Uncovering mechanisms of host recognition, host finding and host specificity

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ABSTRACT

Insect diversification is thought to have been catalyzed by widespread specialization on novel hosts—a process underlying exceptional radiations of phytophagous beetles, lepidopterans, parasitoid wasps, and inordinate lineages of symbionts, predators, and other trophic specialists. The fidelity of such interspecies partnerships is often posited to arise from sensory tuning to host-derived cues, a model supported by studies of neural function in host-specific model species. Abundant literature on parasites also suggest that extrinsic factors, namely dispersal mechanisms and aggressiveness/acceptance from novel hosts, externally enforces host specificity. Here, I first review what is known about host specificity, why it arises and how it is controlled, and then explore how these factors influence the biology of myrmecophiles, the intimate symbiotic associates of ants. I then test the mechanisms of host specificity by investigating the chemosensory basis of symbiotic interactions between a myrmecophile rove beetle and its single, natural host ant species. I show that host cues trigger analogous behaviors in both the ant and myrmecophile. Cuticular hydrocarbons—the ant's nestmate recognition pheromones—elicit partner recognition in the myrmecophile and execution of ant grooming behavior that achieves chemical mimicry. The myrmecophile also follows host trail pheromones, permitting inter-colony dispersal. Remarkably, however, the myrmecophile performs these same adaptive behaviors with non-host ants separated by up to ~100-million years and shows minimal preference for its natural host over non-host ant species. Experimentally validated agent-based modelling supports a scenario in which specificity is enforced by physiological constraints on dispersal, and negative fitness interactions with alternative hosts, rather than via sensory tuning. Infrequent realization of

latent compatibilities of specialists with alternative hosts may facilitate host switching, and the persistence and diversification of seemingly specialized clades over deep time.

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JMW participated in idea generation, literature search, figure making, and writing of this review article.

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JMW helped with project conception, collected all the data, performed all analysis, generated figures, and drafted the manuscript.

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NOMENCLATURE

Myrmecophile. Directly translated, myrmecophile means "ant lover." The term refers to a wide array of species that associate with ants and rely on them for some facet of their biology, whether facultatively or obligately.

Ethology. The study of animal behavior using lab and field approaches. It has an eye for naturalistic behaviors, understanding the functional reason for behavior, the causal stimuli eliciting the behavior, the developmental trajectory influencing the behavior, and the evolutionary origins of the behavior (Tinbergen's four questions).

Neuroethology. Similar to ethology, but with an emphasis on comparative and evolutionary reasoning to understand the relationship between the nervous system and the diverse behaviors of animals.

Ecological Fitting. The idea that interactions between species in an environment may arise not from coevolution, but broadly distributed organisms happening to find themselves in new environments, and surviving only if they happen to fit into the novel environment.

Important species

D. coriaria. *Dalotia coriaria*, the greenhouse rove beetle, is a free-living outgroup to the myrmecophile beetle lineages studied here. It provides a control organism to test whether specialized behaviors of the ant associated beetles are derived rather than ancestral.

L. apiculatum. Sister species to *L. occidentale*, and host of *S. schmitti* and *S. dispar.* Never found to host *S. lativentris*.

L. luctuosum. Sister species to *L. occidentale*, and host of *S. schmitti. L.* luctuosum is sympatric with *L. occidentale* but never found to host *S. lativentris*.

L. occidentale. The velvety tree ant, *Liometopum occidentale*, is a dominant species in the Angeles National Forest, and hosts a number of myrmecophiles as guests. This is the host of *S. lativentris* and is the primary ant of study for this thesis.

S. lativentris. *Sceptobius lativentris* is the primary study organism in this thesis. It is an obligate myrmecophile intimately integrated into the nests of the velvety tree ant *L. occidentale*. It is host specific, and has never been collected with any other ant species.

S. schmitti. Sister species to *S. lativentris* found in the colonies of *L. luctuosum* and *L. apiculatum*.

Chapter 1

HOST SPECIFICITY OF ORGANISMAL ASSOCIATIONS, MYRMECOPHILE BIOLOGY

"My mind leads me to speak now of forms changed into new bodies … from the worlds beginning to the present day." – Ovid's *Metamorphoses*

Computers, flies, or beetles?

When I interviewed for the biology program at Caltech in January of 2017, Joe Parker had yet to start as a professor. A highlight of my visit was seeing the lab space of Michael Dickinson; as I recall, I was the only applicant from the biology program interviewing with him that year and he generously spent way more time with me than our designated interview slot, showing me the wind tunnel, laser cutter, and behavioral rigs that would later inspire my project. I had worked on beetles as an undergraduate (with the delightful Jon Harrison, looking at the constraints on insect body size imposed by their respiratory system), but thought I would surely move away from these charismatic organisms in grad school. Micheal mentioned a new professor who worked on what sounded like almost too-cool-to-be-real beetles that had evolved to look like ants and live inside their colonies. Still, I had spent much of undergrad building my computational skills and had in mind that I should probably get very serious in my PhD and move on from fun organisms like beetles, becoming a stoic computational biologist working on more human related topics. I spent a few months working in Lior Pachter's lab doing computation but found myself longing for a more hands-on project (though getting bit by thousands of ants was hardly what I had in mind then). The siren calls of a charismatic organism, of basic biology, of the study of how animals evolve to interact with each other beckoned. When it came time to take a turn in Michael's lab as a rotation student, he offered me a couple of options: I could work with a great postdoc on fruit fly navigation or strike up a collaboration about beetle behavior with this basement-dwelling new professor. Honestly, 'beetle' was all I needed to hear to go with Joe, despite the basement situation (his lab was still under construction). There was also the promise that working on behavior would let me use machine learning and vision approaches, which gave me a sense

of comfort and consolation that I wasn't giving up on my computational aspirations. I had the privilege of reading up on a mind-blowing group of beetles (Staphylinids) that repeatedly evolved to live with ants, changing their behavior, chemistry, and morphology to suit colony life. The photographs in Joe's 2016 review on myrmecophiles and 2017 paper on convergent evolution of army ant symbionts demonstrated just how real these organisms were, and how they represented the finest system to explore evolution of social interactions any biologist could dream of.

The problem: what mediates specialization/specificity in organismal interactions?

Species richness and ecosystem diversity arises partly due to the complex web of associations between different organisms, many of which specialize to interact with few other members of their environment. In particular, the diversification of symbiotic lineages (whether parasitic, commensal, or mutualist) is thought to have been catalyzed by widespread specialization on novel hosts—a process demonstrated in insects by the exceptional radiations of phytophagous beetles, lepidopterans (*1*, *2*), parasitoid wasps (*3*, *4*), and inordinate lineages of symbionts, predators and other trophic specialists (*5*). The fidelity of such interspecies partnerships is often posited to arise from sensory tuning to host-derived cues (*6–9*), a model supported by studies of neural function in host-specific model species (*10–14*). However, other mechanisms, like the host response to symbionts, reduced survival with wrong hosts, or dispersal strategies also may play into the specificity of interspecies partnerships (*15–17*). In section 1 of this chapter, I review some pertinent examples organismal associations from across the tree of life, and emphasize what we know about host specificity, why it arises, and how it is maintained through ecological and evolutionary time. I will then review some evidence of the widespread host switching exhibited by even the

most intimately associated organisms despite these mechanisms, and the implications of host switching for the long-term success of lineages. In section 2, after outlining these general principles of host association, I will review literature on myrmecophiles, a highly successful and diverse group of organisms with rich and intricate social interaction with ants. Myrmecophiles beautifully illustrate general principles underlying symbioses, and my own thesis work greatly contributes to our understanding of the dynamics of host association in these organisms.

Section 1: An overview of host specificity, why and to what degree it exists, what mechanisms maintain host specificity, and why it breaks

Why be host specific?

Why do animals specialize or become host specific at all? On first look, it might seem like being a generalist would afford numerous advantages: there are plentiful resources to exploit, there is little concern of co-extinction with your host organism, and there are more niches to inhabit. However, there are numerous lineages, especially amongst parasites, exhibiting extreme host specificity. One compelling reason for host specificity comes from Bernays' ground-breaking study of phytophagous insects, beautifully reviewed in (*6*). Bernays argues that generalists often make sub-optimal decisions for critical tasks like locating suitable resources for feeding and oviposition as compared to closely related specialist lineages (*6*). Evidence comes from aphids, lepidopterans, and grasshoppers, among others. Specialists tend to waste less time searching for/assessing resource options, spending more uninterrupted time feeding or finding dispersal and oviposition sites more quickly (*6*). In a complex world

full of numerous resource options, having a simpler decision task pays off. There is strong evidence that many specialists also specialize on easily identifiable and unambiguous sign-stimuli to release their appropriate behavior and promote speed and efficiency (*6–9*). There are many reasons that the increases in efficiency and speed of host localization may be important, e.g. increased survival of eggs laid on optimal resources, less waisted metabolic resources in food seeking, higher quality nutrition from more easily finding high quality substrate. Critically, Bernays argues that the extreme threat of natural enemies in the environment also selects for as-fast-as-possible sensory processing and decision making in host seeking. She points to some elegant experiments showing that the predation and parasitism rates for Lepidopteran larvae, for example, are astoundingly high in the field, and that much of this risk occurs while the animals are active, foraging/eating, as compared to at rest (*18*). The evolutionary history of the most successful lineages attests to the realized advantages of specialization: lepidopterans (*1*, *2*), parasitoid wasps (*3*, *4*), symbionts, predators and other trophic specialists (*5*, *19*) all exhibit massive radiations in deep time, illustrating their importance.

How specific are host associations?

So, there are some clear advantages to being a specialist over generalists, but just how specific are associations between organisms? I will now look at some examples of the degree of host specificity of associations, and some of the factors that govern this extreme specificity. In (*20*), Poulin and Keeney review some important considerations and literature about host specificity. The advent of widespread use of molecular tools for phylogenetics has revealed that morphological or other phylogenetic methods historically underestimated the degree of host-specificity of organisms (*20*). They point to several studies showing

extreme specificity of the associations of parasitic nematodes with their bird hosts (*21*), cichlid fish with their gill parasites (*22*), Digenea flukes with trochid snails (more on flukes later) (*23*), parabasalid protists with their amphibian or mammalian or reptilian hosts (*24*), and avian mites with their hosts on the Galapagos (*25*). Illustrative of the pervasiveness of specialization was the discovery that what was previously thought to be a generalist parasitoid fly turned out to be a group of several related species with high host specificity (*26*). The apparent lack of specificity was illusory, due to misidentified cryptic species. Even with thousands of potential host caterpillars in their environment, these flies use one or a-few-closely-related hosts. These are a mere snapshot of the surfeit of examples of extreme specialization and specificity of associations in nature. It is important to note, too, that the degree of host specificity depends partially on the metric used to measure it. Poulin and others have argued that the consideration of host specificity should expand beyond simply enumerating a list of species a given symbiont associates with, but should also consider the phylogenetic range of the host species, as well as the hosts used by related symbionts (*20*, *27*, *28*). The narrower the phylogenetic range of hosts per species/within a species group, the higher the specificity. With this filter in mind, many lineages still exhibit extreme host specificity, where whole lineages specialize on very narrow ranges of closely related other species.

What controls host specificity, how is it maintained?

The benefits of specialization previously described are numerous, but what prevents organisms from ending up host switching (likely unbeknownst to themselves) to new associates in their environment? I will now describe the two-sided dance between associates that erects multiple layers of barriers host-switching. These consist of 1) neural/recognition

specialization, 2) dispersal/encounter probabilities with new hosts, 3) compatibility/acceptance with new hosts, and 4) access of associates to conspecifics for reproduction. There is further evidence that these barriers to host switching become rapidly stronger when associates are limited to few hosts, suggesting they are also continually self-reinforcing, and are far from mutually exclusive mechanisms.

Associate/symbiont neural specialization as mechanism of host specificity

One straight forward way to maintain a specific host is for an associate to evolve a neural/behavioral specialization to respond only to host cues. As mentioned before, many studies have found specialist phytophagous insects and parasitoids specialize on particular odor cues produced by their host (*6–9*). Such animals often respond only to the volatiles of their food source/hosts, or at least exhibit strong preferences. Some of the best evidence for the mechanisms underlying sensory specialization come from the literature on Drosophilids, where highly advanced molecular tools allow not only ethological but also neural circuit level dissection. One such example comes from the well-studied split between the generalist *D. melanogaster* and *D. sechellia*, a specialist on noni fruit (*14*). They find differential tuning of an odorant receptor in *D. sechellia* to the volatile cues generated by noni fruit, as well as differences in central circuit connectivity in *D. sechellia* in processing odors. Together, these changes in odorant receptor tuning and changes in central circuits likely underpin *D. sechellia* specialization on noni (*14*). Another pair of Drosophilids (*D. melanogaster* and *D. simulans*) also elucidate how neural specialization can maintain specificity in behaviors/association (*29*). Though this study explores maintenance of mating with the correct species, it provides insight into the mechanisms behind specificity of association. They find that both flies detect the pheromone that differentiates the species, a cuticular

hydrocarbon, but differences in how the signal projects to the neuron that controls mating, changing the valance from attractive to aversive. An example from hawkmoths in Arizona shows that some innate sensory tuning leads to feeding from moth adapted flowers, even in the presence of bat-adapted flowers of higher nutritional value (*30*). Though these moths do exhibit olfactory learning and feed on the bat-adapted flowers too, they maintain attraction to their evolved hosts even in the presence of superior alternate hosts (*30*). Together, these studies provide insight into one major mechanism of host specificity: multiple demonstrated types of neural specialization on signals of hosts gives rise to host-specific responses, which maintains the tightness of the association of one organism with another.

Dispersal/encounter probabilities with new hosts as mechanism of host specificity

How intimate, especially obligate, associates disperse to new hosts has significant implications for their biology, and likelihood of ever encountering a potential alternate host. If dispersal away from hosts scarcely happens at all, or is extremely hazardous, this leads to a very low probability of ever leaving hosts or encountering wrong hosts to switch to, thereby providing a strong mechanism maintaining preventing host switches. Most evidence showing increased host specificity with reduced dispersal ability is inferred from phylogenetic analyses/life history observations, with little direct demonstration of the mechanism. At one extreme in terms of associate transmission, a meta-analysis of the degree of co-phylogeny between hosts and symbionts revealed vertical transmission as a key predictor of stronger accord between host and symbiont phylogenies (*31*). This provides some evidence that the least-mobile of symbionts also have high specificity, namely a narrow phylogenetic range of hosts. Many symbionts follow dispersal cues, which may limit their likelihood of encountering the wrong host, as evidenced by clown fish, which follow odor cues to locate

reefs (*32*), and myrmecophiles that follow volatile odor plumes to find hosts (*33*). Another key illustration of dispersal/life history influencing host range comes from studies on pigeon lice. Wing lice have increased dispersal capabilities via phoresy on bird-parasitic flies as compared to body lice which do not engage in phoresy; the better-dispersing wing lice show less population genetic structure, and an increased range of hosts compared to the body lice (*34–36*). The trend is not universal, however, as another study revealed that one multi-host-using louse exhibits almost no phoresy, though it was also the species that showed the greatest ability to move large distances on the ground away from its host, a dispersal ability that may aid in host switching (*37*). The authors hypothesize that the more mobile louse species might be able to walk between bird nests on the ground to disperse, though this requires more experimental evidence (*37*). Others have found that the body-lice showing the greatest host ranges also parasitize terrestrial, as opposed to arboreal, birds, offering additional evidence that ground based dispersal may offer more encounters with potential new hosts, increasing the likelihood of host switching (*16*). Most lice complete their entire life cycle on hosts, and likely never leave the hosts (*38*). If they do leave hosts to disburse, lice only survive short periods of time away from the host, and efforts to rear them without hosts are largely futile (*38*). Some of the factors likely limiting such dispersal are desiccation, evidenced by the increased diversity of lice in more humid environments (*38*). A study in feather mites found multi-host usage, with limited divergence amongst populations on different species (*39*). They suggest that a higher-than-expected dispersal ability between different hosts limits the specificity of these mites (*39*). It is worth noting that a metanalysis, while still supporting dispersal mechanisms as critical for the population genetic structure, also revealed that limited dispersal is only one driver leading to reproductive isolation (*40*).

