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Aggressive and Non-aggressive Behaviors in Mixed and Non-mixed Dorymyrmex smithi and Dorymyrmex bureni Colonies of Southeastern Georgia, USA

Renee K. Nowicki

AGGRESSIVE AND NON-AGGRESSIVE BEHAVIORS IN MIXED AND NON-MIXED DORYMYRMEX SMITHI AND DORYMYRMEX BURENI COLONIES OF SOUTHEASTERN GEORGIA, USA

by

RENEE NOWICKI

(Under the Direction of Joshua D. Gibson)

ABSTRACT

Dorymyrmex species, 'pyramid' or 'cone' ants, are Dolichoderines distributed across North America. Two relatively well-studied species, *Dorymyrmex smithi* and *Dorymyrmex bureni*, are commonly found throughout southeastern Georgia. These species are part of a socially parasitic relationship while simultaneously having starkly different colony structures. *Dorymyrmex smithi* is a polydomous, polygynous species with colonies that are often expansive, at times having hundreds to thousands of nests per colony. *Dorymyrmex smithi* is a temporary social parasite of *D. bureni*, a monodomous, monogynous species. The specific mechanism of this parasitism is currently unknown. Preliminary behavioral assay data from a limited number of sites showed that *D. smithi* could potentially be a 'unicolonial' species, due to low aggression occurring between adult workers that originated from different colonies. In this study, behavioral assays were conducted with *D. smithi* and *D. bureni* adult workers that were collected from five sites across southeastern Georgia, US. Artificial 'parasitized' mixed species colonies were established in the laboratory by introducing *D. smithi* brood (i.e., eggs and larvae) to groups of *D. bureni* adult workers. The *D. smithi* brood were reared to adulthood and a series of behavioral assays were conducted between *D. smithi* and *D. bureni* from mixed species and non-mixed species colonies. The 'unicoloniality' of *D. smithi* was tested with behavioral assays between workers originating from each of the five sites. These tests showed that *D. smithi* adult workers from

different colonies show very little aggression during interactions. This aggression also did not correlate with geographic distance between sites. The mixed and non-mixed species assays showed that *D. smithi* reared by *D. bureni* maintain aggression with non-colonymate *D. bureni* while simultaneously showing less aggression toward colonymate *D. bureni* and increased aggression toward their former colonymate *D. smithi*. This suggests that these behaviors could be determined by both intrinsic (genetic) and extrinsic (environmental) factors. This work is an important early stage in understanding the mechanisms of temporary social parasitism and the complex polydomy in this *Dorymyrmex* system.

INDEX WORDS: *Dorymyrmex* species, Dolichoderinae, Behavior, Polydomy, Polygyny, Supercolony, Unicoloniality, Aggression, Social parasitism

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RENEE NOWICKI

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RENEE NOWICKI

Major Professor:	Joshua D. Gibson
Committee:	Alan Harvey
	Lissa Leege
	Kevin Loope

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CHAPTER 1

INTRODUCTION

Ants are commonly found throughout the globe and are present on all continents except Antarctica (Hölldobler & Wilson, 1990). This worldwide distribution could be attributed to colonial living, where each ant nest could house hundreds to thousands of individuals (Hölldobler & Wilson, 1990). *Dorymyrmex* species (Hymenoptera: Formicidae: Dolichoderinae), commonly known as pyramid or cone ants, are widely distributed across North and South America (Snelling, 1995). *Dorymyrmex* species in the southwestern United States are understudied with notoriously complicated systematics (Deyrup, 2016; Godfrey *et al.*, 2021; Oberski, 2022), while *Dorymyrmex* species in the southeastern United States (e.g., Georgia, Florida, Alabama, etc.) are relatively well-studied thanks in large part to Buren (1975), Nickerson (1976), Trager (1988), and Deyrup (2016). The following research is focused on two common southeastern US *Dorymyrmex* species, the Usurper Cone Ant *Dorymyrmex smithi* and Buren's Cone Ant *Dorymyrmex bureni*. It should be noted that in the most recent systematic revision of North American *Dorymrymex* species, *D. smithi* was described by Snelling (1995) as having a nationwide distribution from Colorado to Florida, but this is more than likely a species complex (Deyrup, 2016; Oberski, 2022). Deyrup (2016) and Oberski (unpublished) refer to *D. smithi* as *D. medeis* (Trager) in their works.

In the southeastern US *D. smithi* is a ground-nesting ant that constructs large multi-nest (polydomous) multi-queen (polygynous) colonies (Nickerson, 1976; Trager 1988; Graham *et al.*, 2004; MacGown & Hill, 2007; MacGown *et al.*, 2009; Deyrup, 2016). Colonies can be found in open disturbed habitats with sandy soils, such as dunes, unpaved roads, and old agricultural fields (Trager 1988). When present, *D. smithi* colonies are often extremely large and expansive,

sometimes described as “dominating” a habitat (Whitcomb *et al*., 1972; Nickerson *et al*., 1975; Nickerson, 1976; Trager, 1988; Graham *et al*., 2004; Graham *et al*., 2008; MacGown & Hill, 2007; Burrow *et al*., 2021). For example, the Tall Timbers Research Station in Florida had a record count of 2,187 *D. smithi* nests in one plot, with an average number of three nests per square meter (Nickerson, 1976). These large polydomous colonies have complex networks of adult workers that walk rapidly (at times appearing to meander haphazardly) on the soil surface between nests (Trager, 1988; Deyrup, 2016). There is currently no evidence of whether there are subterranean tunnel connections between nests mounds.

Polydomy, Supercolinies, and Unicoloniality

Polydomous and polygynous ant species are behaviorally distinct from monodomous (single nest) and monogynous (single queen) colonies (Ellis & Robinson, 2014). Monodomous and monogynous colonies are territorial and aggressive towards other nests (i.e., colonies) of the same species (d’Ettorre & Lenoir, 2010). In contrast, polydomous and polygynous species are not aggressive between nests that make up a single colony (Debout *et al*., 2007). Low aggression in these polydomous colonies typically only extends within the bounds of a colony at a single location (Wilson, 1971). When low aggression extends beyond the limits of a singular location and these behaviors can be observed between adult workers that originate from colonies separated by great distances which could not be traversed by a single ant, this is known as a supercolony (Tsutsui *et al*., 2000). In some instances, these large polydomous supercolonies are called unicolonial, particularly when the range of non-aggression extends hundreds to thousands of kilometers or between continents (Helanterä *et al*., 2009). An extreme example of this phenomenon is in the Argentine ant, *Linepithema humile*. This species is highly invasive and has spread throughout the globe (Human & Gordon, 1996). In their non-native range, when

individual Argentine ants from different continents (e.g., Europe and North America) act non-aggressively towards one another in behavioral assays, these behaviors suggest that these ants come from the same unicolonial colony (Torres *et al*., 2007). The difference between being a supercolony or unicolonial largely depends on the scale at which a population is studied. Wilson (1971) described unicolonial populations as having multiple queens and low dispersal rates, with infrequent (i.e., rare) nuptial flights, and no clear colony boundaries across great distances from tens to thousands of kilometers. Helanterä *et al*. (2009) reiterates this definition and defines a unicolonial population as a population that consists of a single colony which spans over large distances. Helanterä (2022) notes that unicoloniality is a suitable description for a population or species, not a single colony. Essentially, if a species is truly unicolonial, then aggression should not be observed between ants of the same species within a population. Recently, unicoloniality has been studied extensively in invasive species that appear to be unicolonial outside of their native range (e.g., *Linepithema humile* and *Wasmannia auropunctata*; Helanterä *et al*., 2009; Breton *et al*., 2004). Unicoloniality is less commonly encountered in native species. One notable species that appears to be unicolonial in its native range is *Formica paragulubris*, which is native to the Swiss Jura Mountains (Holzer *et al*., 2006). Intraspecific behavioral assays within and between the polydomous supercolonies of *F. paragulubris* showed little to no aggression, with the farthest distance between two colonies being 72 km. In southeastern Georgia, preliminary behavioral assays between *D. smithi* colonies from a limited number of sites show similar extremely low levels of aggression (unpublished data), which prompts further investigation to determine if there is a boundary to this lack of aggression.

Social Parasitism in *Dorymyrmex*

The natural history of *D. smithi* becomes more complex with the fact that this species is a temporary social parasite of *D. bureni*. In contrast to the large polydomous *D. smithi* colonies, *D. bureni* is a single-nest (monodomous), single-queen (monogynous) species. *Dorymyrmex bureni* colonies can be found in the same disturbed sandhill habitats as *D. smithi*, but *D. bureni* appears to be more tolerant of urban areas, as their nest mounds can be found protruding from cobblestone walkways, grass lawns, and at the edges of concrete sidewalks (Deyrup, 2016; personal observation). The temporary social parasitism of *D. bureni* colonies has been observed in the field by means of discovering *Doyrmyrmex* colonies that have adult workers of both species (i.e., mixed species nests) exiting and entering the same nest mound with no obvious aggression occurring (Buren *et al*., 1975; Nickerson, 1976; Trager, 1988; Deyrup, 2016). Outside of these mixed species parasitized colonies, both species were observed to be antagonistic towards one another; Buren *et al*. (1975) and Nickerson (1976) observed that *D. smithi* workers tended to hold their ground while *D. bureni* workers would retreat when interacting with each other in the field. It should be noted that both *Dorymyrmex* species can be easily distinguished from one another by coloration alone, as *D. smithi* is brown-black while *D. bureni* is yellow-orange.

