The Effect of Intra and Interspecific Pheromones on the Behavior of Different Formicine Ant Species

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Abstract

Pheromone trails are known to play an important role in influencing the behavior of many social insects. Ants in particular use them for navigation and as a way to direct other members of their colonies to a specific resource. Here, we created artificial pheromone trails of *Formica subsericea* and used them to monitor the behavioral responses of four different groups of ants: a source colony of *F. subsericea,* a second colony of *F. subsericea*, *Formica pergandei,* and *Camponotus herculeans*. We observed the strongest positive response in the slave-making ants *F. pergandei* and the strongest negative response in the second colony of *F. subsericea*.We propose that these results reflect the ecological relationships between the treatment groups rather than the phylogenetic relationships. Slave-making ants as social parasites need to use heterospecific pheromones to find hosts, and F. subsericea ants avoid intercolonial pheromone trails, as an encounter with another colony would likely result in hostility.

It has been well-established that ants use a variety of navigational techniques. Evidence supports the theory that ants primarily use visual landmarks and path integration, or the use of vectors to determine the relative distance and direction that an organism has traveled which allows it to reach the original point directly, even if the path itself was long and indirect (Judd and Collett 1998; Collett and Collett 2002). In addition, they use magnetism (Slowik et. al, 1997; Abraçado et. al, 2008), internal pedometers (Wittlinger et. al 2007), and the position of the sun (Wehner 2003) to travel between their colonies and various resources. Ants also lay pheromone trails as they forage to navigate and to direct other ants of the same colony to valuable resources (Cook 1971). These pheromones serve many additional purposes, one of the most important being to signal danger to nestmates (Wilson and Pavan 1959). Given that the complex social system of ants relies on intra and intercolonial pheromone signals, we sought to explore the extent and the ways that ants use foreign pheromone trail networks. In particular, we investigated whether or not ants are able to exploit foreign foraging trails, which could allow them to increase the efficiency with which they locate resources.

It is unknown whether or not closely related species of ants are able to use each other's pheromone trails to forage. Blum showed that ants within the Attini tribe follow artificial intergeneric pheromone trails (1964), but Wilson and Pavan showed that Dolichoderinae pheromone trails are species-specific (1959). Different species of ants use different glands within their abdomens, such as the venom, Dufour, and pygidial glands, to secrete their species-specific pheromones (Morgan 2009), including pheromones that elicit foraging or alarm behavior (Wilson and Pavan 1959). While many pheromones are species- or even colony-specific, they may also signal information such as territories to ants of other colonies.

The Formicinae subfamily of ants is one of the few that is subject to social parasitism (Hölldobler & Wilson 1990). Slave-making ants drive out adults and queens of the host colony, and the pre-raised pupa chemically imprint on the slave-makers once they eclose (Stuart 1988; Jaisson 1991). As adults, the enslaved worker ants care for the slavemaker brood and forage outside the slavemaker nests (Apple 2014). The slave-making ants we used, *Formica pergandei*, are a species of obligate slave makers, meaning their nest is made up of a majority of enslaved ants and that the slaves are entirely responsible for foraging (Apple 2014).

Here, we investigate the response of ants from two different colonies of *Formica subsericea*, *Formica pergandei* (slave-making ants), and the more distantly related *Camponotus herculeans* (carpenter ants), to pheromone trails made from the abdomens of *Formica subsericea*. *F. subsericea* are easily identifiable and very abundant near our our work station in the West Sparrow building of the University of Michigan Biological Station, which is why they were chosen for this study. The other species of ants are also commonly found near our research site and were chosen based on their phylogenetic relationships to *F. subsericea*, with *F. pergandei* being a close relative and *C. herculeans* being more distant. The purpose of this study was to determine how intra- and interspecific pheromones affect foraging behavior of formicine ants. Based on phylogenetic relatedness, we should expect the strongest foraging response from the *F. subsericea* colony from which the artificial trail was made, and decreasingly strong responses from the other *F. subsericea* colony, the *F. pergandei,* and the *C. herculeans,* respectively.

Methods and Materials

All ants were collected from the University of Michigan Biological Station (UMBS) in Pellston, MI, which is also where the study was conducted. The specimens used to make the pheromone trail were collected and frozen on July 30, August 2, and August 9th, and were used for every trial. Following a modified version the artificial trail technique established by Wilson (1959), we created a "pheromone cocktail" by crushing the abdomens of two ants to every 100 µL of dichloromethane (CH_2Cl_2).