Though highly speculative, recent evidence suggests that higher proclivity for exploration correlates with diversification in cichlid fish (*41*), and perhaps a similar mechanism may be at play for symbionts, where increased exploration/dispersal allows colonization of new hosts; maintaining high dispersal might facilitate using multiple hosts. In any event, there evidence from many systems points to limited dispersal ability to new hosts as an important mechanism preventing host switches, and hence maintaining host specificity.

Compatibility/acceptance with new hosts as mechanism of host specificity

Even if an organism can disperse to new potential hosts, this new partner may or may not accept the associate, or their biology may be incompatible. Rejection by an otherwise compatible host is another mechanism which maintains host specificity. For phytophagous insects, incompatibility with chronic and induced toxins limit their ability to host switch/feed on new plants (*42–44*). In the case of two animals interacting, aggression against the associate can preclude a host switch. For example, parasitoids attempting to lay their eggs inside aphids face variable aggressive rejection from the aphids (*45*). Aphids kick, rotate away from, push the antennae, and secrete alarm/defensive compounds, which prevent the parasitoids from affectively parasitizing non-host species (*45*, *46*). Some hover flies exhibit extreme specificity, likely driven by rejection from any alternate hosts (*47*). These fly larvae live inside of ant nests and eating brood but must exit the nest to disperse, laying their eggs at the nest opening where the eggs are susceptible to attack from ants (*47*). When Elmes et al. transferred eggs to different nests of the correct species but 20 kilometers distant, ants nearly unilaterally attacked and killed them (*47*). This aggressive rejection by the ants is likely a critical mechanism enforcing the extreme specificity of these fly populations. One study in feather lice directly tested whether defense against parasites might limit host

switching. They found that when they inserted a spacer inside the beak of host birds that prevented effective preening, lice could successfully parasitize hosts but that intact preening successfully defended against lice, preventing host switching (*48*). Brood-parasitic cowbirds are locked in an evolutionary arms race with songbirds; non-host songbirds recognize and reject cowbird eggs, an effective defense that may limit host usage (*17*, *49*).

These specificity-enforcing mechanisms of recognition/defense/attack of would-be-parasites by potential hosts mirror how immune responses from potential hosts also give rise to incompatibilities between organisms. In avian fleas, the more generalist species tend to use bird hosts with weaker immune system responses, suggesting that, without specialization, the birds with strong immune systems exclude the generalists (*50*). The fleas exploiting the birds with the strongest immune responses were also the most host specific (*50*); perhaps specializations needed to thwart the immune response also make fleas no longer compatible with other hosts, though this is just one hypothesis. Work on *Daphnia* and its parasites has demonstrated that few genomic loci in hosts control whether bacterial parasites can infect hosts; even within the species, alternate genotypes of *Daphnia* effectively defend against the parasite, rejecting it (*51*). More recent work has identified the genetic basis for these host defenses that prevent infection (*52*). Rejection by hosts via defense undoubtedly widely prevents parasite host switching on both ecological and evolutionary timescales and is a potent mechanism underpinning host specificity.

Access of associates to conspecifics for reproduction as mechanism of host specificity

Even if associates recognize a host, can disperse to it, and the host accepts it, it still needs to find a mate to complete its lifecycle, which introduces another barrier to host switching. Direct evidence for this hypothesis is sparse, but several systems provide illustrative

examples. The bird cherry-oat aphid offers a striking case. This aphid overwinters on bird cherry trees, and observations of dispersal suggests that less than 1% of animals successfully complete this dispersal, which raises the question of why the aphid doesn't use multiple hosts to increase this survival rate (*15*). Ward argues that the host trees provide locations for reproductives to meet to mate, and otherwise the animals would not find each other in their environment, though concrete experimental evidence is lacking (*15*). Though not directly probing mate rendezvous, others have found aphids unable to complete their lifecycle, or at least showing reduced fitness, on alternate hosts (*53*). More evidence for the importance of meeting points for mating comes from Monogea, helminths parasitizing fish (*54*). Some of these worms exhibit extreme sparseness in their environments, with just a few parasites found on a low percentage of their fish hosts (*54*). Without niche specialization, the likelihood that such a sparsely distributed parasite would locate mates is extremely low. Maggot flies exclusively mate on or near to the fruit of their host trees (*55*), and experimental evidence in the field demonstrates that, even within populations of the same species that have different host preferences, this association of mating with host leads to a strong gene-flow barrier (*56*). Additionally, females deposit chemicals on host fruit which increase the residence time of males in the area for mating, further coupling mating with host location (*57*). Together, these show a close association of host with mate finding; mating opportunities apart from the host would be severely curbed, limiting the possibility of host switching. Bat flies offer a case of close specificity, even in the face of ample other potential hosts (*58*, *59*). These parasites live on the skin of bats, often in roosts where several species of bat frequently come into contact, and yet usually only use a single host species (*58*). Dick and Patterson suggest that mate finding happens on/near host bats, and that this, along with immune defenses from alternate

hosts, maintain the specificity, though again concrete evidence is scarce (*59*, *60*). After eclosing from their pupal case (which is placed on the ground, not on hosts), flies first found host bats for a blood meal before mating, closely coupling mating with hosts (*60*). It is worth noting that the mating-rendezvous mechanism generally presupposes that other mechanisms, like neural specialization for food localization in the maggot flies, leads to highly patchy distributions of mates near to hosts, making hosts necessary as meetup points. I would argue, though, that this does not preclude mating-rendezvous as its own mechanism maintaining host specificity, as it instantiates an extreme penalty to any associates which forgo their stringent partner association; their fitness drops to zero when they fail to locate mating opportunities on what otherwise might be a compatible alternate host.

How isolation into few hosts leads to reinforcement of specificity

So far, I have outlined four mechanisms that help maintain host specificity, namely 1) neural/recognition specialization to host, 2) low dispersal/encounter probabilities with alternate hosts, 3) lack of compatibility/acceptance with new hosts, and 4) lack of access to conspecifics for reproduction on alternate hosts. Each of these mechanism act in concert to limit the ability of a given associate to switch host. The isolation of populations of associates via these mechanisms likely gives rise to rapid specialization that further enforces their specificity. The recent (~29k years) establishment of a population of *D. yakuba* on an island environment lead to rapid adaption/specialization on the noni fruit, including evolution of detoxification genes, olfactory attraction, and aversion to mating with non-island population (*61*). Another striking example of rapid specialization comes from ento-parasitic nematodes. When artificially restricted to a single host for a mere three years in lab culture, one species of nematode lost the ability to parasitize one of its four drosophilid hosts (*62*). Others have

found evidence of rapid evolution in a moth species after a host shift (*63*). In a mite species, others have found specialization allowing detoxification of a lab-enforced host in a mere 25 generations, additionally indicating the speed at which specialization can be achieved (*64*). Together, these examples illustrate that on ecological and evolutionary timescales, the previously described mechanism maintaining host-specificity can easily lead to ratcheting specialization and increased host specificity as populations are ever more isolated, leading to the strengthening of existing and development of new barriers to host switching.

Ecological fitting, rampant host switching, the paradox of the promiscuity of specialists, avoidance of co-extinction

Though I have so far outlined the benefits of specialization and the mechanisms that maintain host specificity, nonetheless these maintenance mechanisms often break, leading to host switching on ecological and evolutionary timescales. I will outline one leading model for the rampant host switching seen in nature and argue that host switching is an important for the evolutionary stability of organismal associates.

Ecological fitting: are interacting species really specialists?

Janzen proposed a provocative principle called 'ecological fitting' to explain how no co-evolution was necessary to form even highly complex associations between organisms (*65*). Janzen writes that "almost all the ecology I see around me [in Costa Rica] could quite easily come to be with virtually no evolution having occurred," pointing out that many of the species interacting in intricate ways in a given environment have ranges that extend far beyond a given field site, such as saturniid moths which extend from Canada to Costa Rica, with no evidence that they evolved their traits in their Costa Rican range. Such is true for numerous species; they associate with enumerable other organisms using the same traits

across huge ranges of varied environments. Janzen suggests that many of these associations have nothing to do with co-evolution, but rather, organisms often show up in a new environment due to range expansions with a suit of traits evolved in a different context, and the ones that happen to fit in that environment survive and reproduce with little evolutionary change. He calls this process 'ecological fitting,' whereby complex assemblages of species form in an environment when the existing traits of these organisms happen to fit together, allowing them to survive and found populations in a new place. This framework of ecological fitting provides a powerful explanation for host switching in associated organisms: if the mechanisms underpinning host association (neural/recognition, encounter/dispersal, compatibility/bypassing host defense, and ability to find mates) happen to work with a novel host, the barriers to host switching fall. Modelling work suggests that ecological fitting as a model can readily explain many host switches, even to sub-optimal alternate hosts (*66*). Natural experiments also demonstrate remarkable cases of host switching to compatible alternate hosts. Liver flukes have a multi-host life cycle, using both snail and ungulates as hosts at points in their development. They have realized a range expansion from North America to Europe via ecological fitting with entirely different species of both snail and ungulate (*67–69*). Despite an extremely complex life cycle with multiple hosts, the flukes double-host-swapped, a powerful example of how fitting can give rise to highly specialized associations with little or no co-evolution. Invasive species also provide examples of ecological fitting giving rise to specialized-looking associations. Invasive ant species tend and defend another species of invasive mealybug, a mutualism arising in a novel environment not native to either associate (*70*). A similar observation has been made in another invasive ant with aphid, where a mutualism has arisen recently between two invasive

species that do not share co-evolutionary history (*71*). The widespread issue of invasive phytophagous insects further highlights the prevalence of host switching, impacting native populations of plants and other insects (*72*, *73*). Over the last century, cowbirds have leveraged new host songbird nests as well, allowing a range expansion along Caribbean islands (*74*). These, as well as numerous other examples, demonstrate how fitting with novel hosts supports host switching on ecological and evolutionary timescales despite the mechanisms which usually maintain host specificity.

Why might such rampant host switching matter for organisms, particularly those which obligately associate with another species? An organism exhibiting extreme host specificity is also at great hazard of co-extinction with its host, jeopardizing its long-term evolutionary success. Colwell et al. review the dynamics of host usage, switching and co-extinction, and recount the story of the passenger pigeon, which is illustrative of the hazard of a specialist (*75*). For decades, it was thought that the specialist lice of the passenger pigeon went extinct with their host (*75*). However, when molecular phylogenies finally revealed the actual closest relatives of the passenger pigeon, researchers discovered that the louse was still thriving on this alternate host (*75*). These lice very directly dodged co-extinction via usage of multiple hosts/host switching. They also point to an example of a rare herbivorous insect which was once thought to only feed on another rare and endangered host, putting it at risk of imminent co-extinction. Later work, however, revealed that the insect also feeds on a common congeneric plant, suggesting multi-host usage/host switching may protect this species from co-extinction hazard (*76*). These provide a couple of concrete examples of the hazards faced by highly specialized lineages which host switching may mitigate. Some of the most successful and old lineages of specialized symbionts show extreme host switching in their

lineage, giving further support to the idea that host switching may be an important component of long term success for associations between organisms (*77–79*). Realizing a host switch on ecological timescales likely critically contributes to lineage success on evolutionary timescales.

Section 2: A review of myrmecophiles, a model of social symbiosis, specialization, and host specificity

Ants place a tremendous selective pressure on the organisms around them and create exploitable, resource rich niches for species which can accommodate or attenuate ant aggression (*80*). Rove beetles sport a suite of pre-adaptations giving rise to repeatable evolutionary trajectories to ant association (*81*). They brandish a defensive tergal gland which bolsters survival in proximity to ants, opening up the ant periphery as a new niche and providing a re-programmable chemical manufactory for ant-manipulating compounds. They wield cells to coat their body surface in pheromonal cues readily modified to mimic ants. They exhibit a rich sensory-behavioral repertoire readily evolvable to facilitate ant association. Here, I will review patterns in ant exploitation used by rove beetle symbionts and suggest that repeatable evolutionary trajectories give rise to nearly identical convergent strategies for ant association. I discuss how reprogramming exocrine glands, behavioral repertoires, morphology, and sensory apparatus together provide repeatable and evolutionarily stable strategies for myrmecophily. Using the framework outlined in section one of this chapter, I also highlight what we know about the dynamics governing host usage, specificity, and switching, critical factors to understand myrmecophile biology.

Neural specializations for colony association: eavesdropping for dispersal and recognition cues, a mechanism of host specificity

Well-defended, gland bearing rove beetles have repeatedly evolved to not merely sporadically associate with ants but sensory specialization to eavesdrop on long-range colony localization cues shared by many ant species. These neural specializations of attraction to host ant cues is a first mechanism governing host specificity of these myrmecophiles. Several independent lineages of rove beetles convergently evolved to follow the chemical foraging trails of ants, ranging in the specificity of their response from strict recognition of only host trail and avoidance of others to following the trails of several species or even subfamilies (*82*, *83*). Other species reportedly follow plumes carrying ant colony odors (often alarm pheromones) (*33*, *84*). Diverse ant species often produce similar or identical components in their trail or alarm pheromones, and the use of common ant cues to find colonies may allow relatively unspecialized beetles to exploit many different ant species across landscapes. Lineages with generally applicable localization strategies enjoy success through ecological fitting, expanding to new niches where their existing adaptations fit with novel interaction partners. Though the precise neural-mechanisms for ant-localization are not known, shifting tuning of odorant receptors in other insects, sometimes with downstream neural changes, lead to attraction to new odor cues (*14*, *85*). Modifications in odorant receptor tuning and downstream wiring appear to be easily evolvable and widespread mechanisms for localization and specialization. A similar mechanism likely readily gives rise to colony localization in rove beetles. Easily evolvable sensory adaptations to common long range ant cues provide rove beetles with a widely applicable toolkit to exploit ant niches.