The first published report of temporary social parasitism between these two species was from Buren *et al*., 1975. A plot of land in the experimental farm area of University of Florida in Gainesville, Florida with many nests of both species was studied and all nest locations were mapped from June 1 – 26, 1975. In the first 10 days (June 1 – 10) five mixed species nests with both *D. smithi* and *D. bureni* workers were found clustered near each other. A sixth mixed species nest (which was approximately 1m away from next nearest mixed species nest) was

excavated on 23 May 1975 at the same location. The excavated colony had 326 *D. bureni* and 726 *D. smithi* adult workers. The only queen ant that was collected from the excavated colony was a dealate *D. smithi* queen. All pupae collected that were mature enough to identify were *D. smithi*. Between June 10th and 26th, four of the five original mixed species nests were replaced by *D. smithi* nests, and more interestingly there was an increase in the number of *D. smithi* nest entrances in the same vicinity. These newer *D. smithi* nests appeared to spatially fill in the gaps between the previous *D. bureni* nests and the subsequent mixed species nests.

The Buren *et al*., 1975 publication is the only formal study to date that thoroughly recorded these mixed species nests. All other records of parasitized nests are notes or observations of their occurrences in the southeastern US. Nickerson (1976) lists seven different mixed species nest localities in Florida. Trager (1988) recounts his own personal observations of *Dorymyrmex* populations and the several instances of mixed species nests, but he does not list specific localities. Trager (1988) informally reared a small mixed species colony by introducing a *D. smithi* queen (it is not specified where he collected her, and it is unclear if this was a dealate or an alate gyne) into a group of approximately 50 *D. bureni* adult workers. The *D. bureni* workers accepted the queen without aggression, and eventually about 20 *D. smithi* workers were reared within the span of a few weeks. However, this small artificial colony did not persist, and the queen died shortly thereafter.

With these historical observations of mixed species nests, it is hypothesized that the temporary social parasitism of *D. bureni* colonies would occur in a similar manner as temporary parasite-host relationships in other species. The following is adapted from Buren *et al*. (1975) and Buschinger (2009): a *D. smithi* gyne leaves her mother colony for her nuptial flight and insemination. After this flight, the *D. smithi* queen enters a *D. bureni* colony. She then kills the

host queen and leaves the remaining *D. bureni* adult workers alive. These *D. bureni* workers then rear all *D. smithi* brood that are laid by the new *D. smithi* queen. As the *D. smithi* brood pupate and eventually eclose into adult *D. smithi* workers, they eventually traverse outside of the nest alongside other *D. bureni* workers. At this stage the mixed species nest can be easily identified when brown-black ants (*D. smithi* workers) are interacting with yellow-orange ants (*D. bureni* workers) with no obvious aggression (e.g., biting, killing, etc.). Eventually, this parasitized mixed species nest transitions to a *D. smithi* nest when all remaining *D. bureni* workers die.

Nestmate Recognition

The specific behaviors which occur in these mixed species nests are currently unknown. Identifying these behaviors in non-mixed and mixed nest environments is a crucial first step to further study this mechanism of temporary social parasitism. The temporary social parasitism in this *Dorymyrmex* system is possible because both species recognize the other as nestmates in these mixed species colonies (Buschinger, 2009). Accurately discriminating between nestmates and non-nestmates is crucial to prevent internal conflict and ensure colony survival (Leonhardt *et al.*, 2016; Sturgis & Gordon, 2012; Holzer *et al*., 2006; Lenoir *et al*., 2001; Guillem *et al*., 2014; Cini *et al*., 2020). Nestmate recognition can be observed via qualitatively assessing behavioral responses between ants (Vander Meer & Morel, 2019). Behaviors can be categorized broadly as aggressive or non-aggressive during standardized behavioral assays (Krapf *et al*., 2019). In most ants, and more broadly social insects, nestmates interact with each other non-aggressively while simultaneously acting aggressively towards non-nestmates (Chapuisat *et al*., 2004). In these social systems, ants rely on chemical cues to communicate with one another; these chemical cues, called cuticular hydrocarbons (CHCs), are the basis of an ant's ability to determine who is friend or foe (Lenoir *et al*., 2001). Each species of ant has a unique CHC profile, which is a

combination of specific compounds, that can vary between colonies depending on the geographic location and environment (Kather & Martin, 2012).

Hypotheses

In this study, *D. smithi* brood and adult workers, and *D. bureni* adult workers, were collected from five sites across southeastern Georgia, US. These ants were transported from the field into the laboratory and maintained in artificial colonies. Several behavioral assays were conducted to answer questions regarding the potential unicoloniality of *D. smithi* and the mechanisms of temporary social parasitism between the two species. The following is a summarized list of all assays and their respective hypotheses.

Is the sampled southeastern Georgia *D. smithi* population unicolonial? Assays were conducted between *D. smithi* adult workers originating from the same and different sites. Can *D. smithi* colony boundaries be defined?

1. The southeastern Georgia *D. smithi* population is unicolonial. There will be little to no aggression between ants from different sites across all sampled sites. This aggression will suggest that the colonies sampled are from the same colony/supercolony, and are therefore one unicolonial population.
2. The southeastern Georgia *D. smithi* population is not unicolonial. There will be aggression between ants from different sites across all sampled sites. This aggression will suggest that the colonies sampled are from different colonies/supercolonies, rather than one unicolonial population.

What are the behavioral mechanisms of temporary social parasitism between *D. smithi* and *D. bureni*? All assays were conducted between ants from the same site and

replicated across multiple sites: *D. bureni* from different colonies; *D. smithi* and *D. bureni* from the same mixed nest and these *D. smithi* with the *D. bureni* that they were reared by, and the *D. bureni* that did not rear them; and *D. smithi* reared by *D. bureni* with the sister *D. smithi* from the same originating parent colony that did not interact with *D. bureni*. How will the ants in mixed nests alter their behaviors?

1. The *D. bureni* from different colonies will be aggressive towards each other. This aggression will support that *D. bureni* are a monodomous species that exhibits aggression between colonies.
2. Mixed *D. smithi* and *D. bureni* will not be aggressive towards each other because they were reared in the same nest. This lack of aggression will support that there is little to no aggression in parasitized mixed nests.
    a. If *D. smithi* and *D. bureni* are acclimating to their reared nest environment, then both mixed nest species will interact equally with non-mixed nest *D. bureni* and parent colony *D. smithi*.
    b. If *D. smithi* and *D. bureni* are not acclimating to their reared nest environment, then both mixed nest species will interact unequally with non-mixed nest *D. bureni* and parent colony *D. smithi*.
3. Mixed *D. smithi* will change their behavior towards their parent colony members. This change in behavior will support that rearing environment influences behavior.
    a. If mixed *D. smithi* interact aggressively with their parent colony *D. smithi*, then the mixed colony environment is dissimilar to the

parent colony. The mixed *D. smithi* and *D. bureni* will both interact aggressively towards parent colony *D. smithi*.

b. If mixed *D. smithi* interact non-aggressively with their parent colony *D. smithi*, then the mixed colony environment is not dissimilar to the parent colony. Mixed *D. smithi* will recognize both mixed *D. bureni* and parent colony *D. smithi* as nestmates.

4. Mixed *D. smithi* will accept all *D. bureni* as nestmates. This change in behavior will support that mixed *D. smithi* are broadly adaptive to be non-aggressive towards all *D. bureni*.

CHAPTER 2

METHODS

Field Collections and Husbandry Protocols

Ants were collected between September 2022 – May 2023 from five sites in southeastern Georgia (Table 1): Ohoopee Dunes Wildlife Management Area (OHD), Metter (MET), Georgia Southern University campus (GSU), Ft. Stewart-Hunter Army Airfield (FSW), and Oatland Island Wildlife Center (OAT). Cheese puffs (CHEETOS® Crunchy Cheese-Flavored Snacks) were used as bait to attract ants. From each site, four adult worker samples were collected: two *D. smithi* and two *D. bureni*, each consisting of approximately 150 ants. All samples were collected at least 10 meters apart from each other. All *D. bureni* samples were assumed to be from separate monodomous colonies, while the two *D. smithi* samples from each site were assumed to be two separate collections from the same polydomous colony. Ants were collected near nest entrances with an aspirator and a 9-dram plastic vial (Bioquip, Rancho Dominguez, CA) or a 50mL conical tube (Thermo Fisher Scientific, Waltham, MA).

