint on total number of mites ( $\chi^2_{1,3} = 1658.95$ ;  $p < 0.001$ ) such that timepoint was positively correlated to total number of mites (Figure A.1). This indicates that throughout our sampling periods, there was an increase in the level of mites, but the more polyandrous colonies had a reduction in total mite load.

To adjust for differences in colony size, mites per bee were calculated by dividing the number of mites, which scales with colony size, by bee population. In checking the distribution of our data through histograms (Figure A.2), mites per bee were 9<sup>th</sup> root transformed to meet the assumptions of the generalized linear model (Figure A.3). Shapiro Wilcox test was used to determine normality ( $w = 0.992$ ;  $p = 0.622$ ). Using a generalized linear mixed model with a Gaussian error structure, we found that polyandry had a significant effect on the number of mites per bee ( $\chi^2_{1,4} = 6.78$ ;  $p = 0.009$ ), and was negatively correlated with number of mites. Timepoint had a positive effect ( $\chi^2_{1,4} = 101.15$ ;  $p < 0.001$ ) on the number of mites per bee present in the colonies and was positively correlated to the number of mites per bee. Therefore, mite numbers predictably increased over time, but within timepoint polyandry had a consistent depressing effect on per capita mite numbers (Figure 3). Gaussian generalized linear mixed models were graphically inspected for goodness of fit (Figure 4).

Finally, we assessed the source colonies of our drones using a generalized linear mixed model with a Gaussian error structure. Polyandry was excluded as it was not a predictor of sperm source, and our colonies were treated as random factors. We found no evidence that any specific sperm source significantly decreased mite load (Table 1).

## DISCUSSION

In this study, we instrumentally inseminated honey bee queens to determine the effect of polyandry on colony fitness. Our findings demonstrate that increasing levels of polyandry reduce *Varroa destructor* mite loads, adding to a growing body of literature on the benefits of polyandry to honey bee colony health. Previous studies assessing honey bee health in terms of queen mating number have included a narrower range of insemination values [16; 42], and no work has approached the range of polyandrous values provided here.

Our results provide support for the genetic variance hypotheses, particularly the subset of hypotheses related to colonial resistance to parasites and pathogens (hypothesis (5) in the introduction). Our findings also challenge what theory predicts is the maximum at which the benefits of polyandrous behavior occur, as phenotypic benefits extended beyond what we would expect based on an average mating asymptote of  $m=6$ . Either phenotypic effects accrue additively, apparently uncoupled from the asymptote predicted by classic GV theory [65], or else rare alleles, however rare individually, nevertheless occur in high enough numbers that their acquisition produces a linear effect.

As part of our investigation of the rare allele model, we assessed the source colonies of drones to determine if there was a relationship between any drone source and the level of mites per bee. We found no evidence that any drone colonies were associated with mites per bee (Table 1). This is inconsistent with the idea of a rare allele model, in which queens' mate with multiple males to capture rare specialist alleles in the population. Support for the rare allele model would show a significant effect of some, but not all, of the source colonies within the population at reducing mites per bee.

Although we found no support for the rare allele model, there are potential routes by

which such a mechanism could express. Moritz et al (49) first proposed the idea of rare “royal” subfamilies, in which emergency queens reared during a supersedure event were reared from rare “royal” subfamilies, which were undetected or present at low frequency in the worker population. Withrow and Tarpy (93) further tested the “royal” patriline theory by genotype analysis of sampled workers and found that cryptic subfamilies are common across colonies and frequently undetected in traditional tests of queen mating number and colony composition. Thus, in addressing the rare allele model, genotype analysis may be necessary to fully reject the rare allele model.

Genotype analysis is also useful for appraising the uniformity of progeny expression by patrilines in a queen’s spermatheca. During copulation, drones ejaculate into the female’s sexual tract, the bursa copulatrix [2]. When a queen returns from her mating flight, she begins storing the sperm in her spermatheca [6; 35]. Therefore, there may be considerable variation in the amount of sperm a queen receives from a focal drone, either due to lost semen during her mating flight [95] or by sperm competition [30]. Differences in drone contribution may thus provide an unknown source of variation for queen mating levels.

To our knowledge, no other studies have investigated a range of insemination levels comparable to those we used here. We used a geometric progression in mating number to examine a wide range of polyandry more economically, and despite this still showed only additive change in phenotype. This begs the question how phenotype can accrue additively while alleles accrue non-additively. What mechanisms obtain this? At what point does phenotype asymptote, if not  $m=6$  as theory predicts? Unfortunately, our design is insufficient to these questions.

In any case, it seems likely that tradeoffs between costs and benefits of polyandry are a delicate balance between individual- and colony-level fitness. The known constraints to

mating number include predation rates as well as sexually transmitted diseases that are unlikely to completely disappear. Assessing when the benefits of polyandry asymptote is necessary to further informing our understanding of the evolution of this mating behavior in the western honey bee.

