Cuticular Hydrocarbon Melting Temperatures and Quantity of *Linepithema humile* in California

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ABSTRACT

Argentine ants (*Linepithema humile*) are invasive ant species to California. Argentine ants are so successful in competing native ant species and extending their habitat ranges that they spread their footprints across California. Argentine ants experience different levels of desiccation stress in their habitats. The result of desiccation assay conducted by the Tsutsui Lab at UC Berkeley indicated that Argentine ants from different colonies across California have different desiccation resistance. To cope with various desiccation stress, Argentine ants alter physical property and composition of Cuticular hydrocarbons (CHCs). CHCs are functioned as a layer of desiccation barrier for Argentine ants. Yet, how Argentine ants manipulate CHCs to resist body water loss is barely studied. I specifically investigated two CHC properties that are likely varied among Argentine ants from different colonies and hence cause variation in desiccation resistance. I examined CHC melting temperature and CHC quantity measured by weigh from different Argentine ant colonies in California. The correlations between the two CHC properties and desiccation resistance were analyzed. I discovered a negative correlation between CHC melting temperature and desiccation resistance. No correlation was found between CHC quantity and desiccation resistance. The conclusion to the research was that the higher CHC melting temperature a colony of ants has, the weaker desiccation resistance they have, and ants that have higher desiccation resistance do not necessarily have a larger amount of CHCs.

KEYWORDS

Insect Chemistry, Desiccation Resistance, Desiccation Barrier, Argentine Ant

INTRODUCTION

Linepithema humile, Argentine ant is native to South America and has invaded around the world (Human and Gordon 1999). Argentine ants can thrive in Mediterranean-like climate, i.e., warm dry summers and cool moist winters around the world (Wetterer et al. 2009). A place like California that is Mediterranean climate provides suitable habitats for them. Argentine ants have a unique surpercolony structure: individuals within these nests behave amicably toward individuals from other nests in the same colony-supercolony (Tsutsui and Suarez 2003). Supercolony structure is very beneficial for Argentine ants with respect to invasion and competition. This unique colony structure not only increases population density but also increase the spatial boundary for Argentine ants in non-native range (Tsutsui and Suarez 2003). In the absence of aggression and territoriality for nestmates and high aggressiveness towards native ant species, more resources can be directed to colony growth, the domination of food and nesting resources, and the displacement of native ants in direct, aggressive encounters (Holway 2018). Argentine ants are also urban and agriculture pests in California. In agricultural systems, Argentine ants are most commonly found in areas with disturbed habitats and some soil moisture (Cooper et al. 2008). Moreover, Argentine ant is associated with out breaks of phloem-feeding insects such as mealybugs, scale and aphids, which the ants protect from natural enemies; in exchange, the ants collect the sugar rich food source (honeydew) excreted by the phloem-feeder (Buckley and Gullan 1991).

Colonies of Argentine ants are found throughout California. Each Argentine ant habitat has different local climates from others, and different local climates impose different desiccation stress on Argentine ants. Insects and other terrestrial arthropods are susceptible to water loss because of their small size (Gibbs 2003). To adapt various temperature and humidity in different habitats, efficient body water conservation becomes critical for Argentine ants. How well Argentine ants can prevent their body water loss is defined as desiccation resistance. And rates of water loss of insects and other terrestrial arthropods are very dependent upon temperature (Gibbs et al. 1998).

Cuticular transportation is one of the main mechanisms for Argentine ants to exchange body water with surrounding environment. Argentine ants use Cuticular hydrocarbons (CHCs) to form a desiccation barrier to maintain water balance and prevent desiccation (Chung and Carroll 2015). CHCs help prevent water loss for social insects since CHC has a certain degree of

permanency for materials such as, gas, solid and liquid. CHCs are presented in a waxy form and coating around Argentine ants' bodies. Changing the characters of CHCs is an efficient way for Argentine ants to resist dry stress by altering their phenotypic traits. However, how CHCs affect desiccation resistance of Argentine ants are not yet studied. Therefore, studying CHC properties is a good way to do so. Differences in composition or amounts of CHCs should affect rates of water loss (Gibbs 2003). Apparently, change in CHC quantity is the direct result of change in CHC amounts. Regrading change in CHC composition, temperature is one of the parameters to measure the change. The ability of a CHC layer to prevent desiccation depends on its composition, which in turn determines its melting temperature (Chung and Carroll 2015). Both CHC properties, CHC melting temperature and quantity are promising to look at.

Researchers have studied fruit flies (*drosophila)*, particularly the correlation between desiccation resistance and the composition, physical properties or amounts of cuticular hydrocarbons is not consistently observed (Gibbs 2003). Mean hydrocarbon chain length increased at higher temperatures, but cuticular lipid melting temperature (Tm) did not (Gibbs et al. 1998). It will be fascinating to see if there is any relationship between the amount and melting temperature of CHC and desiccation resistance for Argentine ants.

Desiccation assay is an experimental design to identify desiccation resistance of different Argentine ant colonies in California by comparing surviving hours of ants in the assay. Desiccation resistance is measured by how many hours ants survived. I used the desiccation assay data from the Tsutsui Lab at UC Berkeley and plotted the data into a bar graph that shows the visual presentation of differences among colonies (Figure 1). As the graph illustrates, each colony in five colonies has different surviving hours under the same air treatment during the desiccation assay. More importantly, disparities in desiccation resistance among Argentine ant colonies were discovered.