Once arthropods associate near ants, they likely have increased selective pressure to improve survival in frequent ant encounters requiring partner recognition systems and chemical-behavioral strategies to thwart ant aggression. Local cues become salient to release appropriate beetle behaviors for symbiosis. Many lineages of myrmecophiles have convergently evolved to groom the body surface of ants to steal the gestalt colony odor and thereby blend in (*81*, *86*, *87*). Initiating such behavior requires recognition of a suitable grooming partner and attraction to that partner instead of the aversive response that most insect exhibit when they encounter ants. To maintain robust partner recognition, though, the recognition system used by the beetle must also accommodate variability in host cue. Even within the lifetime of a single ant nest, chemical profiles shift with diet and environment (*88*). Over time, shifts occur within regional populations (*89*). Beetle recognition must be sufficiently versatile to accommodate the changes in host cues over different timescales. This may select for consistent cues that ants chronically produce, e.g. common cuticular hydrocarbons. Recognition based on CHCs, though not described in rove beetles, appears widespread in insects for variously specific partner recognition, e.g. parasitic wasps, mate recognition, etc. (*90–92*). The widespread use of CHCs as recognition cues suggests it is an easily evolvable sensory-behavioral modification for symbiosis. The widespread convergent evolution of similar ant-grooming behaviors in rove beetles suggests it is also easily evolvable, perhaps by modifying existing self-grooming behaviors. A similar recognition mechanism may also underlie other convergent symbiotic behaviors. For example, rather than flee from ants, some rove beetles selectively deploy compounds which calm ants. As with recognition of grooming partners, such a behavioral change requires recognition of host and swapping of escape/defense behaviors with specialized symbiotic strategies. Together,

specialization of myrmecophiles to ant derived cues, both dispersal and contact-recognition, offer one layer of mechanism which leads to specificity of partner associations, but others are also at play.

Other mechanisms maintaining host specificity in myrmecophiles

If myrmecophiles use general cues to locate their hosts and are beetle-recognition compatible with numerous ant species, neural specialization alone cannot explain the extreme host specificity often seen between myrmecophiles and ants. How does myrmecophile host specificity on ecological timescales arise? We suggest that both aggressiveness/defense by would-be new ant hosts excludes beetles, coupled with the beetles limited dispersal capabilities, and symbiont degeneration due to parasitic life history together produce host specificity. Blue butterflies provide a striking example of host-aggression-enforced specificity. Larvae of *Maculinea* drop from foliage and *Myrmica* ants pick them up and bring them back to the nest (*93*). Many *Myrmica* species will perform this adoption behavior, but there are drastic differences in survival in different ant species' nests (*93*, *94*). Recent evidence suggests that caterpillar cuticular hydrocarbon matching to their would-be host is one key variable influencing survival rate, illustrating how specific host usage can emerge from aggressive exclusion by host rather than an active process where the symbiont interacts with only a single host (*95*). In rove beetles, the dual host using *Lomechusa publicollis* is attracted to odors associated with many species of *Myrmica*, but the *Myrmica* themself display variable responses to the beetles attempting to enter ant nests, from no aggression to attacking the beetle (*96*). Variable survival in response to aggression would bias beetle host usage towards less aggressive interaction partners. In addition to aggressive

responses from would-be partners limiting host usage, myrmecophile' limited dispersal ability limits exposure to other ants, reducing the likelihood of host switching. In the trail following context, for example, beetles often display very high stringency in their trail following, deviating far less from ant trails than the ants themselves (*82*, *83*). If symbionts use ant trails for dispersal (some are wingless), they may never deviate far enough from the host ant niches to encounter another ant species to engage with. On top of that, the most host specific myrmecophiles rarely remain away from ants at all, meaning that if they encountered a new species of ant, they will be with an entourage of host ants at the same time. This may lead to extreme levels of aggression as the ants attack one another, leaving the beetle caught in the crossfire and killed.

Additionally, the combination of degeneration due to relaxed selection in symbionts as well as a convergent trend that myrmecophiles rapidly die when removed from host colonies suggest that myrmecophiles get locked into association with ants, making a reversion to free living life untenable and penalizing even short dispersal-type-excursions away from hosts. Parasitic ants lose the complexity of their olfactory receptor repertoire when they become obligate associates, a pattern that may also hold in other symbionts (*97*). A reduced olfactory system makes the performance of usual insect behaviors, from foraging to dispersal and mating, more difficult outside the context of the ant symbiosis. Some rove beetle symbionts have wholly lost their eyes or wings, presumably due to relaxed selection similar to the convergent eye loss in cave dwelling organisms (*81*, *98*). Akre and other have also observed that myrmecophiles often die very quickly when removed from ant nests (*86*). These insects often appear very stressed and frantically active when away from their host, so they may waste substantial metabolic resources in their panic to return to the host (*86*). Social isolation

stress has been observed in other insects (*99*, *100*); myrmecophiles may have a heightened social isolation stress response as compared to other insects. Social immune factors are also often robust in ant nests, so beetles may be vulnerable to infections from endosymbionts or external pathogens away from the nest (*101*, *102*). Myrmecophiles often have a reduced set of waterproofing cuticular hydrocarbons to facilitate symbiosis, but this also may leave them vulnerable to desiccating when away from their host (*103*). The actual mechanism behind myrmecophile death when removed from host nests is unknown and would provide another key insight into the symbiosis. Whatever the mechanism, the spatial tightness of the symbiosis/limited dispersal ability, loss of sensory apparatus to navigate away from ants, and death-upon-departure together may limit closely-ant-associated beetles from leaving host ant nests long enough to encounter other ant species at rates high enough to survive and host switch.

Host usage and host switching

Despite these mechanisms promoting host specificity, myrmecophiles follow the same trend to host switch as other symbionts/parasites, a trend that we suggest stems from the use of common ant pheromones for partner recognition and which allows expansion to new niches and avoidance of co-extinction with hosts. Myrmecophile host switching appears from ecological to evolutionary timescales, and the most species rich lineages of myrmecophiles tend to show the most rampant and extreme host switching, suggesting the ability to host switch is an advantageous trait increasing lineage success over deep time. Clades with extreme host specificity often show low species richness and may sit in an evolutionarily precarious position susceptible to co-extinction with their hosts. Some species of blue butterfly myrmecophiles illustrate the tenuous situation of extreme specialization. In

the UK, changes in grazing led to taller grasses, reducing soil temperature, and causing a shift in the predominant *Myrmica* species. The butterfly larvae could not accommodate the new *Myrmica*, leading to a population crash (*104*). This illustrates how extreme specialists can falter from even small perturbations in host range. On a larger geographic scale, though, even this delicate, locally host-specific blue butterfly uses multiple *Myrmica* (*105*). This suggests host switching can occur within geographically and genetically clustered populations that specialize in different regions on different hosts (*105*). Aleocharine rove beetles also have numerous species-poor lineages of specialist myrmecophiles which use few or single species of host ants (*81*, *106*). These lineages often display signs of co-evolution with their hosts. For example, *Ecitophya* and *Ecitomorpha* co-vary in color with workers of similar size in their host colonies (*77*, *86*, *107*). Even here, species of these myrmecophiles

Figure 1.2. A *Camponotus* licks the trichomes of a *Xenodusa*, triggering adoption behavior instead of aggression against the beetle.

have switched host to other species of *Eciton*, with CO1 sequencing suggesting that ants first diverged before the beetle branched and switched to a new species of host. Over long timescales, Lycenids in the genus *Acrodipsas* provide an example of a radical ancestral host switch between ants of subfamily Dolichoderinae to Myrmicinae (*79*). *Lomechusa* and *Xenodusa*, related rove beetle myrmecophiles (Fig 1.2), illustrate a striking convergent host switch. Both species live with *Formica* ants in the summer but switch hosts during the winter, *Lomechusa* to an ant of a different subfamily, and *Xenodusa* a different tribe (*33*, *96*, *108*). This suggests that these two Lomechusini have association strategies widely applicable to shared ant biology since they swap to distantly related ant nests within a single year of their lifecyle. Pselaphine rove beetles provide another striking example of rampant host switching. The group appeared soon after ants began their ancient rise to dominance, and use at least 5 subfamilies of ants as hosts (*77*). Host switching appears as a pattern amongst many widely divergent myrmecophiles, suggesting its importance for the success of symbionts over deep time. Though extreme host specialization does occur, most successful, species-rich clades of myrmecophiles instead show evidence of widespread host switching on local to evolutionary timescales. An inability to accommodate a new host may lead to extinction of specialist clades, leading to selection/a survivorship bias for myrmecophile groups with host switching potential. Convergence of host switching likely stems from the similarity in ant cues across diverse lineages. Even as host switching appears common, many of the myrmecophiles mentioned do show host specificity on ecological or local scales.

S. lativentris: a host specific symbiont embodying multifaceted myrmecophile biology

S. lativentris, the unassuming guest beetle of the dolichoderine ant *L. occidentale*, embodies the many fascinating aspects of myrmecophile biology I have previously

described. It has lost its defensive gland, and barrels undefended into crowds of ants. It is obligate, living full time inside of colonies, and dies rapidly when removed from host nests. It is highly host specific, and has never been found with any species of ant besides its single host (*109*). Ethological work has shown it spends an inordinate amount of time grooming host ants, presumably to acquire the gestalt colony odor and hence blend in as a nestmate (*110*). Personal observations in the field indicate that the beetle adroitly follows ant trails, even in the absence of ants on the trail. Not only does *S. lativentris* exhibit a canonical suite of myrmecophile characteristics, but it is also abundant and easy to collect in ant nests in the Angeles National Forest. For my PhD, I set out to understand the biology of this little beetle. How does it recognize host ants for its behavior, and are these recognition cues host specific? If not neural specialization what else might governs the host specificity of *S. lativentris*? What can *S. lativentris* tell us about host recognition, usage, specificity, and evolution? What follows is the story of this little beetle, and its host specificity.

Chapter 2

DEVELOPING *S. LATIVENTRIS* AS A MODEL TO STUDY INTERSPECIES ASSOCIATIONS

« Le choix heureux d'un animal … suffisent souvent pour résoudre les questions générales les plus élevées. » - Claude Bernard

"For such a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied" – August Krogh

Camera, arena, animal – my foray into neuroethology

While still working from my desk in Michael Dickinson's lab, I started building some arenas to monitor the interaction between ants and a free-living rove beetle called *D. coriaria*. Spoiler: the free-living beetle doesn't much like being stuck in a gladiatorial-style showdown with a menacing-and-razor-mandible-bearing ant. We called the initial arena the Coleosseum, though the ant never seemed to look to us researchers for a thumbs up or thumbs down to decide life or death of the beetle. Besides watching these gruesome interactions, the bigger goal was to use the arena, piloted with the plentiful and lab-reared *D. coriaria*, to reconstitute the symbiotic behaviors exhibited by a very special symbiotic beetle. *S. lativentris* is one of those mind-blowing myrmecophiles that lives full time inside of ant nests. Whereas the free-living beetles meet dreadful aggression from ants, the symbiont wends its way between, around, and even on top of them unharried. More than that, it recognizes its host ant, climbs atop it, latches onto an antenna, and uses its highly bristled tarsi to groom the ant (a fellow grad student, Tom Naragon, has since definitively shown the beetle steals ant pheromones to adopt the colony odor). Inter-species social interactions like this are of immense interest to the evolutionary biologist: how do you take a free-living animal and reprogram its existing behavioral-sensory systems to facilitate a symbiosis? How does their biology give rise to the extreme host specificity we see from them in nature? We thought that the *S. lativentris*-*L. occidentale* system might just provide the perfect tool to investigate such questions, but first we needed to test whether we could reconstitute behaviors in lab. I learned quickly that a fairly straightforward approach, with proper implementation, would do just the trick: camera, arena, animals. I remember the first time I saw the myrmecophile that I would spend the next six or so years studying: it

was running around with ants in a Rubbermaid® bin. It turned out that the little beetle was super happy to put on a show of its behaviors. Pretty much as soon as I placed a beetle in the Coleosseum, it climbed right up on the ant and started grooming it. From there, I was off to the races. I scaled up to multiplexed arenas to video many beetles behaving at once. I used deep learning machine vision tools to analyze the videos. I developed tools to reconstitute the beetle's trail following behavior in the lab, and then expanded the throughput of that assay. All the while, I collected these beautiful beetles in the field, got scores of ants in my long hair, and began to learn something new about these creatures.

Tractability of S. lativentris for laboratory study

I first tackled whether the *S. lativentris-L. occidentale* system as a model to study social symbiosis was tractable. Different types of model systems have different requirements

Figure 2.2. An *S. lativentris* following host trail on the bark of a nest tree in the Angeles National Forest.

necessary for their use. Traditionally, the list of requirements includes being easy to rear in the lab, having short generation time, and being genetically accessible. The particulars of our system required a different set of criteria for its usefulness. Myrmecophiles are often rare and difficult to collect, so being able to easily acquire animals on a regular basis is critical. Generally, with social symbioses, the associations between organisms are so intricate and delicate that merely reconstituting the behavior in a lab context is a primary concern. Specialist myrmecophiles are reportedly so sensitive to disturbances in their nest context (*86*) that rearing them in the lab is unlikely; merely keeping them alive and happy for an extended period of time in the lab is the key criterion on this front. Given the complex life cycles in many symbionts (*86*, *104*, *108*), access to the germline for genetic tools is unlikely, so having any type of molecular manipulation at all (e.g. RNAi) is a powerful tool. In this chapter, I will demonstrate how I developed the *S. lativentris*-*L. occidentale* system for use in behavioral studies.

S. lativentris: prevalence, seasonality, collection

After scouting canyons in the Angeles National Forest near to Caltech, we found that *S. lativentris* is very abundant in ant nests. Though we have not systematically generated a count of beetles or ants for nests, I often collected dozens of beetles per nest. We found that almost every large colony of *L. occidentale* we surveyed had beetles. Due to its proximity to campus, I primarily collected near to the parking lot of Chaney trail and along Millard creek in Altedena, CA (34.2163413, -118.146500). My other main collecting site is near Gould Mesa Trail camp, along Gabrieleno trail, also near to a creek (34.2222252, -118.1785464). *L. occidentale* builds nests in the bases of oak (*Quercus*, especially *Quercus agrifolia* at our

Figure 2.3. Some fundamentals of collecting *S. lativentris* and its host ant from the field.

collecting locations) and bay trees (*Umbellularia californica*), though I have also seen infrequent evidence of ants nesting in evergreen trees. Collecting strategies depend on time of year and weather conditions. During colder weather and early in the spring, beetles generally walk on the trees housing the ant nest, often near the opening. When ants are not

very active, blowing into an undisturbed nest often leads to a surge of aggressive ants pouring out of the nest along with *S. lativetris*. In warmer/dryer conditions (especially during the summer) sifting leaf litter near to ant nests and along foraging trails generally is the most productive. Collecting expeditions gave as few as zero to as many as a couple hundred beetles per colony per day. Beetles were captured via aspirator and placed with host ants into falcon tubes with two KimWipes moistened with a few drops of water. We found that *S. lativentris* was plentiful, and available most of the year for collecting. We have collected it every month except December (though I expect it is possible to get beetles in December); they are much more difficult to find October-January.

