GENETIC BASIS OF BODY SIZE VARIATION IN *FORMICA LEMANI*

By

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Abstract

Current genomic methods allow us to determine how genes shape phenotypic traits. Here, we are interested in the genetic basis of body size variation in ants. To conduct this research, we measured the head widths of workers, males, and gynes from colonies of *Formica lemani* ants. We then extracted DNA and conducted genomic sequencing for each measured ant in order to associate genetic variation with phenotypic variation. We found a strong association between genetic regions with low recombination rates and gynes and males with significantly reduced body size. This pattern resembles a similar trend identified in a related species, *Formica cinerea*, wherein the homologous genetic region controls a similar queen miniaturization phenotype. Our study adds to this line of research by adding an additional species and expanding to previously uninvestigated castes.

Acknowledgements

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1 | Introduction

Genes contribute to organismal phenotypic diversity, but we rarely know the genetic basis of traits and are consistently learning more thanks to technological and research breakthroughs (Kent et al. 2019). One of the exciting discoveries linking the genetic basis of phenotypes is the association between complex traits and genes that are locked together in suppressed regions also known as supergenes (Purcell et al. 2014; Yan et al. 2020).

Supergenes are groups of tightly clustered genes passed down from generation to generation that are able to partially bypass the crossing over event that happens during meiosis **(**Gutiérrez-Valencia et al. 2021). During meiosis, a process known as crossing over happens where pairs of chromosomes exchange genetic information prior to gamete formation. Supergenes have evolved to underlie complex traits, where specific combinations of loci are favored by selection (Keller et al. 1998; Navarro-Dominguez, Beatriz et al. 2022).

The underlying effect of supergenes can be seen in the white throated sparrow where a supergene codes for males that can have a white or tan plumage (Tuttle et al. 2016). Depending on the color of plumage the male white throated sparrow has, its mating strategy will differ from being polygamous to monogamous, respectively. Bringing the focus closer to the focus of this research, supergenes can also underlie colony eusocial structure, i.e. the number of queens per colony, in a variety of ant species (Kay et al. 2022).

Ants are **eusocial** species that exhibit traits such as division of labor among the different castes in the colony, shared responsibility in handling numerous tasks needed to take care of the brood, and having multiple generations living in the same colony at the same time (Hölldobler and Wilson 1990). Every individual ant's fitness is dependent on the survival and reproductive success of the colony as a whole (Boomsma et al. 2014).

Like other *Formica* species, my species of focus *Formica lemani* exhibits variation in colony queen number within a single colony (Seppä et al. 2009). A **monogyne** colony consists of one reproducing queen whereas a **polygyne** colony is centered around multiple queens capable of reproduction. Previous research has shown that within the genus *Formica*, a 'social' supergene on chromosome 3 is associated with colony queen number (Purcell et al. 2014; Brelsford et al. 2020) (Table 1). Members of monogyne colonies exclusively carry the M haplotypes whereas individuals from polygyne colonies contain at least one P haplotype (Table 1). Several other ant genera have independently evolved similar supergenes that underlie colony queen number (Wang et al. 2013; Kay et al. 2022; Table 1). We also take a look at haplotypes we named 9a and 9r from a coding region on chromosome 9 that has an influence on body size in *Formica* ants. Interestingly, while different species exhibit a similar phenotypic polymorphism, the genotype distributions associated with the monogyne or polygyne social forms vary among species (Table 1).

Table 1. A comparison of the supergene genotypes across other ant species that evolved independently besides *F. lemani* and shows how the genotypes at the supergenes are associated with the type of colony social form (monogyne vs polygyne). The monogyne genotype for *C. niger* was unable to be found since not much is known about the past of the 2 social morphs.

Monogyne Genotypes	**Species**	**Polygyne Genotypes**	**Citations**
m_a/m_d or m_a/m_a	*Formica glacialis*	p/m_a or p/m_D	Lagunas-Robles et al. 2021
m/m	*Formica francoueri*	m/p	Pierce et al. 2022
m/m or m	*Formica selysi*	p/p or p/m	Purcell et al. 2014
m_A/m_A or m_D/m_A	*Formica cinerea*	m_D/p_1 or p_1/p_1 or m_A/p_2 or m_D/p_2 or p_1/p_2 or p_2/p_2	Scarparo et al. 2023
m/m	*Formica neoclara*	m/m or p/m or p/p	McGuire et al. 2022