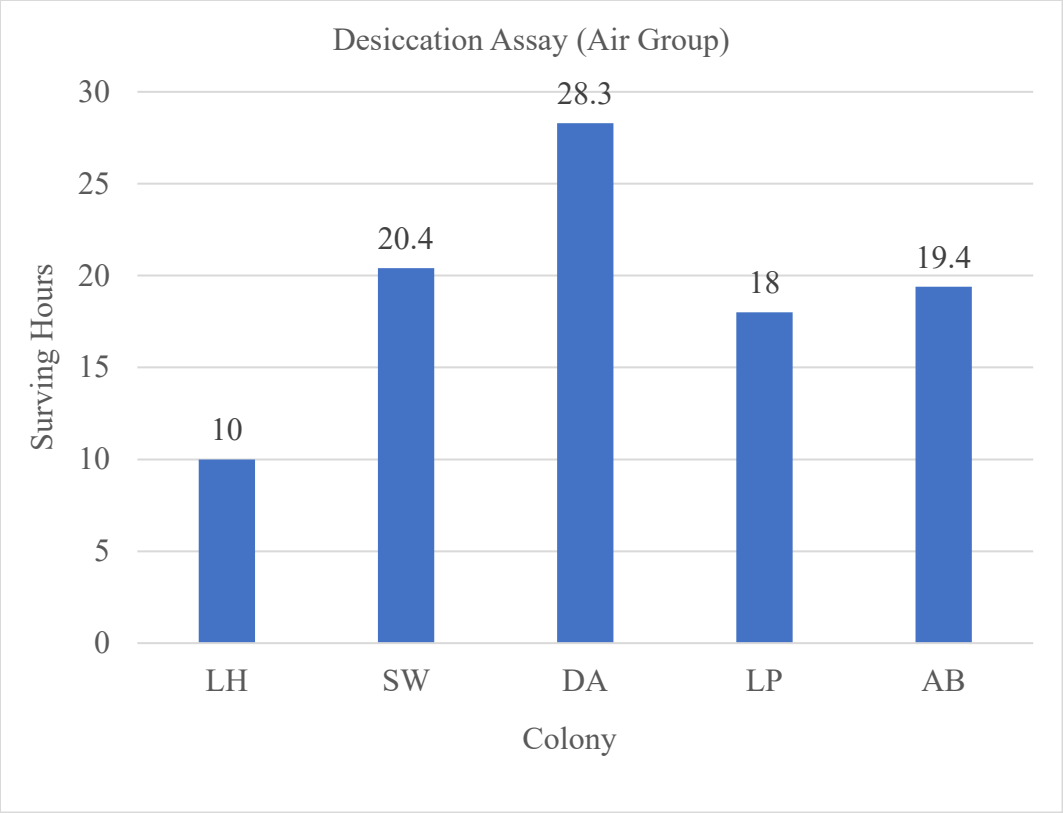

Figure 1. Desiccation Assay Results for five colonies. Data from the Tsutsui Lab at UC Berkeley.

According to the result of desiccation assay conducted by the Tsutsui Lab at UC Berkeley, Argentine ants from different colonies have different resistance to desiccation. Desiccation level is expected to be more intense in high temperature and low humidity area than in low temperature and high humidity area. Studies on *Drosophila* have shown, "Water conservation is critical to the ecological success of desert *Drosophila*" (Gibbs 2003). Similarly, Argentine ants living in a hot and dry area have differences on conservation of body water from the ants living in a warm and humid area, and the differences could be behavioral or physiological. If the differences depend on behavioral difference, population are very susceptible to environmental changes on water resources. Changes of temperature and humidity often happen in nature so population would not survive long in a hot and dry area. Additionally, ant population need to survive long enough to find the water resource when they are first introduced to a new land. Hence, physiological difference must play an important role for ants to survive in a hot and dry area. What is more, changes of temperature in habitats have profound effects on physiological process (Gibbs et al. 1998). Ant's production of cuticular hydrocarbon is a physiological and phenotypic response to

low humidity in dry environment. The quantity and composition of CHC would likely change based on the surrounding temperatures, which should affect rates of water loss.

Overall, the central research question in the thesis is whether CHC properties are responsible for the variation in desiccation resistance among Argentine ant colonies throughout California. The CHC properties that I studied were CHC melting temperature and CHC quantity measured by weight (ng). In other words, I tried to verify if the melting temperature and quantity of CHC were correlated with desiccation resistance. I hypothesized that both CHC properties were positive correlated with desiccation resistance: desiccation resistance would become stronger as the CHC melting temperature and quantity would become higher.

METHODS

Study system

To find out whether CHC are properties responsible for the variation in desiccation resistance among Argentine ants (*Linepithema humile)* throughout California, I specifically looked at two CHC properties: CHC melting temperature and CHC quantity measured by weight (ng). There is logical reason of why I chose the two CHC properties. CHC acts as desiccation barrier for Argentine ants. For CHC melting temperature, if the desiccation barrier does not get melt easily, it can prevent body water loss more efficiently. Same reason for CHC quantity. If desiccation barrier is composed of large amounts of hydrocarbons, the desiccation barrier would become thick so body water loss can be reduced as well. My hypothesis to the question was that I expected positive correlations between both two properties and desiccation resistance.