Experimental Protocol

We collected ten live specimens from each of the colonies being tested. We gathered our first samples of *F. subsericea* outside of the West Sparrow building (N45.559°,W84.675°) on August 8 and our second samples of *F. subsericea* from a different colony outside of East Sparrow (N45.559°,W84.675°) on August 9. Our *F. pergandei* specimens were taken from outside the Hungerford building (N45.559°,W84.675°) on August 9. The *C. herculeans* ants were collected from the UMBS volleyball court (N45.559°, W84.674°) on August 10. All specimens were tested immediately after being captured.

Each ant was caught individually by hand in a microcentrifuge tube before its first of two trials. The experiment was performed on a set-up made of two braided 18 gauge aluminum wires wrapped on each side around the neck of identical 750 mL glass bottles. The wire was 1.17 meters long with masking tape markers one meter apart. The entrance to the maze was marked with masking tape exactly between the end markers. A small wire loop sat at the entrance, wherein the microfuge tubes were placed and opened, allowing the ants to crawl onto the wire.

Every 15 minutes, the pheromone cocktail was applied to one side of the maze using a natural hair paint brush. Once the ant walked onto the wire, a two-minute timer was started, and the position of the ant - whether it was on the side with the pheromone or without - was recorded

every five seconds for a total of 24 positions per trial. We performed paired trials in which each ant was tested twice - the pheromone being on different sides of the maze for each trial. The wire was cleaned with alcohol wipes between trials.

Data Analysis

All data analysis was done using R Studio version 3.3.2 and Microsoft Excel 2016. We performed a Tukey test to compare the proportion of time that each group of ants spent on the pheromone side of the maze (Figure 1). Additionally, a Chi-squared test was used to compare the total amount of times each group hit the markers on the pheromone and the non-pheromone ends of the maze (Figure 2). Because the ants' behavior seemed to change between the first and second trials, we also compared the total amount of times each marker was hit by each group for both trials separately (Figure 3).

Results

A significant difference between the proportion of time spent on the pheromone side of the y-maze was found between the second colony of *F. subsericea* and the slave-making ants, *F. pergandei* (Figure 1; p=0.0226999). No statistically significant differences were found between the average amount of time spent on the pheromone side of the y-maze for any of the other groups of ants (Tukey test; p>0.1861088).

A Chi-squared test revealed that the source colony of *F. subsericea* hit the positive end marker more often than the negative end marker (x^2=6.4, df=1), but there was no significant difference found between the number of times any of the other groups of ants hit the positive and negative markers (FSUB2: x^2=1, df=1; PERG: x^2=0.2, df=1; CAMP: x^2=2, df=1).

A second Chi-squared test showed no significant differences between the number of times each group of ants hit the pheromone and the non-pheromone markers between trials

(FSUB1: pheromone - x^2=1, df=1, non-pheromone - x^2=1, df=1; FSUB2: pheromone - x^2=2.67, df=1, non-pheromone - x^2=1.43, df=1; PERG: pheromone - x^2=2.125, df=1, non-pheromone - x^2=0.2, df=1; CAMP: pheromone - x^2=0.5, df=1, non-pheromone - x^2=0, df=1).

Discussion

Our data show a significant difference between the average proportion of time that the second colony of *F. subsericea* and the *F. pergandei* individuals spent on the side of the maze with the pheromone (Figure 1). This suggests that the groups have adapted different behavioral responses to the pheromones of the *F. subsericea* source colony. These responses differ from those predicted in our hypothesis; the second *F. subsericea* colony responded negatively, and the *F. pergandei* colony responded the most positively. These behaviors reflect ecological relationships as opposed to the phylogenetic relationships used to form our original hypothesis.

Through an ecological lens, it is expected that conspecific individuals would respond negatively to the pheromones of another colony. If they responded positively, and followed a trail back to the other colony, they would likely be killed, and certainly would not contribute to the fitness of their own colony. It also seems adaptive that *F. pergandei* slave-making ants would respond positively to all pheromones of other *Formica* ants, as they are obligate slave-makers and require enslaved ants to do most if not all foraging (Apple 2014). This lack of foraging ability in the *F. pergandei* ants could indicate that slave maker ants specialize on following interspecific rather than intraspecific pheromones.

