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Behavior and Chemical Signals as Markers of Colony Identification in Argentine Ants (Linepithema Humile)

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BEHAVIOR AND CHEMICAL SIGNALS AS MARKERS OF COLONY IDENTIFICATION IN ARGENTINE ANTS (LINEPITHEMA HUMILE).

by

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(Under the Direction of Joshua D. Gibson)

ABSTRACT

Argentine ants, *Linepithema humile*, are a highly successful invasive species around the globe and are especially prominent in states such as California and the southeastern United States. *L. humile* have a unique form of unicoloniality, called “supercolonies”. *L. humile* can detect colonymates through scent markers in their outer cuticle. With these chemical markers, ants will exhibit high aggression if they smell different from one another. In my study, I performed aggression assays among ten different nest sites and analyzed their CHCs through gas chromatography mass spectrometry, or GC-MS, analysis. For my behavior results, while within-nest interactions displayed low aggression as I expected, I also observed one potential colony composed of three of the collected nests. Through GC-MS Analysis, I was able to detect 58 unique CHC compounds within the ten nests samples but was not able to determine any statistically significant patterns among the data to help further explain the unexpected behavior seen between nests that were friendly towards one another, despite being far in distance. I was able to observe that the samples collected show high variation not only between the nests collected, but between samples derived from within the same nest. The high variation present in my study may indicate that the colonies in Georgia present a more complex relationship between CHCs and colony identity than seen with other introduced colonies such as California, and that it

is likely that some much smaller subset of these CHC compounds are involved in colony recognition.

INDEX WORDS: Argentine ant, Behavior, Chemical ecology, Cuticular hydrocarbons

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by

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CHAPTER 1

INTRODUCTION

The Argentine ant, *Linepithema humile*, is a unicolonial invasive species that was introduced into the United States, Europe, and Japan in the 1800's from subtropical regions of South America (Hartley et al. 2006, Helanterä et al. 2009, Latty et al. 2017, Newell and Barber 1913, Sato et al. 2017). *L. humile* has been heavily studied in southern Europe, New Zealand, Spain, and California (Vogel et al. 2010, Roura-Pascual et al. 2009, Corin et al. 2007, Wetterer and Wetterer 2006). *L. humile* are a unicolonial species with an exaggerated form of polydomy and polygny, meaning that one colony consists of multiple nests with more than one queen (Helanterä et al. 2009). This allows *L. humile* to invade a variety of habitats such as agricultural and urban areas causing economical damage and ecosystem disruption (Silverman and Brightwell 2008). *L. humile* takes unicoloniality to an extreme by producing large colonies known as supercolonies, which consist of multiple physically separate nests occupying territories that can span many kilometers, and even span across multiple continents. These nests contain numerous reproductive queens and there is little to no intracolonial aggression within these supercolonies (Tsutsui and Case 2001, Helanterä et al. 2009). Queens stay within the confines of their own natal colonies, meaning that they do not go outside the colony to mate, rather they mate within their own colony (Moffett 2012). In order to expand, workers and other queens "bud off" by moving together from the original nest to new sites nearby and allowing supercolonies to expand as far as the environmental conditions deem possible (Moffett 2012, Suarez et al. 2001). Their invasive success is partially due to their flexible supercolony structure.

While these colonies can span kilometers and even across oceans, it does not mean that different *L. humile* colonies get along. If an ant from one colony interacts with an ant from

another colony, they will exhibit aggressive behavior towards one another that can be anything from flaring their mandibles towards one another, to long grappling fights that can lead to legs and antennae being dismembered, or mortality of another ant (Suarez et al. 1999). It has been observed that ants from introduced ranges will almost always show unicoloniality, while ants from native habitats will exhibit intercolonial aggression towards nests that are further away geographically (Suarez et al. 2008). In California, the global supercolony always initiated aggression towards the other colonies, except in the presence of the secondary colony, Sweetwater, in which case the two colonies initiated aggression at an equal frequency (Tsutsui et al. 2003). Four colonies have been detected in Japan where all same-nest behavioral assays exhibited non-aggressive behavior and the nests from different colonies exhibited aggressive behavior (Sunamura et al. 2007). Despite having their colony members potentially being geographically distributed across the globe, *L. humile* can recognize their nestmates through scent, specifically through their cuticular hydrocarbons.

Nestmate recognition pheromones are contained in L. *humile*'s cuticular hydrocarbons (Brandt et al. 2009). All insects produce cuticular hydrocarbons (CHC's), which are waxy chemicals that coat the outside of their bodies and are used to prevent desiccation and microbial infections (Brandt et al. 2009). CHC's have other uses, such as: species recognition, chemical mimicry to parasitize other organisms, mate recognition, and nest/colony membership identification (Brandt et al. 2009). CHC profiles consist of highly complex mixtures of straight chain carbons such as alkanes, alkenes, and branching methyl alkane groups (Guerrieri et al. 2009). Studies done on *L. humile* populations in California have identified ~70 specific compounds that make up their CHC profile, which showed consistency of CHC compounds identified across samples from the same colony; however, only having 5-6 different colonies in

California makes it difficult to determine the role of individual compounds in colony recognition versus their role in adaptation to local environmental conditions (Buellesbach et al. 2018). While it is known that there are correlations between different colonies and their CHC profiles, evidence supports that no single compound is solely responsible for nestmate recognition and implies the blend of certain compounds being important (Brandt et al. 2009). It has also been found that various factors can affect their CHCs, such as climate, time of year, time in lab settings, and the food they eat (Brandt et al. 2009, van Wilgenburg et al. 2010, Buellesbach et al. 2018, van Wilgenburg et al. 2022). The majority of colony recognition research with *L. humile* has occurred on the 5-6 colonies that span across the California coast. Of these colonies, a massive supercolony has been identified between San Francisco and San Diego, along with smaller colonies in southern California (Tsutsui and Case 2001). In *L. humile's* native range, there is a high level of genetic diversity between distinct colonies, but within introduced ranges, this genetic diversity is greatly decreased and is highly homogeneous, with the southeastern United States being an exception (Suarez et al. 1999). In the southeastern United States, existing data suggests that there are many more colonies compared to anywhere else in the invaded range (Buczkowski et al. 2004, Gibson lab unpublished data). While these colonies appear to be smaller than the large supercolony in California, they can still span several kilometers (Buczkowski and Silverman 2006, Helanterä et al. 2009, Gibson lab unpublished data).