I focused on six Argentine ant colonies in California. They are Albany bulb and Davis at Northern California; Lake Hodges, Sweet Waster, Los Penasquitos and Lake Skinner at Southern California respectively. All these Argentine ant colonies have different local climate and weather. Hence, Argentine ants that live in these habitats presumably have different desiccation resistance to cope with local desiccated stress. And CHC as desiccation barrier, is the mechanism for Argentine ants to alter their desiccation resistance.

CHC Extraction

To extract CHCs, I collected approximately 2500 ants from each study colony in the total of six colonies. I extracted CHCs separately from the six colonies of ants. The way of how I estimated 2500 ants was to collect ants in a 50mL conical vial (2500 ants took up 3.75ml volume in a conical vial). After ant collection was done, I put the conical vials in freezer so the collected ants would freeze to death. I transferred the dead ants from conical vials to glass vials one by one for each sample colony. I ejected 2500 µL hexane solution that can wash off CHC from ants' bodies in the glass vials and put them on a shaker for ten minutes so CHCs from ants' body would completely come off. Next, I pipetted the hexane/CHC solution through silica gel columns that can absorb all the polar compounds (CHC is a non-polar compound) so only pure CHC/hexane solution went through the column. I dried out the hexane in each CHC sample with nitrogen air and resuspended them with 25µL hexane. I took 5µL solution out and saved it for CHC quantity analysis and used the remained 20µL solution for CHC melting temperature analysis.

Melting Temperature Analysis

I transferred the 20 µL hexane/CHC solution to a melting temperature capillary. I put the capillary in a vacuum chamber to dry out the hexane with only pure CHCs left. I repeated the same procedure for all the colony samples. To observe and record the melting temperature of the pure CHC sample, I put the capillary into the melting temperature apparatus. What the apparatus does was that it heated up the CHCs inside the capillary in a set rate and allowed me to record the CHC starting melting temperatures and ending melting temperatures. I determined the CHC melting temperature by observing the phase change of the CHCs inside the capillary. I conducted the same procedure to observe and record the CHC melting temperature for all CHC samples, and I had one sample for each colony.

CHC Quantity Analysis

I used Gas chromatography–mass spectrometry (GC-MS) from the Tsutsui Lab to obtain the chemical profiles of all CHC samples. GC-MS is an instrument that gave me chromatograms and tell me about the chemical profiles of the CHC samples that I put in the instrument. For example, each peak in the Albany Bulb chromatogram represented a unique type of hydrocarbon within the CHC sample and the area under a peak was the abundance of that specific type of hydrocarbon (Figure 1). I dissolved CHC samples in 10µL C-12 standard hydrocarbon that has a known quantity, 75ng/µL. Essentially, what I did was to sum up the areas under all the peaks in a chromatogram of a CHC sample. I compared the total peak areas with the area of a C-12 standard (Figure 1 & Figure 2). By comparing them, I got the area ratio of the CHC sample to C-12 standard, and I used the area ratio to calculate the total weight of the CHC sample. I proceeded the same workflow for all CHC samples, and I had one sample for each colony.