?	*Cataglyphis niger*	M/P (queens)	Lajmi A. et al. in prep.; Kay et al. 2022
B/B or B	*Solenopsis invicta*	B/B or B/b	Wang et al. 2013

Different social forms contain individuals with different body sizes where members of polygyne colonies are on average 10% smaller than individuals from monogyne colonies (Keller 1993). Within ant societies there exists 3 main castes: workers, males, and gynes. The majority of the members in any given *Formica* colony are workers that carry out numerous tasks including but not limited to caring for the queen, caring for brood, gathering food and resources, and defending the colony from attackers. Worker ants are all female and diploid organisms that possess two copies of each chromosome. Unlike workers, male ants only possess one set of chromosomes making them haploid. Upon mating with the queen, male ants will die off within a couple of weeks. Finally, gynes are females born with wings that have the potential of becoming queens once they successfully mate. After mating outside the colony, the gyne will lose her wings and complete her transition into the role of being a reproductive queen that either joins a pre-existing colony if she has less body fat or ventures off to independently start her own monogyne colony if she has more body fat that will enable her to survive alone until the first batch of offspring hatch (Keller et al. 1989).

For this study, we investigated body size variation in workers, gynes, and males of the ant *F. lemani.* We predict that body size of *F. lemani* individuals will depend on the social form of the natal colony. Moreover, we expect that this body size variation may be influenced by the social supergene on chromosome 3 and/or by a second, recently discovered supergene on chromosome 9 that influences extreme queen size dimorphism in the related species *Formica cinerea* (Scarparo et al. 2023). We also think that there could be a specific coding region on chromosome 9 very closely related to a coding region on chromosome 3 and that they may be

co-inherited together to code for ants that display a similar social form and effect on body size reduction. To obtain the necessary data to investigate patterns of body size variation, I measured head widths, assisted with DNA extraction in preparation for genomic sequencing, and analyzed genotype-phenotype associations.

The research's purpose is to shed more light on the possible genetic influences of the social chromosome 3 and chromosome 9 on body size differences of *Formica* ants belonging to either a monogyne or polygyne social form. We hope to find a similar underlying influence between a supergene and ant body size in our research on *F. lemani* where samples that have a certain group of genes found on chromosome 3 & 9 will be smaller than their counterparts that have a different group of genes on the same coding region.

2 | Materials and Methods

2.1 | Study Material

Belonging to the family of Formicidae, *Formica lemani* ants are a common ant species in high elevation environments of Europe. They are classified as a polymorphic species where their social structure can either be monogyne or polygyne (Hölldobler and Wilson 1990). *F. lemani* specimens from 34 colonies were collected in 2020 from Bosco Gurin, Switzerland.

2.2 | Data Collection

We measured the head width of individuals belonging to the worker caste (N = 161), male caste (N = 108), as well as the gyne caste (N = 65) using a Leica DMC 2900 light microscope and accompanying Leica Application Suite version 4.12 software. I configured the light microscope to have a 25x times magnification to view each ant sample's head closely under the microscope. Each individual ant was carefully placed on a specimen holder and maneuvered underneath the microscope lens in an orientation that maximized the width from eye to eye

across the front of the head (Figure 1). The method that I'm using in the Purcell lab is appropriate in collecting data because it allows us to gather lots of data points that we can use to construct visual representations of the hundreds of *Formica lemani* ant samples that have been gathered and studied. Our method of measuring the head width from the widest point of the ant's head because we determined head width to be an appropriate proxy for body size. The head measurements were done in collaboration with undergraduate student Carolina Gonzalez who also worked on the same project with me. Gonzalez measured 22 workers, 68 males, and 65 gynes.