Our findings that ants within a genus (*Formica*) show varying responses to heterospecific or intercolonial pheromones differ from Blum's (1965) which show ant genera within a tribe (Attini) all responding positively to intergeneric pheromones. This difference could be caused by

the differences in experimental methods. In his experiment, Blum noticed that Attini ants would use artificial intergeneric pheromone trails made from extracted poison glands, but would not use intergeneric pheromone trails in the field. He proposed that the ants may add some specificity component to their trails, possibly through their anal glands, that was absent in his experimental trials. Using our modified method, whole abdomens which included the anal glands were used to make the pheromone trails. Our inclusion of the other glands could explain why our results differ from Blum's. Blum also noticed in his field observations that these different genera within the tribe attini would forage next to each other, even crossing paths, without showing aggression toward one another. This shows an ecological behavior different from those displayed by our *Formica* ants. The differences in behavior could also exist simply because we tested different organisms on a different taxonomic level.

Our data also show that the only significant difference in the total number of times a group hit the positive or negative response end markers was in the *F. subsericea* source colony (Figure 2). This suggests that although the second colony of *F. subsericea* and the colony of *F. pergandei* tend to show negative and positive responses, respectively, they are not as invested in finding the end of the original *F. subsericea* pheromone trail as are individuals of that colony. When individuals of the *F. subsericea* source colony encounter the artificial trail, a behavioral response is triggered to find the end of it, which should either be a resource or their colony. While the other groups recognize the trail, they do not recognize it as their own and do not have any behavioral mechanisms to reach its end. We would expect that the slave-making *F. pergandei* individuals would hit the positive end marker significantly more than the negative marker, as finding the end of any *Formica* trail would likely convey higher fitness to their colony. We see no significant difference in the number of times slave-making individuals hit the

positive and negative end markers, and are unable to explain this result. *C. herculeans* individuals did not display any strong response to the *F. subsericea* pheromone. This finding provides support for our original hypothesis in that ants show a weaker response, if any, to the pheromones of more distantly related phylogenetic groups. The observed behavior of *C. herculeans* individuals was unique to the groups we tested in that they turned around much less frequently than any of the *Formica* ants. In the *C. herculeans* trials, individuals would appear to start walking in a direction and continue in that direction until they left the maze and were recollected.

We designed our experiment with paired trials for each ant, switching the side of the wire to which the pheromone trail was applied to strengthen our data and eliminate any variability caused by preferences for one side of the maze over the other. Ants captured in microcentrifuge tubes showed alarmed responses directly after capture, but settled down in the minute or less before being tested. By the second trial, often over an hour later, the ants were much less active, and often would not leave the tube without external stimulus, such as us blowing lightly into the tube. Being held in microfuge tubes for long periods likely changed the behaviors of the ants and for this reason, we separated the total number of times individuals hit an end marker into 1) the times the marker was hit in a group of ants' first trial and 2) the number of times the marker was hit in that group's second trial (Figure 3). This way the change in behavior from the first trial to the second trial was quantified. A Chi-squared analysis revealed no statistically significant differences between trials. However, our qualitative observations were consistent with the trends that are apparent in Figure 3. The lack of statistical significance could be due to our relatively small sample sizes, as time was limited as well as the number of ants that could be tested. The non-source *F. subsericea* group hit the positive marker 5 times in the first trial and only once in

the second trial. The *F. subsericea* source colony hit the positive marker 6 times in the first trial and only 3 times in the second trial. The *C. herculeans* group also decreased in the number of times it hit the positive marker from the first trial to the second trial. Interestingly, the *F. pergandei* group hit the positive marker once in the first trial and 4 times in the second trial. We do not know what caused this change in behavior, but it could be related to the memories of the individuals. For example, the increase seen in the *F. pergandei* group could possibly be explained if they have stronger positive responses to pheromones or other signals with which they are familiar. Similarly, the source group of *F. subsericea* could have lost interest in following the trail to its end if they already knew it did not end at their colony.