Handling of S. lativentris in the lab

Housing beetles

In order to keep *S. lativentris* in the lab, I found it essential to store beetles with lots of well-fed ants from the same colony that the beetles came from. To do this, I always collected a large number of worker ants with the beetles in the field, usually by placing leaf litter on the ant nest opening, which the ants would swarm on, and bagging/bringing back this high-ant-density material. Upon return to the lab, beetles were placed with host ants into ~10 inch x 10 inch Rubbermaid boxes with a Fluon barrier (2/3 water, 1/3 Insect-a-Slip) painted on the sides to avoid escape. Animals were provided a feeder of hummingbird nectar (4 parts water, 1 part nectar) and a test tube setup with water and cotton balls to provide moisture. Specimens housed as such survived from a few hours to several weeks in lab.

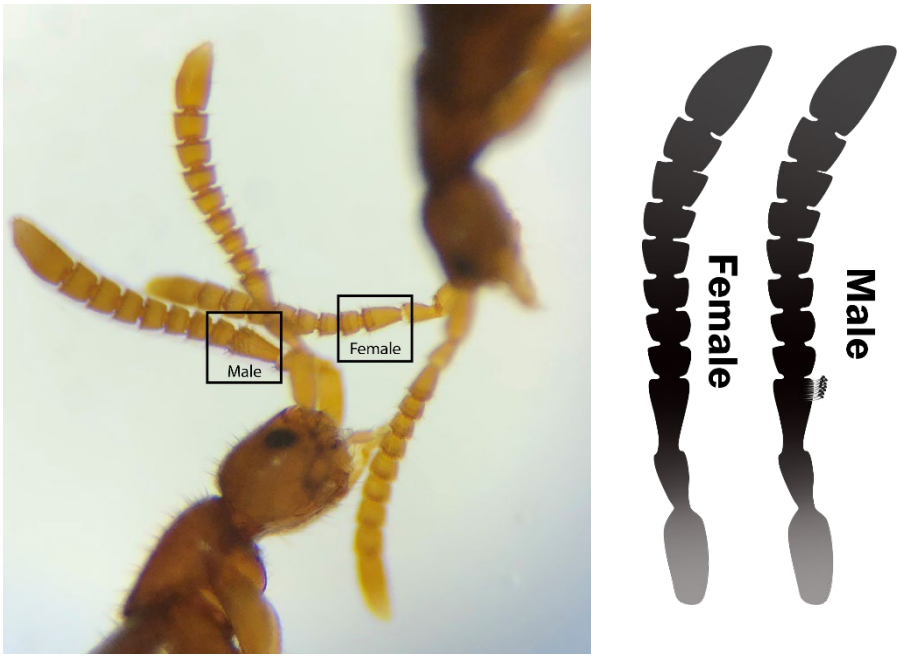

Figure 2.4. *Sexually dimorphic antennae of S. lativentris.* I used the high density of spatulate bristles on the third antennal segment of *S. lativentris* males to differentiate them from females, which lack this patch of bristles.

Sexing beetles

I could readily sex *S. lativentris* for experiments based on a dimorphism in the antennal bristles. Male beetles have a high density of spatulate bristles on their third antennomere, whereas females have few such bristles. *S. lativentris* tends to be sensitive to most any perturbation away from the ants, and I exclusively used ice as an anesthetic for beetles sexing under the microscope as this seemed to have the least negative impact on beetles. I would often house male and female beetles apart with no ill effects on beetle survival.

Grooming behavior: reconstitution and analysis in high throughput

S. lativentris grooms host ants to steal their nestmate recognition pheromones (CHCs), its most intimate social behavior. A single researcher previously studied this grooming in *S. lativentris*, showing it spend a large amount of its total time budget performing this behavior (*110*). Here I will describe the methodology I developed to reconstitute and study the grooming behavior in detail in lab.

Figure 2.5. *Grooming behavior of S. lativentris*. The beetle approaches a host ant, climbs on its back, usually approaching from the rear, latches onto the base of the ant antenna, and grooms the ant body surface, often for protected bouts.

Building grooming rigs

Arena construction 1

I built an array of circular arenas and demonstrated that the beetle would robustly perform its grooming behavior in this setup. To avoid vision influencing behavior, I took a precaution

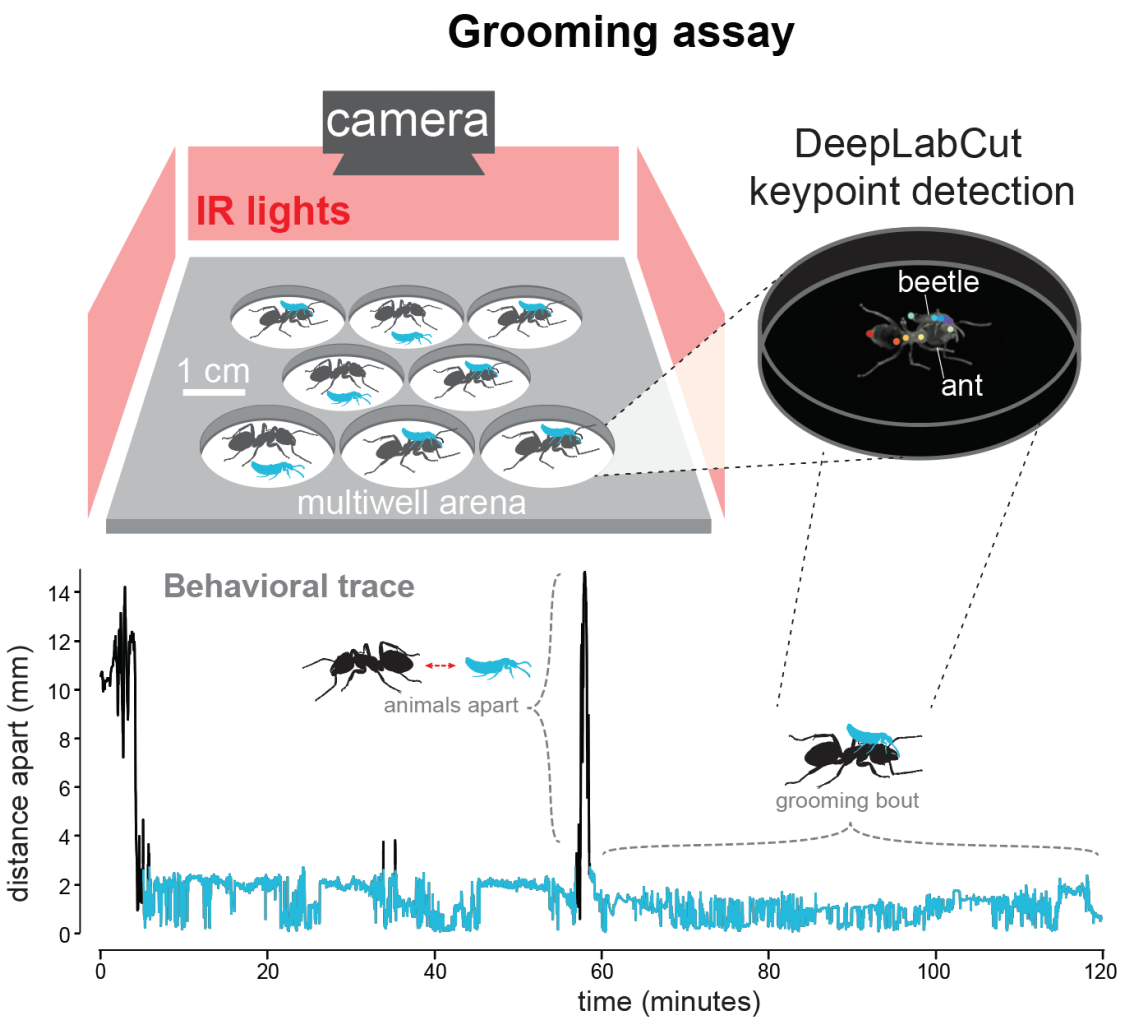

Figure 2.6. *A framework for reconstituting and analyzing S. lativentris grooming behavior in the lab.* I built multiplexed behavioral arenas consisting of 2cm diameter wells and placed a single beetle with a single ant or other animal and recorded the interaction. The beetle robustly performed grooming behavior of its host in this setup. With DeepLabCut, I annotated five keypoints on the beetle and interactor. With these keypoints, I calculated the distance between animals during the trial, and annotated grooming bouts as protracted (30 or more seconds) when the animals were within 3mm of each other.

to avoid light pollution. I constructed behavioral arenas out of 1/8th inch infrared transmitting acrylic (Plexiglass IR acrylic 3143) which transmits far red and infrared while blocking visible light. Arenas consisted of a base layer of finely wet-sanded acrylic (to provide texture for beetles to walk on) a layer with multiple two-centimeter round wells, and a top layer to keep animals inside the arena. I used a few variants of these 2 cm arenas throughout the data collection period, one with fixed well shape and two with a sliding design to allow a particular start time for insect interactions. Behavioral interactions were run at 25 C in a dark incubator with door closed, in a behavior room with lights off behind a blackout curtain to further ensure that the insects were operating in the dark. Arenas were backlit with a custom built IR850nm led PCB and diffused with a semi-opaque white acrylic sheet.

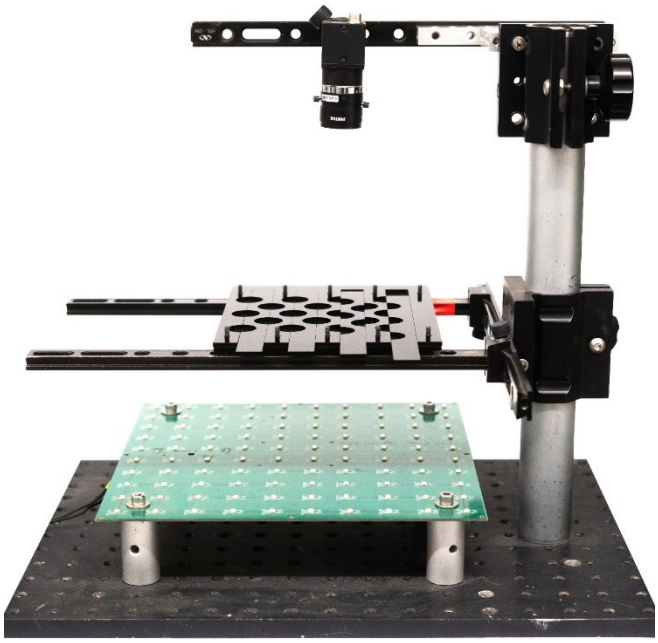

Figure 2.7. *One iteration of the grooming arena*. The arena is illuminated from below with IR LEDs and monitored from above with a camera. The arena itself is composed of layers of IR transmitting acrylic, so all interactions are happening in the dark for the insects. The arena wells are composed of half-circles; when loaded, the circles are staggered relative to each other to separate the animals, and one half can slide into place, letting the animals interact after they wake up from the ice used as an anesthetic before loading.

Recordings of interactions were made using a Flir machine vision camera (BFS-U3-51S5M-C: 5.0 MP) at 3 frames per second with a Pentax 12mm 1:1.2 TV lens (by Ricoh, FL-HC1212B-VG), for 6 hours.

Arena construction 2

Later, I built a similar arena as above but designed with side lighting, higher frame rate, and higher resolution per experimental well to better maintain visibility of the beetle when grooming during the trials and provide more information-rich behavioral data. To do this, I built an 8-well arena with similar design as mentioned above, with a base layer of sanded IR acrylic, a wall layer with eight 2cm circular arena cutouts, a ceiling of static dissipating acrylic with a rim of IR acrylic, and a second roof of IR acrylic. We constructed an aluminum frame to hold the arena, side mounted IR flood lights (Univivi U6R), and camera (BFS-U3-

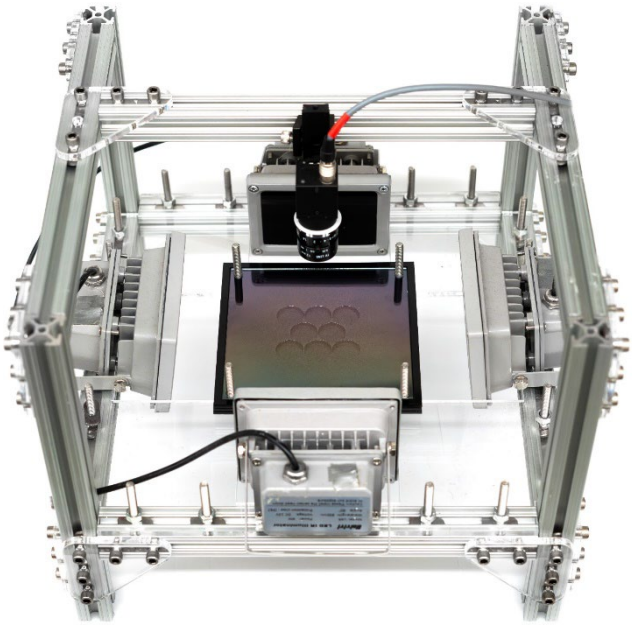

Figure 2.8. *Another iteration of the grooming arena.* The arena is illuminated from the sides and monitored from above with a camera. The arena is once again built with acrylic layers, but has no sliding mechanisms, as keeping animals apart at the beginning of trials was unnecessary for most experiments.

51S5M-C: 5.0 MP), recording at 60 frames per second. We used an Arduino based external trigger to maintain the frame rate of the camera. We placed the arena in a temperature-controlled incubator set to 18 degrees C. With a thermal camera, we determined that the arena itself, heated by the IR lights, was around 21 degrees Celsius during the trials.

Loading and prepping behavioral experiments

I used male *S. lativentris* for my grooming experiments. I isolated beetles in container with two moistened KimWipes for 30 minutes–1 hour before loading into behavioral arena. Beetles and interactor ants/insects were chilled on ice for 10 minutes before loading into 4 C chilled arena in the 4 C refrigerator to keep them awaking and escaping. Note that cooling the arena is important for experiments, loading into a room temperature/warm arena seemed to stress the beetles and ants much more than a cool arena, reducing the likelihood that the beetles would perform the grooming behavior. I then placed the loaded arena into the incubator setup as described above and began recording. In the case of moving arenas, I slid together the arena pieces after *S. lativertis* started moving around its arena well, ~10 minutes after beginning the loading process.

Analysis of grooming behavior runs

DeepLabCut for grooming arena analysis

One of the primary tools I used to analyze the grooming behavior of the beetles was DeepLabCut (*111*) to track the position of the animals during trials. I used a model with five labeled points on the *S. lativentris* and five labeled points on each interactor I put the beetle with. I trained a single model to detect the key points, regardless of the interactor type (Fig 2.9). I added additional training frames for each interactor I added to the dataset, using the

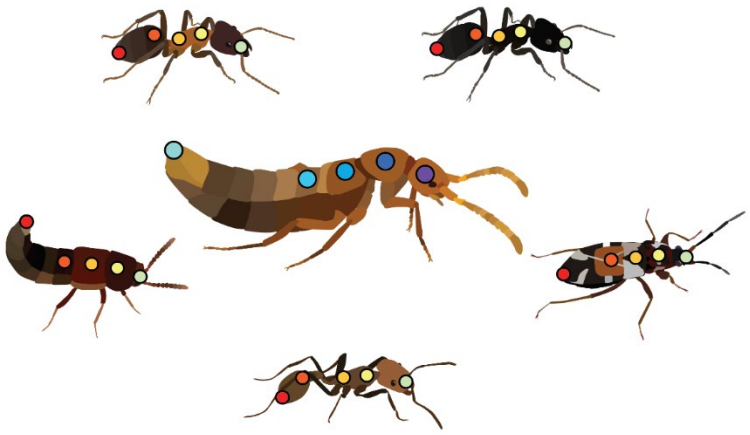

Figure 2.9. I trained a single DeepLabCut model to annotate beetle and interactor position during trials. Shown are the ~key point locations used for the various interactors and for *S. lativentris*.