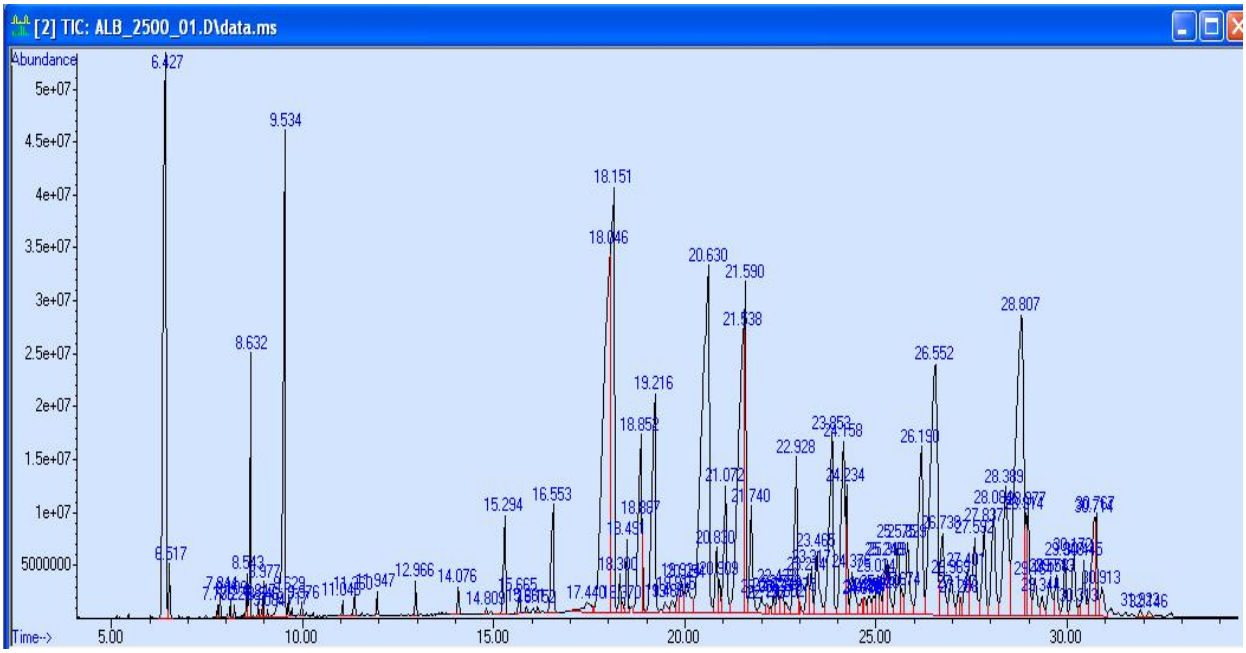

Figure 1. Albany Bulb Chromatogram.

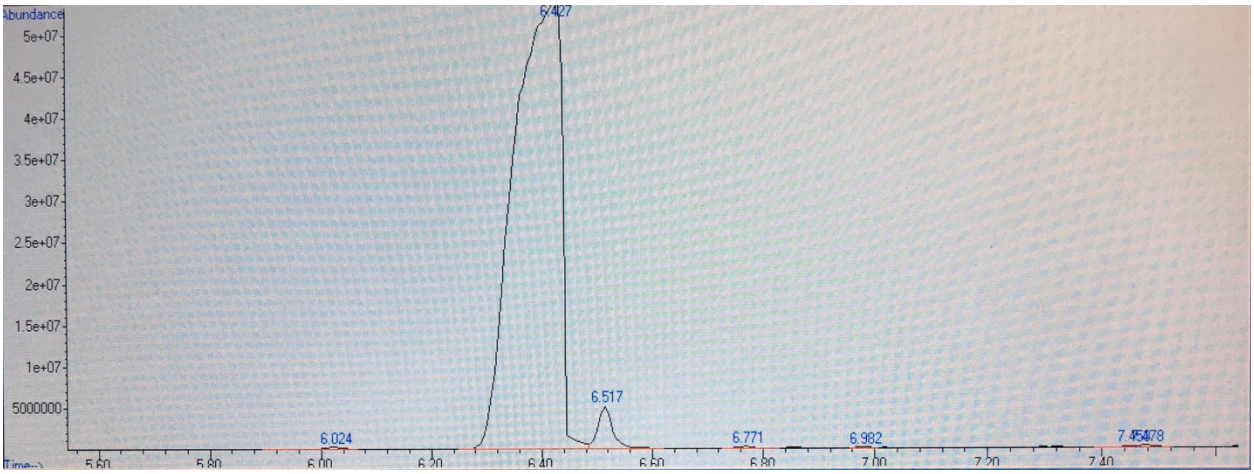

Figure 2. The peak and area of C-12 Standard.

Data analysis methods

For melting temperature analysis, I recorded the starting and ending melting temperatures for each CHC sample from each colony 15 times and stored the data in Excel Spreadsheet (Figure 3). From the starting and ending melting temperatures, I calculate midpoint melting temperatures that were a better representation of the temperature data (Figure 4). To integrate the midpoint melting temperature data from all CHC samples, I plotted the data in a boxplot where x-axis had different ant colonies, and y-axis had CHC midpoint melting temperature. In order to connect the CHC melting temperatures back to desiccation resistance, I plotted a scatterplot that had CHC midpoint melting temperatures from melting temperature analysis against surviving hours from desiccation assay and that was grouped by different colonies. I also added a best-fit line in the scatterplot to show the trend of the data point. To verify the correlation statistically, I ran a Pearson correlation test with the CHC midpoint melting temperatures and surviving hours.

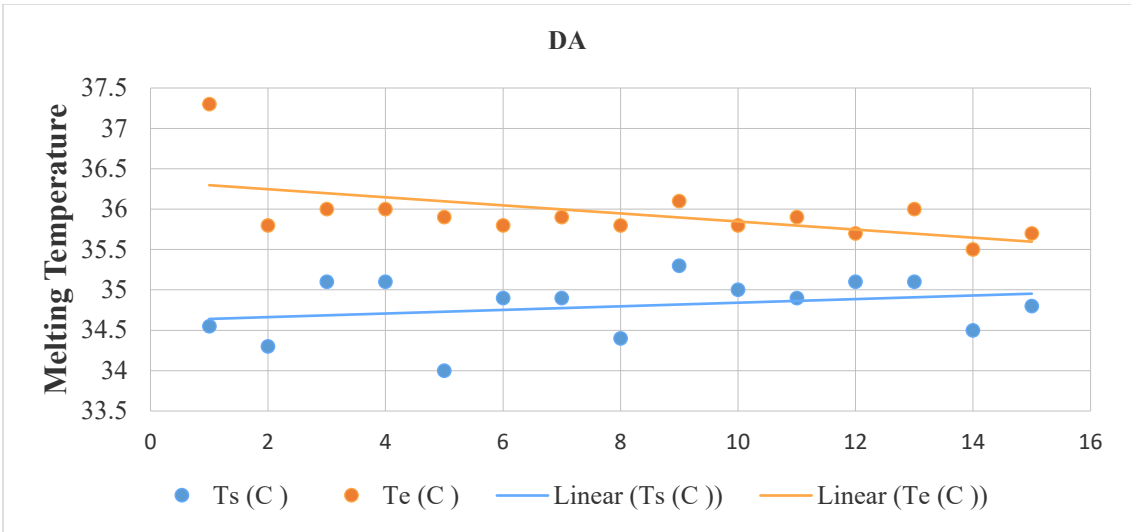

Figure 3. CHC starting and ending melting temperatures of Davis colony sample.