Limitations

Several problems with our experiment, stemming from our limited resources and experience, could have distorted the data and any meaning found therein. A continuous problem throughout our experiment was the testing apparatus itself. While we tried to base our techniques and methods off of those developed by E.O. Wilson (1959), we did not have access to a long glass runway like the one used in his study, and did not have the time or expertise to dissect the ants to extract just their poison glands. Using dichloromethane as Wilson describes for the production of the artificial pheromone trail, our testing substrates were virtually limited to glass, stainless steel, and aluminum. This is due to the chemical properties of DCM, which is a strong solvent that will dissolve most other materials that could have been used for the construction of our maze. For our purposes, aluminum wire was the most inexpensive and its malleability made it ideal for our use. However, the wire would shake from any vibrations in the testing environment, which would often knock the ants off of the wire mid-trial. Furthermore, many ants would either walk or fall off of the wire even without any vibrations. These behaviors were

difficult to interpret in the context of our question, and made our data as a whole more difficult to interpret.

Once again because of our limited resources, we chose to compromise Wilson's artificial trail technique of dissecting the ants for a much faster and easier technique of grinding up whole abdomens in dichloromethane. As found by Wilson and Pavan, the abdomens of ants contain several glands which produce different pheromones, including alarm pheromones (1959). We tested the effect of our modification using Blum's circle trail technique (1965) with our control source colony of *F. subsericea*. We found that *F. subsericea* individuals would follow a trail sourced from their own colony, even if it was made using whole abdomens. This encouraged us to complete our experimental trials using our modified technique, which likely affected our results. Additionally, Blum speculates that the composition of trail pheromones are fairly generic, and that a specificity component is added separately, possibly through the anal glands. If this is true, our methods would differ from Blum's in that our pheromone cocktail would include this specificity component while his would not. This could completely change the results of the experiment, if the non-source colony of *F. subsericea* is able to use the source colony's pheromones but choose not to because the specificity component is included. Also, we made our cocktail from individuals of the *F. subsericea* source colony collected and frozen on three occasions. This differs from Wilson's artificial trail technique where glands are dissected from fresh ant sources. Ants we used to make the cocktail were dead for a week at the most before we used them, and their pheromones could have weakened or deactivated in that time.

In the cited papers authored by Wilson, nothing was written of the methods with which he handled and transported his specimens. After experimenting with different methods of collecting and testing ants, we found the most effective way was to capture individuals from their

colony in a microfuge tube and then test them immediately. Once the ants had spent more than a minute or so in the microfuge tubes they would become much less active and would seldom leave the tube without some stimulus such as us blowing on them. We do not know if capturing the ants elicited alarm responses, but if they did the ants' behavior would certainly be affected. Presumably, an ant in frenzy would not follow foraging trails.

Finally, we were limited by our experience in that we could not confidently determine the caste of the ants we were testing. While it is likely that we would find foragers near the colony, if we tested an ant of a different caste it could have a completely different reaction to our pheromone cocktail.

Further Investigations

Many of the quantitative and qualitative observations made throughout this experiment provided the basis for a number of future investigations that could be done on this topic. For example, during our informal field observations we noticed that members of the source colony of *F. subsericea* tended to forage within a range of approximately 2 meters from their colony. A second *F. subsericea* colony nearby had been usurped by *F. pergandei* and we noticed that the *F. subsericea* members of the colony seemed to forage within a much larger range -- approximately 10 meters from their colony. While it is known that pupa of the usurped colony chemically imprint on the slave-making ants upon hatching (D'Ettorre 2002), it would be interesting to see if this has any effect on their production of pheromone trails and, in turn, their foraging behavior. Slave-making ants mimic their host's pheromones as camouflage (D'Ettorre 2002), so it would be interesting to see if they then use that host's pheromone while inhabiting the usurped colony, or if the host slaves are able to use the original pheromones specific to that slave-making colony. Since parasitism is a strong selective pressure, it would be interesting too to investigate if ants

species that are frequently parasitized have more diverse pheromonal components, or use novel pheromones more frequently.

