EVALUATING POLYANDRY MANIPULATION AND INTERCOLONY GENETIC MIXING ON HONEY BEE COLONY STRENGTH AND RESISTANCE TO *VARROA DESTRUCTOR*

by

John Francis Menz

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AND INTERCOLONY GENETIC MIXING ON

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John Francis Menz

Approved: _____

Deborah A. Delaney, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____

Jacob L. Bowman, Ph.D.
Chair of the Department of Entomology and Wildlife Ecology

Approved: _____

Mark W. Rieger, Ph.D.
Dean of the College of Agriculture and Natural Resources

Approved: _____

Douglas J. Doren, Ph.D.
Interim Vice Provost for Graduate and Professional Education and
Dean of the Graduate College

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This thesis is dedicated to the Lugo- Morinigo family of San Juan Bautista, Paraguay and especially to Alejandro Lugo, my first partner-in-crime beekeeping with wild, Africanized honey bees.

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ABSTRACT

Apis mellifera L., the European honey bee, is a social insect that is vital to managed pollination services in the United States. Honey bee health and colony survival are challenged by the novel parasite *Varroa destructor* (Anderson & Trueman 2000). Honey bee queens are naturally polyandrous, mating with an average of 12 males. Hyper-polyandrous queens, created via artificial insemination, have shown increased resistance to *Varroa* mites. This project combined a hyper-polyandry treatment with a genetic treatment of bees selected for Varroa Sensitive Hygienic (VSH) trait, an additive rare resistance trait. The combination of these two intrinsic forms of resistance resulted in colonies with lower *Varroa* mite levels and these findings offer support to the rare-allele hypothesis for the evolution of extreme polyandry in honey bees. Additionally, an applied field method was evaluated to simulate the benefits of hyper-polyandry via brood mixing: the manual sharing of immature bees between a set of colonies to increase genetic diversity without artificial insemination. Brood mixing did not have a significant effect on any measure of colony strength and the lack of similar results to relevant tests of hyper-polyandry in the United Kingdom may be due to the homogeneous genetic structure of the United States honey bee population and indicates a lack of heterogeneous genetics when sourcing queen stock from individual honey bee queen breeders.

Chapter 1

INTRODUCTION: POLYANDRY AND COLONY HEALTH IN *APIS MELLIFERA* L.

1.1 Importance of honey bees to United States agriculture

European honey bees, *Apis mellifera* (L. 1758), provide substantial pollination services in natural ecosystems and are responsible for 13% of total global flower visits (Hung et al. 2018). They contribute even more to crop agro-systems where honey bees are the single most common visitor to the 35% of agricultural crops dependent on insect pollination (Klein et al. 2007; Garibaldi et al. 2014). Honey bees are an important managed pollinator in the United States and their services annually contribute $17 billion to the US agricultural economy (Morse and Calderone 2000; Calderone 2012). Since the 2009 valuation of honey bee services by Calderone (2012), the honey bee-dependent almond acreage in the United States has increased 56% indicating that demand for and value of honey bee pollination services have continued to rise (USDA 2019). There has been concern that the increase in demand for commercial pollination will outstrip the available supply of honey bee colonies (Aizen & Harder 2009) due to the increasingly difficult economic model for beekeepers that struggle with high colony loss rates that have hovered between 30 and 40% since 2006. These losses represent double the acceptable rate as reported by beekeepers. Survey data from 2018-2019 show the highest overwintering loss rate (37.7%) in 12 years (Bruckner et al. 2019). Beekeepers have been able to maintain colony numbers from year to year via splitting of survivor colonies, however this current model will be

unsustainable if colony losses continue to increase as observed in 2018-2019. Efforts to reduce colony losses are necessary to combat these concerning trends.

1.2 The threat and control of *Varroa destructor* (Anderson & Trueman 2000)

Honey bees in the United States suffer from a variety of health pressures including pesticide exposure and lack of forage (Brosi et al. 2017). The primary global threat to honey bee survival has been the parasitic mite *Varroa destructor* (Anderson & Trueman 2000; Genersch 2010; Dietemann et al. 2012) which not only feeds directly on honey bee fat bodies (Ramsey et al. 2019), which are vital to insect metabolism and immune function (Arrese & Soulages 2010), but also vectors and increases virulence of viral pathogens that devastate colonies within two years without intervention (Rosenkranz, Aumeier, and Ziegelmann 2010). The *Varroa* mite is held accountable as the root cause of disease in honey bee colonies and the major factor in colony demise as shown in studies of over-wintering loss (Guzmán-Novoa et al. 2010; Kielmanowicz et al. 2015).

Beekeeper intervention is essential to control *Varroa* mites and prevent colony death. The mites reproduce inside the sealed brood cells of honey bees. Mite populations are relatively low in colonies after winter when there is little brood present. However, as the season progresses the ratio of mites to bees increases exponentially to its highest point in autumn without treatment or control (Traynor et al. 2016). Multiple avenues of control have been developed since the arrival of *Varroa* to the United States more than 30 years ago, but none are currently sustainable (Rosenkranz et al. 2010; Dietemann et al. 2012). Synthetic acaricides have been successful in suppressing colony mite levels, however, acaricide resistance has been documented for several products (Elzen et al. 2000; Rinkevich et al. 2017; Rinkevich

2020). The most effective and widespread acaricide compound in use, amitraz (Haber et al. 2019), has shown adverse interactions with agrochemicals that reduce colony health (Johnson et al. 2013), can reduce individual bees' ability to tolerate viral infections (O'Neal et al. 2017), and permits near constant exposure to both bees and mites because of accumulation in beeswax (Johnson et al. 2009; Traynor et al. 2016). Within the first month of 2020 there were several authenticated reports of amitraz-resistant *Varroa* across the United States (Rinkevich 2020). Calls for judiciousness in synthetic acaricide use stem from the mite's extreme propensity to evolve resistance due to its short generation times and nearly clonal reproductive cycle (Rosenkranz et al. 2010; Beaurepaire et al. 2017; Rinkevich 2020). Fumigation with formic and oxalic acids has proven an effective treatment that is unlikely to select for resistant mites but is highly temperature sensitive, which gives it a narrow therapeutic index and limits repeat use due to queen mortality risks. Breeding bees for resistance was regarded as the most sustainable route for controlling *Varroa* (Dietemann et al. 2012), however, use of selectively-bred resistant honey bee stock has not become the silver bullet solution because the inbred lines must be maintained via artificial insemination requiring frequent replacement of queens (Danka et al. 2012). Furthermore, the presence of basic resistance behavior, as with other Integrated Pest Management (IPM) control options, does not preclude the need to chemically treat colonies with high rates of mite infestation (Spivak & Reuter 2001).

Along with breeding resistant bees, preventative IPM measures can be exceptionally useful in suppressing mite population increase, reducing the number of chemical treatments needed per season, and thereby conserve acaricide effectiveness (Delaplane et al. 2005; Danka et al. 2012; Beaurepaire et al. 2017; Lodesani et al.

2019). Despite this being the most sustainable future for honey bee management and *Varroa* control, non-chemical methods are associated with higher rates of colony loss (Haber et al. 2019; Underwood et al. 2019), displaying the need for additional, effective IPM methods that can be integrated into current beekeeping operations without additional costs.

A significant body of research has delved into the evolutionary mechanisms that have enabled social insects to cope with parasite and pathogen pressures, with the hope that it might yield insight into the control of maladapted parasites like *Varroa destructor*. The evolution of **polyandry**, the practice of females taking multiple male partners, has received extensive attention especially in honey bees, a managed social insect of economic importance. The focus of this project was to assess the benefits of increasing levels of polyandry in honey bee colonies in conjunction with selected *Varroa* resistance behavioral traits, via either intrinsic or simulated means. A background on honey bee mating biology, colony genetic diversity, and their function in resistance to parasites and pathogens is necessary to understand the mechanisms underlying the potential utility of exploiting increased levels of polyandry.