ResNet50 as the network architecture. The final network was trained on around 2300 frames from more than 200 videos. This network achieved an error of 2.53 pixels for the training data, and 4.45 for the test data, which represents an error of less than 1/5th of a mm within the arena (less for most videos). If no detection for a given animal was present in a frame, we used linear interpolation from the last known position to the next known detection position to fill in the gaps. We calculated the distance between the beetle and the other interactors during the trial, and considered an interaction a grooming bout if the beetle was within 3mm of the ant for at least 30 seconds.

YOLOv8 for preference assay analysis

To test whether *S. lativentris* showed a preference for grooming its host ant over other ants, we placed a single host ant and either a single sister ant (*L. luctuosum*) or a divergent ant (*V. andrei*) with a single beetle in an arena well. To assess the preference for one ant or another, we determined the amount of time the beetle spent grooming each ant during a two- or six-hour trial. For analysis, we thinned behavioral videos to one frame per 16.7 seconds. We

used YOLOv8 for detection and bounding box generation of the location of each ant and each beetle during the behavioral trial. For this, we extracted frames uniformly from each behavioral trial video (10 per video for the *L. luctuosum* analysis for a total of 480 frames from 48 trials, or 30/31 per video for the *V. andrei* analysis for a total of 481 frames labeled) and split the data into 85% training data and 15% validation data. We generated labeled data with a bounding box per animal in with CVAT (https://www.cvat.ai/). We trained with YOLOv8's default settings (epochs: 100, patience: 50, batch: 16, imgsz: 640, lr0: 0.01, lrf: 0.01, momentum: 0.937, weight_decay: 0.0005, warmup_epochs: 3.0, warmup_momentum: 0.8, warmup_bias_lr: 0.1, etc.) (Fig 2.10, 2.11 for training results). We then performed detection on all frames of the thinned behavioral videos. For each frame, we took the highest confidence detection for each animal type per frame. If no detection for a given animal was present in a frame, we used linear interpolation from the last known position to the next known detection position to fill in the gaps. We calculated the distance between the beetle and the other interactors during the trial, and considered an interaction a grooming bout if the beetle was within 3mm of the ant for at least 90 seconds. To estimate the amount of time grooming each individual ant type, we eliminated ambiguous grooming bouts where the beetle was within 3mm of both ants. We then summed the time spent grooming just one or the other of the ants unambiguously. We subtracted the groom times to get a differential groom time estimate.

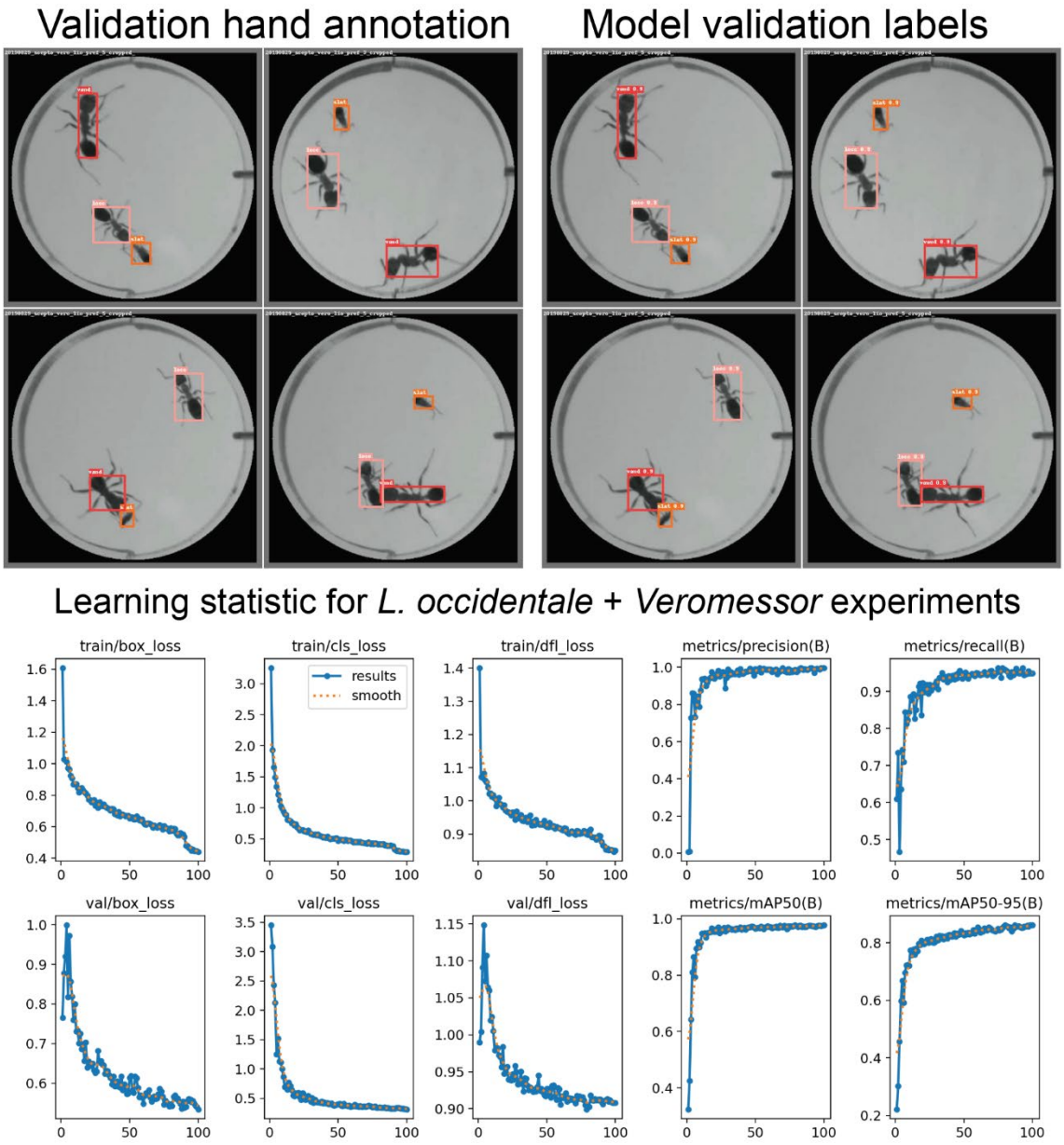

Figure 2.10. *Training data, and training statistics for YOLO object detection models for preference assay analysis of L. occidentale vs Veromessor*. On the top, example bounding boxes for animals hand annotated with CVAT, and the bounding boxes generated by the trained model. On the bottom, information of the effectiveness of training the model.

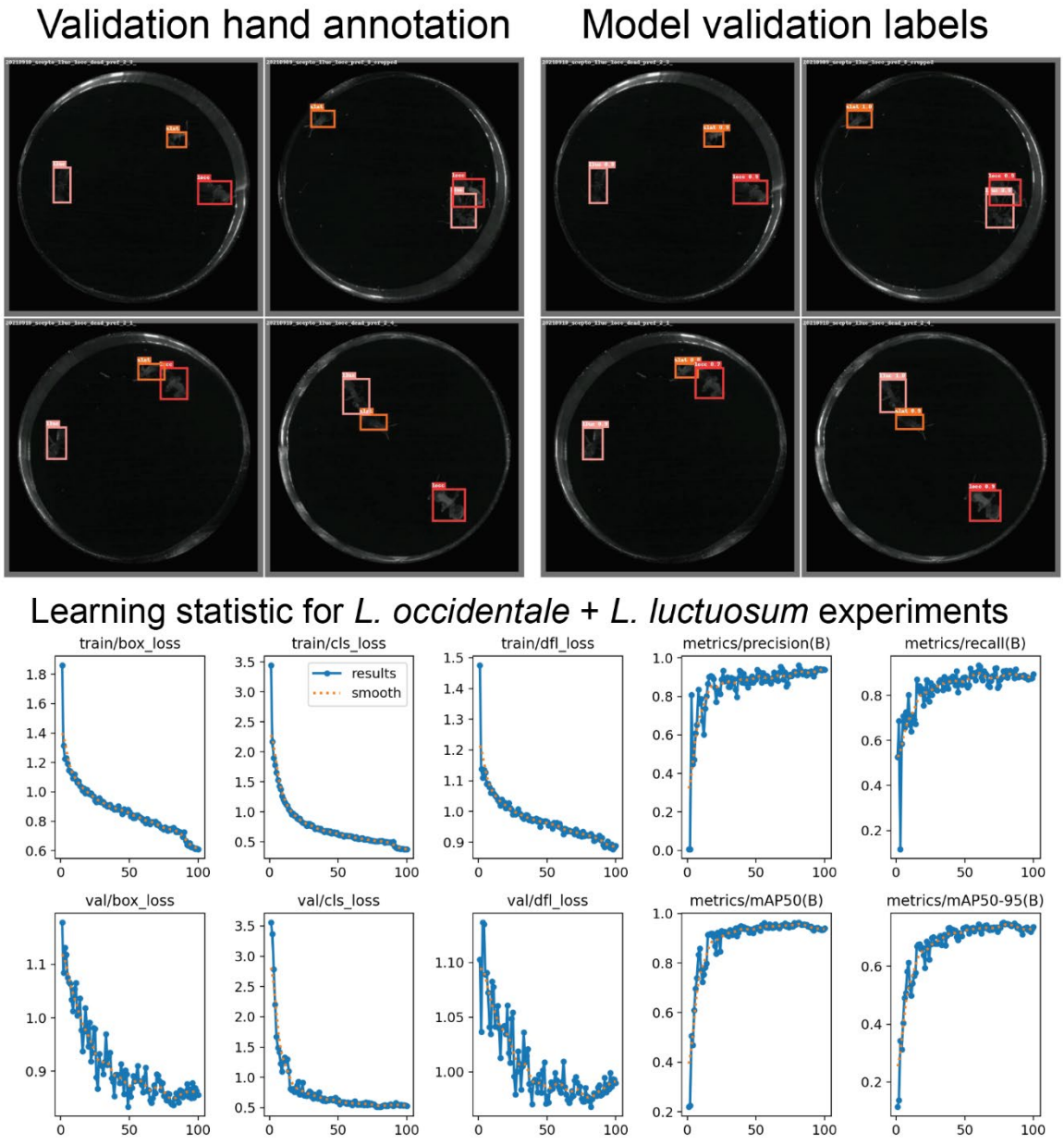

Figure 2.11. *Training data, and training statistics for YOLO object detection models for preference assay analysis of L. occidentale vs L. luctuosum.* On the top, example bounding boxes for animals hand annotated with CVAT, and the bounding boxes generated by the trained model. On the bottom, information of the effectiveness of training the model.

Trail following behavior: reconstitution and analysis in high throughput

In addition to the grooming behavior, we noticed in the field that beetles appeared to follow ant trails in nature, possibly for dispersing to new nests, since the beetles are wingless. I first needed to establish that the beetle was following a chemical trail, as opposed to just staying in the vicinity of ants who themselves were following trail, and then uncover the chemicals governing this symbiotic behavior.

Figure 2.12. Trail following behavior of *S. lativentris*.

Ant laid trail arena

In order to assay trail following ability and specificity of *S. lativentris*, I constructed a large (~16x20 in) open field behavioral arena, once again enclosed on IR transmitting acrylic. To provide a naturalistic ant-trail stimulus, I allowed a lab colony of *L. occidentale* to lay down a trail in the arena, with a large sheet of filter paper covering the bottom of the arena and acting as a diffuser for the IR 850nm strip backlights (Fig 2.13). After starving the ants for 2 days, I connected the colony to the arena environment, with a foraging object (sugar water) available at a distal region of the arena. Within the free field arena, I placed barriers to force the ants to lay a trail with specific geometry (Fig 2.13). After allowing the ants to lay trail for 12 hrs, I disconnected the ant colony, filled the arena with CO2 to knock out the ants, and

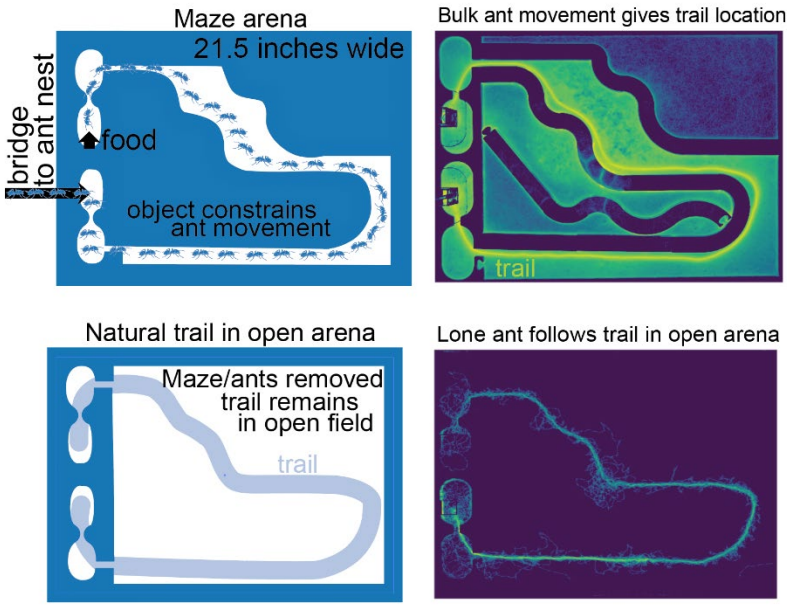

Figure 2.13. *Assay design to probe natural trail following.* I attached a starved queen-bearing ant colony to the entrance of a behavioral arena with a foraging object at the end of a obstacle-filled space. The ants laid foraging train in the arena. After many hours, I removed the ants and the obstacle from the arena and observed that a single ant would still follow the bulk-movement-pattern-of-ants from when the obstacle was in place, indicating that this was the position of the ant trail. Other animals could then be tested for similar trial-following ability.

removed all ants from the arena. After removing the ants, I removed the barrier that forced the ant trail geometry, placed the trail-laden filter paper back into the arena, and placed a *S. lativentris* into the arena. To quantify trail following, I correlated net movement from frame-to-frame of the beetle in the arena with ant flow at that position in the arena. Frame-to-frame beetle or ant movement was calculated based on thresholding the difference between subsequent frames to find locations of flow.

In addition to quantifying net movement of the beetles via frame-to-frame difference, I also performed blob tracking on beetle position throughout a behavioral trial. For this, I performed median filtering on a set of frames from the beetle-walking-in-trail-arena video to construct a background frame. With OpenCV, I performed blob detection background subtracted frames from the video. The median position of the blob was used to make a trajectory for beetle position in the arena.