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Figures

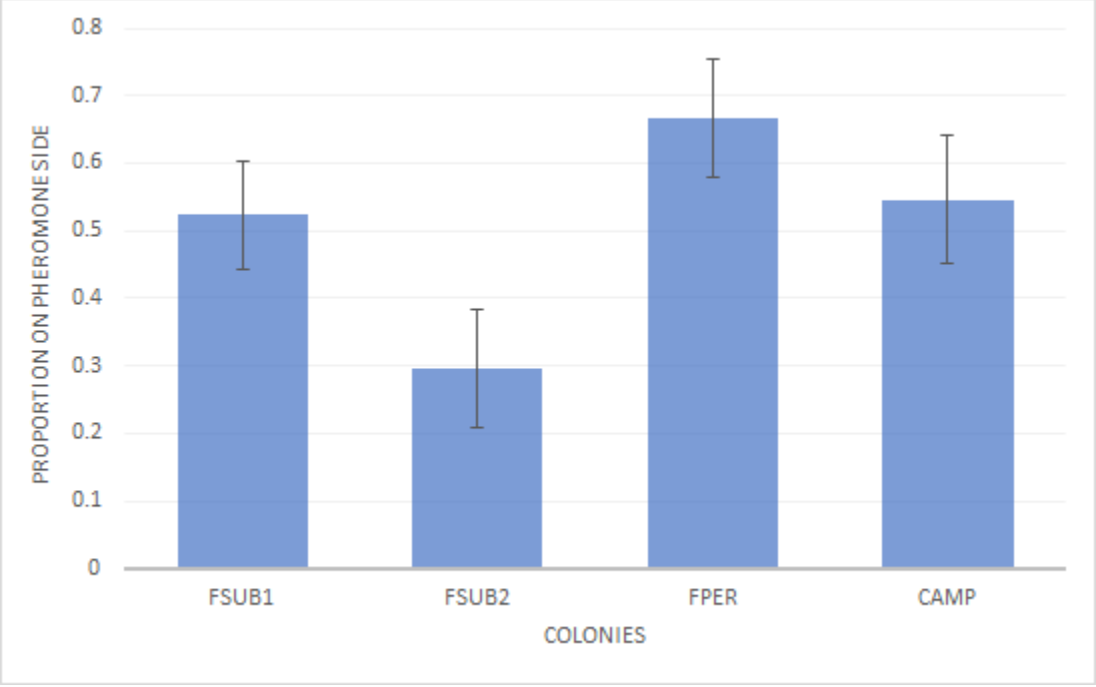

Figure 1. The proportion of time each species spent on the pheromone side of the y-maze (FSUB1 = *F. subsericea* control colony; FSUB2 = Second colony of *F. subsericea,*; FPER = *F. pergendei*; CAMP = *Camponotus herculeans*). A significant relationship was found between the second colony of *Formica subsericea* and the slave-making ants, *Formica pergandei* (Tukey test; p=0.0227367).