For transport from field sites to the lab, ants were placed into plastic containers (Cut Comb Honey Container, 4-5/16″ x 4-5/16″ x 1-3/8″, 14oz, Pioneer Plastics, Dixon, KY) with a coat of Insect-a-slip (Bioquip, Rancho Dominguez, CA) painted on with a damp foam brush on the inner walls to prevent ants from walking up the sides. Small holes were drilled into the container lids for air circulation. A small piece of fine mesh nylon fabric cut from a nut milk bag (Superior Quality Nut Milk Bag, Utopia Kitchen, Plainview, NY) was hot glued overtop the small holes. Moist soil (excavated on-site) was sprinkled into all containers to prevent desiccation during transport.

Dorymyrmex smithi brood were collected from each site by excavating a shallow hole (between approximately 7 – 30cm deep) with a shovel near the entrance of an active *D. smithi* nest. The shovel was inserted within an approximate 15cm radius around the nest entrance and angled towards the entry hole in a shallow manner to excavate brood from the upper chambers of the colony, which are located just below the soil surface. Multiple nests were excavated if necessary to collect a minimum of 200 *D. smithi* brood per site. In the field, one of the two *D. smithi* adult worker samples was randomly chosen to be "S1". The excavated brood were placed into the same collection container as the S1 adult workers when the ants were transported from the field site to the lab. The *D. smithi* adult workers sample that did not come in contact with brood were designated as "S2" and were transported in a separate container from the S1 adult workers and *D. smithi* brood. The *D. bureni* adult workers from the two colonies samples were transported to the lab in separate collection containers.

Upon arrival at the lab, approximately 10 adult ants from each nest sample were frozen at −20°C and stored for future cuticular hydrocarbon analyses. The remaining ants and soil were placed into 6-quart 14″ x 8″ x 4.88″ plastic boxes (referred to as "nest boxes" henceforth), which had a coat of Insect-a-slip applied to the upper inner wall. Adult worker field samples (n = 20) were placed into their own designated nest box. The interspecific mixed nests were simulated in the lab by adding approximately 150 *D. smithi* brood (designated as "SB1") to one of the two *D. bureni* adult worker samples collected from the same site immediately after arriving in the lab from the field. The *D. bureni* nest box which was given *D. smithi* brood was chosen at random and designated as "B1"; the other *D. bureni* nest box which did not have *D. smithi* brood was designated as "B2". Excess *D. smithi* brood that were not immediately added to B1 nest boxes were placed into another nest box with other *D. smithi* adult workers that were collected from the

same site ("extra brood" nest boxes henceforth). The *D. smithi* adult workers in these extra brood nest boxes were used as a supplemental source of *D. smithi* ants. The S1 and S2 *D. smithi* nest boxes at the start of laboratory husbandry had at minimum approximately 200 adult workers each. If the S1 or S2 nest boxes had significant mortality and there were fewer than 50 ants, then *D. smithi* from the extra brood nest box from the same field site were added until there were at least 200 ants. These S1 and S2 nest box adult workers were eventually combined into a single nest box labeled "SB1P" for the mixed species assays. Combining these nest boxes increased the number of available *D. smithi* parent colony adult workers for the mixed species behavioral assays. *Dorymyrmex smithi* brood were removed from the extra brood nest boxes and added to B1 nest boxes if there was significant (>50) brood mortality in the artificial mixed colonies. Some B1 colonies rejected large numbers of brood within days of the initial introduction. Initially, only *D. smithi* pupae were added to the mixed nest boxes in the hopes that this stage would decrease the amount of time it took for *D. smithi* adult workers to eclose. The *D. bureni* ended up discarding most (if not all) of the pupae. Because of this, eggs and larvae were added, which were largely accepted and reared to adulthood. Due to the initial pupae mortality, *D. smithi* brood (i.e., eggs and larvae) were subsequently re-sampled from the field and introduced to B1 laboratory colonies as needed (Table 1).

All nest boxes were placed on top of a masonry brick in a 28-quart 23″ x 16.25″ x 6″ plastic box, which also had a coating of Insect-a-slip applied to the upper inner walls. Soapy water was poured into the 28-quart containers up to approximately 0.5cm below the nest boxes; the soapy water acted as a moat to contain any ants that escaped their nest box enclosures. Soapy water was added to the 28-quart containers as needed due to evaporation. All nest boxes had one 100 x 15mm petri dish that had a thin layer of dental plaster in the bottom half. The dental plaster

was hydrated with DI water prior to being placed in the nest boxes. The lid and base of the petri dishes were both spray-painted black to prevent light from entering the enclosure. A small hole was drilled into the side which allowed ants to move freely in and out of the dish. These petri dishes served as artificial dark nesting sites for the adult workers and any brood collected. Each nest box also had two glass culture tubes, one 16 x 100mm tube filled with tap water, and one 12 x 75mm tube filled with a 20% sugar water solution. A small piece of cotton pulled from a cotton ball was stuffed into the top of both tubes to prevent spillage. The ants could freely drink both liquids through the soaked cotton barriers. Ants were fed small portions of scrambled egg (approximately <5g) 2 – 3 times a week to allow *ad libitum* feeding. Egg was used as a protein source because there is evidence that ants can acquire cuticular hydrocarbons from insect prey, such as crickets (van Wilgenburg *et al*., 2022). It was important to feed the ants a non-insect protein source because all specimens were frozen after behavioral assay experiments and stored for future cuticular hydrocarbon analyses.

Behavioral Assays

Behavioral assays were conducted one-on-one in one Insect-a-slip lined well of a 12-well cell culture plate (Greiner). Two 12-well plates were taped together and closed shut like a book, which dropped one ant from the "top" well into the corresponding "bottom" well that housed the other ant. Up to six wells were used at a time (Figure 1a). All ants were randomly assigned to a top or bottom well each replicate (Figure 1b). For the *D. smithi* intraspecific assays (within and between-site experiments), approximately 25 ants were aspirated from each S1 and S2 nest box from all sites. For the intraspecific *D. smithi* within-site assays, workers from the S1 and S2 were used; between-site assays used workers from two S1 nest boxes that originated from different sites. These ants were placed into empty 100 x 15mm petri dishes with piece of damp

cotton. Insect-a-slip was applied to the inner walls of each dish. For the *D. smithi – D. bureni* mixed interspecific assays, there was no 24-hour isolation period. All sites were used for the *D. smithi* intraspecific within and between-site assays. Sites OHD, MET, and GSU were used for all mixed species assays except for SB1–SB1P. Sites FSW and OAT were only used for SB1-SB1P due to mortality of *D. bureni* adult workers in the 'B1, SB1' nest boxes prior to the behavioral assay dates (Figure 1a).

Dorymyrmex smithi intraspecific within and between-site assays were completed twice – once with ants that were collected from September – December 2022 ("winter" henceforth), and once with ants that were collected in May 2023 ("summer" henceforth). The *D. smithi* ants that were used in the winter behavioral assays were not used in the summer assays and vice versa. The winter behavioral assays were conducted February – March 2023 and the summer behavioral assays were conducted May – June 2023. All nest boxes were kept in the Georgia Southern University (Statesboro, GA) insectary at 22.5°C. Mixed species nest boxes were moved into an incubator set to 23°C on 17 June 2023. The incubator temperature was increased several times due to slow *D. smithi* adult eclosion rates. These temperature changes were on 9 August (set to 25°C) and 7 September (set to 28°C). All mixed nests were removed from the incubator during the mixed behavioral assays, which were conducted November – December 2023.

Approximately 5 minutes before each assay, all ants were dropped into their corresponding top or bottom wells, as a brief acclimation period. Ants without obvious injuries or missing appendages were selected for the assays. Once the 12-well plates were shut, a timer was set, and assays were run for 10 minutes. Footage of each 12-well plate was recorded using a Canon EOS 6D Mark 2 dSLR camera with a Canon 100mm f/2.8L Macro lens (resolution 1920 x 1080). After each assay all ants were immediately frozen and stored at -20°C (Figure 1b). Ant

behaviors were assessed and blindly scored from the recorded footage using an ethogram (Table 2) at least two weeks after the assays were completed. Each well in the 12-well plate was individually assessed when behaviors were scored. When the footage was reviewed, behaviors were recorded upon contact between the two ants for each well. There was not a set period of time in which behaviors were not assessed after the lid was shut and the top well ants fell into the bottom well. Prior research using this technique did not show aggression between Argentine ants that were from the same colony, and so it was assumed that the lid slamming would not have a major effect on ant behaviors in *Dorymyrmex* assays.