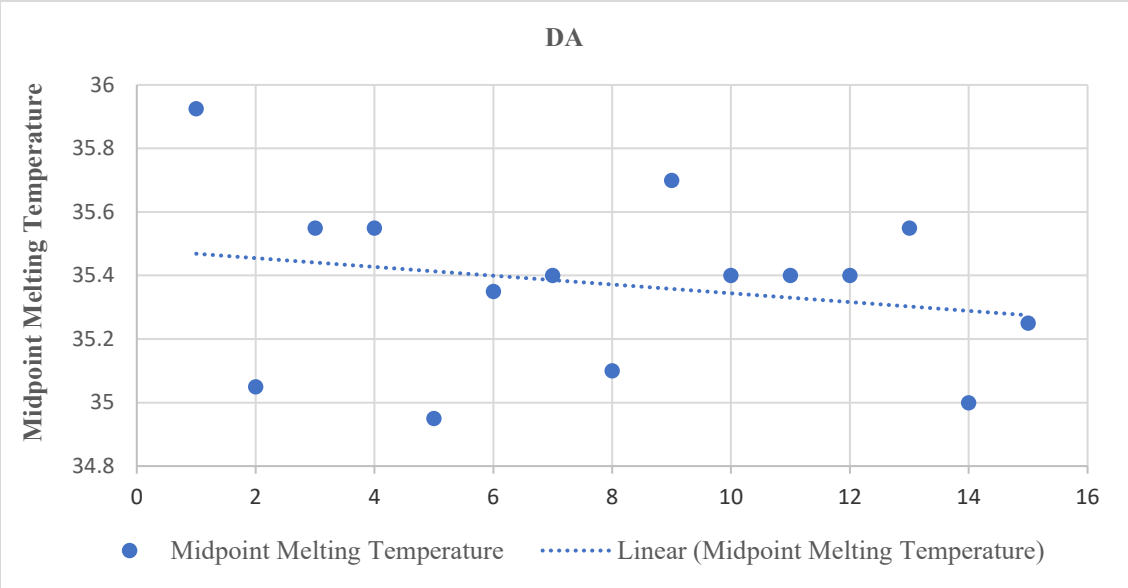

Figure 4. CHC midpoint melting temperatures of Davis colony sample.

For CHC quantity analysis, I used Excel to do all the calculation such as area of CHC sample, area ratio of CHC samples to C-12 standard and CHC sample quantity. I put all the relevant data from the CHC quantity analysis together in a table by using Power Point. To link the CHC quantity to desiccation resistance, I plotted a scatterplot that had CHC quantity against surviving hours with trend line added and that was grouped by different colonies. To verify the correlation statistically, I ran a Pearson correlation test with the CHC quantity and surviving hours.

CHC Quantity Calculation Equation:

Area Ratio= Area of a CHC sample/ Area of C-12

GC-MS Sample Weight= Area Ratio * 75ng/μL (C-12 Standard) * 5μL *$\frac{10\mu L}{5\mu L}$

CHC Sample weight=GC-MS Sample Weight * $\frac{25\mu L}{5\mu L}$

RESULT

Melting Temperature Analysis

My study ant colonies were Davis and Albany Bulb in Northern California; Lake Hodges, Sweet Water, Los Penasquitos and Lake Skinner in Southern California. I decided to exclude Lake Skinner data in the melting temperature analysis because the CHC sample was not viable to gather melting temperature data. I will talk about the reason and details of why I forwent Lake Skinner data in discussion section. Based on the data that I collected from the CHC melting temperature analysis (figure 5.), I discovered that difference of midpoint CHC melting temperatures existed among the five Argentine ant colonies. For example, Lake Hodges ants have the highest midpoint CHC melting temperature, and Davis ants have the lowest midpoint CHC melting temperature. Since I had one sample per colony, the range in each box represents the standard errors instead of standard deviations. Analyzing the data from the melting temperature analysis and desiccation resistance, I found a negative correlation between CHC melting temperature and surviving hours which were the measurement of desiccation resistance (Figure 6.). Davis ants had the lowest midpoint CHC melting temperature but they had the highest desiccation resistance that was determined by the longest surviving hours. The other three colonies except for Los Penasquito showed the same relationship as Davis'. To follow the standard procedure of a scientific experiment, I ran a Pearson correlation test with the melting temperature data and surviving hours. The correlation coefficient was -0.9810196 with a 95% confident interval and the P-value of 0.00313. However, the validation of the Pearson correlation test was debatable because there were only five data points. In conclusion, CHC melting temperature is negatively correlated with

desiccation resistance for Argentine ants in California (i.e. the higher desiccation resistance ants had, the lower CHC melting temperature they had).