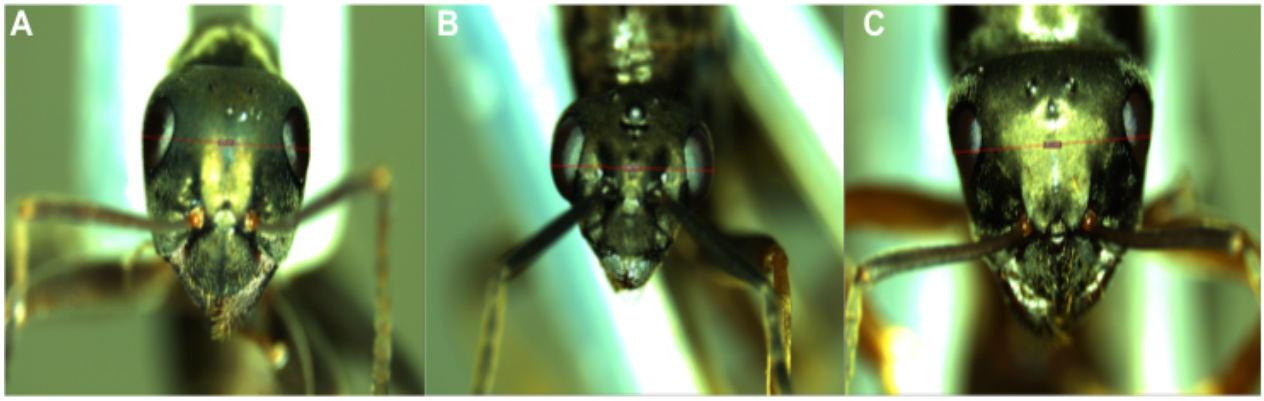

Figure 1. Image shows the images captured using the Leica Application Suite light microscope software to measure the width of a *Formica lemani* worker, male and gyne head. Figure 1A shows a worker ant with a head width of 1.534mm. Figure 1B shows a male ant with a head width of 1.492mm. Figure 1C shows a gyne ant with a head width of 1.812mm.

2.3 | DNA Extraction

We performed DNA extraction from the head and thorax of worker ants and the head of the males and gynes. We dissected each individual using a flame sterilized scalpel and retained the unused portion of the ant as a voucher in 100% Ethanol. The head and thorax or head of each ant would then be individually transferred using a pair of flame sanitized forceps to a new empty

centrifuge tube which is labeled and placed in a custom built styrofoam tray mount system. After repeating this process multiple times until enough samples are mounted, liquid nitrogen is poured down a slope of the mounting system and is allowed to encircle each individual tube but not enter the tubes themselves. Moving quickly, we would use one sterilized pestle per centrifuge tube to grind the ant parts into a fine powder. When we have ascertained that the ant pieces have been ground to the texture of small dust particles, we add a solution of proteinase K and ATL buffer to each individual tube. We then place all the samples into an incubator set at 56^{o}C degrees overnight. We transferred the supernatant to the QIAcube HT/QIAxtractor robot to complete the extraction (QiaAmp 96 extraction kit). We eluted the DNA in 100 µL of buffer EB.

2.4 | Genomic Library Prep and Bioinformatics

The following steps were carried out by PhD student Zul Alam, a collaborator on this project. In brief, we amplified portions of the DNA to analyze specific single nucleotide polymorphisms (SNPs) to look for changes in the DNA genome following a customized ddRADseq protocol (Brelsford et al. 2016). After we obtained the necessary information from the DNA extraction process and RADseq, the data was compiled and organized into useful information using bioinformatic techniques and software. When the data is obtained from the RADseq, what comes out is strands of DNA segments that are not directly usable. We aligned the unknown *F. lemani* sequences to the genome of *F. selysi*, as they are very close on the phylogenetic tree. Using filtering tools such as vcftools in a software program called Unix as well as other tools that look for SNPs in the DNA, informative SNPs are retained while loci with substantial missing data are removed to provide a clearer image of the genetic trend in the samples. After applying the filters minDP (minimum read depth) of 8, max-missing of 0.9 (genotype information must be present in 90% of individuals), and MAC (minor allele count) of 2, our final dataset resulted in 24,439 SNPs. Both chromosome 3 and 9 were excluded for some analyses because we wanted to be able

to find the social form of the samples independently of the effect of the supergenes on chromosome 3 and 9. Separately, we inspected the variation found on chromosome 3 and chromosome 9 to ascertain individuals' genotype at each supergene locus.