The southeastern United States provides a crucial study environment for *L. humile.* Nests in this region are patchy, and Buczkowski et al. (2004) pointed out it is not certain if this is due to the effects of a genetic bottleneck, as proposed in Tsutsui et al. (2000), or ecological mechanisms (such as biotic and abiotic factors) as proposed in Giraud et al. (2002). In previous unpublished work in the Joshua Gibson lab, six *L. humile* nests were collected along a 225 km

transect across Georgia and behavioral assays were conducted. Ants from two sites (BV and GR) were shown to have all non-aggressive interactions out of all cross-site comparisons. Two other nests (SW1 and MC1) also exhibited a low proportion of aggression. All remaining nest sites were mutually aggressive with all other sites, indicating a total of four to five colonies across this single transect (Gibson lab unpublished data, Figure 1). Another study was conducted on three nests sites in Georgia, looking at the CHC profiles of ST1, SW1, and a nest that was around 1.6 km from ST1 (Barrs 2021, Figure 1). Through GC-MS analysis, it was found that ST1 and the nearby nest were shown to have CHC compounds shared in both samples, and not found in the SW1 sample, suggesting that ST1 and the nearby samples were part of the same colony, and the sample from SW1 is part of a different colony (Barrs 2021, Figure 1). Collecting more nests from Georgia, running in-lab aggression assays, and analyzing the collected *L. humile* CHC profiles will add more insight into colony structures in the Southeastern United States and will help to ascertain the role of different CHCs in colony identity signaling.

The goal of my study was to determine the geographical distribution of colonies in Georgia and a better understanding of how their CHC are used as colony identity markers. I hypothesized that there are more *L. humile* colonies in the southeastern United States compared to other introduced regions around the globe such as California, Europe, and Japan. If this is supported, I predict that I will observe high levels of aggression between nests that are from different collection sites and low levels of aggression between nests that are in close proximity. For my hypothesis regarding the chemical data, I hypothesize that CHC profiles are markers of colony identity. If this hypothesis is supported, I predict that ants from nests that exhibit low levels of aggression will have similar CHC profiles and those that exhibit high levels of aggression will have less similar CHC profiles.

CHAPTER 2

METHODS

Identifying Different Colonies Using Aggression Assays Between the Nest Sites Collected

Nest Collection: Ten *L. humile* nests sites were collected in the summer and fall months of 2021 along a transect spanning ~225 km in Georgia (Figure 1). Six out of the ten collection sites were previously discovered in a research project in the summer of 2020 (Unpublished data from Gibson Lab): GR (Griffin, GA), BV (Barnesville, GA), HFE (Jackson, GA), MC1 (Macon, GA), SW1(Swainsboro, GA), and ST1 (Statesboro, GA). The other four collection sites were found within a radius of 1.6 km minimum to 24 km maximum from one of the previous sites: ST2 (Statesboro, GA), SW2 (Swainsboro, GA), MC2 (Macon, GA), and HFW (Jackson, GA). This distance was to ensure that the nests were independent from one another, but close enough that they could potentially be from the same colony (Figure 1).

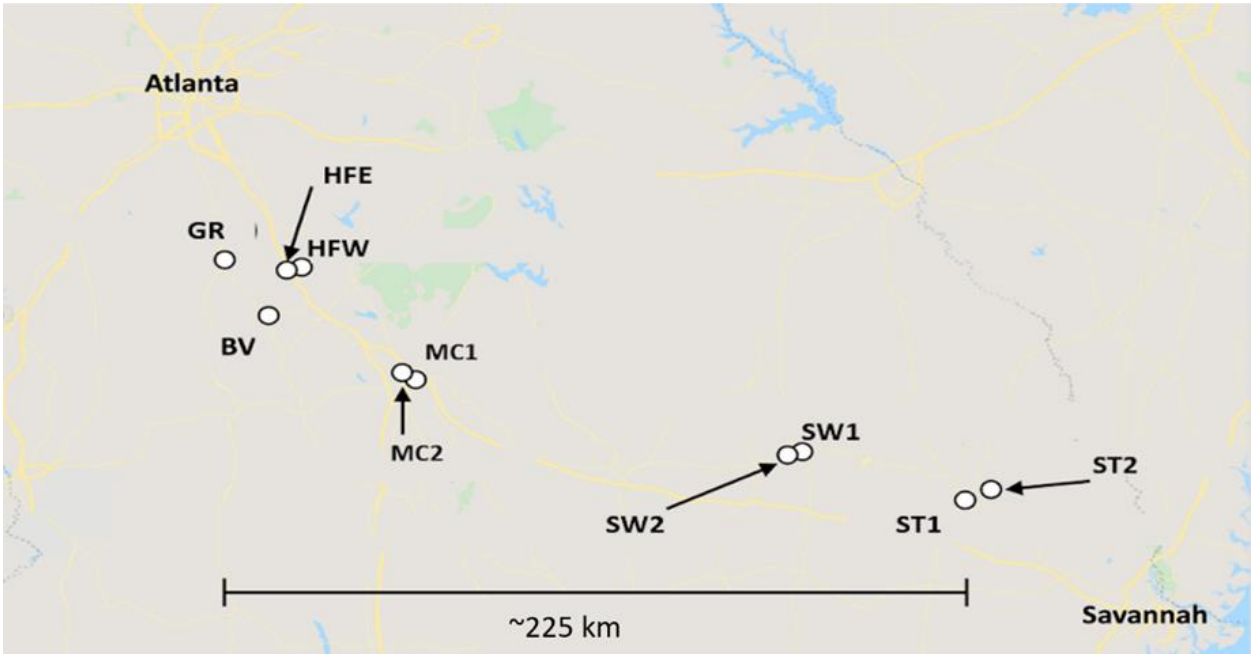

Figure 1. Map of all the nest collection sites. The faint dotted line is the state border between Georgia and South Carolina. The City of Statesboro is located at the sites of ST1 and ST2.