1.3 Multiple mating and colony health in social insects

Honey bee queens are highly polyandrous, meaning they mate with multiple males. The haploid nature of male drones results in distinct groups of highly related workers within a colony known as patrilines and the number of patrilines expressed in a colony is dependent upon the queen's mating number (Estoup et al. 1994). Theoretical models predict that mating with six drones provides 90% of the genetic variation observed in a typical colony (Palmer & Oldroyd 2000), however, natural mating numbers usually range between 12 and 20 (Tarpy et al. 2004). The genetic

variance (GV) hypothesis is the leading rationale for the evolution of this costly and seemingly excessive number of matings (Keller & Reeve 1994). It suggests that even a slight increase in genetic variation from extra matings provides colony benefits (Crozier & Fjerdingstad 2001) in the form of more efficient genetic task specialization (Calderone & Page 1988; Jones et al. 2004; Waibel et al. 2006; Oldroyd & Fewell 2007), increased parasite and disease resistance (Tarpy 2003; Seeley & Tarpy 2007; Simone-Finstrom et al. 2016), greater productivity (Mattila & Seeley 2007; Mattila et al. 2012a) and even improved microbiome diversity and increased representation of beneficial gut bacteria (Mattila et al. 2012b). From this body of research, it stands to reason that further benefits can be reaped from even higher levels of polyandry, but such levels might be impossible to achieve due to the physiological limits of mating. This addition to the GV hypothesis was supported via a test of **hyper**-polyandrous queens (Delaplane et al. 2015).

Delaplane et al. (2015) tested the limits of the GV hypothesis by artificially inseminating queens with pooled semen from up to 60 drones, thereby creating **hyper**-polyandrous queens (mating number ≥30). This technique overcomes the physiological limits to mating number. Their results showed that hyper-polyandrous queens produced colonies with reduced *Varroa* mite levels and increased brood rearing efficiency. Neither the queens nor the drones came from specially selected lines of bees (Delaplane et al. 2015). Hyper-polyandry exploits a caveat to the GV hypothesis that suggests higher mating numbers increase the chances of capturing rare behavioral resistance alleles already present at low levels within a population which allows colonies to better cope with parasite and pathogen pressure (Fuchs & Moritz 1998). For example, resistant individuals may remove infected brood, thereby

preventing infections from spreading or participate more in allogrooming (Spivak & Gilliam 1998). As shown via the positive results of the artificial insemination experiment, the physiological costs of natural mating likely prevent colonies from reaping the benefits of higher mating numbers and the increased representation of rare resistance alleles. Although breeding programs have been successful in elevating the expression of specific resistance alleles in selected lines of honey bees, these resistant lines have not been widely adopted due to difficulty in maintaining expression of these traits at the colony level (Dietemann et al. 2012; Plate et al. 2019). Examples of well-studied resistance traits include hygienic behavior (Evans & Spivak 2010) where bees can detect and remove diseased brood, and Varroa Sensitive Hygienic (VSH) behavior where bees can identify and remove brood that is specifically infested with *Varroa* (Villa et al. 2009). The great multiplicity of resistance tactics is the foundation of social immunity, a phenomenon which refers to the behavioral or physiological traits of individuals contributing to colony-level resistance to parasites or infectious disease (Cremer et al. 2007). Importantly, not all individuals need to display immunity to a disease or expression of a resistance trait for a colony to overcome and survive an infection (Kurze et al. 2016) which is why rare alleles are thought to have benefit even at low levels of expression at the colony level. *Varroa* resistance alleles have already been found in different, unselected populations of honey bees in the United States (Harbo & Hoopingarner 1997) and globally (Mondet et al. 2020). Hyper-polyandry is a potential key to unlock diversified levels of resistance expression at the individual colony-level using already available population-level genetic resources.

This collaborative effort between the Delaplane lab at University of Georgia and the Delaney lab at University of Delaware sought to explore the potential

synergistic effect between high levels of polyandry and selective breeding efforts on overall honey bee colony strength and resistance to *Varroa* mites and also evaluate a cultural Integrated Pest Management (IPM) method of simulating hyper-polyandry without the need for artificial insemination..

Chapter 2

EVALUATING POLYANDRY LEVEL AND A RARE RESISTANCE TRAIT ON COLONY STRENGTH AND *VARROA* MITE LEVELS

2.1 *Varroa*-resistant genetic stock and potential synergy with polyandry manipulation

Significant research has been conducted on the major behavioral and physiological mechanisms that can contribute to a colony that is functionally resistant to parasites such as *Varroa*, and to pathogens such as viruses, bacteria, and fungi (Evans & Spivak 2010; Kurze et al. 2016; Biganski et al. 2018). Honey bee colonies are made up of thousands of individuals that respond to stimuli, stress, and their environment in different ways according to their genetics and their age. Within a colony there may be multiple mechanisms, whether physiological or behavioral, to either resist or tolerate a pathogen stressor with great variation between individuals. This multiplicity of resistance tactics is the foundation of social immunity, a phenomenon which refers to the behavioral or physiological traits of individuals contributing to colony-level resistance to parasites or infectious disease (Cremer et al. 2007). Honey bee breeding programs have shown potential to increase the expression of specific resistance traits like Varroa Sensitive Hygienic (VSH) trait (Danka et al. 2013), which allows a colony to suppress the growth of *Varroa* mite levels. VSH worker bees detect and remove brood infested with reproductive *Varroa* mites.

Hyper-polyandry via artificial insemination is an attempt to boost colony-level genetic diversity specifically by capturing rare alleles for resistance traits such as VSH

trait. Theoretically, applying hyper-polyandry to a population with a known additive resistance trait, such as VSH, should provide further benefits for parasite resistance than the same trait under natural mating conditions. Additionally, this project will build off the initial research on hyper-polyandry by (Delaplane et al. 2015) by directly testing the rare-allele hypothesis for evolution of extreme polyandry.

2.2 Objectives and hypotheses

Objective: Test the synergistic effect of selectively bred VSH honey bees with paired levels of polyandry: One pair representing hypo-polyandry with levels 1 vs. 4 drones and hyper-polyandry with levels 9 vs. 54 drones. The test of polyandry level 54 combined with VSH genetics was a direct test of the rare-allele hypothesis for evolution of extreme polyandry in *Apis mellifera*.

Hypothesis: In both paired polyandry level scenarios, the higher level of polyandry in conjunction with VSH genetics was expected to provide the greatest resistance to *Varroa* mites and result in generally stronger colonies. The test of polyandry level 54 combined with VSH genetics was expected to indicate rare-allele effects compared to polyandry level 9 and both levels of wild-type (control) genetics.

2.3 Methods

The following field trials in Georgia and Delaware sought to compare multiple levels of polyandry in conjunction with the selected VSH trait.

2.3.1 Test of hyper-polyandry and selected stock, Georgia 2018

Two levels of polyandry via artificial insemination were tested against and in conjunction with wild-type (WT) bees that had not undergone any specific breeding for *Varroa* resistance traits and bees selected for Varroa Sensitive Hygienic (VSH)

trait at the Delaplane Honey Bee Lab at University of Georgia, Athens during summer 2018. Maternal genetic diversity between experimental colonies was controlled to reduce variation between experimental colonies. Eighty "supersister" queens who share the same drone father and are therefore hyper-related, were reared from a WT queen artificially inseminated (AI) with one WT drone. Due to the high level of post-AI mortality in preliminary trials, a large surplus of supersister queens was reared and inseminated with the target of producing 40 successfully inseminated queens.

Many previous studies had already sought to demonstrate the benefits yielded by general genetic diversity, therefore effects from paternal genetic variation were controlled for drone fathers by using multiples of drones from the same source colonies. The semen used for artificial insemination was collected from 9 drone source colonies headed by VSH queens sourced from Johnny Thompson Apiaries (Mississippi) established in the spring and 9 WT drone source colonies from preexisting, unselected stock at University of Georgia.