2.5 | Colony Social Forms

To infer colony social form, we calculated within colony pairwise relatedness using COANCESTRY 1.0.1.10 (Wang 2010). This analysis is strongly sensitive to sample size, thus, we kept only colonies with at least 5 diploid individuals (workers and gynes). Within monogyne colonies, we expect to find exclusively full-siblings (relatedness = ~0.75), or half-siblings (relatedness = ~0.25), while within polygyne colonies, we expect most of the individuals to be unrelated (relatedness = ~0). We organized the different social forms as follows: we called colonies with all pairwise relatedness estimates greater than or equal to 0.6 as monogyne monoandrous (= colonies with only one queen mated with a single male); we called colonies with at least 40% of pairwise relatedness estimates greater than or equal to 0.6, but none less than 0.2 as monogyne polyandrous (=colonies with only one queen mated with 2 or more males); colonies with less than 40% of the pairwise relatedness values greater than or equal to 0.6, but none less than 0.2 as inbred polygyne (=colonies with two or more related queen); colonies with at least one pairwise relatedness estimate equal to or less than 0.1 as polygyne (= colonies with 2 or more unrelated queens).

2.6 | Statistics

We used R studio to turn the large amount of gathered data into visual representations that can be used to scan for patterns in the body size dimorphism of the *Formica* from different colonies as well as different social forms. Some packages used in R studio to create the graphs include ggplot2, readxl, and magrittr. We specifically used the built-in function of ggplot2 geom_boxplot() to create the boxplots. We used the readxl package to read excel sheets that we

imported into R studio to be used for creating the boxplots. We used magrittr to organize the genotypes in the order we wanted them to be as seen in figures 2A, 2C, and 2E. We performed independent linear mixed model analyses for chromosomes 3 and 9, to test whether individuals within the caste exhibit different body sizes based on their supergene genotypes. We used colony as a random effect and genotypes as a fixed effect. We also fitted linear mixed models to test whether the body sizes of workers, gynes, and males differ across colony social forms. For all linear mixed models, we used the R package lme4 (Bates et al. 2014). Pairwise p-values were obtained after performing Tukey post hoc tests using the emmeans function in R (Lenth et al. 2019).

3 | Results

Previous analysis done by collaborators Dr. Giulia Scarparo and Zul Alam on *F. lemani* have shown that there are 4 haplotypes on chromosome 3 called M1, M2, P1, and P2 with 10 different combinations of genotypes. Two haplotypes were found on chromosome 9 called 9a and 9r with the genotype combinations of 9a9a, 9a9r, and 9r9r. The P2 haplotype on chromosome 3 and the 9r haplotype on chromosome 9 are co-transmitted together. They are almost always passed down together in the same individual. From previous research there is an imperfect association between the 4 haplotypes on chromosome 3 with colony social form. M1and M2 haplotypes are associated with the monogyne social form whereas the P2 haplotype is associated with the polygyne social form. An exception occurs with the P1 haplotype since it is found almost equally in both monogyne and polygyne social forms. From this observation, we can say that the P1 haplotype does not seem to be associated with social form anymore. It is worthy of further investigation to research why P1 has stopped clearly being responsible for social form in *F. lemani.*