Any notable behaviors that occurred after the 10-minute recording period were not recorded or scored. These notable behaviors were described prior to conducting these assays via an ethogram (Table 2) written using other *Dorymyrmex* assay videos recorded from a previous study in the lab. The videos used to describe these behaviors and write the ethogram were chosen blindly at random (i.e., it was unknown if the specific comparisons were between ants that originated from the same site, different sites, same colony, different colony, etc.) Ethograms are commonly used in ant research – after the original ethogram was written, other ethograms were referenced for more detailed descriptions of ant behaviors that are often observed in other species (Krapf *et al*., 2019; Gibson laboratory Argentine ant ethogram). When the *Dorymyrmex* footage from previous research was reviewed, there were notably infrequent aggressive behaviors observed between *D. smithi* ants (within or between site was unknown). Because of this, a fine-scale ethogram was used as an example template for this study (Krapf *et al*., 2019). Using a fine-scale ethogram ensured that subtle non-aggressive behaviors could be described in detail and accurately recorded for each assay. When statistically analyzing the behavior results, these behaviors were lumped into an “aggressive” or “non-aggressive” category. The fine-scale

ethogram was necessary to note and record nuances in *Dorymyrmex* behaviors. This fine-scale also allows later adjustments to which behaviors are included in the "aggressive" or "non-aggressive" categories.

The ethogram is a whole number numerical scale from lease aggressive (-4) to most aggressive (4). Based on previous use of these categories in other studies, initially numbers -4, -3, -2, -1, and 0 were non-aggressive. Numbers 1, 2, 3, and 4 were aggressive. For each assay, the most aggressive behavior was assigned as the final score. If no aggressive behaviors (1 – 4) were observed, then the assay was assigned the least aggressive behavior (-4 – 0). For each assay category (e.g., *D. smithi* within-site assays) the number of assays with each score from -4 to 4 scale were tallied.

The following is a description of each behavior from highest to lowest aggression. These categories are largely based on the ethogram in Krapf *et al*. (2019). In their research, these descriptions were written from behaviors observed in both Myrmicinae and Formicinae assays:

4, Killing: Killing occurs when one or both ants are actively killed by the other at any point in the 10-minute assay. Killing is not accidental. Killing was only assigned as a behavior if the death of an ant was due to the other ant in the assay, not due to handling during the assay itself.

3, Biting: Biting is the physical closing of mandibles from one or both ants onto any body part of the other ant(s). Biting can be brief (milliseconds, i.e., "snapping") to prolonged (several seconds). Prolonged biting was uncommon in *Dorymyrmex* assays.

2, Chasing and Fleeing: One ant actively chases the other, typically with mandibles flared. The fleeing ant clearly runs away in the opposite direction at a frantic pace. Movement

does not appear to be walking slowly. Contact after or during chasing and fleeing does not lead to further aggression (biting and/or killing).

1, Mandible Flaring: At least one of the two ants stands in a wide stance with mandibles stretched out wide laterally at more than 45°. Mandible flaring typically lasts for at least 1 second, but this behavior could also be brief. Mandible flaring is obviously aggressive – the ant with outstretched mandibles is also spreading antennae upwards laterally and maintains the mandible flaring stance when approached or close by the other ant. Non-aggressive mandible spreading is not counted as mandible flaring. An ant may non-aggressively open her mandibles when she is cleaning herself or briefly adjusting her mandibles without spreading her antennae widely.

0, Ignoring: One or both workers do not interact with the other. This lack of interaction could be standing more than 1 mm apart from each other and not facing each other, or actively walking around and not interacting with the other ant on contact. Ignoring is not a lack of touch, but a lack of acknowledgement (e.g., rubbing antennae, pausing during touch).

-1, Close Proximity: Close proximity is when both ants sit close to each other without physically touching. The ants are less than 1 mm apart and facing each other. One or both ants may slowly sway their head and/or antennae laterally side-to-side, but there is no active antennation between workers.

-2, Antennation: One or both workers actively touch each other with their antennae. This behavior can occur face-to-face or on any other body part. During antennation, workers assess whether the other worker belongs to the same colony or not (Krapf *et al*., 2019). Furthermore, during a prolonged antennation time, information on food sources, foraging locations, orientation cues, and trail conditions, among others, might be exchanged between workers (Mc Cabe *et al*.,

2006; Robinson *et al*., 2009; Farji-Brener *et al*., 2010; Czaczkes *et al*., 2014). That is, antennation is meaningful also in context other than nestmate recognition.

-3, Allogrooming: Allogrooming is when one worker cleans the other with their labium. The worker actively allogrooming the other has relaxed (slightly open, less than 20°) mandibles. Allogrooming typically occurs all over the body of the other ant. Allogrooming is difficult to assess unless the labium of one ant is visibly touching the other ant. Allogrooming could be confused with antennation as both behaviors may occur simultaneously.

-4, Trophallaxis: Trophallaxis is the exchange of food or fluids between workers during which chemical cues, growth proteins, and hormones are also transferred (Krapf *et al*., 2019; LeBoeuf *et al*., 2016). In *Dorymyrmex*, it is unclear if true trophallaxis occurs, in the sense that there is an exchange of fluids, however the physical behavior observed fits the description of the mechanism of trophallaxis. This behavior must occur face-to-face. One ant stands still with mandibles spread laterally (less than 90°) and labium protruding. The other ant slightly opens her mandibles and touches the labium of the other ant. It is difficult to determine how much liquid is exchanged between the two, or if any liquid is exchanged at all, but this behavior is distinct and easy to discern from other non-aggressive behaviors. The mandible to labium contact is prolonged and typically lasts 4 – 6 seconds.

For each assay category the number of assays with each score were tallied. These final counts were used for statistical tests. Fisher's exact test (2x2 contingency tables) was used to assess the difference between two assay categories and their proportion of aggressive behaviors (e.g., *D. smithi* intraspecific within-site versus between-site; *D. bureni* (B1) with mixed *D. smithi* (SB1) versus *D. bureni* (B1) with non-mixed *D. bureni* (B2)). For the mixed species assays, only the most informative comparisons were compared statistically (Table 4). A linear regression was

used to test if there was a correlation between observed aggression and distance between sites for the *D. smithi* intraspecific assays. Individual pairings of interest for the *D. smithi – D. bureni* mixed nest interspecific assays were compared post hoc with individual 2x2 contingency tables (Fisher's exact test), and those *p* values were compared to a target α level using the Holm-Bonferroni correction method (Holm, 1979). All statistics were calculated in JMP Pro 17 and Excel. Figures were made with BioRender or in Excel.

Table 1. Site names with corresponding codes, coordinates, and collection dates. Dates are separated by assay. *Dorymyrmex smithi* samples were collected twice from all sites for intraspecific assays, these were all run within the same seasonal collecting event (i.e., adult workers collected in December 2022 were not used in the same assay block as adult workers collected in May 2023). *Dorymyrmex smithi* brood and *D. bureni* adults were collected on the same dates in the summer. Each site required multiple *D. smithi* brood collection days. Each † next to the month indicates an additional day of brood collection.

Site	**Code**	**Coordinates**	***D. smithi* intraspecific assays**	**Mixed species assays**
Ohoopee Dunes Wildlife Management Area	OHD	32.6346, -82.4274	Dec 2022, May 2023	May 2023††, June 2023††
Metter	MET	32.4470, -82.0557	Oct 2022, May 2023	May 2023†, June 2023††
Georgia Southern University, Statesboro Campus	GSU	32.4288, -81.7855	Oct 2022, May 2023	May 2023†, June 2023††
Fort Stewart-Hunter Army Airfield	FSW	32.0739, -81.6573	Sep 2022, May 2023	May 2023†, June 2023†
Oatland Island Wildlife Center	OAT	32.0485, -81.0260	Nov 2022, May 2023	May 2023†, June 2023†