WT supersister queens were divided into 4 treatment groups. Treatments included insemination with either VSH or WT drone semen and insemination with a mixed sample of semen from 9 or 54 drones (see Table 2.1). A sample of 9 drones followed a conservative approximation to natural effective realized paternity frequency of 12 drones (Tarpy et al. 2004). The other treatment of 54 drones represented an increase in mating number treatment by a magnitude of six. This level of polyandry is beyond the highest naturally occurring mating number of 40 drones in *A. mellifera scutellata* reported by Tarpy et al. (2010). As in Delaplane et al. (2015), a 54-drone semen sample from the same source colonies that supplied the polyandry level 9 sample allowed for a test of rare-allele effects that would otherwise be

impossible through natural mating, whereas conservative numbers around 20 and 30 drones might have been confounded by simple increases in genetic diversity.

Table 2.1: Treatments and initial colony sample sizes for the 2018 Georgia hyper-polyandry field trial.

		Polyandry level	
		9 drones	54 drones
Genetics	VSH	4 Queens	9 Queens
	WT	11 Queens	10 Queens

Pooled semen samples for the polyandry level 9 treatment were made using 1 drone from each of the 9 WT or 9 VSH drone source colonies that had been previously established. Pooled semen samples for the polyandry level 54 treatment were made using 6 drones from each of the 9 WT or VSH drone source colonies. Virgin supersister queens emerged in 5-frame nucleus colonies started with 1.5 kg of adult worker bees from Rossman Apiaries (Moultrie, Georgia). Queens were inseminated with their respective treatments 6-8 days after emergence using standard insemination protocol following Harbo (1985) and Delaplane et al. (2015). Data collection began 6 weeks after queens had demonstrated successful egg laying and the package workers had expired. Adult bee population, brood area (cm^2), *Varroa* mite numbers, and comb construction (cm^2) were assessed for each colony beginning in July and at 4-week intervals until November for a total of 4 assessment points. See Table 2.2 for methodology of each measurement taken.

The Effective Realized Paternity (ERP) – the number of patrilines represented in a colony adjusted for proportional representation – was determined for 18 of the GA colonies in order to ground truth the hyper-polyandrous treatment's resulting

effectiveness and also provide a fixed covariate for data analysis. See Chapter 3 for methods and results of ERP determination.

Table 2.2: Measures of colony strength taken during assessments for Georgia (2018) and Delaware (2019) polyandry level field trials and Delaware (2019) brood mixing field trial. All assessments were conducted at 4-week intervals.

Variable	**Methodology**	**Reasoning and citation**	**Unit**
Adult worker bee population	Visual summation of proportions of adult bees on deep Langstroth frames by two trained assessors. Final score is average of assessors.	Proportion to be converted via regression model to colony population. 1215 adult bees/fully covered deep frame side provides population estimate. (Burgett & Burikam 1985; Delaplane et al. 2013)	Number of bees
Brood production	Visual summation of proportion of capped brood on frame by two trained assessors. Final score is average of assessors.	Surface area of one side of deep Langstroth frame is 880 cm^2, giving total area of brood (Delaplane et al. 2013).	cm^2 brood
Comb construction	Each colony provided with empty frame with 2.5 cm wax starter strip. Drawn comb area measured after 48 hours.	Comb building is an energetically costly activity, area of comb built is proxy for strength. (Mattila & Seeley 2007).	cm^2 comb/48 hours
Varroa mite count	48-hour plastic board left to accumulate fallen mites under a bottom screen.	Fallen mites are counted.	Number of mites

2.3.2 Test of hypo-polyandry and selected stock, Delaware 2019

A second field trial was conducted during the summer of 2019. WT virgin supersister queens were managed and inseminated in a manner identical to the 2018 Georgia field trial (see methods 2.3.1) however with sub-optimal levels of polyandry, or "hypo-polyandry". Single-drone inseminated queens were reared to fulfill the lower polyandry level treatment, which in this case was monogamy. For the higher polyandry level, queens were inseminated with 4 drones. Two drones in each sample shared a colony of origin. In parallel with the 2018 Georgia trial featuring normal- and hyper-polyandry, this trial sought to compare depressed levels of polyandry (4 drone insemination) with monogamy (single drone insemination) in conjunction with a treatment of differing genetic origin of drones (WT vs. VSH) to assess rare allele effects at suppressed mating levels (see Table 2.3). These adjusted polyandry levels were used due to the failure of 12 of the 15 VSH drone source queens, possibly due to generally weak stock or stressful shipping conditions. WT supersister virgin queens were inseminated in July at the Delaney Bee Lab at the University of Delaware Newark Research Farm. WT drones were sourced from University of Delaware colonies while VSH drones came from colonies headed by VSH queens sourced from Johnny Thompson Apiaries (Mississippi). Adult bee population, brood area (cm^2), *Varroa* mite numbers, and comb construction (cm^2) were assessed for each colony beginning in August and at 4-week intervals until October for a total of 3 assessment points. Refer to Table 2.2 for methodology of each measurement taken.

Table 2.3: Treatments and initial colony sample sizes for the 2019 Delaware hypo-polyandry field trial.

		Polyandry level	
		1 drone	4 drones
Genetics	VSH	6 Queens	4 Queens
	WT	8 Queens	7 Queens

2.3.3 Statistical analysis

All tests for statistical significance were conducted in JMP® Pro 14.0.0 (SAS Institute Inc.). The 2018 Georgia and 2019 Delaware field trails were analyzed separately due to the difference in polyandry level treatments assigned and due to differences in climate and timing of assessments. The square root of data for bee population, cm^2 brood, mite count, and cm^2 comb was taken to meet assumptions of normality. Univariate mixed ANOVAs were conducted for bee population, cm^2 brood, mite count, and cm^2 comb. The fixed effects assigned were genetic treatment (WT vs. VSH), polyandry level (DE: 1 vs. 4; GA: 9 vs. 54), and the interaction of genetics and polyandry level. Individual colonies were treated as random repeating measurements across the 4 assessment time points for Georgia and 3 time points for Delaware. The continuous covariate ERP was included for 18 of the Georgia colonies for all strength measures. For mite counts, bee population and cm^2 brood were assigned as covariates. Cm^2 comb was measured during only one assessment for Delaware and was therefore analyzed via standard least squares ANOVA with the same fixed effects as Georgia but with no random effect nor covariate.

All ANOVA results are presented in Table 2.6 and results were considered significant at the $\alpha < 0.05$ level. Least squares means and 95% confidence intervals were calculated for all measures and are displayed untransformed in Table 2.7.

2.4 Results

2.4.1 2018 Georgia hyper-polyandry trial results

Colony losses occurred across all treatment groups throughout the season (Table 2.4).

Table 2.4: Georgia 2018 hyper-polyandry trial colony sample size by month and treatment of genetics (VSH vs. WT) combined with polyandry level (9 vs. 54).

	Sample sizes during assessment months			
Genetics/Poly#	**July**	**August**	**September**	**November**
VSH/9	4	4	2	2
WT/9	11	9	9	9
VSH/54	9	6	5	5
WT/54	10	10	9	9

GA bee population

Polyandry level had a significant effect on bee population ($p<0.05$) with colonies receiving a polyandry level 54 treatment showing larger populations.

GA cm^2 brood

No significant effects from either genetic or polyandry level treatments were observed on cm^2 brood.

GA mite count

No significant individual effects from either genetic or polyandry level treatments were observed on mite counts, however, there was a significant effect of their interaction ($p<0.05$). Lower mite levels were observed when increasing polyandry level from 9 to 54 only for VSH genetics. The opposite trend was found for WT genetics (Figure 2.1).