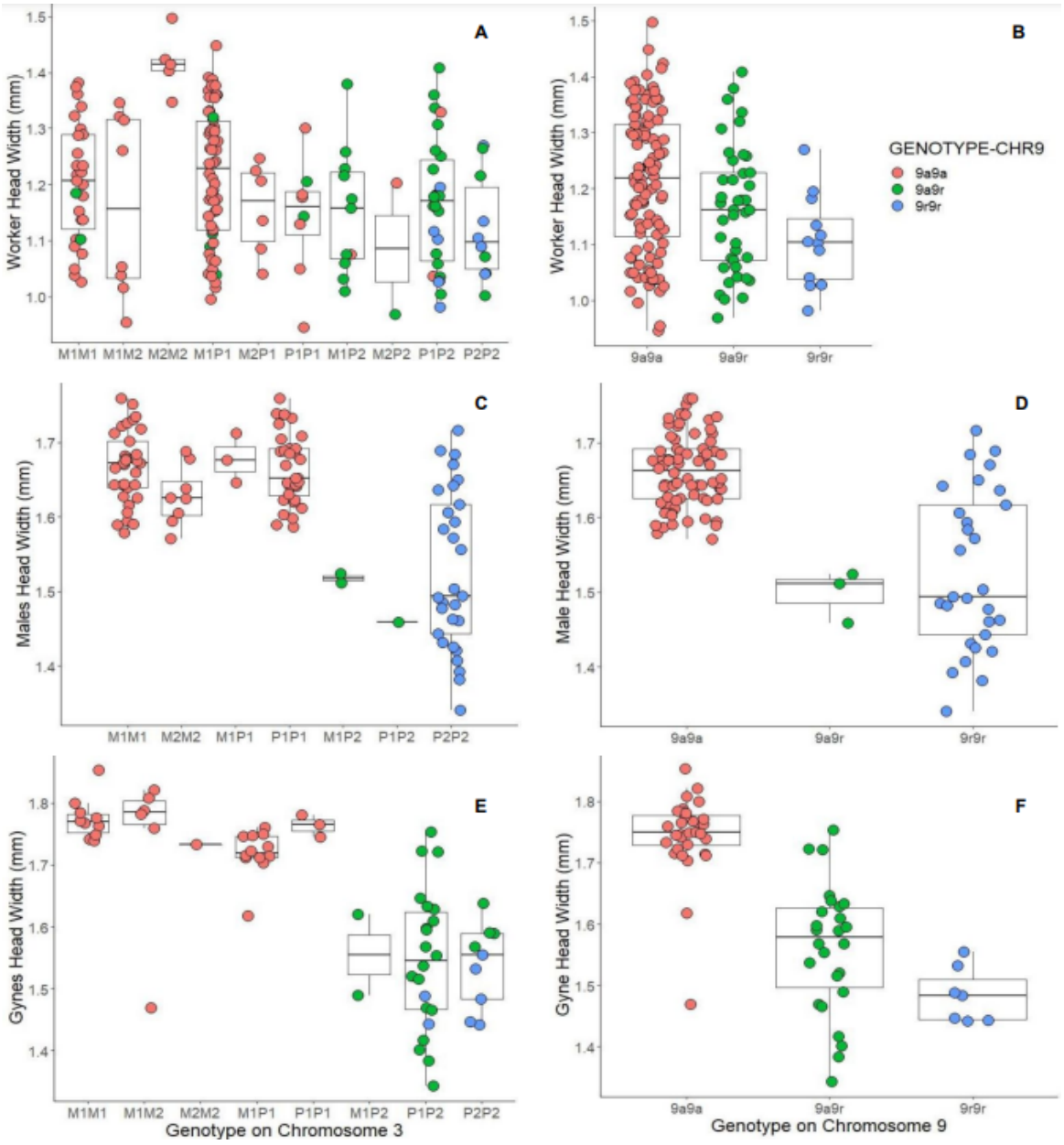

Figure 2. We compare the head width as a proxy for body size of the 3 castes in *Formica lemani* (workers, males and gynes). In observing the results of the 9r haplotypes on chromosome 9 we observe a body size shrinking effect in the presence of a 9r haplotype on *F. lemani* with the exception of worker ants. We observe that workers who do not have the 9r haplotype vs. those that do have it don't show a statistical significant difference between head widths. (A). Male *F. lemani* ants without a 9r haplotype either have no difference in head width size or can be larger than their counterparts if they were homozygous for the 9r haplotype (C&D). Gynes who do not have the 9r haplotype have an average head width that is larger than gynes with a 9r haplotype (E). We observe the body size shrinking effect of the 9r haplotype on chromosome 9 in *F. lemani* as ants with it are on average smaller than those without it. It can be seen that males without the 9r haplotype have an average head width that is larger than the average male head width of ants that contains 1 or 2 9R haplotypes in their genotype (D). We can also observe that gynes that do not have the 9r haplotype have an average head width that is larger than that of the average head diameter of gynes that have at least one copy of the 9r haplotype (F). We observed a small number of diploid males therefore we labeled the x-axis with diploid labels but the majority of the homozygotes are expected to be haploid.

3.1 |Genetic Basis of Body Size Variation

A general trend of smaller body size was observed in the boxplot data sets with samples that had either a P2 haplotype or 9r haplotype with the exception of worker ants. Boxplots in figures 2A, 2C, and 2E showed a relationship between the measured head width of *F. lemani* workers, males, and gynes and supergene genotypes on chromosome 3. Boxplots in figures 2B, 2D, 2F showed a relationship between the measured head width of *F. lemani* workers, males, and gynes and genotypes on chromosome 9. Looking at Figure 3 below, we can observe that samples with the 9r haplotype are on average smaller than their counterparts except for the worker caste.

Table 2. This table shows the significant p values across the ant castes to find the relationships of the genotypes on chromosome 9. The values were calculated using linear mixed models. The significant p-values are in bold.