Secondarily, our results convincingly show that more polyandrous colonies have less per-capita mites, which may occur through a few different mechanisms. A simple interpretation of the GV hypotheses would suggest that polyandrous behavior evolves due to genotypic variation in the colony, promoting resilience (and resistance) to parasites and pathogens [77]. However, could synergistic interactions between workers leading to further hygienic behavior also play a role? Previous work has demonstrated that colonies headed by queens inseminated with multiple males are healthier than colonies headed by a single inseminated queen due to an increase in the flow of nutrients into a honey bee colony and the rate it is distributed among colony members [21]. Support has also been found for the better recruitment and signal communication of foragers of genetically diverse colonies when compared to their less genetically diverse counterparts [28].

Emergent properties may also provide explanation for the phenotypic benefits that appear to accrue past  $m=6$ . Emergent properties refer to the creation of order out of pre-existing individuality [15]. For the honey bee, the superorganism provides a level of organization that would not be possible at the level of individual. Resistance to mites with increasing polyandry may be an artifact of unknown structural elements that emerge out of complexified sociality, elements that could obtain in genetically diverse eusocial organisms in general.

Using a range of artificial insemination treatments has demonstrated that the level of mites per bee decreases with increasing levels of polyandry, and that instrumental



insemination can deliver this benefit. Investigating low cost alternatives to deliver the benefits of artificial insemination may be valuable for future research. Polyandry, delivered through artificial insemination, may be important to mitigate the negative impacts of parasites and pathogens within honey bee colonies. Investigating the performance of honey bee colonies at different levels of polyandry has potential to completely change traditional trait-based beekeeping. While beekeepers often select for traits like temperament or honey production, these may come at a cost to colony health. By understanding the mechanisms by which honey bees have marshalled genetics to solve problems in evolutionary time, we can better deliver those mechanisms to maximize colony productivity in managed systems.

## FIGURES

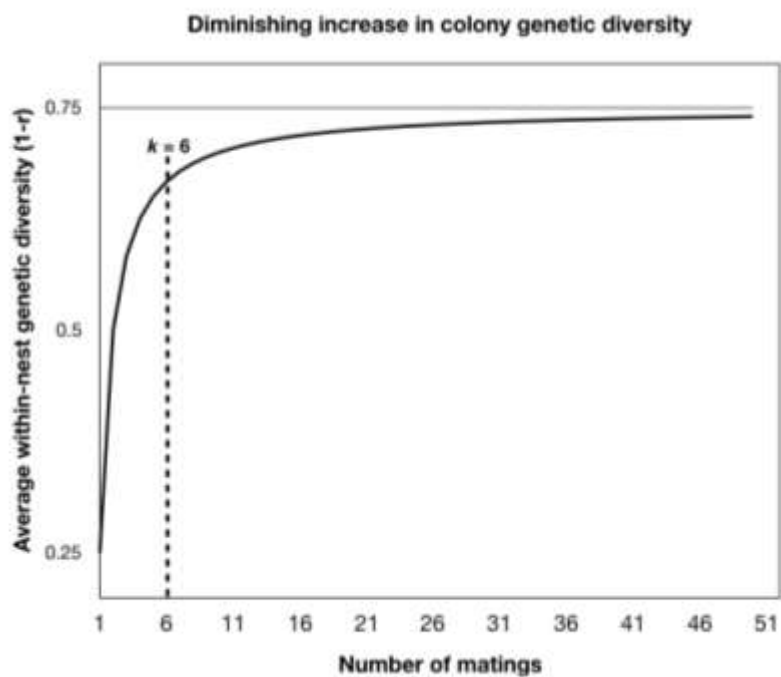


Figure 1: Theory shows that over 90% of the change in within-colony genetic relatedness is explained by a queen's first 6 matings, but the species routinely reach beyond this asymptote and mate, on average with 12 males and some up to 77 [93].



Figure 2: Packages of bees were installed in single deep 10 frame super. Mites were collected using sticky screens in the bottom of the colony.