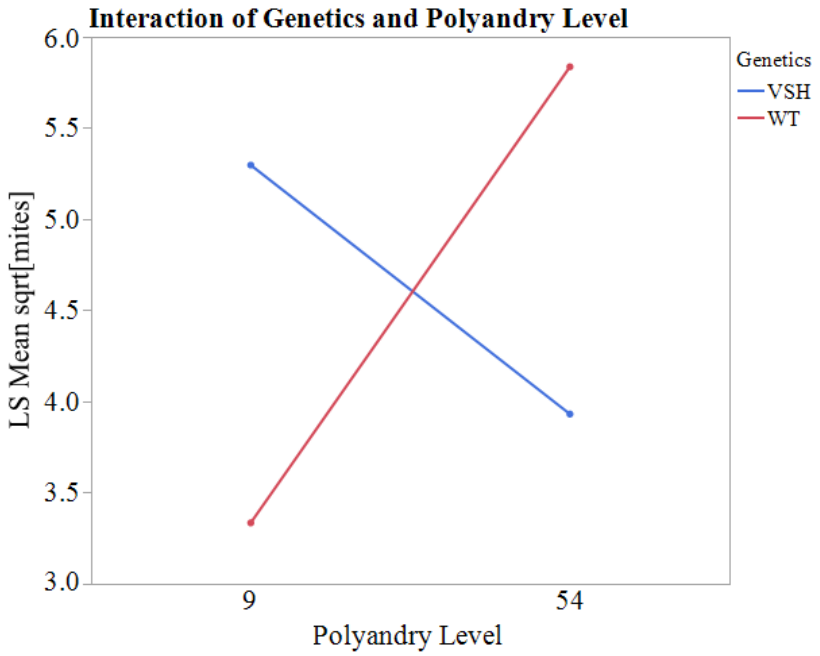

Figure 2.1: Interaction plot of the least squares means of the *Varroa* mite count for Georgia 2018 field trial colonies receiving both a genetic treatment (VSH vs. WT) and polyandry level treatment (9 vs. 54).

GA cm^2 comb

A significant effect of polyandry level ($p<0.01$) and its interaction with genetics ($p<0.05$) was observed on area (cm^2) comb constructed. Greater cm^2 comb was observed at the higher polyandry level 54. VSH genetics had a slightly greater effect on comb area constructed at the higher polyandry level than did WT genetics, however, the overall effect of genetics was not significant ($p = 0.22$).

2.4.2 2019 Delaware hypo-polyandry trial results

Two colonies were lost after the first assessment in the VSH/1 treatment group (Table 2.5).

Table 2.5 Delaware 2019 hypo-polyandry trial colony sample size by month and treatment of genetics (VSH vs. WT) combined with polyandry level (1 vs. 4).

	Sample sizes during assessment months 2019		
Genetics/Poly#	**August**	**September**	**October**
VSH/1	6	4	4
WT/1	8	8	8
VSH/4	4	4	4
WT/4	7	7	7

DE bee population

No significant effects from either genetic or polyandry level treatments were observed on bee population.

DE cm^2 brood

Polyandry level had a significant effect on cm^2 brood ($p<0.01$), with greater brood area observed at the higher polyandry level 4.

DE mite count

No significant treatment effects were observed for mite counts; however, polyandry level had an approximately significant effect with a p-value of 0.0507. LS mean mite counts were lower for WT colonies.

DE cm^2 comb

Comb construction area was significantly affected by genetics ($p<0.05$), polyandry level ($p<0.01$), and their interaction ($p<0.05$). Greater cm^2 comb was observed with WT genetics and polyandry level 4.

2.4.3 ANOVAs and LS means for Georgia and Delaware field trials

Table 2.6: ANOVA results for the effects of genetics (VSH vs. WT), polyandry level (DE: 1 vs. 4; GA: 9 vs. 54), and interactions (G*P) on colony strength measures analyzed separately for Delaware and Georgia field trials. Effective Realized Paternity (ERP) is a continuous covariate for 18 colonies in the Georgia trial only. Significant results are **emboldened** ($p<0.05$).

	Delaware			Georgia		
	df	F	P>F	df	F	P>F
			bee population			
Genetics	1,21.7	1.03	0.3214	1,10.7	3.95	0.0729
Poly Level	1,21.7	1.36	0.2563	1,	8.50	**0.0104**
G*P	1,21.7	0.33	0.5727	1,11.5	0.03	0.8671
ERP	-	-	-	1,15.4	7.92	**0.0128**
			cm^2 brood			
Genetics	1,19.6	0.00	0.9592	1,7.3	1.78	0.2228
Poly Level	1,19.6	10.12	**0.0048**	1,13.5	4.06	0.0642
G*P	1,19.6	0.63	0.4364	1,7.8	1.60	0.2419
ERP	-	-	-	1,13.1	4.21	0.0608
			mite drop over 24 hours			
Genetics	1,22.1	1.28	0.2702	1,9.2	0.00	0.9711
Poly Level	1,22.6	4.26	0.0507	1,18.2	0.13	0.7259
G*P	1,21.7	0.03	0.8659	1,10.1	6.97	**0.0245**
ERP	-	-	-	1,19.1	1.67	0.2120
bee pop	1,65	3.05	0.0857	1,33.2	2.97	0.0941
cm^2 brood	1,50.4	8.88	**0.0044**	1,35.4	1.80	0.1879
			cm^2 comb			
Genetics	1,21	7.03	**0.0149**	1,6.1	1.80	0.2274
Poly Level	1,21	12.83	**0.0018**	1,7	14.17	**0.0070**
G*P	1,21	7.97	**0.0102**	1,6.1	13.08	**0.0109**
ERP	-	-	-	1,7.6	7.99	**0.0233**

Table 2.7: Untransformed least squares means, degrees of freedom (df), and 95% confidence intervals for the effects of genetic and polyandry level treatments for colony strength measures in Delaware and Georgia. Different letters in the same row indicate significantly different treatment group means ($p<0.05$).

LS Means for genetic and polyandry level treatment effects

		LS Mean	df	Lower 95%	Upper 95%	LS Mean	df	Lower 95%	Upper 95%
		Delaware							
		VSH				WT			
Genetics	bee pop.	9208	22.4	7439	11166	10396	20.5	8920	11985
	cm^2 brood	1059	19.9	779	1383	1069	19.2	856	1305
	mites	9.08	22.6	4.73	14.83	5.92	20.6	3.19	9.49
	cm^2 comb	17.18 **a**	21.0	0.44	58.22	96.84 **b**	21.0	49.65	159.64
		Georgia							
	bee pop.	9111	13.5	7249	11187	7096	7.3	5965	8325
	cm^2 brood	2385	10.0	1738	3136	1925	3.7	1468	2444
	mites	21.29	11.3	10.35	36.15	21.03	6.0	13.04	30.93
	cm^2 comb	36.19	6.1	12.04	73.31	58.55	6.0	37.72	83.93

		LS Mean	df	Lower 95%	Upper 95%	LS Mean	df	Lower 95%	Upper 95%
		Delaware							
		Poly 1				Poly 4			
Polyandry level	bee pop.	9123	23.2	7617	10765	10487	20.5	8706	12434
	cm^2 brood	797 **a**	20.2	587	1039	1370 **b**	19.2	1080	1695
	mites	4.80	23.8	2.19	8.44	10.59	20.8	6.10	16.32
	cm^2 comb	9.90 **a**	21.0	0.05	36.75	117.49 **b**	21.0	55.58	202.31
		Georgia							
		Poly 9				Poly 54			
	bee pop.	5456 **a**	17.1	3594	7706	11199 **b**	11.9	8723	13983
	cm^2 brood	1504	14.9	839	2361	2910	9.0	2060	3906
	mites	18.63	18.1	5.66	39.11	23.86	14.2	9.66	44.36
	cm^2 comb	6.15 **a**	7.7	0.31	30.46	125.17 **b**	6.0	64.11	206.47

2.5 Discussion

The results indicate that introduction of rare alleles via hyper-polyandry did increase resistance to *Varroa* mites. This was observed specifically in the Georgia field trial where genetics and polyandry level had a significant interaction. VSH bees at polyandry level 9 had higher mite levels than polyandry level 54 VSH bees while the reverse was seen for the WT genetic treatment (Figure 2.1).