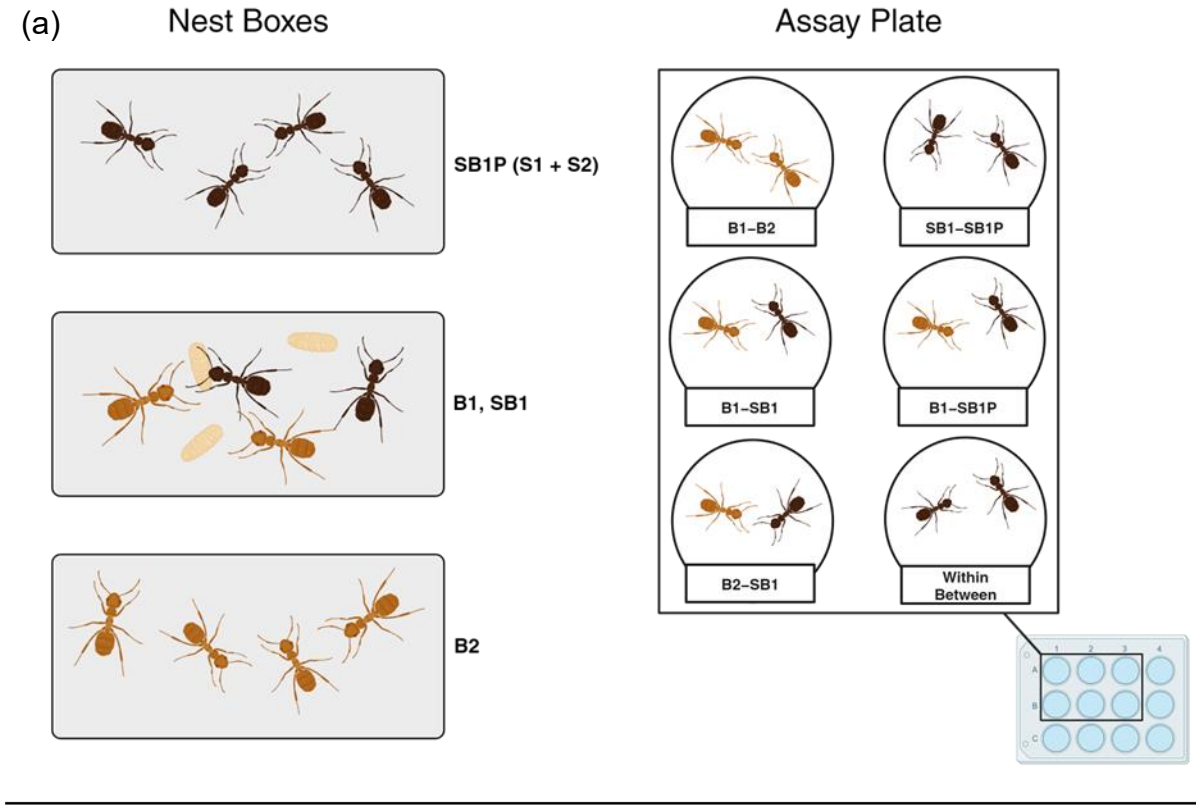

Figure 1. Ant husbandry and behavioral assay protocols. **(a)** Nest boxes for adult workers and *D. smithi* brood. **(b)** Graphic representation of transferring ants into top or bottom wells of the 12-well plates. Six wells were used at a time. Behavioral assay footage was recorded for 10 minutes. Ants were frozen and stored at -20°C immediately after

Table 2. Ethogram used for scoring ant behaviors. A single behavior was assigned to each assay. Tests were scored based on the most aggressive behavior. If no aggressive behaviors were observed (i.e., none at a rating of 1 – 4), then the assays were assigned a final score at the least aggressive level. This ethogram was adapted largely from Krapf *et al.*, 2019.

Aggression Level	Numerical Scale	Behavior	Description
Aggressive	4	Killing	One or both ants engage in behavior that results in death of either or both.
Aggressive	3	Biting	Biting is a physical contact of mandibles to any part of the opponent's body. Biting could be brief (i.e., snapping) or prolonged (i.e., grappling).
Aggressive	2	Chasing and fleeing	One ant actively chases the other, typically with mandibles flared. The fleeing ant runs away quickly at a frantic pace (i.e., does not appear to be walking slowly). Contact does not lead to further escalation in aggression.
Aggressive	1	Mandible flaring	One or both ants stands still with mandibles aggressively flared. Does not lead to chasing or further excitement.
Non-aggressive	0	Ignoring	Ignoring is the lack of interaction between ants. Ants can be standing still or moving.
Non-aggressive	-1	Close proximity	Close proximity is when the ants stand next to one another ≤1mm apart without physically touching.
Non-aggressive	-2	Antennation	Antennation is the touching of antennae to any part of the body for a prolonged period (i.e., greater than a few seconds).
Non-aggressive	-3	Allogrooming	Allogrooming is when one ant cleans the other via licking the other with their tongue. The ant being groomed may stand still or move slowly.
Non-aggressive	-4	Trophallaxis	Trophallaxis is the exchange of food or fluids between workers. One or both ants must have their mandibles spread with their tongue protruding. This behavior must occur face-to-face.

CHAPTER 3

RESULTS

Behavioral assay results are summarized in Table 3.

Intraspecific *D. smithi* Assays

Dorymyrmex smithi from different sites were significantly more aggressive towards one another than *D. smithi* from the same site when all behaviors were accounted for (Figure 2a; Fisher's exact test, $p < 0.0001$), as well as when only contact aggression behaviors are taken into consideration (Figure 2b; Fisher's exact test, $p < 0.0001$). However, both the within-site and between-site assays had relatively low proportions of aggressive behaviors overall. There was no significant differences in observed aggression between the winter and summer season collecting events. There was no correlation between observed aggression and distance (km) between sites. (Figure 2c,d, d; $R^2 = 0.004$, $F(1, 8) = 0.0346$, $p = 0.857$).

Table 3. Observed non-aggressive and aggressive behavior counts for all assays.

Species	Assay Test	Non-aggressive	Aggressive	Total
D. smithi	Within	198	2	200
D. smithi	Between	321	78	399
D. bureni	B1−B2	23	24	47
D. bureni, *D. smithi*	B1−SB1	30	14	44
D. bureni, *D. smithi*	B2−SB1	16	28	44
D. bureni, *D. smithi*	B1−SB1P	13	35	48
D. smithi	SB1−SB1P	13	17	30

Mixed Species Assays

The statistical differences between individual comparisons varied (Figure 4, Table 4). SB1–SB1P (mixed nest *D. smithi* versus the parent colony *D. smithi*) showed significantly more aggression than *D. smithi* from between different sites (Figure 4a; Fisher's exact test, $p < 0.0001$); B2–SB1 (*D. bureni* which did not rear *D. smithi* brood against *D. smithi* adults that were reared by *D. bureni*) showed significantly more aggression than B1–SB1 (*D. bureni* against *D. smithi* the colony reared; Figure 4b; Fisher's exact test, $p = 0.0052$); B1–SB1P (*D. bureni* that reared *D. smithi* against *D. smithi* from the parent colony) showed significantly more aggression than B1–SB1 (*D. bureni* against *D. smithi* the colony reared; Figure 4c; Fisher's exact test, $p = 0.0001$).

Table 4. Fisher's exact test (FET) *p*-values and Holm-Bonferroni (HB) α levels for all test comparisons of interest, listed in order of significance (top, most significant; bottom, least significant).

Test Comparison			FET *p*-value	HB α level
SB1−SB1P	↔	Between	<0.0001	0.0071
B1–SB1	↔	B1–SB1P	0.0001	0.0083
B1–SB1	↔	B2–SB1	0.0052	0.01
B1–SB1	↔	SB1−SB1P	0.0541	0.0125
B1–SB1P	↔	SB1−SB1P	0.1492	0.0167
B1–B2	↔	B2−SB1	0.2902	0.025
B2–SB1	↔	SB1−SB1P	0.6304	0.05

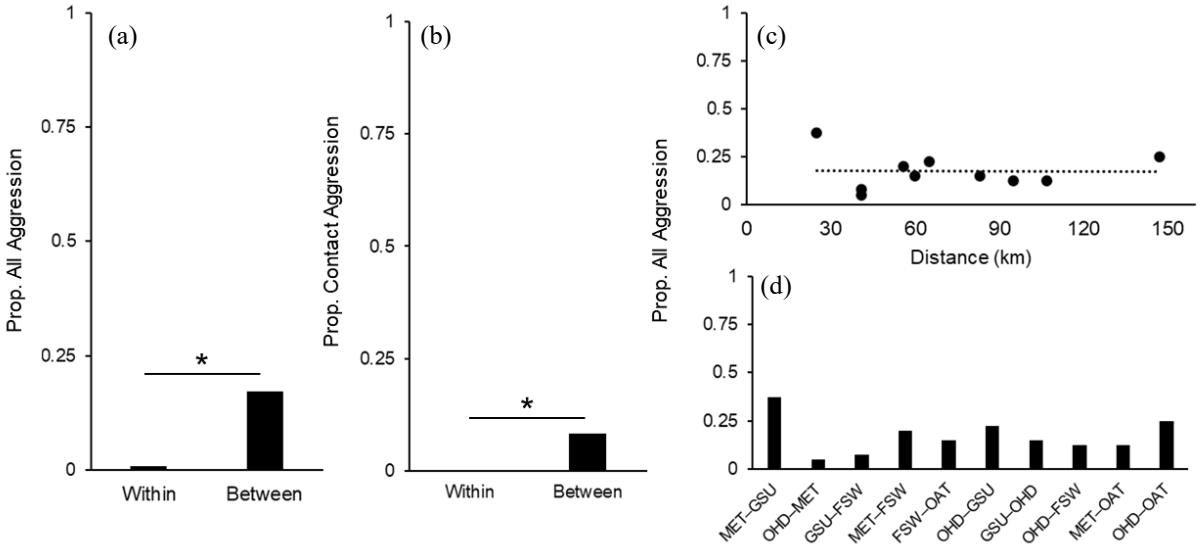

Figure 2. *Dorymyrmex smithi* intraspecific assay results. Asterisks indicate $p < 0.05$ significant differences. **(a)** Proportion of aggressive behaviors observed in intraspecific *D. smithi* assays with adult workers collected from within the same site (n = 200) and between different sites (n = 399). Panel is combined data, all behaviors. **(b)** Proportion of contact aggression behaviors (i.e., biting and killing) **(c)** Proportion of aggressive behaviors observed versus distance (km) between sites; within-site assay data were excluded. **(d)** Proportion of aggressive behaviors observed versus between-site locality comparisons. Bars are ordered from the shortest distance between sites (left, MET–GSU, 25km) to the farthest distance between sites (right, OHD–OAT, 147km); n = 40 for all between-site comparisons except for GSU–FSW (n = 39). MET = Metter; GSU = Georgia Southern University; OHD = Ohoopee Dunes Wildlife Management Area; FSW = Fort Stewart-Hunter Army Airfield; OAT = Oatland Island Wildlife Center.