All ten nests were collected using the same method. 5-gallon buckets (Lowe's) coated in Insect-a-slip (Bioquip Products, Rancho Dominguez, CA) were used to keep the ants from escaping and transport the collected nests. Once a nest was found, gardening trowels were used to scoop up ants and brood, along with the dirt and leaf litter containing the nest. Once enough ants were collected (anywhere from 10,000 to 20,000 ants), a damp paper towel was placed over the leaf litter/soil to keep the nest collection from drying out. The lid to the 5-gallon bucket was also secured on top of the bucket during travel.

The ants in the 5-gallon buckets were brought back into the lab and then flooded. Flooding consists of dripping water into a large plastic container, which holds not only the ants collected, but the dirt and leaf litter that was dug up with them. A paper bridge is provided for the ants so they can move themselves and their brood into a second clean container, which consists of black petri dishes with dental plaster added to serve as nesting sites and two silos for liquids: one with fresh water and one with 20% sugar water. The water and sugar water silos were made from 50 ml conical tubes (Fisher Brand) that were inverted and have small holes drilled around their base to facilitate access to the liquid. The lids of the conical tubes were molded into an open petri dish filled with dental plaster to create a sturdy base for the silos. Flooding the ants into a clean plastic container makes ant collection more feasible during trials. In addition to the water and sugar water, the ants were fed frozen crickets cut in half three times per week.

Aggression Assays: Aggression assays were conducted in November 2021. Evidence suggests that aggression assays conducted with more ants (instead of one ant from one collected nest versus another) can help minimize false negatives (Roulston et al. 2003). For this experiment, aggression assays were executed using three ants vs three ants in twelve well plates

(Greiner Bio-One). Six of the twelve wells were used for each run of the assay, allowing six comparisons to be made at once. The duration of the aggression assays were ten minutes; videos were taken for the full duration of the trials. Ten replicates were run for each pairwise assay. All pairwise combinations were tested, including within-nest pairs, for a total of 550 trials. Recordings of the assays were later reviewed blindly (viewer did not know which site the ants were from) and notes on their behavior were recorded using the following behavior, in decreasing order of aggressiveness: grappling, biting, mandible flaring, avoidance/recoil, avoidance, and antennation. These sets of behaviors were based on the aggression assays run in Suarez et al. (1999) and Suarez et al. (2002). Notes were taken during the trials to note if any of the ants escaped the well plates. The highest form of aggression seen during trials was then assigned after viewing the trial recordings. If the trials were seen having aggressive behavior involving a physical altercation (biting and grappling), that trial was given a "yes" for aggression. If the trial was seen having non-physical behavior (mandible flaring, avoidance/recoil, avoidance, or antennation), that trial was given a "no" for aggression. The proportion of aggressive behavior shown for each trial was put into a proportion out of ten, where zero equals zero out of the ten trial replicates exhibited no aggression and one equals ten out of the ten trial replicates exhibited aggression.

Determining the Differences in CHC Profiles of Collected Nests

CHC Extraction: In order to assess the different compounds making up the CHC profiles, 100 ants from each collection were freeze-killed. The frozen ants were put into a 2 ml vial with 200 µl of hexane. The vial was swirled for ten minutes at 180 r/min. The hexane and CHC solution was extracted with a glass Pasteur pipette and transferred into a plastic insert (250µL; Agilent) which was then placed back into the original 2 ml vial. The Hexane/CHC

solution was then evaporated down using a slow flow of nitrogen gas. CHC's were resuspended using 12 µl of hexane spiked with 7.5 ng/µL of dodecane (N-Dodecane >99%, Sigma Aldrich) to be used as a dodecane standard in order to calculate the mass (ng) of each CHC compound.

Running CHC Extractions on the GC-MS: Following the protocol in Barrs (2021), the CHC's extracted in hexane were analyzed with a gas chromatograph-mass spectrometer (GCMS: QP2010S, Shimadzu Corporation, Kyoto, Japan). The entire sample, 12 µL, was taken out of each CHC/hexane vial and was manually injected into the GC-MS with a 50 µL syringe (ACE Glass Incorporated, Vineland, NJ), and the sample was run for 30 minutes with a final temperature reaching 300^{o}C in splitless mode. Compounds extracted from the samples were separated using an XTI-5 column (30 m x 0.25 mm x 0.25 µm, Restek GC Columns, Restek Corporation, Bellefonte, PA). After the samples were run, the retention graphs were analyzed in GCMSsolution, Postrun Analysis software version 4.50 (Shimadzu Corporation, Kyoto, Japan) and in OpenChrom® version 1.4.0 (Lablicate GmbH, Hamburg, Germany), which provided the identity and area under the curve of all the individual CHC compounds that are contained in the CHC profile. For CHC analysis, five samples for each nest collection were run, making a total of 50 CHC profiles analyzed. CHC compounds were identified using the retention times, diagnostic ions, and the similarity search library in the GC-MS Post Analysis Software Shimadzu, and in OpenChrom. CHC compounds were grouped into n- alkanes, n- alkenes, and methyl branched alkanes. Using the area under the curve, I calculated the mass (ng/µL) of each identified compound. Calculations were based off of the n-dodecane internal standard (7.5 ng/µL) that was used to determine the values of each peak identified using the formula:

$$x = (124.357 * D)/A$$

In this formula, x is the ng/µL singular CHC compound identified within the 12 µL sample imputed in the GC-MS; 124.357 comes from multiplying the n-dodecane standard (7.5 ng/µL) by 12 µL; D is the area of the dodecane peak found in the sample's retention graph; and A is the area of the singular CHC compound identified within the 12 µL sample.

Data Visualization and Statistical Analysis

A heat map matrix was produced to give a visual of the aggression proportions. A table of every compound identified was created to give a visualization of the distribution of compounds across samples. A non-parametric multidimensional scaling test, or NDMS, was run (Rstudio version 3.3.0+) to create a two-dimensional plot to help visualize how individual samples cluster regarding the relative mass of their alkenes, alkanes, and methyl branched alkanes. To investigate if compounds were identified consistently across samples from the same nest, I binned compounds based on the number of samples they were found in. A bar graph of the average mass of these compounds within each bin was used to visualize if there is any relationship between mass and compound identification frequency. Kruskal-Wallis tests also were run (JMP version 16.0) to see if there were any statistical differences between nest sites in the masses of each category of CHCs; alkanes, alkenes, and me-alkanes, as well as the total amount of CHCs. A linear Regression was run to determine if there is a correlation between the total mass of CHCs (ng/µL) in each sample and the total number of compounds found in each of those samples (JMP version 16.0).