Multi-well trail arena

To probe the particular chemicals relevant for trail following, I also developed a multiplexed assay to test beetle behavior in response to artificially applied trails. In particular, I built an arena with nine square wells of 3.5 inches by 3.5 inches, and painted ant extracted

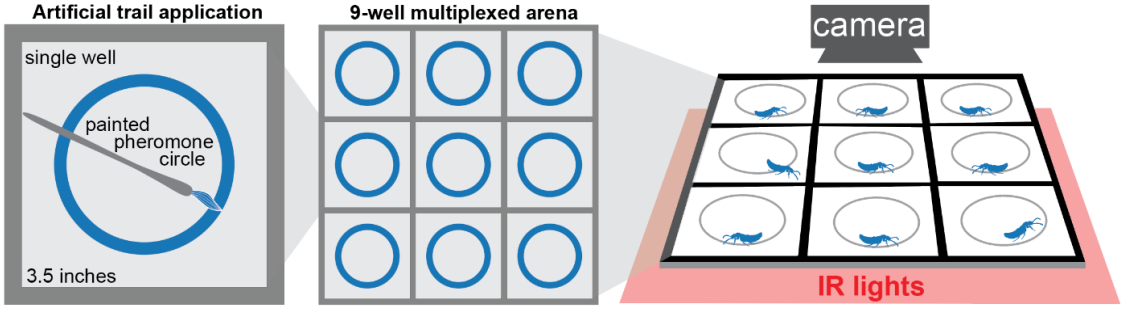

Figure 2.14. *Assay to test particular ant chemicals as trail pheromones.* I painted circles of different pheromones onto a ground glass surface in a multiplexed arena and monitored animal movement responses to different chemicals.

chemicals to a ground glass surface and monitored beetle activity in response to the applied chemicals. The arena was constructed out of stacked layers of acrylic and glass as follows: 1) the base floor is ¼ inch clear acrylic 2) an 1/8th inch thick layer of Plexiglass IR acrylic 3143 to block visible light 3) an IR acrylic layer with a 12 inch by 12 inch opening that fits a 12 inch by 12 inch square of 1/8th inch thick glass with a ground surface to provide grip for beetles to walk on it 4) an opaque white acrylic layer with nine wells of 3.5 by 3.5 inches with fluon applied to the walls to prevent insects from walking on them/climbing to the roof 5) an 1/8th inch layer of static dissipating acrylic as a roof 6) an 1/8th inch layer of IR acrylic to block visible light 7) a 1/4th inch layer of clear acrylic to weight down the ceiling and keep it flat. The layers were all held together by screws affixed to a metal frame and backlit with IR 850nm strip LED backlights. The arena was monitored with a FLIR machine vision camera (BFS-U3-16S2M-CS: 1.6 MP) with a Pentax 12mm 1:1.2 TV lens (by Ricoh, FL-HC1212B-VG). Trials were two hours long.

Analysis of multi-well trail arena

To analyze the resulting videos, we used OpenCV. We first performed cropping on the video to extract the individual wells from the experiment. We warped the individual square well to square them and set to a constant resolution of 320x320 pixels per square well. We then constructed a background frame via median filtering on a set of images from the given well. Then, we background subtracted for each well and used the OpenCV blob detection

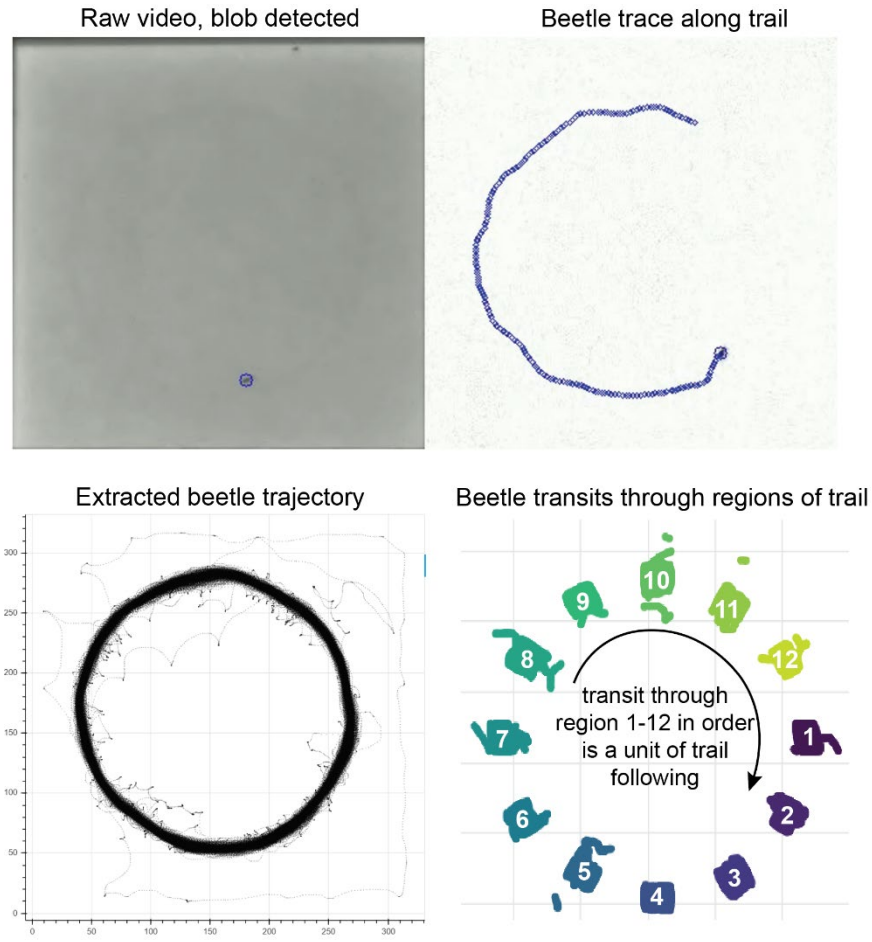

Figure 2.15. *Analysis of beetle behavior in circle-trail assay.* I performed blob detection on raw videos after performing background subtraction. With the resulting trajectories, I found bouts within the video where the animal transited though regions of interest along the trail in order, without reversing direction or double transiting through a section. This approach stringently and extremely accurately provided the sections of the trajectory where the beetle walked in the circular arcs around the arena.

method to threshold/find the beetle. The position of the beetle in the well was saved. To calculate the degree of trail following observed in the trial, we used the position information given by the blob tracking and looked for circular arcs within the animal trajectory. To do this, we defined regions of interest as sections along the circular applied trail of approximately 0.5 cm along the circle and diverging from the trail of about 0.5 cm farther or closer to the circle center. We defined twelve such regions per circle, at intervals of 30 degrees along the circle (Fig 2.15). We then tracked the instances where an animal traversed through these twelve regions sequentially from one to the next for an entire revolution of the circle. Each such traversal counted as a single circular trail following event. We then calculated the distance traveled while the animals were traversing these circles and plotted these distances.

Preference assay in trail arena

In order to test whether *S. lativentris* prefers trails of its host ant over its sister ant species, we used a variant of the multi-well trail arena. We made approximate concentration matches of bulk extract from the host ant *L. occidentale* or the sister ant *L. luctuosum*. To do this, we integrated the region of a GCMS trace (GCMS methods described elsewhere) representing the iridoid fraction of the trace. We then made a dilution to $1/5^{th}$ the concentration so we had comparably low and high concentration extract for the host and sister ant. Based on total ion count of iridoid chemicals, we diluted the bulk extracts to match concentration. We then painted abutting lobes of semi-circular trail with low or high concentration of extract from the two different ant species. We then placed a single beetle in each arena well with the variable high-low concentration trails and recorded their movement through a two-hour trial. For this assay we used the same setup as described above for the multi-well trail arena, but

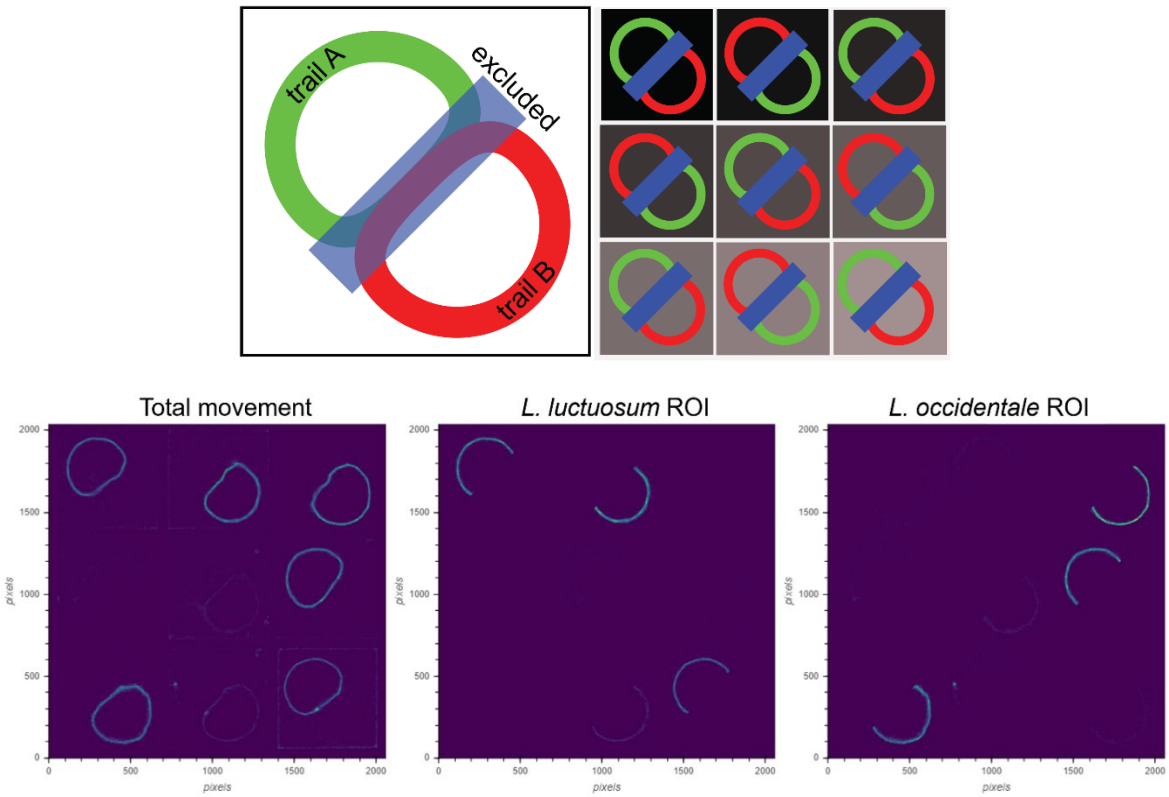

Figure 2.16. *Analysis of multiplexed preference assay for trail chemicals.* I painted chemicals from different species at different concentrations as semi-circular and abutting lobes. I calculated bulk movement of the beetle during the trial by subtracting subsequent frames from the behavioral trial. I used manually defined regions of interest to extract the bulk beetle movement on the different arms of the trail (excluding the middle section where trails overlapped) and summed the movement on particular trails, then subtracted these values to get a differential in trail following.

with a BFS-U3-63S4M-C 6.3 MP camera with a Pentax C61232KP 12mm F1.4 Manual Lens with Lock Screw. To quantify the results, we calculated movement of the animals during the trial as the sum of pixel difference between subsequent frames for the whole experiment. We then used manually defined regions of interest as the arms of the trail lobes belonging to either species. We summed the total movement in these trail regions and subtracted these values to see the difference in total movement during the trial on one trail or the other, which

is our preference index for this experiment. Depictions of the movement histogram and the movement histogram for a given trail lobe ROI are shown (Fig 2.16).

Chemical fractionation for probing of chemicals

For fractionations, we collaborated with Jocelyn Miller at UCR. I made a bulk extraction of many thousands of *L. occidentale* workers, with a concentration estimated of around 50-100 ants per ml. Stock solution is stored at -20C. Jocelyn concentrated 50 ml just to dryness by rotary evaporation and took the residue in 5 ml hexane. He prepared a vacuum flash chromatography column from a 10 ml sintered glass funnel filled with 230-400 mesh flash chromatography grade silica gel. He packed the silica gel bed with hexane, pulling the solvent through with vacuum, and loaded the hexane solution of concentrated ant extract onto the column, rinsing on with hexane. He eluted the column sequentially with:

a. 3 x 12 ml hexane
b. 3 x 12 ml 5% cyclohexene in hexane
c. 2 x 12 ml ether
d. 2 x 12 ml EtOAc

This accomplished the fractionation, leaving saturated hydrocarbons in fraction 1 and 2, unsaturated hydrocarbons in fractions 5 and 6, and more polar compounds in fractions 7 and 8. He combine Frac 1 and 2, 5 and 6, and 7-10, and adjust the total volume of each to 10 ml, or 250 AE/ml. We used the polar and non-polar fractions for our experiments.

GCMS analyses

Analysis of GCMS data happened manually in the "GCMS Postrun Analysis" software by Shimadzu, the manufacturer of the GCMS we used. I also did some analysis with the pyteomics package in python, after exporting the GCMS files to mzXML format.

Host association: reconstitution and analysis under lab conditions

In-silico model of host switching

We built an in-silico model of beetles interacting with ants based on two core biological observations: intrinsic and extrinsic mortality. Ants attack outsiders, killing them, which we call extrinsic mortality. Beetles die when away from ants, from CHC loss and other isolation related effects, which we call intrinsic mortality. We produced an agent-based model to see how parameters like level of aggressiveness of other ants, distance between ant colonies, and rate of death when away from ants influence the ability of the beetle to switch from one colony to another. We start with a NXN grid of variable size which represents the forest floor that beetles and ants will navigate through. We initiate colony A and colony B (different ant species) at opposite corners of the forest grid. All beetles start with colony A, and all ants start at their respective colony locations. The beetles also start with a supply of CHCs, one for recognition and one for resistance to dying from desiccation, which they lose both of when away from ants. At each step, ants move to one of the four squares it directly touches with equal chance (when close to the its nest) or with higher chance to move back towards the nest (when farther from the nest). When ants encounter the ants of the other species, if they are outnumbered in the square they die. If they are of equal number, which species wins is determined by a coin flip. At each time step, the beetles also move in the arena, and lose their CHCs at a constant rate. When beetles encounter an ant with the same CHCs it has (at the start, its host CHCs) it gains both recognition and desiccation resistance CHCs to full. When beetles encounter an ant with opposite CHCs as its own, it has a chance to be killed in the encounter, but if it survives it fills all its CHCs and changes its recognition CHC ID to

the opposite ant. The odds that the beetle survives the encounter depends on the number of wrong CHC ants present, and the amount of the wrong ID CHC it has, given by

$$\text{odds killed} = \frac{1}{\left(1 + 50 * e^{-0.5*\left(1+\frac{\#\text{ ants}}{10}\right)*\text{aggressivness}_{\text{ant}}*\text{CHC}_{\text{beetle}}}\right)^2}$$

When ants or beetles die, they are reset at their starting colony, to keep the number of animals in the simulation constant. The simulation runs for 1000 steps, and the outcomes recorded. This 1000-step run is done 100 times per set of parameters to get averages for the results. We ran the simulation with variable CHC loss rates, variable size of forest arena, and variable aggressiveness of ants to see how these parameters influence how often/why beetles die in the simulation, whether they get the CHCs from the non-host ants, and whether they moved to the ant nest on the other side of the arena.