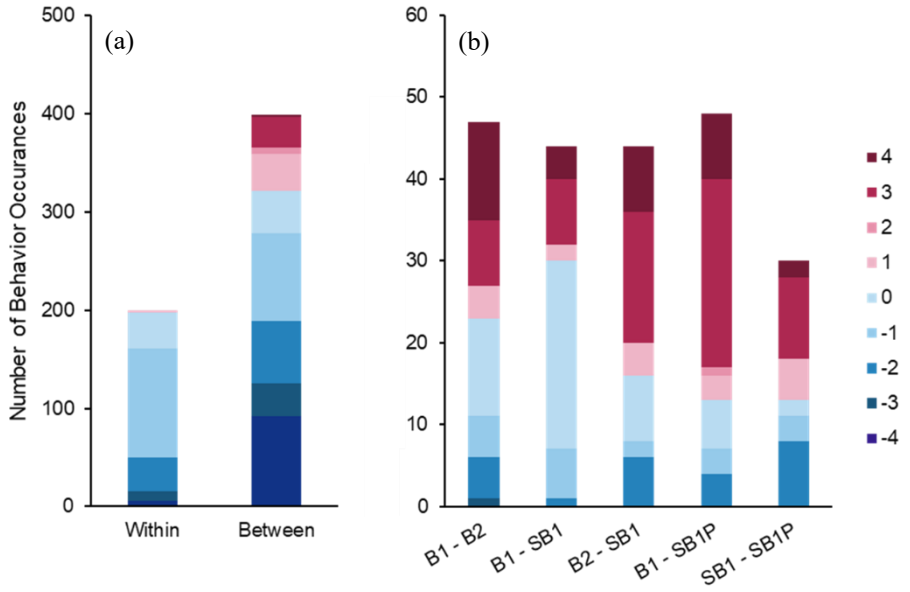

Figure 3. Stacked bar graphs showing the total number of each scored behavior for **(a)** intraspecific *D. smithi* assays and **(b)** mixed species assays. Warm-toned (red and pink) colors represent aggressive behaviors. Cool-toned (light and dark blue) colors represent non-aggressive behaviors. The numerical scale corresponds to the behavior categories in Table 2.

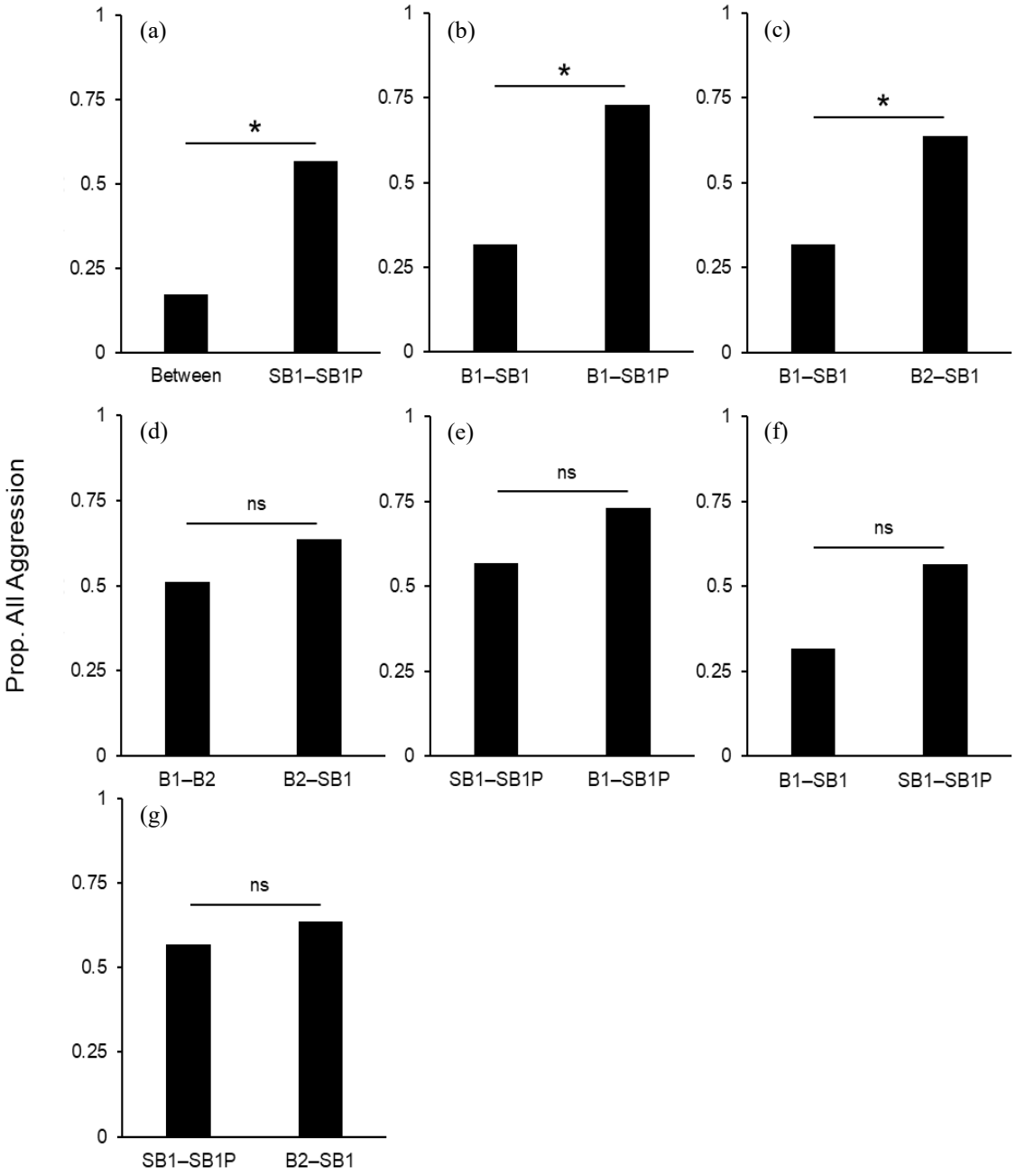

Figure 4. Proportion of observed aggressive behaviors in mixed and *D. smithi* intraspecific between-site assays. Asterisks indicate $p < 0.05$ significant differences (Fisher's exact test, Holm Bonferroni correction). Each panel is a comparison between two mixed species assays. NS indicates no significant different. Sample sizes were as follows: B1 – B2, n = 47; B1 – SB1, n = 44; B2 – SB1, n = 44; B1 – SB1P, n = 48; SB1 – SB1P, n = 30.

CHAPTER 4

DISCUSSION

This research is the first laboratory study where behavioral interactions between *D. smithi* and *D. bureni* were qualitatively and quantitatively assessed. *Dorymyrmex smithi* exhibited low aggression within the same site and between all sites. Aggression was observed significantly more frequently between ants from different sites than between ants from same site, but this between-sites proportion of aggression was still extremely low at a frequence of only 0.17 (Figure 2a). Most of the aggressive interactions that occurred between sites were low levels of aggression (i.e., mandible flaring; Figure 3a). When only contact aggression is taken into consideration, aggression between sites is even less common with an observed frequency of 0.08 (Figure 2b). The very low level of aggression between sites that are more than 140 km apart coupled with *D. smithi* polydomy and polygyny may suggest that this species is unicolonial. Unicoloniality is largely described in the invasive ranges of species such as the Argentine ant, *Linepithema humile*, and the tawny crazy ant, *Nylanderia fulva* (Helanterä, 2022). Unicoloniality in native species on the same magnitude as *D. smithi* are far less common; this study has shown that *D. smithi* is not only non-aggressive to the extreme on a local level, but also at a larger geographic scale. Currently there are few other known species which exhibit a similar level of unicoloniality. Behavioral assays support unicoloniality in two *Formica* species (*Formica paralugubris* and *Formica exsecta*). Holzer *et al*. (2006) collected adult workers from different colonies across the Swiss Jura Mountains and conducted intraspecific aggression assays. Similarly to *D. smithi*, *F. paralugubris* adult workers demonstrated low aggression at local and regional scales, the greatest distance between two colonies being 72 km. However, unlike *D. smithi*, *F. paralugubris* aggression is correlated with geographic distance; but this noticeable

difference could be due to the Swiss Jura Mountains having more complex topography. The distance between peaks and isolation of the colonies due to elevational changes could act as 'sky islands' (Favé *et al*., 2015) that decrease gene flow between supercolonies, but nuptial flights could negate this as reproductive flights might not be limited by distance with some ant species having reported nuptial flight distances of over 1000 km (Stukalyuk *et al*., 2022). In contrast to the Swiss Jura Mountains, southeastern Georgia (specifically regions below the Fall Line), has incremental changes in elevation between 75 – 600 feet (Miller & Robinson, 1995). These small changes in elevation may facilitate more gene flow between supercolonies in southeastern Georgia than in the Swiss Jura Mountains.