CHAPTER 3

RESULTS

Behavior Analysis

All within-nest pairwise trials exhibited low aggression with the highest proportion of aggression being 0.2 for GR vs GR, MC1 vs. MC1, and ST1 vs. ST1 (Table 1). The highest proportion of aggression in between-nest comparisons, where all ten trials showed aggression, included ST1 vs. HFW, ST2 vs. HFE, and MC1 vs. ST2 (Table 1). Nearly all of the between-nest comparisons showed proportions greater than 0.2 (Table 1)

Cuticular Hydrocarbon Analysis

The mass of each CHC compound found within each sample was put into a table (Table 2). A total of 58 CHC compounds were identified. There was not a single compound that was found across all samples. One of the five samples of ST1 was removed from data analysis since the sample's mass averaged 100x higher than the rest of the mass samples, making it an outlier. There was also not one CHC compound that was found within the same nest across all samples (Table 2).

Cluster Analysis

Using the masses of the alkanes, alkenes, and me-branched alkanes per sample, a Nonmetric Multidimensional Scaling plot (NMDS) was produced (Figure 2). Each nest collection is assigned their own color and polygons are used to connect all of the samples from each designated nest (Figure 2).

Individual Compound Analysis

A bar graph was created to look at the mean mass of CHC compounds binned by the frequency of those compounds. 58 individual CHC compounds were identified between all 49

samples (Table 2). 38 of these compounds were only found within 1-5 of the 49 samples. Two compounds were identified within the largest bin of 26-30 of the 49 samples (Figure 3). There was not a single identified compound that was found within all 49 samples (Figure 3) nor was any single compound identified across all five samples from any given nest (Table 2).

Cuticular Hydrocarbon Group Analysis

A Shapiro-Wilks test was used to test for normality in the mass of the alkanes, alkenes, and me-branched alkanes for each sample. The data was not normally distributed (Alkanes; $W = 0.67224$, p-value = 0.0001274) (alkenes; $W = 0.79669$, p-value = 0.003334) (me-branched alkanes; $W = 0.57171$, p-value = 1.417e-05). Four non-parametric, Kruskal-Wallis tests was used to determine if there were any significant differences between the masses (ng/μL) of the alkanes, alkenes, me-alkanes, as well as the total sum of CHCs. A critical p-value of 0.05 was used to determine significance: there were no significant differences between nest sites in mass of compound groups, nor total CHC mass (Table 3).

Four box and whisker plots were created to give a visual representation of the Kruskal-Wallis Tests (Figure 4, Table 3). Figure 4A shows the box and whisker plots of the mass of all combined CHCs per ant between each nest collection. MC2 had the lowest mass of combined CHCs per ant identified and ST1 had the highest mass (Figure 4A). Figure 4B shows the box and whisker plots of the mass per ant of alkanes between each nest collection. SW2 had the lowest mass of alkanes identified and ST1 had the highest mass (Figure 4B). Figure 4C shows the box and whisker plots of the mass per ant of alkenes between each nest collection. HFW had the lowest mass of alkenes identified and MC1 had the highest mass (Figure 4C). Figure 4D shows the box and whisker plots of the mass of me-alkanes between each nest collection. HFE had the lowest mass of me-alkanes identified and ST1 had the highest mass (Figure 4D)

Technical Sensitivity Analysis

A linear regression was run between the total CHC mass and the number of compounds within samples. My data showed no significant correlation between the total CHC mass and the number of compounds (p-value= 0.1103, Figure 5).

	GR	BV	HFW	HFE	MC2	MC1	SW2	SW1	ST1	ST2
GR	0.2	0	0.5	0.9	0.6	0.7	0.9	0.9	0.3	0.2
BV	x	0.1	0.7	0.8	0.3	0.7	0.6	0.6	0.6	0
HFW	x	x	0.1	0.8	0.6	0.8	0.6	0.6	1	0.3
HFE	x	x	x	0.1	0.8	0.9	0.4	0.8	0.6	1
MC2	x	x	x	x	0.1	0.6	0.2	0.5	0.5	0.4
MC1	x	x	x	x	x	0.2	0.8	0.6	0.7	1
SW2	x	x	x	x	x	x	0.1	0.1	0.7	0.9
SW1	x	x	x	x	x	x	x	0.1	0.8	0.6
ST1	x	x	x	x	x	x	x	x	0.2	0.5
ST2	x	x	x	x	x	x	x	x	x	0

Table 1. Heat map of the proportion of aggressive behaviors in the aggression assays between the ten nest collections. Zero = no aggressive interactions shown within the ten trials between the paired nests. One = all ten trials show aggression within the trails between the paired nests. Nests are organized geographically from west to east.

Site	GR					BV					HFW					HFE					MC2					MC1					SW2					SW1					ST1				ST2				
Nest #	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
C7 n-alkane					2																				2																				160				
C7 n-alkene												3																																					
C7 me-alkane														8																																			
C8 n-alkene	18	176		21		787	29		31	29	11				16		21		89	9		7	14	24	9		18		449	17	96						32	18	84	6	13	8		16	85	39	
C9 n-alkene			13				1							90					10	9																2				2	23								
C11 n-alkene																									7																								
C13 n-alkane															39																				51											55		
C13 me-alkane																																		266													
C14 n-alkane									78	105														92															107		64							
C14 me-alkane					5						47																			121				88													
C15 n-alkane				17	5				310	112	258													158	488					526						116	104			106			508		521				
C15 n-alkene												0																187						5									22				
C15 me-alkane						270								39							19									734			16			31												
C16 n-alkene		60		211							15																				49										60							
C16 n-alkene													101																							4			34									
C16 me-alkene																				66								739					690			597											83	
C17 n-alkane					105																				40														17								
C17 n-alkene		56		13	59		3		104	14									25	5													52			27											
C17 me-alkane								12			4	1	20	35		13	25	11				14						44	282		21	20				22				9			4	46				
C18 n-alkane						442								400																	14																
C18 n-alkene	55																													24																	
C18 me-alkane	2887																																															
C19 n-alkane		1023						134		117	144		198	48		31		16					369	4	613			432	2312	4		206				65		393		8	1446	255	136		91		105	696	9	3
C19 n-alkene	189			82				286							529		31			525	432	693				23					148	777		10		372				164	7			773	271					
C19 me-alkane			1		45		33												66																4													
C20 n-alkane	124				412			28																											4													
C20 n-alkene																									32					140																	
C20 me-alkane									53		2	2								16	496					4				11								83										
C21 n-alkane																							13						52									220											
C21 n-alkene									15																																							
C21 me-alkane												20																		67																		
C22 n-alkane						247			99						34											8					57												14						
C22 n-alkene																					31																												
C22 me-alkane												5			37													126	67							29						16				760				