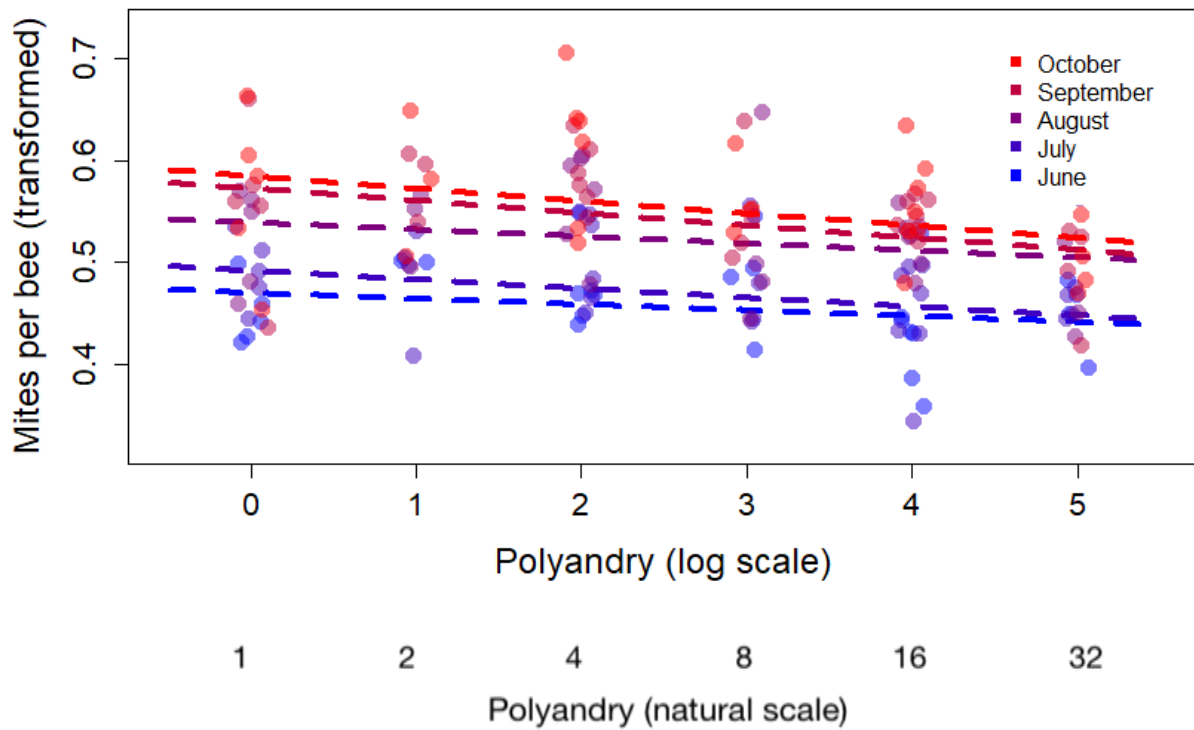


Figure 3: The relationship between the number of mites per bee and the level of polyandry. The number of mites per bee decreases as levels of polyandry increase. This graph depicts our polyandry treatments (over our 5 timepoints depicted by dotted lines). We also included our natural scale of insemination values below our original x-axis.

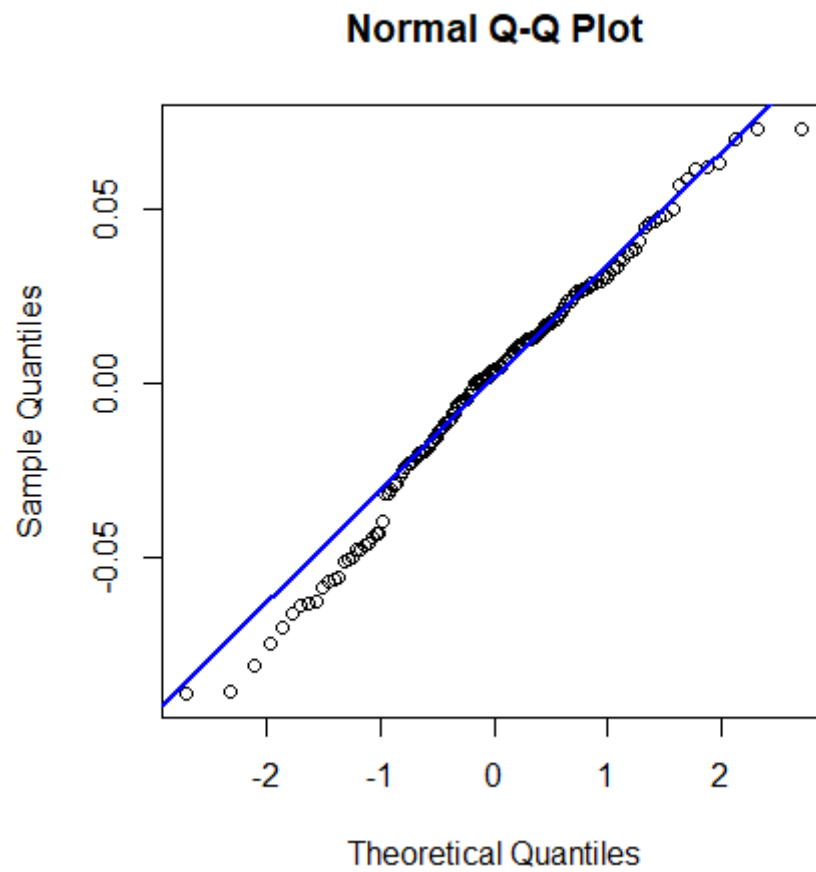


Figure 4: Gaussian generalized linear mixed models were graphically inspected for goodness of fit. Mites per bee data appear to be reasonably distributed with no clustering.