	Workers	Males	Gynes
9a9a to 9r9r	0.0770	**<0.0001**	**0.0002**
9a9a to 9a9r	0.2462	**0.0134**	**0.0075**
9r9r to 9a9r	0.4871	0.7191	**0.0052**

3.2 |Workers Measurements

Looking at the social chromosome 3 of *F. lemani* workers, we used a linear mixed model test and observed a statistically significant difference in body size between M2M2 individuals and individuals carrying other supergene genotypes ($p < 0.006155$). M2M2 workers are 23.5% bigger than all other workers (Figure 2A). The P2 haplotype was not significantly associated with a reduction in the body size of workers. When we looked at the pairwise difference results between the 3 different genotypes on chromosome 9, we did not find a significant difference between 9a9a workers and workers that had two copies of the 9r haplotype ($p<0.0770$) (Table 2).

According to their social form, workers in polygyne colonies were smaller than their counterparts in monogyne colonies. We ran a linear mixed model test and found that samples from inbred polygyne colonies were statistically different from monogyne monoandrous colonies ($p = 0.001$) and monogyne monoandrous were statistically different from polygyne colonies ($p = 0.0081$) which helps prove the body size reduction in polygyne colonies was indeed seen.

3.3 |Males Measurements

We saw a significant difference in body size on chromosome 3 among the 7 different genotype combinations seen in male ants ($p < 0.001$). In every instance where there was a statistically significant difference between body size using the statistical threshold of $p < 0.05$, we saw the presence of at least one P2 haplotype that made the males with the P2 haplotype smaller than those without the haplotype (Figure 2C). Unlike in worker ants, the pairwise difference results between the genotype combinations on chromosome 9 showed a statistical difference between males with the 9r haplotype versus those that did not have the 9r haplotype ($p < 0.0001$) (Table 2). Males with two copies of the 9r haplotype were 8% smaller than their counterparts that did not have a single 9r haplotype (Figure 2D).

Looking at their social form, males in polygyne colonies did not appear to have a statistical difference in body size compared to their counterparts in monogyne colonies ($p = 0.05569$).

3.4 | Gynes Measurements

Similar to the results we found in *F. lemani* males, gynes also exhibited a statistically difference in body size on chromosome 3 among the 8 genotype combinations ($p < 0.008858$). There were 6 instances of statistical difference we observed when looking at pairwise differences on chromosome 3 genotypes that showed a consistent pattern of smaller gyne body size with the presence of one or 2 of the P2 haplotype (Figure 2E). Gynes with the P1 haplotype and no copies

of the P2 are not significantly different from gynes without any P haplotypes. Gynes with at least one copy of 9r haplotype are significantly smaller than gynes that did not have the 9r haplotype ($p = 0.0002$) (Table 2). Indeed, 9r9r gynes were 14.9% smaller than 9a9a gynes, while 9a9r gynes are 10.3% smaller than gynes without the 9r haplotype (Figure 2F).

According to their social form, gynes in polygyne colonies were smaller than their counterparts in monogyne colonies. To test this, we ran a linear mixed model test and found that samples from monogyne monoandrous were statistically different from polygyne colonies ($p = 0.0166$) which helps prove the body size reduction in polygyne colonies was indeed seen.

3.5 | Mismatches

There is an imperfect association between the body size-reducing haplotype 9r and the P2 haplotype that didn't follow our hypothesis of the 9r and P2 haplotypes being co-inherited together. Individuals that showed mismatched haplotype pairing numbered 8. Contrary to the expected co-inheritance of the P2 and the 9r haplotypes, these few examples had a P2 haplotype in their genotype but were homozygous for the 9a haplotype.

3.6 | Effect of Social Form on Body Size Variation

We observed 2 types of social forms that *F. lemani* exhibits namely monogyny and polygyny. We can further subdivide the monogyny colonies into two categories namely monogyne monoandrous and monogyne polyandrous. We can further subdivide the polygyny colonies into two categories: a polygyne colony and an inbred polygyne colony. For the worker ants we had 87 samples from monogyne colonies and 76 samples from polygyne colonies. For the male ants we had 60 samples from monogyne colonies and 38 samples from polygyne samples. For the gyne ants we had 26 samples from monogyne colonies and 36 samples from polygyne colonies.