Site	GR					BV					HFW					HFE					MC2					MC1					SW2					SW1					ST1				ST2				
Nest #	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
C23 n-alkane					34	277										89					27	27					7										138	626				7			23					
C23 me-alkane	4	334	27				21	65			33	16							36				35	1				95	126		13	56	77			35	7		24			3			296				6
C24 me-alkane	6					37			9												14								149							18													
C25 n-alkane				7		161		17					58	360			12				7					4				11					4					3			48			14	2	
C25 me-alkane																8						14						35												9	42							
C26 n-alkane	4		5					7										15			11					4		26	18	14	19			6						2	29			8				
C26 n-alkene										86																																						
C26 me-alkane		52					9				23		4			6							37					11								18		2										
C27 n-alkane						1310		18										101				17	361										286	73					3171	28		645			39	9	25	
C27 me-alkane			20													14																																
C28 n-alkane				2		169															6	8				3				12		10			8			4	99	8							
C28 me-alkane		319							11							9																											3					
C29 n-alkane						46															7																												
C29 me-alkane																8																							86		6			9	9			
C30 n-alkane																				181																												
C30 me-alkane																																																
C31 n-alkane			5											142		62				702												43																
C31 me-alkane																			19																														
C33 n-alkane																																									28							15	
C34 n-alkane																																										39							8	
C34 me-alkane																																											47							
C35 n-alkane																																										35							
C37 n-alkane																																																51	
C37 me-alkane																																											69	1024						
C38 n-alkane																																										9			725					

Table 2. Table of the mass (ng/ant) of every CHC compound identified in each sample. The cells that are left empty signifies where a CHC compound is not found. Samples are organized from west to east in terms of nest site location. Samples are grouped together by nest collection site.

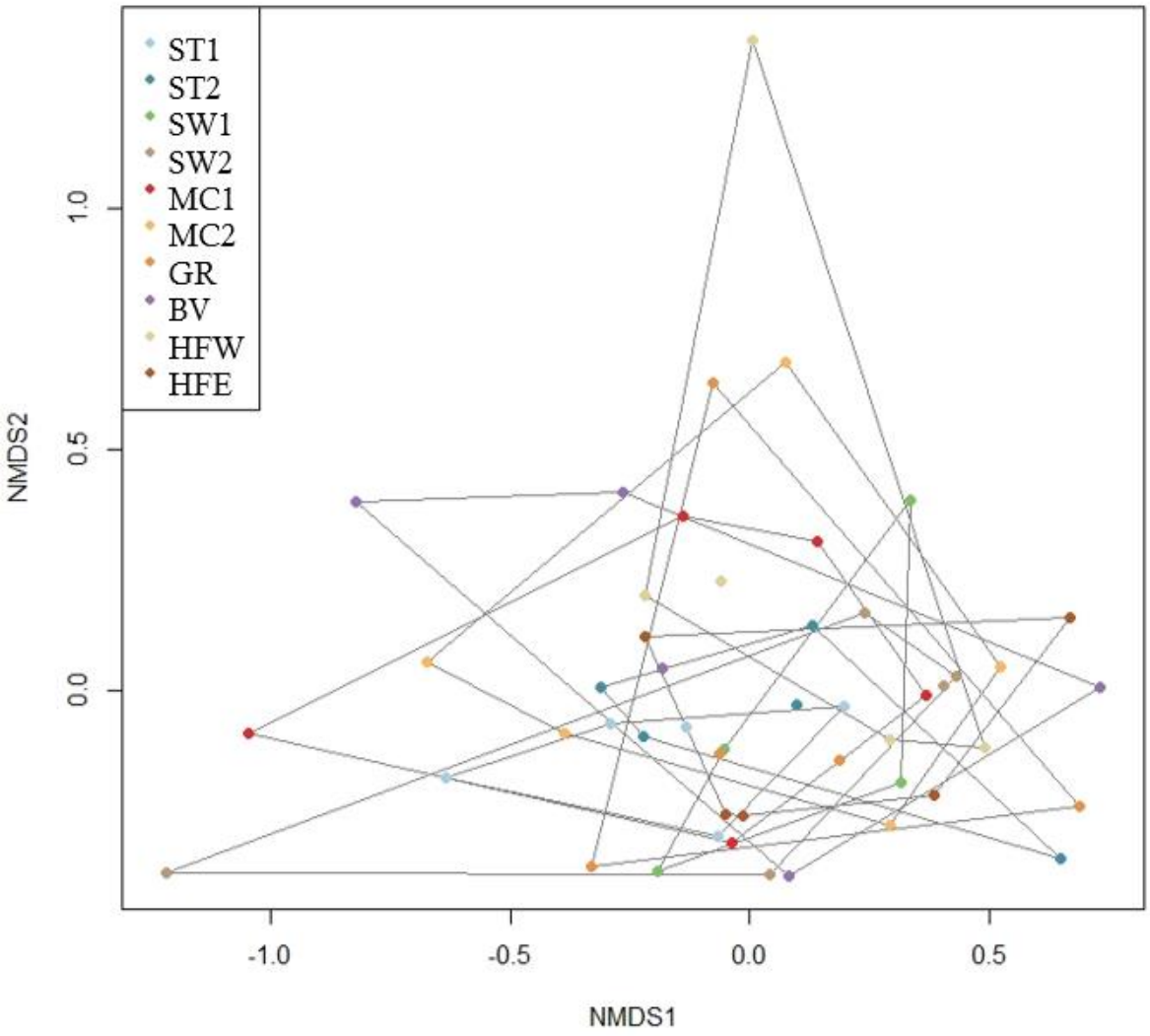

Figure 2. NMDS plot features 49 samples of all ten nests. Points are based in the masses of the CHCs making up the three compound groups: alkanes, alkenes, and me-alkanes. Polygons connect the samples belonging to each nest.