## TABLES

| <b>Drone Source Colonies (Effect)</b> | <b>Chi-square</b> | <b>p-value</b> |
|---------------------------------------|-------------------|----------------|
| Timepoint                             | 101.15            | <.001          |
| L8                                    | 0.47              | 0.494          |
| TO21                                  | 1.32              | 0.25           |
| LHILL 71                              | 0.91              | 0.341          |
| FARM 9                                | 0.15              | 0.696          |
| F2                                    | 1.51              | 0.219          |
| F22                                   | 0.02              | 0.893          |
| ED32                                  | 0.94              | 0.334          |
| BHILL 34                              | 0.68              | 0.411          |
| TO 1                                  | 1.02              | 0.312          |
| RED 1                                 | 0.2               | 0.657          |

Table 1: Depicts our analysis of sperm source colonies. Generalized linear mixed model with a Gaussian error structure was used to model the data. Polyandry was removed as a predictor variable and colony was treated as a random factor. Analysis indicates that no drone source colony deviated from a null expectation of non-difference in its effect on per-capita mite load.

## APPENDIX

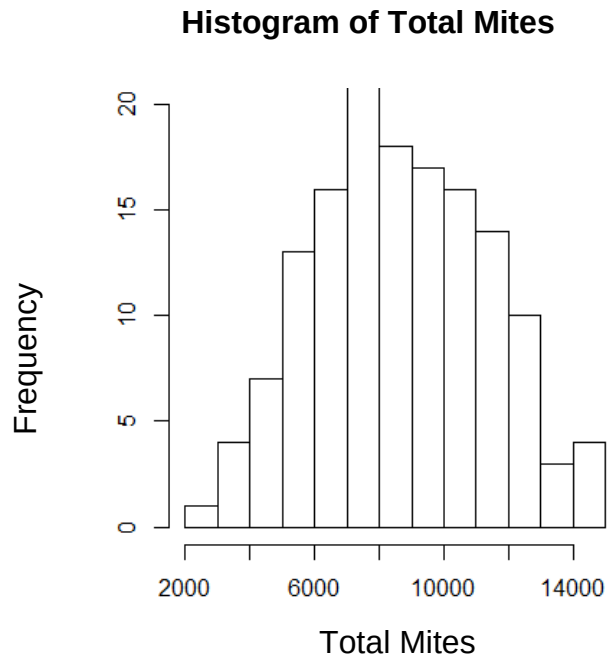


Figure A.1: Histogram of total number of mites. Relatively well distributed data with no need for transformation.

**Histogram of Mites per Bee (untransformed)**

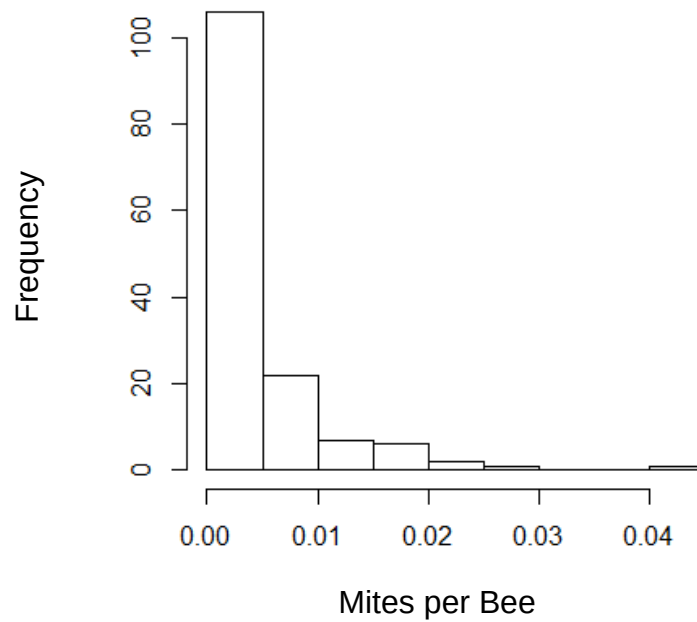


Figure A.2: Histogram of untransformed mites per bee. Our data is skewed to the left, which indicates abnormally distributed data. We visually assessed the different transformations to see which would be closer to normal distribution. Normal distribution follows a bell curve.