It is clear that *D. smithi* adult workers that would otherwise never encounter one another in the field are not overtly aggressive towards each other. As suggested previously, this lack of aggression is likely due to genetic and environmental (i.e., colony cuticular hydrocarbon profiles) factors. Behaviors are rooted in genetic and chemical similarities (Drescher *et al*., 2007; Brandt *et al*., 2009). The number of queens in a *D. smithi* colony, and more specifically where those queens originate from, likely impacts the genetic diversity of that colony. If a colony has many queens that were not born in said colony, then the genetic diversity is likely higher, and that could in turn cause that colony to be less aggressive towards other *D. smithi* colonies with similar genetic diversity. It is difficult to say at this time what the shared genetics would be both within and between *D. smithi* colonies. Currently the number of queens per *D. smithi* colony are unknown. Nickerson (1976) excavated over 107 *D. smithi* nests and collected queens from only five of them. The maximum number of queens collected from one single nest was 16, which were collected between 4 – 13 cm below the soil surface. In this current research, at least 30 nests were excavated and only four queens were collected, three of which were excavated at the

Ohoopee Dunes Wildlife Management Area, and two of these queens were collected from the same nest. All queens were collected in May 2023 and there were no obvious signs of queens in colonies that were excavated between September – November 2023 (i.e., no eggs were found during excavations). It is not yet known how far reproductives (both gynes and males) can disperse, but they may be able to reach other nearby colonies. Analyzing an ant species CHC profile can aid in identifying an ant's colony of origin, which in turn can determine how individuals would behaviorally interact with others from different locations (Buellesbach *et al*., 2018). Identifying a species' CHC profile is essential when studying its behavior across different colonies and geographic locations (Dettner & Liepert, 1994). Cuticular hydrocarbons are likely a primary reason for the behaviors observed in the mixed species artificial 'parasitized' nests.

Dorymyrmex smithi from non-mixed colonies show low aggression between sites and virtually no aggression within the same site (Figure 2a, b), but this changes significantly with *D. smithi* reared by *D. bureni* (Figure 4). There is no significant difference in the aggression of workers from the parent colony of the mixed nest *D. smithi* (i.e. their sisters) toward the *D. smithi* or the *D. bureni* from the mixed nest (Figure 4e) – the parent colony *D. smithi* do not recognize mixed colony *D. smithi* as being different from their nestmate mixed colony *D. bureni*. It should be noted that this comparison had a smaller overall sample size (SB1 – SB1P, n = 30; B1 – SB1P, n = 48) which may have decreased the ability to statistically detect a difference. Simultaneously, *D. bureni* from non-mixed colonies do the same – they are interacting with both *D. smithi* and *D. bureni* from mixed nests equally aggressively (Figure 4d). Mixed *D. smithi* behaviors appear to be an intermediary stage between non-mixed *D. bureni* and *D. smithi* colonies. Aggression between mixed *D. smithi* and parent colony *D. smithi* is not significantly

different from mixed *D. bureni* and parent *D. smithi* interactions (4e), nor do they differ significantly from mixed *D. bureni* and *D. smithi* interactions (4f).

It is also important to note that there is still aggression present in these mixed nests, significantly more aggression than the within-site *D. smithi* interactions, which are the most comparable comparison (Figure 3b). This result supports previous observations by Buren *et al*. (1975) that mixed colonies in the field seemed to disappear relatively quickly after discovery. They hypothesized that over time, as the mixed nest ratio shifts from majority *D. bureni* to *D. smithi*, antagonism between the two increases. At this time, it is not possible to declare any certainty in how this aggression may escalate. Buren *et al*. (1975) also noted that there were likely multiple queens in the mixed nests which could have accelerated the transition from several mixed *D. smithi – D. bureni* nests to a newly established *D. smithi* polydomous colony. In this current study queens were not present in the colonies that were used for behavioral assays, so there are no conclusions to be made about how dealate queens may influence the behaviors of both *D. bureni* and *D. smithi* workers in mixed nests, as queens typically differ in their CHC profiles to workers in colonies which could change a colony's chemical profile overall (Sprenger & Menzel, 2020).

Adult workers of both species and *D. smithi* brood were successfully collected from the field, transported to the lab, and maintained for several months. Establishing reliable husbandry standards is an essential step for continued behavioral research in this system. Studying the behaviors of this system in a laboratory setting is in its infancy. There are many avenues to take with this research in the future. Adult workers of both species were collected from the field and immediately frozen for CHC profile analyses, which can be used to identify the baseline CHC profile of both species. Microsatellite molecular markers in the nuclear genome are also being

used to determine how the colonies may differ genetically. The markers are based on Argentine ant primers that have been successfully amplifying *D. smithi* DNA. Using these primers saves a significant amount of time, but at this moment it is unknown whether these markers vary within *D. smithi*. If they vary, these markers can be used to assess gene flow between sites and could greatly inform us as to where alate females and males are travelling between sites and if this contributes to the low aggression between sites.

Other questions to explore for this *Dorymyrmex* system include but are not limited to: What is the CHC profile of *D. smithi* queens before and after mixed colony establishment? What are the ecological, behavioral, genetic, and chemical differences and/or similarities between *D. smithi* colonies and invasive species that have similar supercolonial strategies? What are the exact behavioral mechanisms of the transition from *D. bureni* nests to mixed species colonies to *D. smithi* polydomous colonies? What factors (e.g., genetic, environmental) effect the aggression of a *D. smithi* colony to other *D. smithi* and *D. bureni* colonies? Specifically, the GSU and MET colonies in the intraspecific *D. smithi* site-to-site assays had the highest observed aggression, but these sites are the closest together geographically.

Dorymyrmex smithi and *D. bureni* in southeastern Georgia present a new and exciting intersection between supercoloniality and social parasitism in ant behavioral research. The results from this study suggest that genetic and environmental factors likely play a role in what behaviors (aggressive versus non-aggressive) were observed in interactions between *D. smithi* from different colonies and both *D. smithi* and *D. bureni* in artificial mixed nest settings. Overall, this work is an important early stage in learning more about the behavioral mechanisms of temporary social parasitism and the complex polydomy in this *Dorymyrmex* system.

REFERENCES

Brandt, M., van Wilgenburg, E., Sulc, R., Shea, K. J., & Tsutsui, N. D. (2009). The scent of supercolonies: the discovery, synthesis and behavioural verification of ant colony recognition cues. *BMC biology*, *7*, 1-9.

Breton, J. L., Delabie, J. H., Chazeau, J., Dejean, A., & Jourdan, H. (2004). Experimental evidence of large-scale unicoloniality in the tramp ant *Wasmannia auropunctata* (Roger). *Journal of insect Behavior*, *17*, 263-271.

Buellesbach, J., Whyte, B. A., Cash, E., Gibson, J. D., Scheckel, K. J., Sandidge, R., & Tsutsui, N. D. (2018). Desiccation resistance and micro-climate adaptation: cuticular hydrocarbon signatures of different Argentine ant supercolonies across California. *Journal of chemical ecology*, *44*, 1101-1114.

Buren, W. F., Nickerson, J. C., & Thompson, C. R. (1975). Mixed Nests of *Conomyrma insana* and *C. flavopecta*—Evidence of Parasitism (Hymenoptera: Formicidae). *Psyche: A Journal of Entomology*, *82*(3-4), 306-314.

Burrow, A. K., Crawford, B. A., & Maerz, J. C. (2021). Ground cover and native ant predation influence survival of metamorphic amphibians in a southeastern pine savanna undergoing restoration. *Restoration Ecology*, *29*(7), e13410.

Buschinger, A. (2009). Social parasitism among ants: a review (Hymenoptera: Formicidae). *Myrmecological News*, *12*(3), 219-235.

Chapuisat, M., Bocherens, S., & Rosset, H. (2004). Variable queen number in ant colonies: no impact on queen turnover, inbreeding, and population genetic differentiation in the ant *Formica selysi*. *Evolution*, *58*(5), 1064-1072.

Cini, A., Branconi, R., Patalano, S., Cervo, R., & Sumner, S. (2020). Behavioural and neurogenomic responses of host workers to social parasite invasion in a social insect. *Insectes Sociaux*, *67*, 295-308.

Czaczkes, T. J., Schlosser, L., Heinze, J., & Witte, V. (2014). Ants use directionless odour cues to recall odour-associated locations. *Behavioral Ecology and Sociobiology*, *68*, 981-988.

d'Ettorre, P., & Lenoir, A. (2010). Nestmate recognition. *Ant ecology*, 194-209.

Debout, G., Schatz, B., Elias, M., & Mckey, D. (2007). Polydomy in ants: what we know, what we think we know, and what remains to be done. *Biological Journal of the Linnean Society*, *90*(2), 319-348.