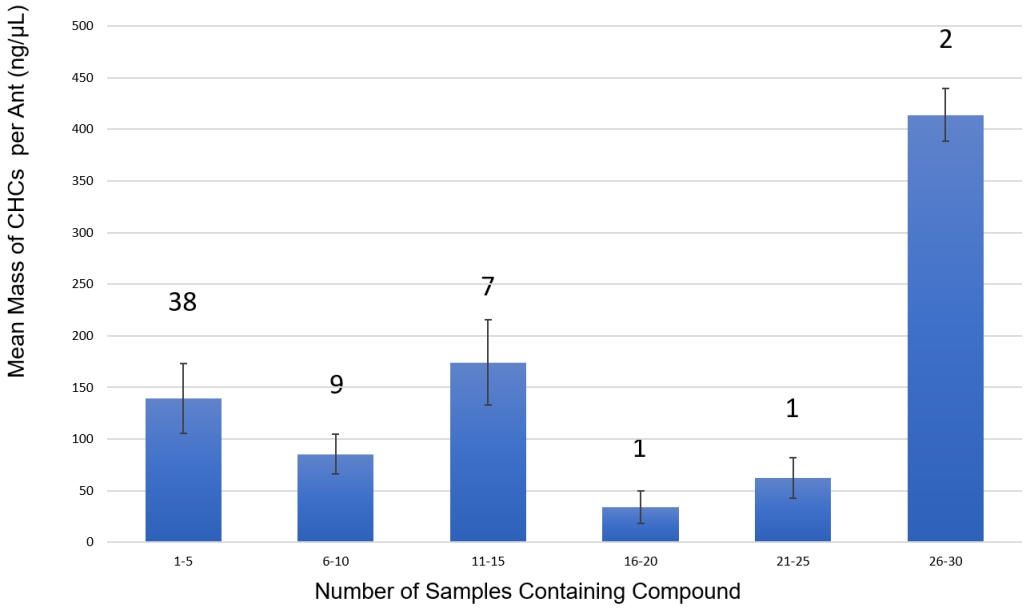

Figure 3. Binned groups of the number of samples containing an individual compound against the summed mass of CHCs per ant (ng/µL). Error bars represent the standard error of the mean of each bin. Numbers above the bar indicate the number of individually identified compounds contained within each bin.

Kruskal-Wallis Test

Group	Chi-Square	df	Prob>ChiSq
Totals	7.5687	9	0.5781
Alkanes	10.3823	9	0.3204
Alkenes	4.6289	9	0.8654
Me-Alkanes	3.4003	9	0.9463

Table 3. Non-parametric Kruskal-Wallis Tests run on the alkanes, alkenes, me-alkanes, and total amount of CHCs (ng/µL) per ant. A Shapiro-Wilks test was used to determine if the data was normally distributed before running the Kruskal-Wallis test. Critical p-value of 0.05 to determine statistical significance. No significant differences were found within all four tests.