Delaware VSH colonies overall showed poor performance compared to WT colonies in the 2019 Delaware hypo-polyandry field trial. LS mean mite count was higher for VSH bees than WT. Although genetics did not have a significant effect, it was at least expected that VSH genetics would show some improvement for mite resistance over WT genetic at any level of polyandry. In Georgia, LS mean mite count was higher for VSH than WT genetics at the lower polyandry level (9). Although this difference in mite levels was also not significant, the trend of VSH having lower resistance to mites at depressed polyandry levels suggests a possibility that VSH stock is particularly susceptible to low mating numbers due to the genetic bottleneck introduced by breeding efforts. When selecting for a specific trait such as VSH, other traits and overall genetic diversity are inevitably lost in the process (Meixner et al. 2010; Leclercq et al. 2017) perhaps resulting in colonies with expression of VSH trait but lacking in overall robustness that results in colonies that build less comb – an indication of weaker colonies that was observe for Delaware VSH colonies – and are less resistant to *Varroa* at the colony level despite their specialized behavioral traits. Hyper-polyandry might be especially important for selectively bred lines of bees to counteract loss of diversity from trait selection.

The apparent negative effect of hyper-polyandry on WT colonies' resistance to *Varroa* did not mirror results from Delaplane et al. (2015) in the United Kingdom.

This result may reiterate the issue of homogeneous genetic diversity in the US honey bee breeding population addressed by Delaney et al. (2009). Regionally homogeneous US populations may lack the opportunity for rare resistance alleles to emerge in contrast with the heterogeneous honey bee population across the European home range, which display more unique regional genotypes (Leclercq et al. 2018) and may therefore have greater opportunity to capture rare resistance traits when hyper-polyandry is induced. In the Georgia field trial, however, polyandry level significantly improved colony population and comb building, two colony strength measures that were not shown to improve with increased polyandry level in the UK hyper-polyandry study.

Chapter 3

GENOTYPIC GROUND-TRUTHING OF PATERNITY IN HYPER-POLYANDROUS COLONIES USING MICROSATELLITE MARKERS

3.1 Patriline determination for *A. mellifera* colonies

Standardized methods of determining effective realized paternity (ERP), the number of patrilines expressed in one colony adjusted for proportional representation of each patriline, have been developed and widely used for *A. mellifera* (Estoup et al. 1994; Tarpy et al. 2004; Tarpy et al. 2010; Tarpy et al. 2015). The ERP was calculated for the artificially inseminated queens from a subset of the Georgia field trial colonies to demonstrate the effectiveness of artificial insemination for introducing above average levels of polyandry. This was a test to observe potential threshold levels of paternity frequency that can be expressed by a single queen inseminated with a representative sample of 54 drones and serve as a proof of concept for creating hyper-polyandrous queens via artificial insemination.

3.2 Objective and hypothesis

Objective: Assess effectiveness of artificial insemination to create hyper-polyandrous colonies with high patriline expression.

Hypothesis: ERP within colonies will show little variation from the prescribed insemination treatments for each queen. Mean ERP for each treatment group will be significantly different.

3.3 Methods

One hundred fifty adult worker bees were collected from each of the 34 colonies in the Georgia field trial. A 50-worker subsample was taken from 8 colonies in the control group (insemination number = 9) and 10 colonies in the hyper-polyandrous group (insemination number = 54).

Table 3.1: Number of genotyped colonies from each treatment group Georgia 2018.

		Insemination number	
		9 drones	54 drones
Genetics	VSH	3 colonies	4 colonies
	WT	5 colonies	6 colonies

DNA was extracted from the right hind leg for all 900 honey bee specimens using a Qiagen DNeasy Blood & Tissue Kit. Hind legs were thoroughly cut up with micro-scissors and digested with proteinase K in a 56°C water bath for 3 hours, with sample agitation every hour. Extracted DNA was stored at -80° C until microsatellite amplification. Ten variable microsatellite loci were screened for all samples using two multiplexes, Plex 1 and Plex 2. Plex 1 included loci A107, A113, AP043, A024, and A006; Plex 2 included loci A28, A88, AP66, AP81, and B124 (Shaibi, Lattorff, and Moritz 2008; Delaney et al. 2009). Both plexes were amplified for one cycle at 95°C for one 7 min, 30 cycles of 95°C for 30 sec, 54°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 60 min. A 10 µl final reaction volume containing 5 µl of PCR Master Mix (Promega, Madison, WI), 1.0-2.5 µl of fluorescent dye-labeled primer, 0.9 µl of nuclease-free water and 2 µl of DNA extract per sample was analyzed at the University of Delaware Sequencing and Genotyping Center at the

Delaware Biotechnology Institute with an Applied Biosystems 3130 XL Genetic Analyzer via capillary electrophoresis.

Microsatellite repeat sizes were scored using Geneious Prime® 2020.0.5 software (Biomatters Ltd.). The number (N_0) and frequency (p_i) of full sibling patrilines was determined using raw microsatellite data in the software program COLONY 2.0.6.5 (Jones & Wang 2010). The observed patriline number (N_o), proportion of each patriline within each sample (p_i), and the number of worker bees in each respective sample (n) were used to calculate ERP (m_e) with the following equation (Nielsen et al. 2003; Tarpy et al. 2004; Tarpy et al. 2015):

$$m_e = \frac{(n-1)^2}{\sum_{i=1}^{N_O} p_i^2(n+1)(n-2)+3-n}$$

Figure 3.1: Calculation of Effective Realized Paternity (m_e) for a honey bee colony based on observed patriline number (N_o) and patriline frequencies (p_i) (Tarpy et al. 2015).

Statistical analysis was conducted in JMP® Pro 14.0.0 (SAS Institute Inc.) to compare group means between the two levels of polyandry. Levene's test indicated heterogeneous variances between the hyper-polyandrous and control groups ($F_{1,16}$ = 5.42 , p = .033) which made the data unfit to be compared via ANOVA. To test for significant differences between the group means, the non-parametric Welch test for unequal variances was used.

3.4 Results

Hyper-polyandrous colonies had higher ERP numbers ($\bar{x}$ = 26.51±1.97, N = 10) than control colonies ($\bar{x}$ = 9.11±2.20, N = 8). Welch's test found a significant difference between the hyper-polyandrous and control group means ($F_{1,11.8}$ = 41.56 , p < .0001).

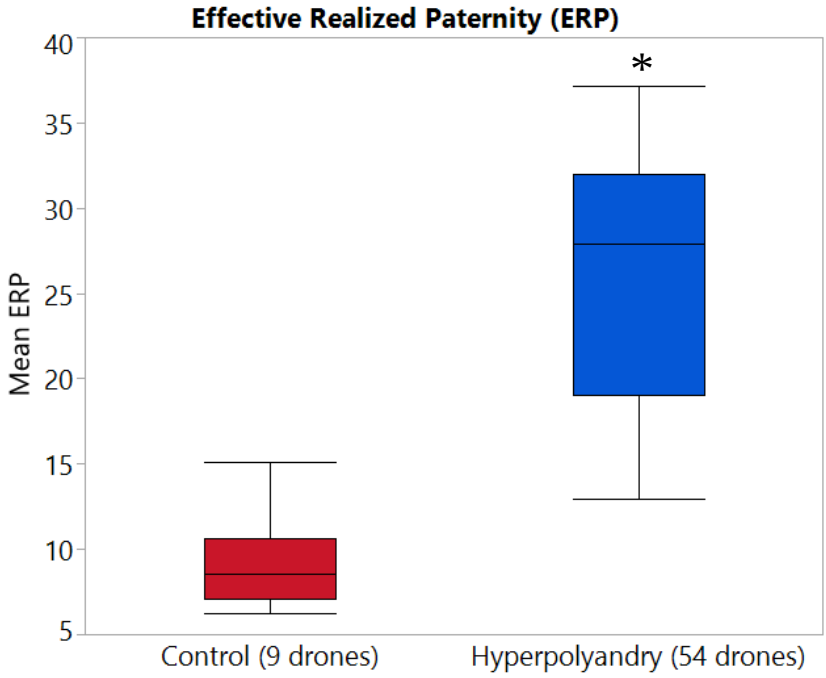

Figure 3.2: Comparison of mean ERP (m_e) between control (polyandry level = 9 drones) and hyper-polyandrous colonies (polyandry level = 54 drones).