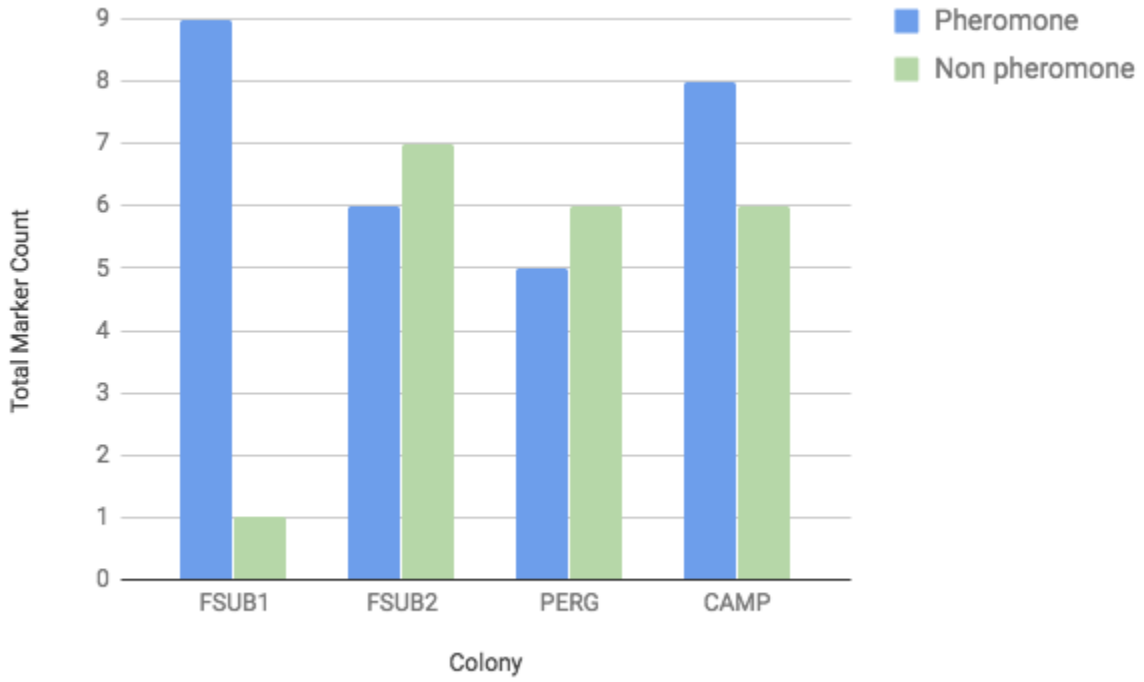

Figure 2. The total number of times members from each group of ants hit the markers at the end of the pheromone and the non-pheromone sides of the y-maze. A significant difference was found between the number of times the source colony of *F. subsericea* hit the positive and negative markers (Chi-squared; x^2=6.4, df=3.841). No significant differences were found between the number of times any of the other groups of ants hit the pheromone and non-pheromone markers (FSUB2: x^2=1, df=1; PERG: x^2=0.2, df=1; CAMP: x^2=2, df=1).

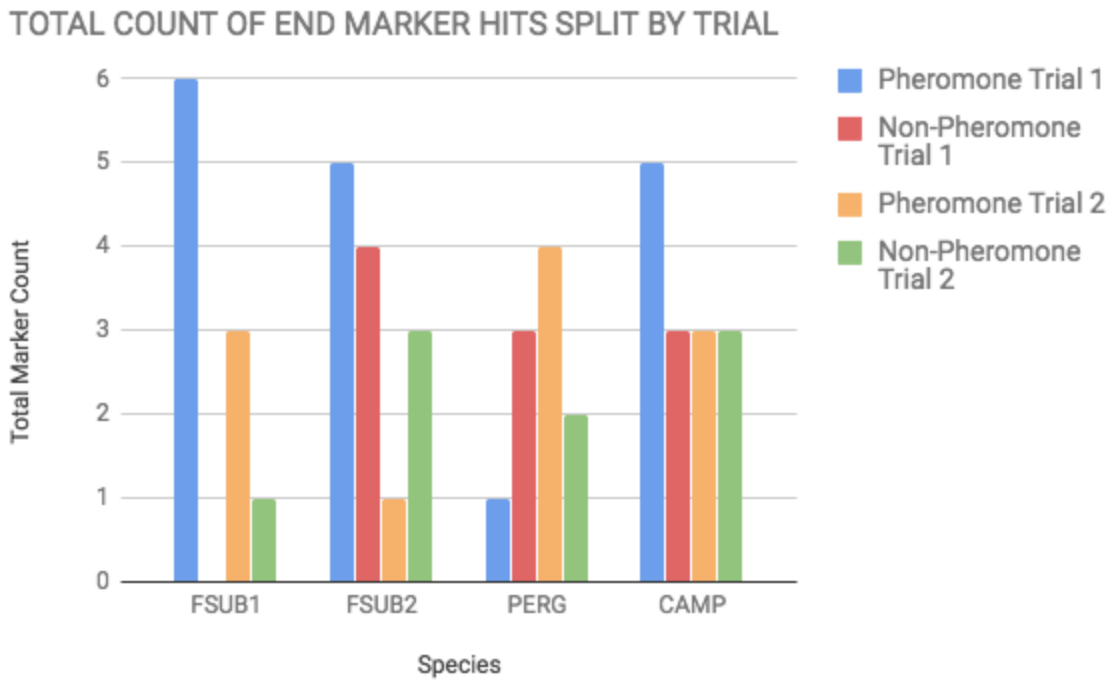

Figure 3. The total number of times each group hit the pheromone and non-pheromone markers split by trial. None of the differences between trials were found to be significant (FSUB1: pheromone - x^2=1, df=1, non-pheromone - x^2=1, df=1; FSUB2: pheromone - x^2=2.67, df=1, non-pheromone - x^2=1.43, df=1; PERG: pheromone - x^2=2.125, df=1, non-pheromone - x^2=0.2, df=1; CAMP: pheromone - x^2=0.5, df=1, non-pheromone - x^2=0, df=1). However, we see a general decrease in the number of times the pheromone marker was hit between the first and the second trial for all species except for the slave-making *F. pergandei*, which hit the pheromone marker more frequently on the second trial.