### Histogram of Mites per bee 9<sup>th</sup> root transformed

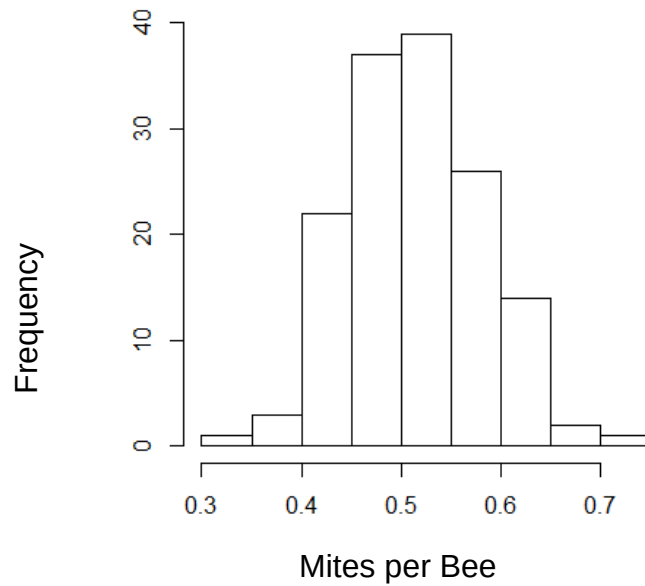


Figure A.3: Histogram of 9<sup>th</sup> root transformed mites per bee. Transforming the data allows us to better meet the assumptions of the model and become closer to being normally distributed. We visually assessed the different transformations to see which would be closer to normal distribution. This transforms our response variable (mites per bee) to  $\text{mites per bee}^{1/9}$ .

## REFERENCES

1. Arnqvist G, Nilsson T. **2000**. The evolution of polyandry: multiple mating and female fitness in insects. *Animal Behaviour*. 60:145-64
2. Baer B. **2005**. Sexual selection in *Apis* bees. *Apidologie*. 36 (2):187-200
3. Bateman AJ. **1948**. Intra-sexual selection in *Drosophila*. *Heredity*. 2:349-68
4. Boomsma JJ, Ratnieks FL. **1996**. Paternity in eusocial Hymenoptera. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*. 351 (1342): 947-75
5. Bourke AFG. **2011**. *Principles of Social Evolution*. Oxford [England], New York: Oxford University Press.
6. Brutscher LM, Baer B, Niño EL. **2019**. Putative drone copulation factors regulating honey bee (*Apis mellifera*) queen reproduction and health: A review. *Insects*. 10(1):8
7. Calderone N, Robinson GE, Page R. **1989**. Genetic structure and division of labor in honeybee societies. *Experientia*. 45:765-7
8. Calderone NW, Page RE. **1991**. Evolutionary genetics of division of labor in colonies of the honey bee (*Apis mellifera*). *The American Naturalist*. 138:69-92
9. Calderone NW, Page RE. **1996**. Temporal polyethism and behavioural canalization in the honey bee, *Apis mellifera*. *Animal Behaviour*. 51:631-43
10. Cobey SW, Tarpy DR, Woyke J. **2013**. Standard methods for instrumental insemination of *Apis mellifera* queens. *Journal of Apicultural Research*. 52:1-18

11. Cole BJ. **1983**. Multiple mating and the evolution of social behavior in the Hymenoptera. *Behavioral Ecology and Sociobiology*. 12:191
12. Crozier R, Pamilo P. **1996**. *Evolution of Social Insect Colonies: Sex Allocation and Kin Selection*. Oxford, UK: Oxford University Press
13. Crozier RH, Page RE. **1985**. On being the right size: Male contributions and multiple mating in social Hymenoptera. *Behavioral Ecology and Sociobiology*. 18:105
14. Darwin C. **1859**. *The Origin of Species by Means of Natural Selection*. London, UK: Cambridge University Press
15. Delaplane KS. **2017**. Emergent properties in the honey bee superorganism. *Bee World*. 94:8-15
16. Delaplane KS, Pietravalle S, Brown MA, Budge GE. **2015**. Honey bee colonies headed by hyperpolyandrous queens have improved brood rearing efficiency and lower infestation rates of parasitic Varroa mites. *PLoS One*. 10 (12): e0142985
17. Desai SD, Currie RW. **2015**. Genetic diversity within honey bee colonies affects pathogen load and relative virus levels in honey bees, *Apis mellifera* L. *Behavioral Ecology and Sociobiology*. 69:1527-41
18. Ding G, Xu H, Oldroyd BP, Gloag RS. **2017**. Extreme polyandry aids the establishment of invasive populations of a social insect. *Heredity*. 119: 381-7
19. Dreller C. **1998**. Division of labor between scouts and recruits: genetic influence and mechanisms. *Behavioral Ecology and Sociobiology*. 43:191-6
20. Dreller C, Fondrk M, Page R. **1995**. Genetic variability affects the behavior of foragers in a feral honeybee colony. *Naturwissenschaften*. 82:243-5