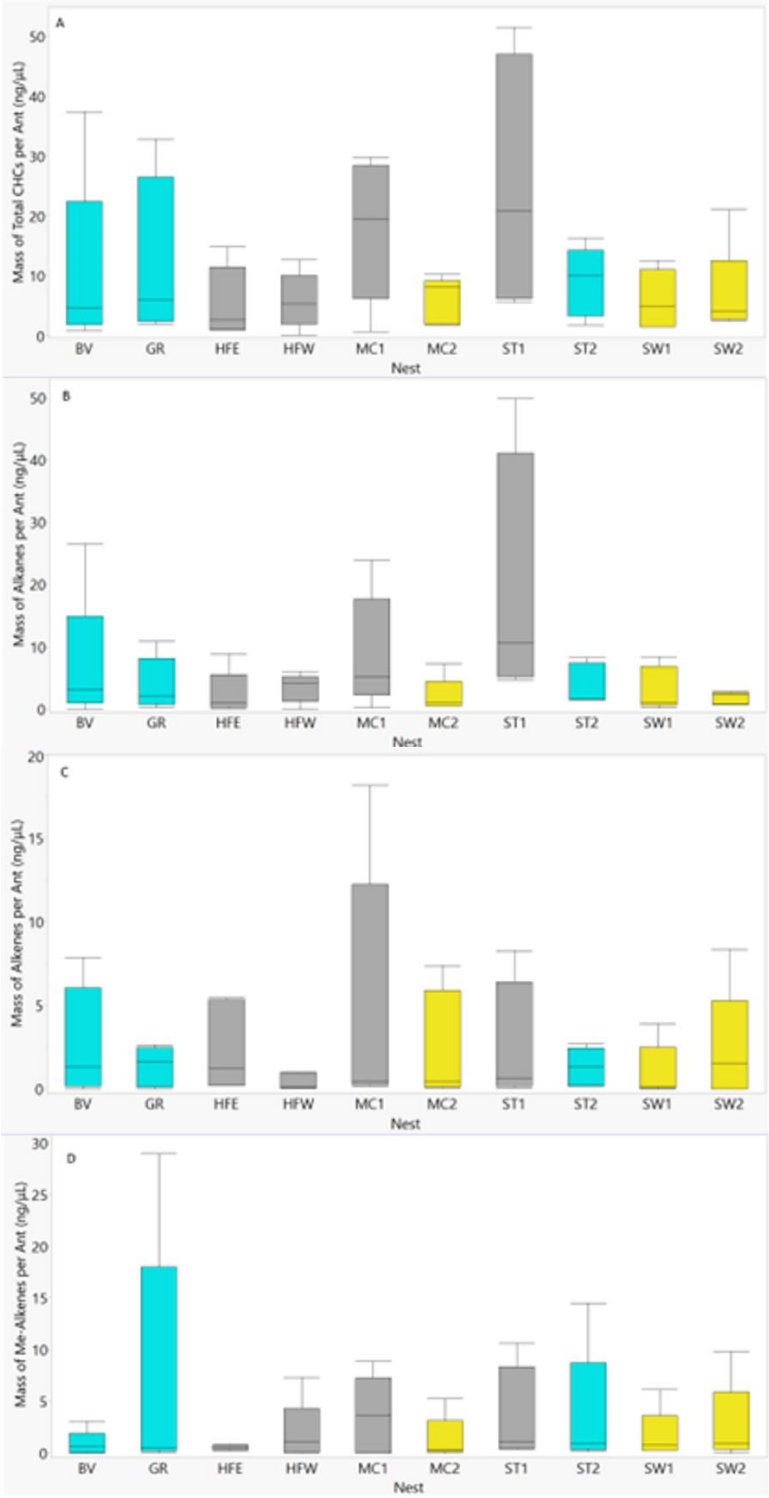

Figure 4. Box and Whisker Plot: Visual of the data found in the Kruskal-Wallis test, composed of mass (ng/µL) per ant of each CHC compound group in each sampled nest. Note: y-axis scales differ, Colors: Blue indicates the three behaviorally non-aggressive nests. Yellow indicates the behaviorally non-transitive nests. Gray indicates the four nests that were behaviorally aggressive towards each pair Whiskers are the maximum (top) and minimum (bottom), box is third and first quartile, line is median. A: Total CHCs, B: Alkanes, C: Alkenes, D: Me-alkanes.

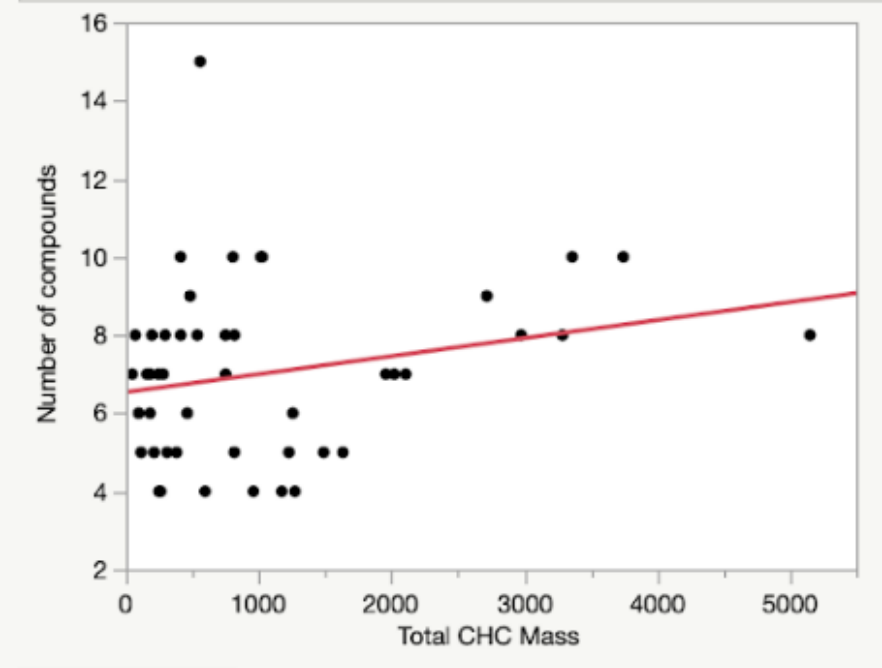

Figure 5. Linear regression of the total CHC mass and the total number of compounds found within samples. y=0.0004x+6.8797. R^2= 0.0247. p-value= 0.1103.

CHAPTER 4

DISCUSSION

In this study, I looked at the intraspecific aggression of *L. humile*, an invasive species in much of the southeast United States. The goal of my study was to determine the geographical distribution of colonies in Georgia and a better understanding of how their CHC are used as colony identity markers. To do so, I collected ten unique nests from a transect across Georgia and I performed aggression assays between nest pairs to gain a better understanding the geographic distribution of colonies along this transect, while also extracting and analyzing their cuticular hydrocarbons to better understand how these relate to colony identity. I hypothesized that there were more potential colonies residing in the southeast than in other introduced areas around the globe. I predicted that there would be high levels of aggression between nests that are from different collection sites and there will be low aggression within nests and between nests that are in close proximity to one another. Only one of my predictions was supported, which was that ants from the same nest showed low aggression.

I expected to see all ten trials for within-nest pairs exhibit no aggressive behavior towards one another. Interestingly, while all within-nest aggression assay trials exhibited low aggression, there were some cases where aggression was shown, and the highest proportion of aggression was 0.2 (Table 1). This could be due to all ten nests in my study being collected in the summer months of 2021, and aggression assay trials not being conducted until November of 2021. Ants that have been in the lab for long periods of time tend to show higher levels of aggression not only towards different nests also contained within the lab but will also show aggression towards the colony that they originally came from in the field. In Suarez et al. (2001), aggression assays were conducted between the four colonies in California, where all within-nest aggression assays

showed no aggression towards one another except for one colony. This colony first showed low aggression, but then after an extended amount of time in the lab, the within-nest trials began to exhibit aggressive behavior towards one another. These trials were removed from the study's overall analysis, and it was suggested that the change in behavior was due to a change in their CHCs. It has also been recorded that other factors such as environmental conditions and diet can affect how a nest behaves towards another nest (Buczkowski & Silverman 2005, Suarez et al. 2002, Liang and Silverman 2000, Buellesbach et al. 2018). The results of van Wilgenburg et al. (2022) showed in the study that ants that were fed crickets showed reduced aggression towards other nests collected. In addition to this, ants fed different varieties of cockroaches also exhibited changes in behavior, showing lower aggression towards nests that were fed the same species of cockroach. In my study, all the ants were fed the same species of cricket, which may have impacted my results.