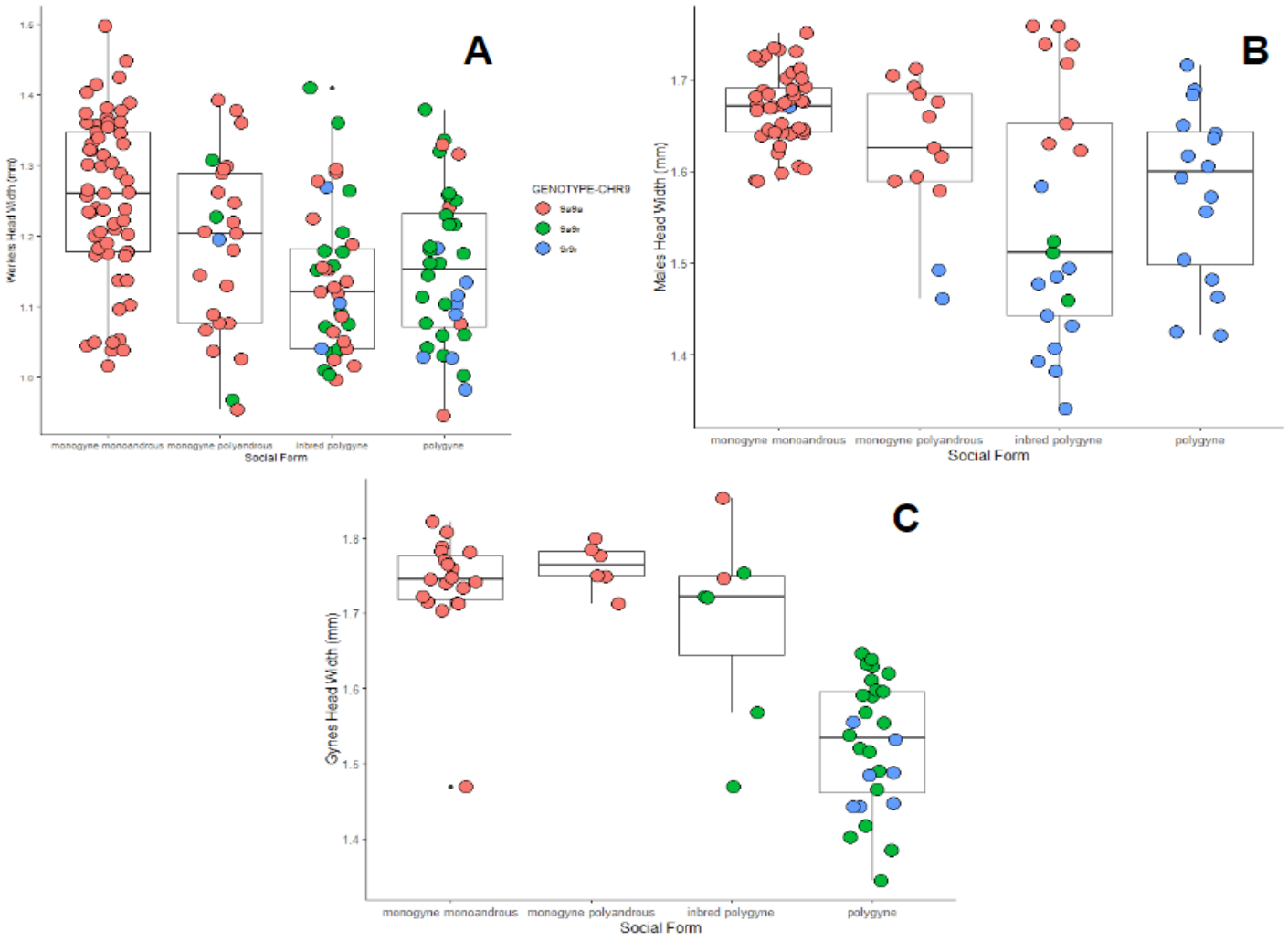

Figure 3. We inferred colony social form for each of the analyzed colonies using pairwise relatedness estimates. Figure 3 shows head width distribution in monogyne monoandrous, monogyne polyandrous, inbred polygyne and polygyne colonies in workers (A), males (B), and gynes (C). Figure 3B shows the social form distribution of male ants. Figure 3C shows the social form distribution of gyne ants. We can see very clearly in Figure 3C that any sample with the 9r9r genotype belonged to a polygyne colony. In figures 3A and 3B we can see a similar result with only a few samples that had the 9r9r genotype belonging to a mongyne colony. Individuals are color coded according to their genotypes on chromosome 9.