3.5 Discussion

These results indicate that a distinct group of hyper-polyandrous queens was successfully created via artificial insemination with pooled samples of drone semen. This suggests that the hyper-polyandrous colonies in the 2018 Georgia field trial were

indeed a distinct group, however, mean ERP for the hyper-polyandrous treatment was far below the actual polyandry treatment level of 54 drones used in the artificial insemination process and ERP was quite variable. Mean ERP for the control group precisely matched the number of drones used in the artificial insemination process with little variation.

Several factors may account for the low ERP for the hyper-polyandrous colonies. Sampling bias in the initial worker subsample and an insufficient sample size of genotyped individuals could have resulted in misrepresentation of the resulting patriline proportions for a colony, thereby skewing the ERP calculation (Tarpy & Nielsen 2002). Additionally, non-detection errors could have occurred, which result when a microsatellite marker is identical between patrilines and thus two separate patrilines are interpreted as one (Tarpy & Nielsen 2002). This is especially likely to have occurred for the hyper-polyandrous colonies as only 9 colonies were used to supply 54 drones – each colony supplied six drones – in the mixed semen samples used during artificial insemination. In the future, paternity analysis of hyper-polyandrous colonies with highly related individuals will need to utilize a larger set of diversified markers to achieve the most accurate ERP calculation.

The variability in ERP for hyper-polyandrous queens indicates the need for improved methods to increase targeted patriline number precision when artificially inseminating queens with mixed semen samples from extreme numbers of related drones.

Chapter 4

BROOD MIXING: EVALUATING THE BENEFITS OF SIMULATED HYPER-POLYANDRY THROUGH A CULTURAL INTEGRATED PEST MANAGEMENT TECHNIQUE

4.1 Simulating hyper-polyandry via brood mixing

Hyper-polyandrous *Apis mellifera* queens have shown promise as a possible avenue of increasing colony resistance to *Varroa destructor* (Delaplane et al. 2015). Hyper-polyandry is induced via artificial insemination (AI); however, this technique is financially and technologically implausible for most beekeepers and requires specialized equipment. Furthermore, AI queens can suffer from reduced longevity (Cobey 2007) making them impractical in the long term for implementing hyper-polyandry as an applied IPM strategy for honey bee health. A more effective method for average beekeepers to create hyper-polyandrous colonies is to manually move frames of brood, or immature bees, between colonies. Brood mixing, as it has been dubbed, overcomes the obstacle of increasing patriline numbers without jeopardizing queen longevity or necessitating intensive technical training and costly equipment. Although it does not generate intrinsic hyper-polyandry, brood mixing simulates the effect of hyper-polyandry by temporarily increasing the number of patrilines through manual transfer of brood frames between sets of colonies. From each colony within a hypothetical set of four colonies, three brood frames are removed and distributed equally so that each colony now contains three donated frames of brood from its neighbors (Figure 4.1).

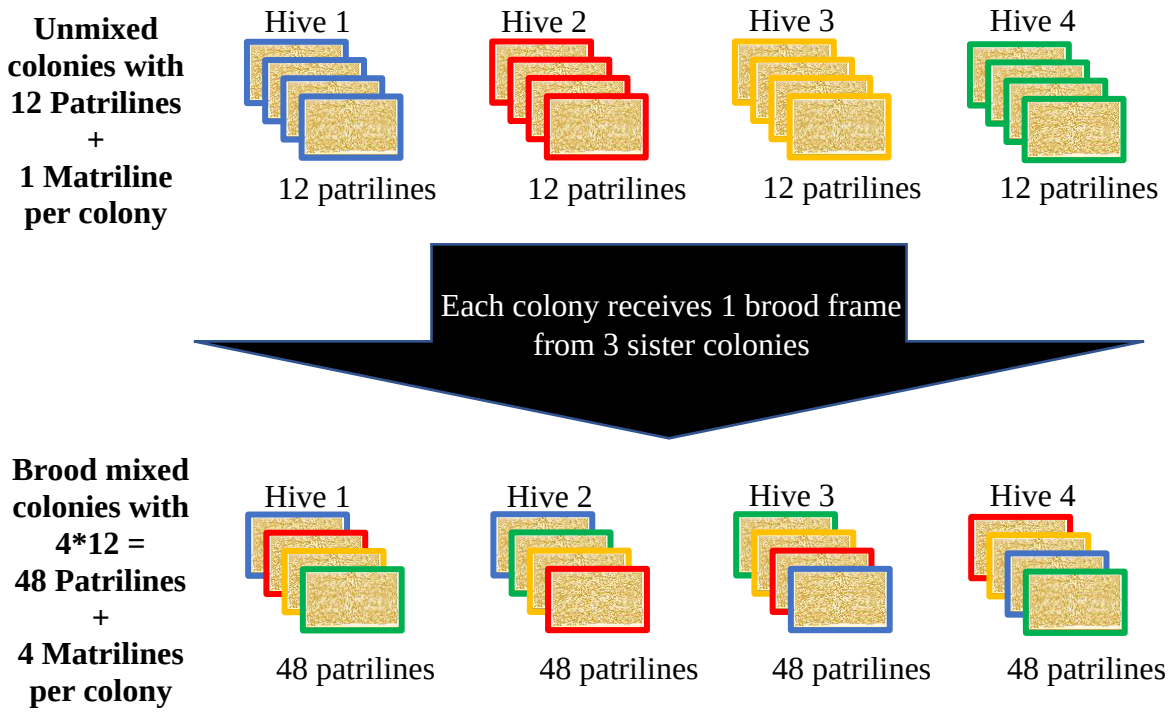

Figure 4.1: Representation of a four-colony brood mixing treatment and resulting colony patriline and matriline structures.

If we assume an average mating number of 12 drones for each queen in this hypothetical group of four colonies, the result after brood frame transfer is 4x12 patrilines = 48 effective patrilines in each of the four colonies in the set. This treatment can therefore be scaled depending on the number of colonies and brood frames available. For example, the brood mixing 'dosage' can be increased or decreased by sharing frames between a set of two colonies (2x12 patrilines = 24 patrilines), or a set of six colonies (6x12 patrilines = 72 patrilines) as was tested in a 2019 field trial at University of Delaware. Additionally, brood mixing also introduces

multiple matrilines into participating colonies, which does not occur with hyper-polyandrous queens.

One key difference from colonies headed by intrinsically hyper-polyandrous queens is that brood mixing's inflated patriline effect will dissipate after the workers from the mixed frames expire about 6 weeks after emergence. Understanding the role of a worker's post-emergence age was key to the assessment protocols for this field trial and is therefore briefly explained. The honey bee colony is a super-organismal collection of individual workers whose collective execution of tasks such as foraging, guarding, cleaning, and brood rearing contribute to the overall function and health of a colony (Seeley 1989). The determining factors of an individual worker's task is a combination of genetic predisposition – known as Genetic Task Specialization (GTS) – and its age – known as age-based polyethism (Calderone & Page 1988). Younger adult bees are involved in care of brood, wax production, and hive cleanliness (Calderone 1998). As workers age, hypopharyngeal glands that function heavily in brood-rearing activity begin to atrophy (Huang & Otis 1989) and workers progress to a new task cohort where they are more likely to exhibit defensive behaviors, such as guarding of the colony entrance. Ultimately, the oldest workers will dedicate their energy to tasks outside the colony, such as foraging for nectar, propolis, and pollen. Therefore, the effect of a single brood mixing treatment will have different effects in separate colony functions as the mixed bees graduate between the task cohorts.