Dettner, K., & Liepert, C. (1994). Chemical mimicry and camouflage. *Annual review of entomology*, *39*(1), 129-154.

Deyrup, M. (2016). *Ants of Florida: identification and natural history*. CRC Press.

Drescher, J., Blüthgen, N., & Feldhaar, H. (2007). Population structure and intraspecific aggression in the invasive ant species *Anoplolepis gracilipes* in Malaysian Borneo. *Molecular Ecology*, *16*(7), 1453-1465.

Ellis, S., & Robinson, E. J. H. (2014). Polydomy in red wood ants. *Insectes Sociaux*, *61*, 111-122.

Farji-Brener, A. G., Amador-Vargas, S., Chinchilla, F., Escobar, S., Cabrera, S., Herrera, M. I., & Sandoval, C. A. (2010). Information transfer in head-on encounters between leaf-cutting ant workers: food, trail condition or orientation cues?. *Animal Behaviour*, *79*(2), 343-349.

Favé, M. J., Johnson, R. A., Cover, S., Handschuh, S., Metscher, B. D., Müller, G. B., Gopalan, S., & Abouheif, E. (2015). Past climate change on Sky Islands drives novelty in a core developmental gene network and its phenotype. *BMC evolutionary biology*, *15*, 1-21.

Godfrey, R. K., Oberski, J. T., Allmark, T., Givens, C., Hernandez-Rivera, J., & Gronenberg, W. (2021). Olfactory system morphology suggests colony size drives trait evolution in odorous ants (Formicidae: Dolichoderinae). *Frontiers in Ecology and Evolution*, *9*, 733023.

Graham, J. H., Hughie, H. H., Jones, S., Wrinn, K., Krzysik, A. J., Duda, J. J., Freeman, D. C., Emlen, J. M., Zak, J. C., Kovacic, D. A., Chamberlin-Graham, C., & Balbach, H. (2004). Habitat disturbance and the diversity and abundance of ants (Formicidae) in the Southeastern Fall-Line Sandhills. *Journal of Insect Science*, *4*(1), 30.

Graham, J. H., Krzysik, A. J., Kovacic, D. A., Duda, J. J., Freeman, D. C., Emlen, J. M., Zak, J. C., Long, W. R., Wallace, M. P., Chamberlin-Graham, C., Nutter, J. P., & Balbach, H. E. (2008). Ant community composition across a gradient of disturbed military landscapes at Fort Benning, Georgia. *Southeastern Naturalist*, *7*(3), 429-448.

Guillem, R. M., Drijfhout, F., & Martin, S. J. (2014). Chemical deception among ant social parasites. *Current Zoology*, *60*(1), 62-75.

Helanterä, H. (2022). Supercolonies of ants (Hymenoptera: Formicidae): ecological patterns, behavioural processes and their implications for social evolution. *Austrian Society for Entomofaunistics*

Helanterä, H., Strassmann, J. E., Carrillo, J., & Queller, D. C. (2009). Unicolonial ants: where do they come from, what are they and where are they going?. *Trends in Ecology & Evolution*, *24*(6), 341-349.

Hölldobler, B., & Wilson, E. O. (1990). *The ants*. Harvard University Press.

Holm, S. (1979). A simple sequentially rejective multiple test procedure. *Scandinavian Journal of Statistics*, 6, 65-70.

Holzer, B., Chapuisat, M., Kremer, N., Finet, C., & Keller, L. (2006). Unicoloniality, recognition and genetic differentiation in a native *Formica* ant. *Journal of Evolutionary Biology*, *19*(6), 2031-2039.

Human, K. G., & Gordon, D. M. (1996). Exploitation and interference competition between the invasive Argentine ant, *Linepithema humile*, and native ant species. *Oecologia*, *105*, 405-412.

Kather, R., & Martin, S. J. (2012). Cuticular hydrocarbon profiles as a taxonomic tool: advantages, limitations and technical aspects. *Physiological Entomology*, *37*(1), 25-32.

Krapf, P., Hochenegger, N., Arthofer, W., Schlick-Steiner, B. C., & Steiner, F. M. (2019). Comparing ant behaviour indices for fine-scale analyses. *Scientific Reports*, *9*(1), 6856.

LeBoeuf, A. C., Waridel, P., Brent, C. S., Gonçalves, A. N., Menin, L., Ortiz, D., Riba-Grognuz, O., Koto, A., Soares, Z. G., Privman, E., Miska, E. A., Benton, R., & Keller, L. (2016). Oral transfer of chemical cues, growth proteins and hormones in social insects. *eLife*, *5*, e20375.

Lenoir, A., d'Ettorre, P., Errard, C., & Hefetz, A. (2001). Chemical ecology and social parasitism in ants. *Annual review of entomology*, *46*(1), 573-599.hei

Leonhardt, S. D., Menzel, F., Nehring, V., & Schmitt, T. (2016). Ecology and evolution of communication in social insects. *Cell*, *164*(6), 1277-1287.

MacGown, J. A., & Hill, J. G. (August, 2007). *Ants collected at Fall Line Sandhills Natural Area, Taylor County, Georgia.* Mississippi Entomology Museum. https://mississippientomologicalmuseum.org.msstate.edu/Researchtaxapages/Formicidaepages/faunal.lists/Fall.Line.SandHills.htm

MacGown, J. A., Hill, J. G., & Deyrup, M. (2009). Ants (Hymenoptera: Formicidae) of the Little Ohoopee River Dunes, Emanuel County, Georgia. *Journal of entomological science*, *44*(3), 193.

Mc Cabe, S., Farina, W. M., & Josens, R. B. (2006). Antennation of nectar-receivers encodes colony needs and food-source profitability in the ant *Camponotus mus*. *Insectes Sociaux*, *53*, 356-361.

Miller, J. H., & Robinson, K. S. (1994, November). A regional perspective of the physiographic provinces of the southeastern United States. In *Edwards, MB comp. Proceedings of the eighth biennial southern silvicultural research conference* (pp. 1-3).

Nickerson, J. C., Whitcomb, W. H., Bhatkar, A. P., & Naves, M. A. (1975). Predation on founding queens of *Solenopsis invicta* by workers of *Conomyrma insana*. *Florida Entomologist*, 75-82.

Nickerson, J. C. (1976). Ecology and behavior of *Conomyrma insana*. PhD dissertation, University of Florida.

Oberski, J. T. (2022). First phylogenomic assessment of the amphitropical New World ant genus *Dorymyrmex* (Hymenoptera: Formicidae), a longstanding taxonomic puzzle. *Insect Systematics and Diversity*, *6*(1), 8.

Robinson, E. J., Richardson, T. O., Sendova-Franks, A. B., Feinerman, O., & Franks, N. R. (2009). Radio tagging reveals the roles of corpulence, experience and social information in ant decision making. *Behavioral ecology and sociobiology*, *63*, 627-636.

Snelling, R. S. (1995). Systematics of Nearctic ants of the genus *Dorymyrmex*. *Contrib. Sci*, *454*, 1-14.

Sprenger, P. P., & Menzel, F. (2020). Cuticular hydrocarbons in ants (Hymenoptera: Formicidae) and other insects: how and why they differ among individuals, colonies, and species. *Myrmecological news*, *30*.

Stukalyuk, S., Akhmedov, A., Stelia, V., Shymanskyi, A., & Netsvetov, M. (2022). Nuptial flight in ants (Hymenoptera: Formicidae). *Serangga*, *27*(1), 152-179.

Sturgis, S. J., & Gordon, D. M. (2012). Nestmate recognition in ants (Hymenoptera: Formicidae): a review. *Myrmecological news*, *16*(400), 101-110.

Torres, C. W., Brandt, M., & Tsutsui, N. D. (2007). The role of cuticular hydrocarbons as chemical cues for nestmate recognition in the invasive Argentine ant (Linepithema humile). *Insectes sociaux*, *54*, 363-373.

Trager, J. C. (1988). A revision of *Conomyrma* (Hymenoptera: Formicidae) from the southeastern United States, especially Florida, with keys to the species. *Florida Entomologist*, 11-29.

Tsutsui, N. D., Suarez, A. V., Holway, D. A., & Case, T. J. (2000). Reduced genetic variation and the success of an invasive species. *Proceedings of the National Academy of Sciences*, *97*(11), 5948-5953.

van Wilgenburg, E., Mariotta IV, M., & Tsutsui, N. D. (2022). The effect of diet on colony recognition and cuticular hydrocarbon profiles of the invasive Argentine ant, *Linepithema humile. Insects*, *13*(4), 335.

Vander Meer, R. K., & Morel, L. (2019). Nestmate recognition in ants. In *Pheromone communication in social insects* (pp. 79-103). CRC Press.

Whitcomb, W. H., Denmark, H. A., Bhatkar, A. P., & Greene, G. L. (1972). Preliminary studies on the ants of Florida soybean fields. *Florida Entomologist*, 129-142.

Wilson, E. O. (1971). *The insect societies* (pp. x+-548).