Cross arena construction

To test the in-silico model of host switching, we constructed an arena which matched the design of the model. Namely, we bult an arena composed of two chambers which we could place beetles and various numbers of ants of two different species, and a twenty-by-twenty grid of connected wells in which the ants could move around and interact (Fig 2.17). We printed the cross arena plate design in a Prusa I3 MK2 3d printer in clear PLA. The base of the piece was 1/8th inch thick, and the wall component also 1/8th inch thick. We used acrylic to sandwich the 3d printed component and provide a ceiling to close the animals in and prevent escapes. In particular, we cut screw holes into a base plate of 1/8th inch clear acrylic, matching holes in two 1/8th inch pieces with cutouts the same dimensions as the arena, and in a top piece of clear acrylic. This made a 4-layer sandwich encasing the arena. We mounted

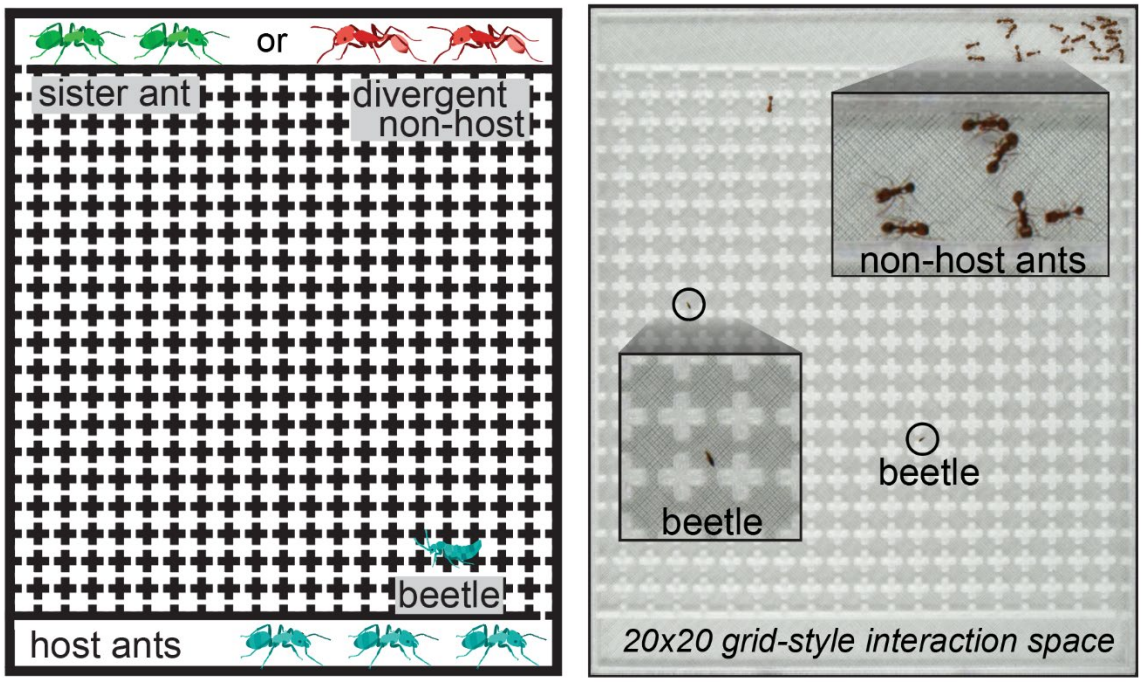

Figure 2.17. *Cross arena for host switching.* I designed an arena with a large grid of connected interaction chambers to observe beetles interacting in a space with multiple ant species.

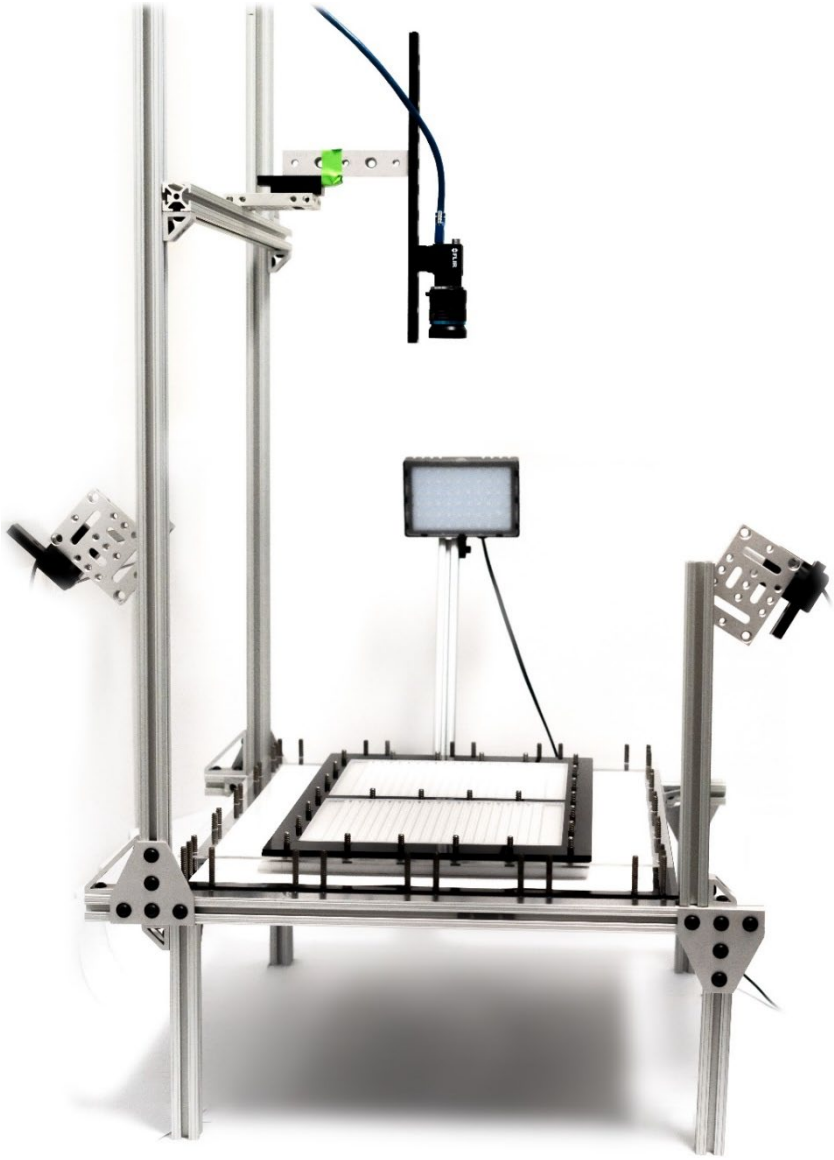

Figure 2.18. *Lighting and frame system for cross and cross-maze arenas.* I used LED photography lights to illuminate the arena from above on all sides. I used a wide-angle lens and high-resolution color camera to record behavior in the arena.

two such arenas next to each other in the same metal frame used for the trail arena experiments (Fig 2.18). We wanted to maintain color information in the trials to help differentiate ants of different species and the beetles, so placed white LED photography lights around the arena on four sides. We mounted a color camera (BFS-U3-200S6C-C: 20 MP, 18

FPS, Sony IMX183, Color, camera) to the frame and with a a 16mm 10MP Telephoto Lens for Raspberry Pi HQ Camera (lens). For experiments, we ran behavioral trials for 24 hours at 5 frames per second. When beetles survived or were physically intact enough, we extracted each beetle and two of each ant type from the run in hexane with a c18 standard for 20 minutes before running samples on the GCMS with the CHC program parameters as described elsewhere.

Cross-maze arena

To test whether beetles could survive/navigate to a new nest of ants without any dispersal cue, we constructed a variant of the above arena in a maze configuration. In particular, only alternating end walls connecting the rows of the arena were left open, forcing the beetles to traverse a distance of ~4 meters at minimum to find a group of ants at the other end of the arena. The ants were behind a size selecting door that would allow the beetle to enter, but too

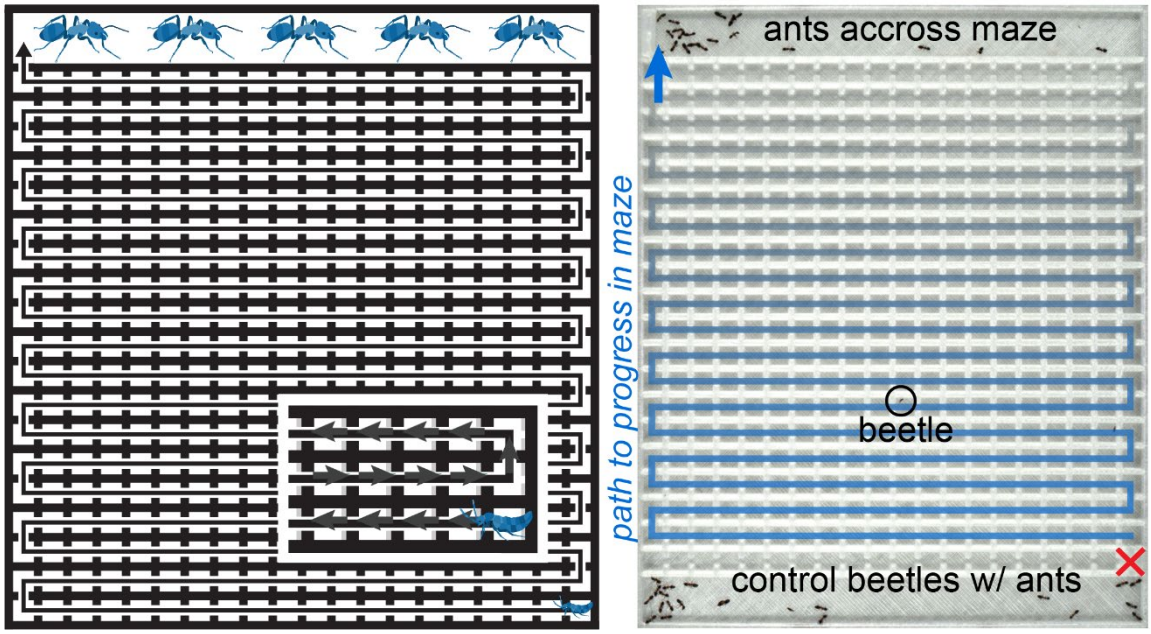

Figure 2.19. *Cross-maze arena to probe dispersal abilities.* I constructed a variant of the cross arena with only a single opening between rows of square arena components (on opposite sides per row). This made a zig-zagging maze with a minimal distance of about 4 meters to cross and access ant hosts.

small for the ants themselves to exit. We ran experiments with beetles in this arena for 24 hours and extracted the animals for GCMS as with the cross arena.

Blob track for cross-maze distance analysis

To analyze the resulting behavioral trials, we used a combination of manual annotation and machine vision. To calculate the distance the beetles moved in search of ants in the maze, we used a blob tracking approach. Using the python implementation of OpenCV, we first cropped each individual replicate (right or left arena), de-distorted with the warp perspective method to square the image/correct for the fish-eye effect from the wide angle lens and downscaled the frame to 20% its original resolution to speed the blob tracking analysis, giving final dimensions of about 500x500 pixels per arena. With the resulting videos, we constructed a background frame using a median filter on ~10 frames taken uniformly at times during the first ~5 hours of the video. After making the background frame, we looped through the downscaled video, background subtracted the frame, detected blobs in the frame, and saved the result. With the outputs of the blob tracker, we ran the detections through SORT to generate IDs for the tracked blobs where possible. This also let us filter out short/spurious trajectories where the blob tracker made wrong/random non-beetle detections, which generated short trajectories. We filtered out trajectories shorter than 20 seconds long, which were likely the spurious ones, and summed up the total distance the beetles in the experiment traveled during the run. We also used the trajectory to locate the farthest point in the maze that either beetle made it during the trial. We also annotated by hand the location that the beetle ended the run at. Together, these gave the total distance traveled, how far they made it in the maze, and where they were at the end of the trial. We correlated the distance traveled with the CHC level from hexane extractions of the beetle from the end of the trial.

Manual curation for death times

For the cross arena and cross-maze arena, we manually measured time to death for beetles in the experiments. To do this, we manually scrubbed through videos and located the last time that the beetle moved in the arena under its own power (ants sometimes moved dead beetles, so merely annotating when beetles cease moving in the arena is not a sufficient criteria).

C h a p t e r 3

THE PROXIMATE RELEASERS OF *S. LATIVENTRIS* SOCIAL BEHAVIORS

"Did the scientist really follow an elegant chain of reasoning in executing his experiments? … Did he not sometimes put the cart before the horse, bang his head against a stone wall, or bury it in the sand?" - Kenneth D. Roeder

Follow the system: failed hypotheses can make for a much more interesting story

When I started my project, we had a pretty clear hypothesis for how the host specificity of the *S. lativentris-L. occidentale* system might work: the beetle would respond only to the pheromones of its host ant, ignoring or rejecting those from others. This would provide a very clear mechanism of specificity, where the beetle had a highly specialized nervous system tuned to its host and only its host. I very quickly found that this was far from the case, and the beetle exhibited symbiotic behaviors in strikingly wrong contexts. Before I discuss these findings and their implications for the system, though, I'll outline how I parsed the likely pheromonal basis for the beetle's recognition system. Using the grooming assays which I had built, I placed many different interactors with a beetle and observed its behavioral response to these interactors. I placed a bunch of different insects with the beetle, and the beetle ignored or avoided these insects (with some critical exceptions I will talk about in the next chapter). I extracted the pheromones off these insects, and they all looked quite different from the host ant. This made me think that the ants body surface pheromones (CHCs, used for nestmate recognition) might play a critical role in the beetles' ability to recognize and groom its host. Some more evidence came when I stripped all the pheromones off a dead ant and observed that this eliminated the grooming behavior. I also took another symbiont of the ant (*P. sonomae*) which has a CHC profile similar to the ant and the *S. lativentris* chased it around in the arena well and tried its best to groom it even as the other animal rebuffed it and tried to use its appeasement gland, appearing to think that the *S. lativentris* itself was an ant! When I presented a dead *P. sonomae* to *S. lativentris* the beetle did groom it since it wasn't being constantly knocked off. I also cut the gaster off an ant to produce a stimulus with only body surface pheromones and not the chemicals in the

anal glands of the ant, which the beetle still very happily groomed. The beetle also groomed a second CHC mimic of the ant, the rove beetle *L. newtonarum*. Together, these showed that the beetle uses host ant CHC to recognize for grooming. A few weeks before the pandemic hit, I showed pretty definitively that *S. lativentris* follows ant trails; I let an ant nest lay trail in an open arena of a couple square feet with a sugar water object, then kicked the ants out and let a lone beetle walk in the arena. Its trajectory matched the ant movement almost exactly! This was one of my n=1 experiments that instantly gave a clear result, which I replicated during the pandemic from my university-owned apartment in after bringing an arena and ant nest to my house. I biked to the field (I didn't own a car and didn't feel safe using rideshare apps during the pandemic!), brought back beetles, and tested their trail following at home. I later got fractionations of the ant pheromones and found that the beetle would only follow the non-CHC polar fraction of the extract (iridoids). These findings, however, only tell a partial tale of this beetle, with much more to come in Chapter 4.