21. Eckholm BJ, Huang MH, Anderson KE, Mott BM, DeGrandi-Hoffman G. **2015.** Honey bee (*Apis mellifera*) intracolony genetic diversity influences worker nutritional status. *Apidologie*. 46:150-63
22. Estoup A, Solignac M, Cornuet J-M. **1994.** Precise assessment of the number of patriline and of genetic relatedness in honeybee colonies. *Proceedings of the Royal Society of London. Series B: Biological Sciences*. 258:1-7
23. Fewell J, Page R. **1993.** Genotypic variation in foraging responses to environmental stimuli by honey bees, *Apis mellifera*. *Experientia*. 49:1106-12
24. Fuchs S, Moritz R. **1999.** Evolution of extreme polyandry in the honeybee *Apis mellifera* L. *Behavioral Ecology and Sociobiology*. 45:269-75.
25. Fuchs S, Schade V. **1994.** Lower performance in honeybee colonies of uniform paternity. *Apidologie*. 25:155-68
26. Gençer HV, Kahya Y. **2019.** Sperm competition in honey bees (*Apis mellifera* L.): The role of body size dimorphism in drones. *Apidologie*. 51:1-17
27. Gerula D, Węgrzynowicz P, Panasiuk B, Bieńkowska M, Skowronek W. **2014.** Performance of bee colonies headed by queens instrumentally inseminated with semen of drones who come from a single colony or many colonies. *Journal of Apicultural Science*. 58:87-97
28. Girard MB, Mattila HR, Seeley TD. **2011.** Recruitment-dance signals draw larger audiences when honey bee colonies have multiple patriline. *Insectes Sociaux*. 58 (1): 77-86
29. Hamilton WD. **1964.** The genetical evolution of social behaviour: I. *Journal of Theoretical Biology*. 7:1-16

30. Harbo J. **1985**. Instrumental insemination of queen bees. *The American Bee Journal*. 1: 197-202
31. Holmes MJ, Beekman M. **2017**. When does cheating pay? Worker reproductive parasitism in honeybees. *Insectes Sociaux*. 64:5-17.
32. Hughes WO, Oldroyd BP, Beekman M, Ratnieks FL. **2008**. Ancestral monogamy shows kin selection is key to the evolution of eusociality. *Science*. 320:1213-6
33. Jones JC, Myerscough MR, Graham S, Oldroyd BP. **2004**. Honey bee nest thermoregulation: Diversity promotes stability. *Science*. 305:402-4
34. Keller L, Reeve HK. **1994**. Genetic variability, queen number, and polyandry in social Hymenoptera. *Evolution*. 48:694-704
35. Koeniger G, Koeniger N, Ellis J, Connor LJ. **2014**. *Mating Biology of Honey Bees (Apis mellifera)*. Kalamazoo, MI: Wicwas Press LLC.
36. Kraus B, Page J, Robert E. **1998**. Parasites, pathogens, and polyandry in social insects. *The American Naturalist*. 151:383-91.
37. Kraus FB, Gerecke E, Moritz RF. **2011** Shift work has a genetic basis in honeybee pollen foragers (*Apis mellifera* L.). *Behavior Genetics*. 41:323-8
38. Kraus FB, Moritz RF. **2010**. Extreme polyandry in social Hymenoptera: Evolutionary causes and consequences for colony organisation. *Animal Behaviour: Evolution and Mechanisms*. 1:413-39
39. Lee GM, McGee PA, Oldroyd BP. **2013**. Variable virulence among isolates of *Ascosphaera apis*: Testing the parasite–pathogen hypothesis for the evolution of polyandry in social insects. *Naturwissenschaften*. 100(3): 229-34

40. Mackensen O. **2014**. Effect of carbon dioxide on initial oviposition of artificially inseminated and virgin queen bees. *Journal of Economic Entomology*. 40:344-9
41. Madsen T, Shine R, Loman J, Håkansson T. **1992**. Why do female adders copulate so frequently? *Nature*. 355:440-1
42. Mattila HR, Burke KM, Seeley TD. **2008**. Genetic diversity within honeybee colonies increases signal production by waggle-dancing foragers. *Proceedings of the Royal Society B: Biological Sciences*. 275:809-16
43. Mattila HR, Seeley TD. **2007**. Genetic diversity in honey bee colonies enhances productivity and fitness. *Science*. 317:362
44. Mattila HR, Seeley TD. **2010**. Promiscuous honeybee queens generate colonies with a critical minority of waggle-dancing foragers. *Behavioral Ecology and Sociobiology*. 64:875-89
45. Mattila HR, Seeley TD. **2011**. Does a polyandrous honeybee queen improve through patriline diversity the activity of her colony's scouting foragers? *Behavioral Ecology and Sociobiology*. 65:799-811
46. Mattila HR, Seeley TD. **2014**. Extreme polyandry improves a honey bee colony's ability to track dynamic foraging opportunities via greater activity of inspecting bees. *Apidologie*. 45:347-63
47. Moritz RF. **1985**. The effects of multiple mating on the worker-queen conflict in *Apis mellifera* L. *Behavioral Ecology and Sociobiology*. 16:375-7