Due to their close proximity, I expected to see the predicted "paired" nests, such as ST1 and ST2, SW1 and SW2, MC1 and MC2 to show low aggression towards one another, thinking that they could be from the same colony. Surprisingly, the only nests pairs that followed that pattern were BV and GR with a proportion of aggression of zero, and SW1 and SW2 with a proportion of aggression of 0.1 (Table 1). There appeared to be three nests that behaved as if they were from the same colony but were on either end of my transect: GR, BV, and ST2 (Figure 1, Table 1). Other unexpected outcomes came from comparisons of nests that were not considered "close proximity" pairs but exhibited relatively low aggression. For example: BV and MC2 had a proportion of aggression of 0.3; ST1 and GR had a proportion of aggression of 0.3; HFW and ST2 had a proportion of aggression of 0.3; and MC2 and SW2 had a proportion of aggression of 0.2 (Figure 1, Table 1). This variation of aggression has been recorded before in

previous unpublished data in the Gibson lab. Previous data between the six colonies (ST1, SW1, MC1, BV, GF, and HFE) displayed a range of proportions of aggression between 0.17 and 0.95 (Unpublished Data from Gibson Lab). Unexpectedly, in the previous data, MC1 was shown to be non-aggressive between SW1 in the past with a proportion of aggression of 0.17, but in my data, MC1 and SW1 had a proportion of aggression of 0.6 (Table 1, Unpublished Data from Gibson Lab). Interestingly, two nests that showed the highest proportion of aggression of the "paired" nests, HFE and HFW that had a proportion of aggression of 0.8, were only ~400 m apart. These nests are separated by the Towaliga River, which could pose as a geographical barrier between colonies.

I also hypothesized that colony identity could be signaled by chemical markers and predicted that ants from the same nest should have similar CHC profiles and nests from different colonies, based on my behavioral analysis results, should have more dissimilar CHC profiles. In my study, this hypothesis could not be supported. The presence and absence of CHC compounds varied tremendously across nests, as well as across samples from the same nest (Table 2, Figure 3). Shockingly, 38 out of the 58 different compounds identified were only found in 1-5 of 49 samples (Figure 3). Even more so, not a single CHC compound was consistently found within the samples from the same nest (Table 2). In past studies, while it has been noted that queens may be missing certain cuticular hydrocarbons relative to the workers from the same nest, such as any Di- or Tri- methyl carbons, worker ants have been noted to have similar CHC compounds across samples, with little variation within nests (Vásquez et al. 2009). In my NMDS plot (Figure 2), if the hypothesis was supported, the five samples from each nest would cluster close together on areas on the plot distinct from other nests. The results from my study did not yield this expected outcome. All my grouped samples overlapped and there were no distinguishable

clusters. There were also no significant differences between nests in the mass of any CHC group (Table 3, Figure 4).

Since there is not a statistically significant pattern between the groups of hydrocarbons between each nest, I wanted to determine if this was due to a technical sensitivity issue which would result in more CHCs being identified in samples with greater amounts of CHCs. However, there was no significant correlation (Figure 5). Barrs (2021) found that the column used in their study and mine does not have the capability to detect the same number of CHCs detected in studies taking place in California (Buellesbach et al. 2018). They found that the column run at 300^{o}C fails to detect C_{40} and it also delays the time between signal peaks of CHC compounds larger than C_{35} (Barrs 2021). In this same study, they found 36 unique CHC compounds, while using the same machine I was able to detect 58 compounds. It should also be noted that my tests only ran for 30 minutes, whereas the Barrs (2021) tests ran for 46.5 minutes. Given these findings, one technical explanation could be that I am not able to detect all the CHC compounds that are present in a sample. In addition to these potential technical issues, other factors such as diet and weather conditions have been shown to affect CHC patterns in *L. humile.* In Buellesbach et al. (2018), the study investigated how CHC profiles change based on desiccation rates due to the environment. It was found that there are more n-alkenes and n-alkanes positively correlated with higher temperatures in the region where they were collected (Buellesbach et al. 2018). Weather conditions were not recorded in my study during field collection, so it is not possible to assess whether this impacted my findings. Regarding diet, as mentioned previously, all ants were fed the same species of cricket and previous studies have shown that this can affect CHC patterns (van Wilgenburg et al. 2022).

Typically, in introduced ranges, *L. humile* colonies exhibit low genetic diversity while native colonies have high genetic diversity. The global supercolony experienced a genetic bottleneck, which produced large colonies with low genetic diversity (Suarez et al. 1999). There is no evidence that this supercolony resides in the southeastern United States and it has been suggested that southeastern *L. humile* colonies have not experienced such a large bottleneck and therefore have higher genetic variation (Buczkowski et al. 2004). One possible explanation for the higher level of genetic diversity could be due to the shipping trade between North and South America (Buczkowski et al. 2004). Savannah, GA is home to the fourth busiest container port in the United States, which has a volume of trade across the eastern seaboard in the United States, including countries in South America (Ramos 2014). Due to the sheer volume of trade within this port, it is possible that there have been multiple introductions of *L. humile* in the southeastern United States resulting in more colonies being present in this region (Buczkowski et al. 2004). This could be the reason behind the highly variable intraspecific aggression within and between the nests that have been collected for this study (Buczkowski et al. 2004).

Conclusions

In conclusion, there are likely many colonies of *L. humile* in the southeastern United States and that their behavioral interactions are more complex than those of colonies in other introduced regions. Going forward, higher sampling within a smaller geographical range could help us further clarify the geographic distribution of these colonies and may help explain the high levels of aggression seen from nests that are close in proximity to one another. In a previous study, the ST1 nest was shown to have low aggression towards a nest that was less than two kilometers away but, in my data, ST1 was shown to be aggressive towards its “pair” ST2, which in turn is 12.5 kilometers apart (Gibson Lab Unpublished Data, Table 1). Since the nests in

Georgia and the Southeast are patchier than in other regions, it is harder to determine a “border” of when one colony ends and when another colony begins. The idea of colony borders has been studied in California (Tsutsui and Case 2001) but has not been looked at in the southeast. HFE and HFW could potentially help better understand the overall geographic distribution of colonies in the southeast, and how the geography and environment can play a role in the high variation seen in the southeast. These two nests are only ~400 m apart, but a river separates the two nest collection sites and they exhibit high aggression (Figure 1, Table 1). There have not been many studies done on *L. humile* in the Southeast, but increased sampling done in close proximity and increased sampling for the CHCs in the region can help us to gain a more detailed understanding of the high behavioral and chemical variation seen in this study.

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