4 | Discussion

4.1 | Size Variation

We observed substantial head width variation within castes. Gynes and males were highly variable, while workers exhibit lower size variability (Figure 2). In general, workers, males and gynes from monogyne colonies were larger than those from polygyne colonies (Figure 3).

4.2 |Overview of supergene variation

The genetic analysis carried out by my collaborators revealed 4 haplotypes on chromosome 3 that we named M1, M2, P1, and P2 and 2 haplotypes of interest on chromosome 9 called 9a and 9r.

4.3 |Relation between supergene variation and size

We were able to distinguish a pattern for ant body size reduction based on the haplotypes P2 and 9r from individuals that did not contain these haplotypes (Figure 2). A notable discovery unrelated to our conjectures on the effect of the 9r and P2 haplotype on body size we discovered was in worker ants where we noticed a phenomenon where individuals with a M2M2 genotype were statistically larger than their counterparts without the M2M2 genotype. This may mean that the M2M2 genotype could be responsible for larger body size in *F. lemani.* However, this result needs to be taken with caution as we only had 5 samples that had the M2M2 genotype that are all from the same colony. Furthermore, the influence of the M2M2 genotype was only observed in worker ants and therefore may only have an effect on workers ants. To confirm this possible association between larger body size and the M2M2 genotype, we need to increase the sample size. On a more broad overview of the patterns we observed in Figure 3, most samples that had either one or two 9r haplotypes tended to belong to a polygyne colony rather than a monogyne one and were on average smaller in all 3 castes across colonies. However, we did observe an exception in the worker ant caste where there was no statistical significance in body size

difference. We think this could have been due to the fact that the social chromosome 3's P2 haplotype is co-inherited with the 9r haplotype. We know this because when looking at the pair graphs of Figure 2C and 2D for males and Figure 2E Figure 2F for gynes we can see that ants with a P2 haplotype tended to also have at least 1 9r haplotype in their genotypes.

4.4 |Additional observations about supergenes

We observed some individuals with a P2 haplotype that lacked the 9r haplotype, and some individuals with the 9r haplotype that lacked the P2 haplotype. Interestingly, all of these "mismatches" were observed in workers (Figure 2A). We speculate that there were not any mismatches in the males and gynes due to the difference in their lifestyles. Specifically, mildly deleterious combinations of haplotypes would be strongly disadvantaged during the independent phase of their life.

4.5 |Comparison to other Formica *species*

Through our study, we observed a similar effect of the homologous chromosome 9 supergene in reducing male and gyne body size in both *F. lemani* and the closely related species *F. cinerea* (Scarparo et al. 2023). The two species showed a different magnitude in the reduction of body size among males and gynes. Specifically in gynes we saw a slightly greater reduction in body size in *F. cinerea* (20%) compared to *F. lemani* (14.9%). In males we saw a similar reduction in body size (~8%). Workers were measured for the first time in *F. lemani* and we observed no difference in size based on the supergene on chromosome 9. We did not see a large difference between the body size reduction percentage for male ants in both *F. lemani* and *F. cinerea.* The body size reduction influenced by the supergene on chromosome did not seem to affect *F. lemani* gynes as much as it affected *F. cinerea.*

5 | Conclusion

After looking at the trends gathered by assembling the data on our many ant samples from monogyne and polygyne colonies, we have observed that the haplotype designated 9r has without a doubt a significant amount of influence on body size reduction in *F. lemani.* For example, we saw as much as a 8% decrease in body size in male ants that were homozygous for the 9r haplotype in comparison to male ants that did not have any copies of the 9r haplotype at all (Figure 2D). Following on a similar trend, gyne ants that were homozygous for the 9r haplotype showed as much as a 14.9% decrease in head width as a proxy for body size compared to gynes that were homozygous for the 9a haplotype.

Through this research, we discovered a genetic factor that influences body dimorphism depending on the social form in the species of study *F. lemani*. We were able to find that a haplotype called 9R, with a few exceptions where it appeared in monogyne colonies, appears mostly only in polygyne colonies was a factor that reduced the average body size of ants across all castes in comparison to samples found in monogyne colonies. This offers exciting new specific information on a haplotype that influences body size in *Formica* ants that adds to the theory of polygyny syndrome discovered in previous research (Kay et al. 2022).

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