48. Moritz RF, Kryger P, Koeniger G, Koeniger N, Estoup A, Tingek S. **1995.** High degree of polyandry in *Apis dorsata* queens detected by DNA microsatellite variability. *Behavioral Ecology and Sociobiology*. 37:357-63
49. Moritz RF, Lattorff HMG, Neumann P, Kraus FB, Radloff SE, Hepburn HR. **2005.** Rare royal families in honeybees, *Apis mellifera*. *Naturwissenschaften*. 92:488-91
50. Neumann P, Moritz RFA. **2000.** Testing genetic variance hypotheses for the evolution of polyandry in the honeybee (*Apis mellifera* L.). *Insectes Sociaux*. 2000: 271-279
51. Oldroyd B, Moran C. **1983.** Heritability of worker characters in the honeybee (*Apis mellifera*). *Australian Journal of Biological Sciences*. 36:323-32
52. Oldroyd B, Rinderer TE, Harbo JR, Buco SM. **1992.** Effects of intracolony genetic diversity on honey bee (Hymenoptera: Apidae) colony performance. *Annals of the Entomological Society of America*. 85:335-43
53. Oldroyd BP, Fewell JH. **2007.** Genetic diversity promotes homeostasis in insect colonies. *Trends in Ecology and Evolution*. 22:408-13.
54. Oldroyd BP, Morag JC, Siriwat W, Thomas ER, Sylvester HA, Ross HC. **1997.** Polyandry in the genus *Apis*, particularly *Apis andreniformis*. *Behavioral Ecology and Sociobiology*. 40:17
55. Oldroyd BP, Rinderer TE, Buco SM. **1991.** Honey bees dance with their super sister. *Animal Behaviour*. 42:121-9.
56. Oldroyd BP, Rinderer TE, Buco SM. **1991.** Intracolony variance in honey bee foraging behaviour: The effects of sucrose concentration. *Journal of Apicultural Research*. 30:137- 45

57. Oldroyd BP, Rinderer TE, Buco SM. **1992**. Intra-colonial foraging specialism by honey bees (*Apis mellifera*) (Hymenoptera: Apidae). *Behavioral Ecology and Sociobiology*. 30:291-5
58. Oldroyd BP, Rinderer TE, Buco SM, Beaman LD. **1993**. Genetic variance in honey bees for preferred foraging distance. *Animal Behaviour*. 45:323-32
59. Oldroyd BP, Smolenski AJ, Cornuet J-M, Wongsiri S, Estoup A, Rinderer E, Crozier RH. **1995**. Levels of polyandry and intracolony genetic relationships in *Apis florea*. *Behavioral Ecology and Sociobiology*. 37:329-35.
60. Oldroyd BP, Smolenski AJ, Cornuet J-M, Wongsiri S, Estoup A, Rinderer E, Crozier RH. **1996**. Levels of polyandry and intracolony genetic relationships in *Apis dorsata* (Hymenoptera: Apidae). *Annals of the Entomological Society of America*. 89:276-83
61. Oldroyd BP, Sylvester HA, Wongsiri S, Rinderer TE. **1994**. Task specialization in a wild bee, *Apis florea* (Hymenoptera: Apidae), revealed by RFLP banding. *Behavioral Ecology and Sociobiology*. 34:25-30
62. Page RE, Metcalf RA. **1982**. Multiple mating, sperm utilization, and social evolution. *The American Naturalist*. 119:263-81
63. Page RE. **1980**. The evolution of multiple mating behavior by honey bee queens (*Apis mellifera* L.). *Genetics*. 96:263-73
64. Page RE, Robinson GE, Fondrk MK, Nasr ME. **1995**. Effects of worker genotypic diversity on honey bee colony development and behavior (*Apis mellifera* L.). *Behavioral Ecology and Sociobiology*. 36:387-96
65. Palmer KA, Oldroyd BP. **2000**. Evolution of multiple mating in the genus *Apis*. *Apidologie*. 31(2):235-248