4.2 Objectives and hypotheses

Objective: Assess the ability of inter-colony brood mixing to replicate the benefits of hyper-polyandry.

Hypothesis: In addition to merely increasing the number of patrilinies in a colony, colonies with mixed brood will experience the beneficial effects of hyper-polyandry when compared to unmixed colonies as represented by stronger colonies and lower *Varroa* mite levels.

4.3 Methods

Twenty-four 1.5 kg packages of bees with naturally-mated Italian queens from Rossman Apiaries (Moultrie, Georgia) were established at the University of Delaware Newark Farm in April 2019. These queens were not supersisters as in previous field trials utilizing artificially inseminated queens, however, they originated from the same queen breeder and likely bred with the same population of drones and could therefore be considered a closed "population" of queens. In July, each colony was divided and reduced to a single 10-frame deep Langstroth box with 6 brood frames. Two sets of 6 colonies were designated as controls. The other two sets of six colonies were designated to receive the brood-mixing treatment, whereby each colony shared one randomly selected frame of brood with the other 5 colonies in their designated mixing group. Each colony then contained brood from 6 representative queens including the original colony. A total of 72 patrilines and 6 matrilines could then potentially be expressed in each mixed colony assuming the group of naturally mated queens had an average effective realized paternity of 12 drones (Tarpy et al. 2004).

Assessment strategy accounting for age-related task cohorts

Using *a priori* knowledge of age-associated tasks, a model was constructed by Lewis Bartlett of University of Georgia to predict the peak saturation of patriline diversity across all age-based task cohorts across all mixed colonies. These predictors informed which days after the brood mixing treatment that specific task-related colony

assessments should be made. The area proportions of eggs, open brood, and capped brood for each frame in each experimental colony – including unmixed colonies to control for handling effects – were recorded by two trained assessors and averaged one day prior to the brood mixing treatment. Based on the age proportions of brood at the start of the experiment, the model predicted Day 23 as the specific diversity peak for guard bee behavior and Day 26 as the peak expression of patriline diversity across all age-related task cohorts. Adult bee population, brood area (cm^2), *Varroa* mite numbers, and comb construction (cm^2) were assessed for each colony on Day 26 after the brood mixing treatment during the predicted peak patriline saturation in August. Two additional rounds of colony strength assessments were conducted at four-week intervals in September and October. See Table 2.2 for methodology of each measurement taken.

Additionally, a one-time measure of colony-level aggression was conducted 23 days after the brood mixing treatment during peak patriline expression within the guard bee task cohort for brood mixed colonies. This test was conducted to assess differences in a colony-level behavioral response between control and hyper-diverse colonies. Aggression level was determined by the proxy of number of guard bees recruited to the colony entrance following exposure to a synthetic pheromone. Following methods described in Pearce et al. (2001), a cotton swab was tacked to the landing board of each colony and an initial photograph (T_0) was taken of the colony entrance. 50 µl of isoamyl acetate (Acros Organics), a major component of honey bee alarm pheromone (Boch et al. 1962), was pipetted onto the swab. After 120 seconds (T_{120}) a second photograph was taken. The total guard bee recruitment was calculated

by subtracting bees present at T_0 from those present at T_{120} and expressed in terms of 100 bees.

4.3.1 Statistical analyses

All tests for statistical significance were conducted in JMP® Pro 14.0.0 (SAS Institute Inc.). Data for bee population, cm^2 brood, mite population, cm^2 comb, and guard bee recruitment were log transformed to improve assumptions of normality. Univariate mixed ANOVAs were conducted for bee population, cm^2 brood, and mite population with brood mixing as a fixed effect and individual colonies as random repeating measurements across the three assessment time points in August, September, and October. For cm^2 brood, bee population was assigned as a fixed covariate. For mite counts, both bee population and cm^2 brood were assigned as covariates.

Cm^2 comb and guard bee recruitment were measured during only one assessment and were therefore analyzed via standard least squares ANOVAs with brood mixing as a fixed effect and bee population assigned as a fixed covariate. All ANOVA results are presented in Table 4.2 and results were considered significant at the $\alpha < 0.05$ level. Least squares means and 95% confidence intervals were calculated for all measures and are displayed reverse transformed in Table 4.3. A regression model was made to characterize the relationship between aggression and bee population (Figure 4.2).

Additionally, standard least squares ANOVAs were conducted for *Varroa* mite counts at each monthly assessment for August, September, and October to assesses differences between brood mixed and control group means at individual time points. Monthly mite ANOVAs are presented in Table 4.4 and reverse transformed LS means and 95% confidence intervals are presented in Table 4.5.

4.4 Results

Two experimental colonies were lost in each of the two treatment groups after the second round of colony assessments in September (Table 4.1).

Table 4.1: Delaware 2019 brood mixing trial colony sample size by month and treatment (control vs brood mixed).

	Sample sizes during assessment months 2019		
Treatment	**August**	**September**	**October**
Control	12	12	10
Brood Mixed	12	12	10

Colony strength measures

Brood mixing did not have a significant effect on bee population, cm^2 brood, mite counts, cm^2 comb, or on guard bee recruitment (Table 4.2).

Table 4.2: ANOVA results for the effects of brood mixing (Delaware 2019) on colony strength measures and guard bee recruitment with bee population and cm^2 brood included as specific covariates for some measures. Significant results are **emboldened** ($p<0.05$).

ANOVAs of brood mixing effect			
	df	F	P>F
		bee population	
Brood mixing	1,21.7	0.297	0.591
		cm^2 brood	
Brood mixing	1,19.1	2.262	0.149
bee pop.	1,33.3	12.955	**0.001**
		mites	
Brood mixing	1,13.4	1.429	0.253
bee pop.	1,24	2.137	0.157
cm^2 brood	1,59.9	8.250	**0.006**
		cm^2 comb	
Brood mixing	1,17	0.026	0.874
bee pop.	1,17	11.062	**0.004**

		guard bees	
Brood mixing	1,21	0.065	0.80
bee pop.	1,21	15.344	**0.0008**

Table 4.3: Untransformed least squares means, degrees of freedom (df), and 95% confidence intervals for the effect of brood mixing on each colony measure during the Delaware 2019 field trial. Different letters in the same row indicate significantly different treatment group means ($p<0.05$).

LS Means for brood mixing treatment effect

	Control				Brood mixed			
	LS Mean	df	Lower 95%	Upper 95%	LS Mean	df	Lower 95%	Upper 95%
bee pop	9746	21.5	8365	11354	10316	21.9	8849	12026
cm^2 brood	2198	18.6	1812.5	2666.4	1802	19.7	1480.1	2195.0
mites	46.0	12.8	32.7	64.5	35.0	13.8	24.7	49.6
cm^2 comb	264.5	17	179.72	389.36	253.7	17	172.36	373.42
guard bees	54.6	21	39.6	75.3	51.6	21	37.5	71.2

Monthly mite counts

A monthly breakdown of mite count ANOVAs for brood mixed and control groups is presented in Table 4.4. No significant differences were found at any time point; however, the significance level of the brood mixing effect and covariate significance did vary from month to month. In August, the significance of brood mixing effect was $p = 0.371$ and cm^2 brood had a nearly significant covariate effect ($p = 0.051$). In September, brood mixing's significance increased to $p = 0.053$ and the bee population covariate was significant ($p<0.01$) and cm^2 brood had decreased in significance ($p = 0.347$). The same trend was seen in October with brood mixing effect decreasing in probable significance ($p = 0.133$), bee population remaining significant ($p<.05$), and cm^2 brood continuing to lack significant effect ($p = 0.385$).