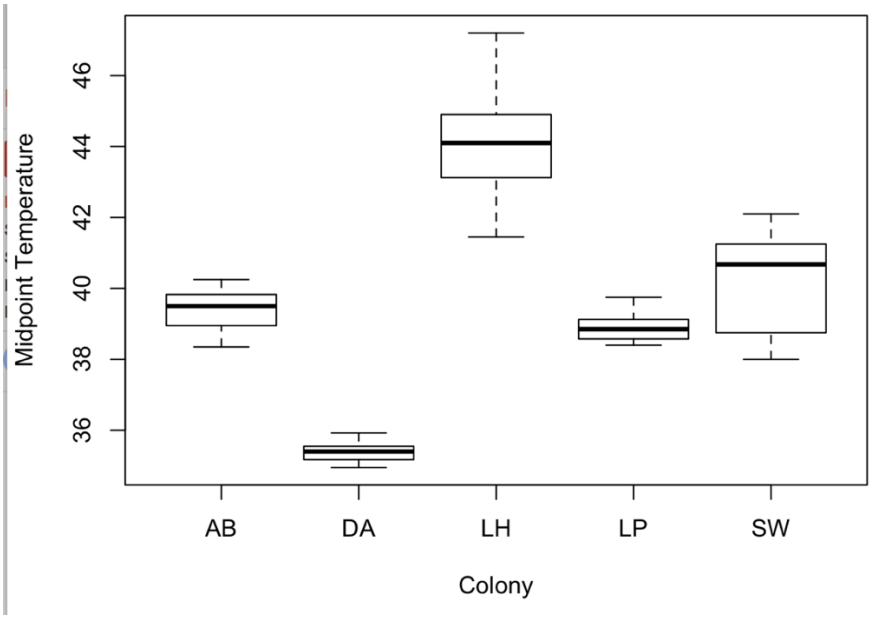

Figure 5. Midpoint CHC melting temperature of the five Argentine ant Colonies. AB: Albany Bulb, DA:Davis, LH:Lake Hodges, LP: Los Penasquito, SW:Sweet Water.

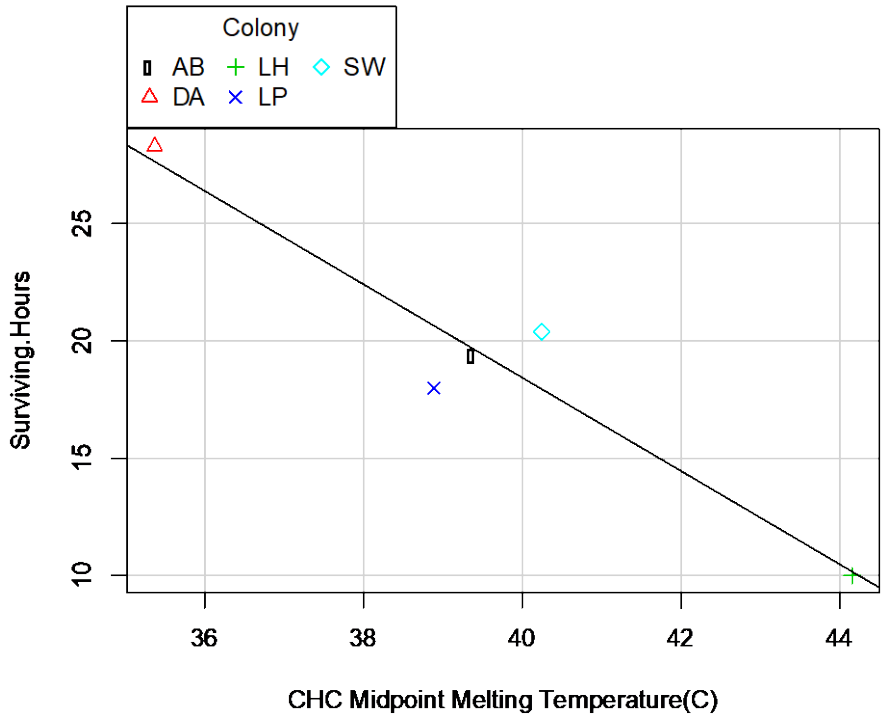

Figure 6. The relationship between midpoint CHC melting temperature of five colonies and surviving hours.

CHC Quantity Analysis

There was no correlation between CHC quantity and desiccation resistance. Lake Hodges had the largest amount of CHC: 74088 ng per sample or per 2500 Argentine ants (Table 1). Davis had the lowest amount of CHC: 44781 ng per sample or per 2500 ants (Table 1). Different colony samples had different area ratio to C-12 standard, which led to different CHC quantity. I did not find any trends indicating a positive or negative correlation between CHC quantity and surviving hours that were the measurement of desiccation resistance (Figure 7). The correlation coefficient was -0.5682137 with 95% confident interval and P-value 0.2394 from the Pearson correlation test. Therefore, there was no correlation between CHC quantity and desiccation resistance. According to the data from the CHC quantity analysis, I concluded that ants that had higher desiccation resistance did not necessarily had larger amounts of CHCs or a thicker CHC layer on their bodies.

Table 1. CHC Quantity Analysis Data CHC area for each colony sample, C-12 Standard weight by volume (ng/ul) and area, area ratio of CHC sample to C-12 Standard, CHC sample quantity and CHC quantity for each ant.