66. Palmer KA, Oldroyd BP. **2003**. Evidence for intra-colonial genetic variance in resistance to American foulbrood of honey bees (*Apis mellifera*): Further support for the parasite/pathogen hypothesis for the evolution of polyandry. *Naturwissenschaften*. 90:265-8
67. Pamilo P. **1993**. Polyandry and allele frequency differences between the sexes in the ant *Formica aquilonia*. *Heredity*. 70:472
68. Parker GA, Birkhead TR. **2013**. Polyandry: The history of a revolution. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 368 (1613)
69. Payne AN, Rangel J. **2018**. The effect of queen insemination volume on the growth of newly established honey bee (*Apis mellifera*) colonies. *Apidologie*. 49:594-605
70. Queller DC. **1993**. Worker control of sex ratios and selection for extreme multiple mating by queens. *The American Naturalist*. 142:346-51
71. R Development Core Team. **2019**. R: A language and environment for statistical computing. R Foundation for Statistical Computing
72. Ratnieks FL. **1990**. The evolution of polyandry by queens in social Hymenoptera: The significance of the timing of removal of diploid males. *Behavioral Ecology and Sociobiology*. 26:343-8
73. Ratnieks FL, Boomsma JJ. **1995**. Facultative sex allocation by workers and the evolution of polyandry by queens in social Hymenoptera. *The American Naturalist*. 145:969-93

74. Ratnieks FL, Helanterä H. **2009**. The evolution of extreme altruism and inequality in insect societies. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 364:3169-79
75. Sato N, Tsuda S-I, Alam MNE, Sasanami T, Iwata Y, Kusama S, Inamura O, Yoshida M, Hirohashi N. **2020**. Rare polyandry and common monogamy in the firefly squid, *Watasenia scintillans*. *Scientific Reports*. 10:1-9
76. Schlüns H, Moritz RF, Neumann P, Kryger P, Koeniger G. **2005**. Multiple nuptial flights, sperm transfer and the evolution of extreme polyandry in honeybee queens. *Animal Behaviour*. 70:125-31
77. Schmid-Hempel P. **1998**. *Parasites in Social Insects*. Princeton, N.J.: Princeton University Press.
78. Seeley TD. **1997**. Honey bee colonies are group-level adaptive units. *The American Naturalist*. 150: 22-41
79. Seeley TD, Tarpay DR. **2006**. Queen promiscuity lowers disease within honeybee colonies. *Proceedings of the Royal Society B: Biological Sciences*. 274:67-72
80. Shaskolsky D. **1977**. Polyandry--a defending factor of the colony against a great number of lethal eggs. In Apimondia symposium on bee biology (1): 67-71
81. Sherman PW, Seeley TD, Reeve HK. **1988**. Parasites, pathogens, and polyandry in social Hymenoptera. *The American Naturalist*. 131:602-10
82. Shykoff JA, Schmid-Hempel P. **1991**. Genetic relatedness and eusociality: Parasite- mediated selection on the genetic composition of groups. *Behavioral Ecology and Sociobiology*. 28:371-6

83. Shykoff JA, Schmid-Hempel P. **1991**. Parasites and the advantage of genetic variability within social insect colonies. *Proceedings of the Royal Society of London. Series B: Biological Sciences*. 243:55-8
84. Tarpy D, Nielsen R, Nielsen D. **2004**. A scientific note on the revised estimates of effective paternity frequency in *Apis*. *Insectes Sociaux*. 51:203-4
85. Tarpy DR. **2003**. Genetic diversity within honeybee colonies prevents severe infections and promotes colony growth. *Proceedings of the Royal Society of London. Series B: Biological Sciences*. 270:99-103
86. Tarpy DR, Page REJ. **2002**. Sex determination and the evolution of polyandry in honey bees (*Apis mellifera*). *Behavioral Ecology and Sociobiology*. 52:143
87. Tarpy DR, Pettis JS. **2013**. Genetic diversity affects colony survivorship in commercial honey bee colonies. *Naturwissenschaften*. 100:723-8
88. Taylor ML, Price TA, Wedell N. **2014**. Polyandry in nature: A global analysis. *Trends in Ecology & Evolution*. 29:376-83
89. Toth AL, Rehan SM. **2017**. Molecular evolution of insect sociality: An eco-evo-devo perspective. *Annual Review of Entomology*. 62:419-42
90. Trivers RL, Hare H. **1976**. Haplodiploidy and the evolution of the social insect. *Science*. 191:249-63
91. Wilson EO. **1971**. *The Insect Societies*. Cambridge, MA: Harvard University Press
92. Winston ML. **1991**. *The biology of the honey bee*. Cambridge, MA: Harvard University Press
93. Withrow JM, Tarpy DR. **2018**. Cryptic “royal” subfamilies in honey bee (*Apis mellifera*) colonies. *PloS One*. 13

94. Woyke J. **1963**. What happens to diploid drone larvae in a honeybee colony.  
*Journal of Apicultural Research*. 2:73-5