Table 4.4: ANOVA results for the effects of brood mixing (Delaware 2019) on mite count measures in August, September, and October with bee population and cm^2 brood included as specific covariates for some measures. Significant results are **emboldened** ($p<0.05$).

	Varroa mite counts		
	df	F	P>F
	August		
Brood mixing	1,20	0.838	0.371
bee pop.	1,20	0.529	0.475
cm^2 brood	1,20	4.307	0.051
	September		
Brood mixing	1,20	4.239	0.053
bee pop.	1,20	8.826	**0.008**
cm^2 brood	1,20	0.927	0.347
	October		
Brood mixing	1,15	2.529	0.133
bee pop.	1,15	5.578	**0.032**
cm^2 brood	1,15	0.802	0.385

Table 4.5: Untransformed least squares means, degrees of freedom (df), and 95% confidence intervals for the effect of brood mixing on mite counts in August, September, and October during the Delaware 2019 brood mixing field trial. Different letters in the same row indicate significantly different treatment group means ($p<0.05$).

	LS means for *Varroa* mites by month							
	Control				Brood mixed			
	LS Mean	df	Lower 95%	Upper 95%	LS Mean	df	Lower 95%	Upper 95%
August	7.9	20	5.0	12.4	10.4	20	6.7	16.4
September	49.2	20	38.0	63.7	33.4	20	25.8	43.3
October	264.9	15	191.6	366.3	186.0	15	132.1	261.8

Colony-level aggression

A positive correlation between the log of guard bee recruitment and log of adult bee population was found. The regression equation was found to be significant ($F_{1,22}$ = 16.24, p < .001) with an R^2 of 0.425 (Figure 4.2).

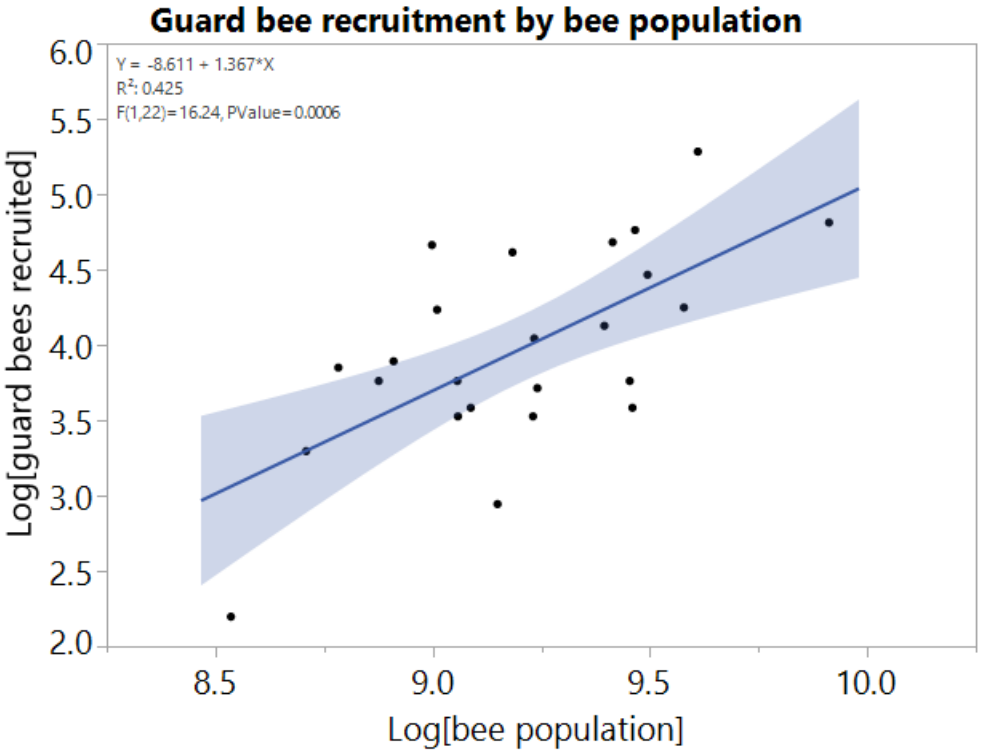

Figure 4.2: Regression model for guard bee recruitment by adult bee population for 2019 Delaware brood mixing field trial.

4.5 Discussion

Brood mixing did not influence aggression when controlled for population. The level of aggression, represented by proxy of guard bee recruitment, showed a

positive correlation with the colony population, similar to previous studies by Schneider & Mcnally (1992) and Wray et al. (2011).

This field trial of brood mixing did not find that inter-colony genetic mixing within a closed US population of honey bees recreates the results of the associated test of hyper-polyandry in the United Kingdom (Delaplane et al. 2015). No significant differences were found between brood mixed and control groups for any measure. In analyzing mite counts at separate time points, significance did increase in September and October with brood mixed colonies showing lower mite counts, but ANOVA results were not significant enough to conclude differences.

Of note when breaking down the mite count analyses into separate time points is the variation in covariate significance. Cm^2 brood had a nearly significant effect ($p = 0.051$) in August while bee population was not significant. This was reversed in September and October when mite levels were overall higher. These trends suggest that the mite count methods could be susceptible to bias based on the specific time within brood cycles at which mite counts are taken, as a significant proportion of mites could be sequestered within brood cells and therefore be undercounted. In the future, field counts of mite levels should look specifically at the proportion of mites within brood cells to improve count precision.

Despite the substantial genetic mixing treatment, which should have theoretically increased patriline number beyond that of the hyper-polyandrous queens used by Delaplane et al. (2015), the brood mixing treatment showed no significant effects. Several issues regarding the genetic structure of the United States honey bee population could explain the dissimilarity between the results of the brood mixing field trial and the United Kingdom hyper-polyandry trial.

In Europe there has been extensive research on conservation of honey bee genetic resources. Populations of managed honey bees were found to have reduced genetic diversity compared to wild populations (Moritz et al. 2007; Jaffé et al. 2010; Meixner et al. 2010; Leclercq et al. 2018). This high level of diversity and heterogeneous regional genetics across the original range of *A. mellifera* may provide greater opportunity for resistance traits to arise and be selected for in Europe, which in turn allowed for a hyper-polyandry treatment to improve resistance to mites (Delaplane et al. 2015). Genetic diversity is an especially relevant issue for US honey bee populations, which suffered an initial bottleneck due to a small European founder population and a second bottleneck as a result of current breeding practices whereby most queens bred in the US each year descend from as few as 600 mother queens mated with a drone source population of unknown diversity (Delaney et al. 2009). Considering that genetic heterogeneity is the potential source of rare resistance traits, the homogeneous nature of the US honey bee gene pool and the narrow population sample that was used for the brood mixing field trial likely could not replicate the benefits of an individual hyper-diverse colony. This brood mixing study may indicate the need for US beekeepers to obtain honey bees that not only have diverse allelic compositions but also to source queens that originate from multiple breeders with distinct genetic stock.

The long-term goals of the brood mixing project were to investigate novel IPM practices to relieve parasite and pathogen pressure on honey bees and reduce the use of synthetic acaricides to conserve their long-term effectiveness. With further research, brood mixing could serve as a complement to traditional breeding practices whereby beekeepers will be able to source queens from multiple breeders and express

desired traits from multiple selected lines simultaneously within individual colonies. This idea has been implemented in other areas of agriculture, for example, the widespread use of grafting in fruit production. Plants of two separate genetic lines are manually grafted together to combine disease resistant rootstock with hearty fruit bearing germplasm thereby reaping the benefits of both disease resistance and high yields (King et al. 2008; Bletsos and Olympios 2008). In an applied analogy to apiculture, one could imagine utilizing the brood mixing technique – or a brood "graft" – between a colony with high resistance to *Varroa destructor* and another colony with genetic predisposition for excellent honey production in order to express both phenotypes in each of the two colonies. Additionally, future field trials must test the number of repeat mixing treatments required across a season to maintain the mixed phenotypes across all age-related task cohorts and thereby sustain any beneficial effects.

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