Colony	CHC area (abundance)	C-12 Standard (ng/ul)	C-12 area (abundance)	Area Ratio	CHC sample quantity (ng/sample)	CHC quantity for each ant(ng)
AB	50433561664	75	3241211214	15.5600	58350	23.3401
DA	35021097667	75	2932689625	11.9416	44781	17.9124
LH	33085060001	75	1674598612	19.7570	74088	29.6355
SW	82430888847	75	4235296269	19.4628	72985	29.1942
LP	1029442294 08	75	4453572922	23.1149	86681	34.67246

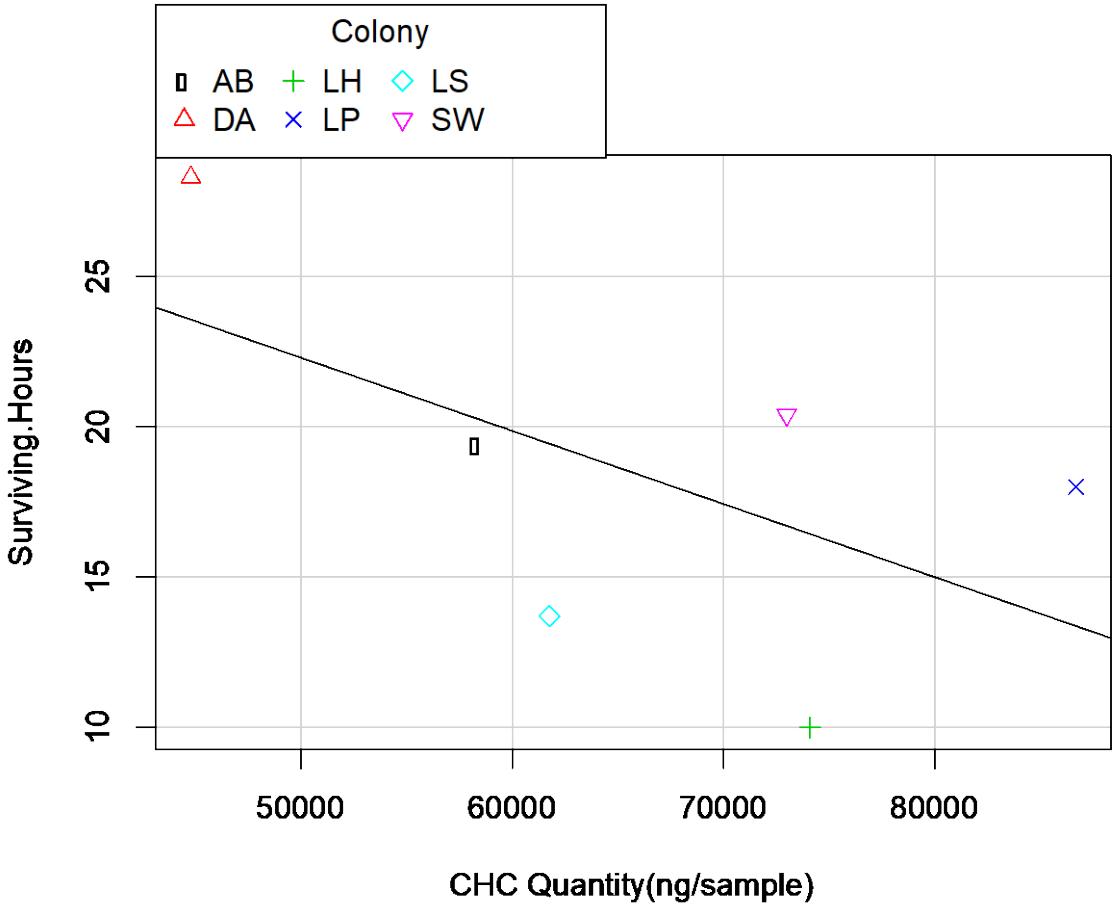

Figure 7. The relationship between CHC Quantity of five colonies and desiccation assay data

DISCUSSION

I discovered a negative correlation between melting temperature and desiccation resistance. Total amount of CHCs did not explain the variation in desiccation resistance among different Argentine ant colonies throughout California because high desiccation resistance did not correlate to high CHC melting temperatures. CHCs are a mixed composition of different types of hydrocarbons. Certain types of hydrocarbons have higher melting temperatures than others. Hence, the total CHCs may not have the same level melting temperature as those hydrocarbons. The melting temperature result was consistent with Gibbs's study on the effects of thermal acclimation on cuticular lipids and rates of water loss of adult D. *mojavensis*. They discovered the mean hydrocarbon chain length increased at higher temperatures, but cuticular lipid melting temperature

(Tm) did not (Gibbs et al. 1998). Insects use and produce CHCs in a similar way. What applies to D. *mojavensis* would likely apply to Argentine ants as well. Therefore, the melting temperature result and Gibbs's result implicate that Argentine ants may increases the production of certain types of hydrocarbons that have high melting temperature but the melting temperature of the total CHC does not increase since CHCs are also used for other purposes like communication.

I found no statistically significant correlation between CHC quantity and desiccation resistance. The result of CHC quantity analysis indicates that the total CHCs cannot explain the variation in desiccation resistance. The quantity (abundance) of specific types of hydrocarbons within CHC is a promising research direction in future. Differences in the composition or amounts of CHCs should affect rates of water loss (Gibbs 2003). A recent study showed that the abundance of specific types of hydrocarbons on different social wasps increased as the surrounding temperature increased: An increase in the percentage of linear alkanes and a decrease in the percentage of branched alkanes were correlated with increased temperature (Balbuena et al. 2018).

Limitations and Future Directions

I measured the number of ants by volume (3.75ml≈2500 ants), which later was found to be not totally accurate because different colonies of ants have different body sizes. The inaccuracy of ant measurement introduced biases into both analyses because bigger ants may produce more CHCs. CHC melting temperature and quantity could vary based on the amount of CHCs. To address the ant measurement issue, ants should be measured by weight. Counting 2500 ants for each colony need to be done, and then we would weigh out the exact 2500 ants to obtain the standard weight of 2500 ants for each colony. We then could use the standard weight to collect 2500 ants for whatever colony in future sampling.

The method of using the melting temperature apparatus to observe and gather melting temperature data had some drawbacks. The experimental design for CHC melting temperature analysis was that I could record the temperatures when the state of the CHCs shift. However, I realized it was not easy to capture and decide the phase shift of CHCs because they are in wax form. Additionally, it was hard to be precise about each measurement for each sample because samples have different volume, which influenced my judgement on when I should record the

melting temperature. I suggested to use Fourier Transform Infrared Spectroscopy to gather CHC melting temperature data because it gives more accurate results and requires less number of ants.

The sampling method in the experimental design had room for improvement in this research. The sample size for both CHC melting temperature and CHC quantity analysis was one for each colony, which was not an ideal setup in this research. Each sample was extracted from 2500 ants. This number of ants could be considered as a combination of multiple sets of ant collection (e.g. five sets of 500 ants). And therefore, one sample with 2500 ants was a representative sampling of the colony population since the focus in the research was the physiological difference (variation in desiccation resistance). However, it may be better if I could collect multiple samples from one colony and run both analyses with all the samples. Lastly, I could take the mean of all the samples from that colony and use the mean value to plot against the surviving hours from desiccation assay.

In this research, the major variable that was not taken into consideration was seasonality variation that potentially influenced CHC production and composition of ants in different colonies. Since I was not able to collect ants in different seasonal periods throughout a year, ants from different colonies were collected in different time. It would be valuable to gather the data for the two analyses under the same seasonal period and interesting to see how the results change as the seasonality changes. I was also not able to extract CHCs from different colonies of ants at the same time; this limitation is why I did not use Lake Skinner sample in CHC melting temperature analysis. Lab condition may very likely alter what kinds of hydrocarbons ants produce and how much hydrocarbons they produce to form their CHCs. As a result, the downside of not extracting CHCs from different colonies of ants at the same time was that the melting temperature and quantity of the CHCs that were extracted late may not accurately reflect the actual state of these properties. For example, B colony of ants that are supposed to have higher CHC melting temperature or quantity than C colony of ants. The result of the two analyses may show B colony has lower CHC melting temperature and quantity than C colony just because I extract CHCs from B colony later than C colony. The reason I excluded Lake Skinner sample for CHC melting temperature analysis is that Lake Skinner CHC sample melt in an unusual low temperature.

To address the seasonality variable, colony samples need to be collected multiple times throughout a year to eliminate the seasonality variable. More elaborately, different colony samples need to be collected at the same seasonal period four times a year so that we can see how Argentine

ants' chemical properties and profiles change over time. To address the concern of sample size, CHC samples for melting temperature analysis and quantity analysis could be collected multiple times from colonies that would be collected in different seasonal periods. Furthermore, CHC samples for melting temperature analysis and quantity analysis should be extracted simultaneously when desiccation assay would be conducted.

Broader Implications

From the results of the CHC melting temperature and quantity analyses, we knew that Argentine ants did not change CHC melting temperature and quantity to cope with dry environment and climate. It is not the physical properties (melting temperature and quantity) of CHCs contributing to the variation in desiccation resistant among Argentine ants in California. I suggested a future research direction of looking into CHC composition. CHC composition may be the key factor of how CHC affects desiccation resistance. In order words, tolerance to temperature variations mostly depend on changing the chemical composition of the cuticle (Michelutti et al. 2018). Michelutti et al. has testified this claim with social wasps. Him and his colleges' research showed that an increase in the percentage of linear alkanes and a decrease in the percentage of branched alkanes [in CHCs] were correlated with increased temperature (Michelutti et al. 2018). Argentine ants have unique super colony structure and can survive in different habitats with different desiccation stress. As the main physiological mechanism for desiccation resistance and the communication cues, CHCs are crucial to Argentine ants. Furthermore, the Tsutsui lab at UC Berkeley has been researching the trade-off between desiccation resistance and communication. As the desiccation stress increases, Argentine ants would use more CHCs to resist desiccation, and less CHCs would be used for communicational recognition. Argentine ants are invasive species to California; studying Argentine ants' CHCs is important to further know about this species, and we could use the information to control their invasiveness.

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