Using S. lativentris to study host specificity and recognition

In the last chapter, I described the methods and apparatus I developed in order to study the association between *S. lativentris* and *L. occidentale*, its host. I will now describe how I used these methods to uncover the story of this myrmecophile, how it recognizes its host ant, and what implications this has for host specificity. All reports of the distribution of *S. lativentris* indicate it is wholly host specific (Fig. 3.1A). It has only ever been found with *L. occidentale* ants, despite sympatry with numerous other ant species, including the closely related sister ant *L. luctuosum* (Fig 3.1B). To probe the recognition space of *S. lativentris*, I began by

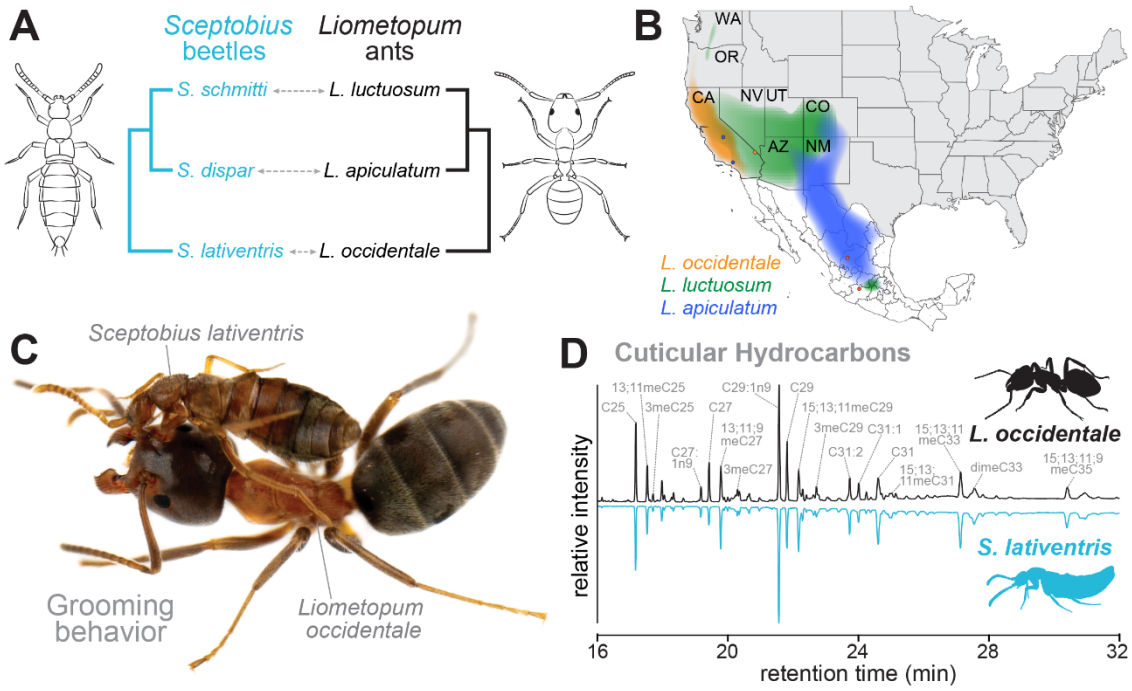

Figure 3.1. *The system: a myrmecophile to study the mechanisms of specialized host recognition and hyper-specific association.* The symbiotic beetles of genus *Sceptobius* live intimately with the particular host *Liometopum* ants (A). *S.* lativentris, in particular, is only ever found with the single species, *L. occidentale*, despites sympatry with the sister species *L. luctuosum* (B). The beetle grooms the host (C) to steal the nestmate recognition pheromones (CHCs) to match the host and gain entrance into the colony (D). We ask a simple question: why is the *S. lativentris* host-specific, and how is this specificity maintined?

investigating the grooming behavior of the beetle (Fig. 3.1C); the beetle uses this behavior to steal the gestalt colony odor (CHCs) of the ant it grooms, and hence perfectly matches its host ants recognition pheromones (Fig. 3.1D). The obvious candidate for the recognition cues the beetle use are these CHCs themselves, which is where I started.

Ant hydrocarbons mediate host recognition and social attraction

To probe the behavioral-sensory space releasing the grooming behavior from the beetle, I used our previously described multi-well grooming assay. I allowed the beetle to interact with wildtype ants, ants ablated in a variety of ways, and other insects. The trail chemicals and alarm pheromones for the ant are produced in glands in the gaster of the ant, so I removed these chemicals by removing the gaster (Fig 3.2A). The beetles groomed the gasterless ants for the same time as wildtype, indicating that the alarm and trail chemicals are not needed to trigger grooming. Since the only other major pheromonal compound produced by the ants are their body surface cuticular hydrocarbons, this strongly implicates CHCs as the releaser

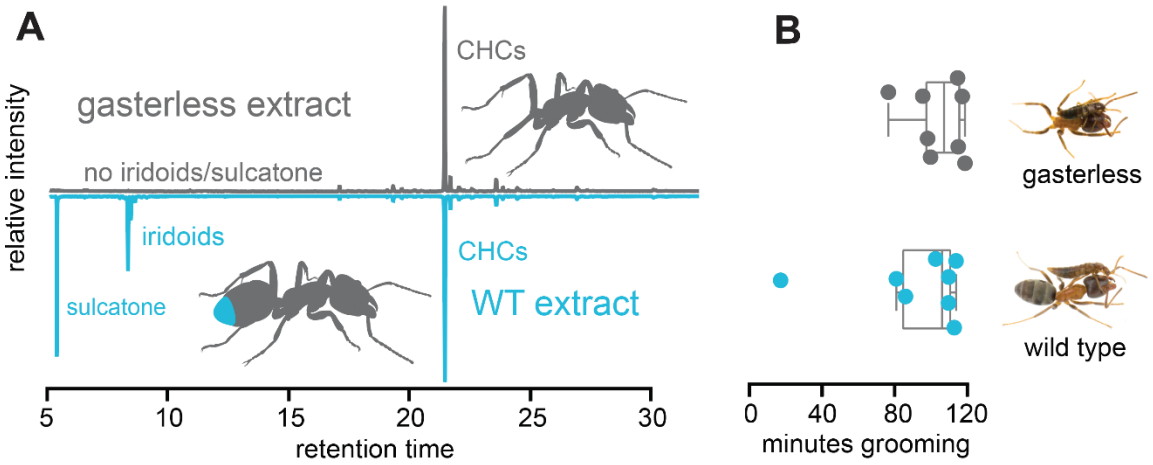

Figure 3.2. *S. lativentris grooms gasterless ants, implicating CHCs as the recogntion pheromone.* Cutting off the gaster of an ant effectivly removes the iridoid and sulcatone components of its pheromones, leaving the CHCs and a living ant that survives several hours (A). The beetle spends similar time grooming the gasterless ant as a wildtype one, during a 120 minute trial (B).

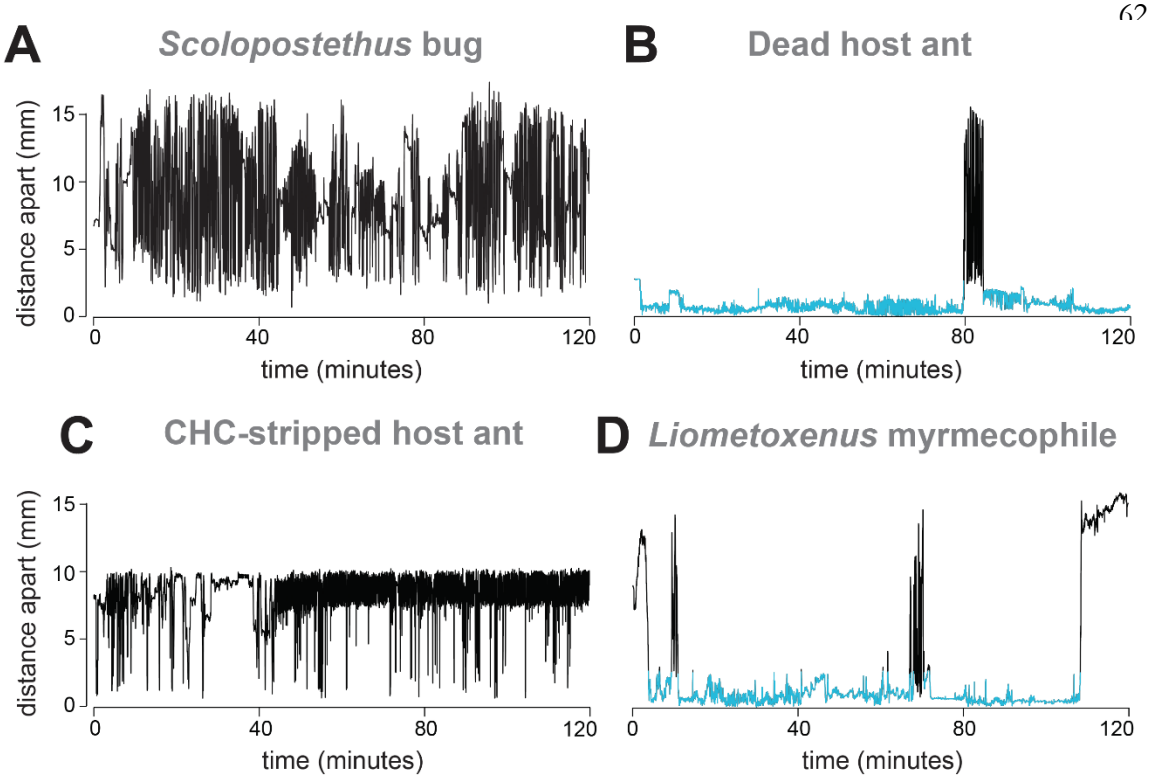

Figure 3.3. *Behavioral traces of S. lativentris interacting with different animals.* In (A) the beetle interacts with a bug, and shows no grooming bouts. In (B) the beetle spends most of the trial grooming a freshly dead ant, indicating movement/signals related to being alive are not neccesary to trigger grooming. However, stripping the CHCs off a dead ant eliminates grooming, further supporting the criticality of CHCs for grooming (C). The beetle also grooms a fellow myrmecophile of the ant host, which matches the CHCs of the ant (D).

of the behavior (Fig 3.2AB). I also found that beetle groomed dead ants, indicating that the beetles do not require a moving/living ant to recognize the host for grooming (Fig 3.3B). The beetles do not, however, groom ants stripped of their body surface chemicals, showing their necessity to elicit a response (Fig 3.3C). When I placed a bug, highly divergent from the host ant, with the beetle, they largely avoided each other, and I saw no signs of grooming (Fig 3.3A). I also provided a variety of other insects to *S. lativentris,* all of which had divergent CHC profiles from the host ant, and none of which elicited the grooming behavior (Fig 3.4). I recovered the grooming behavior when providing *S. lativentris* to interact with other ant

symbiotic beetles, *Platyusa sonomae* and *Liometoxenus newtonarum*, which both mimic the CHCs of the *L. occidentale* ant, but which are phylogenetically highly divergent from ants and each other and hence diverge in cuticle structure, texture, and shape, etc. (Fig 3.3D, 3.4). A summary of the groom times for all the animals which we tested against *S. lativentris* is presented in Figure 3.4. In summary, the beetle grooms anything bearing host ant CHCs, and beetles that mimic the host ant CHCs. I wanted to better understand the body surface chemistry of the organisms interacting with *S. lativentris*, so I extracted the insects and performed GCMS analysis on the CHCs of the groomed vs not-groomed insects. The animals groomed showed substantially more similar CHCs to the host ant as compared to the other

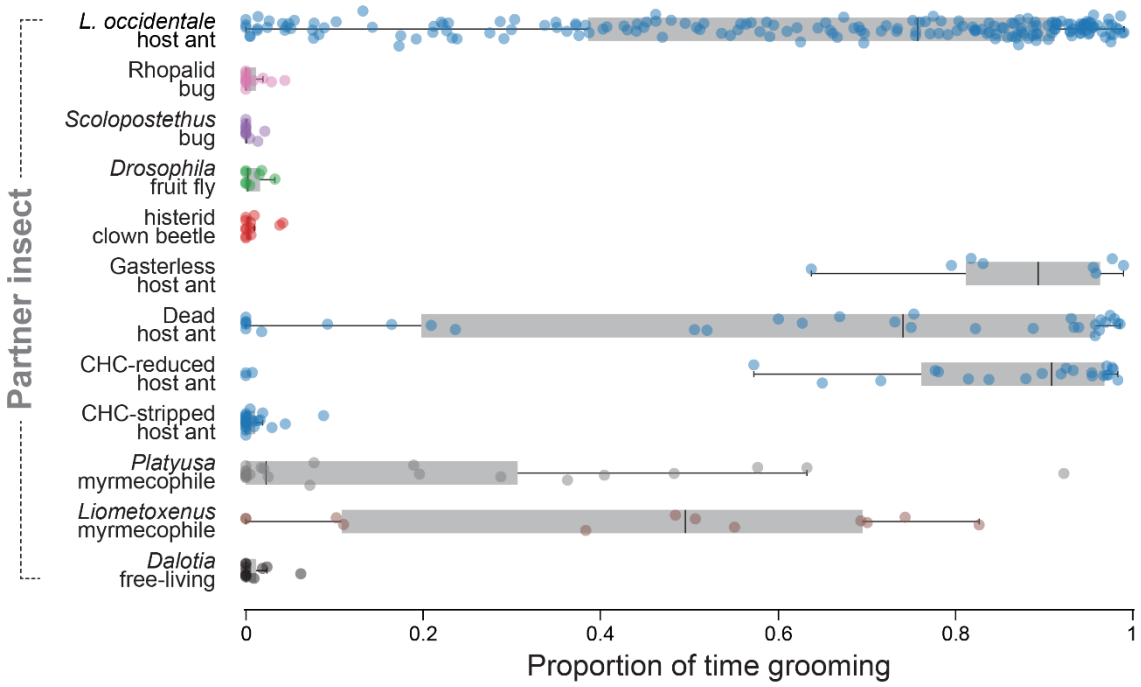

Figure 3.4. *A summary of the grooming proclivity of S. lativentris.* The beetle grooms host ants, even when dead or missing the gaster, but stripping CHCs ablates grooming. The beetle ignores other non-ant insects, except its fellow myrmecophiles from the same host, which also match the CHCs of the host (see Figure 3.5). Together, these experiments demontrate that CHCs are the recognition cue the beetle uses to recognize its host for grooming.

species assayed (Fig 3.5). Together, these data indicate that the ant body surface CHC pheromones elicit the beetle grooming behavior.

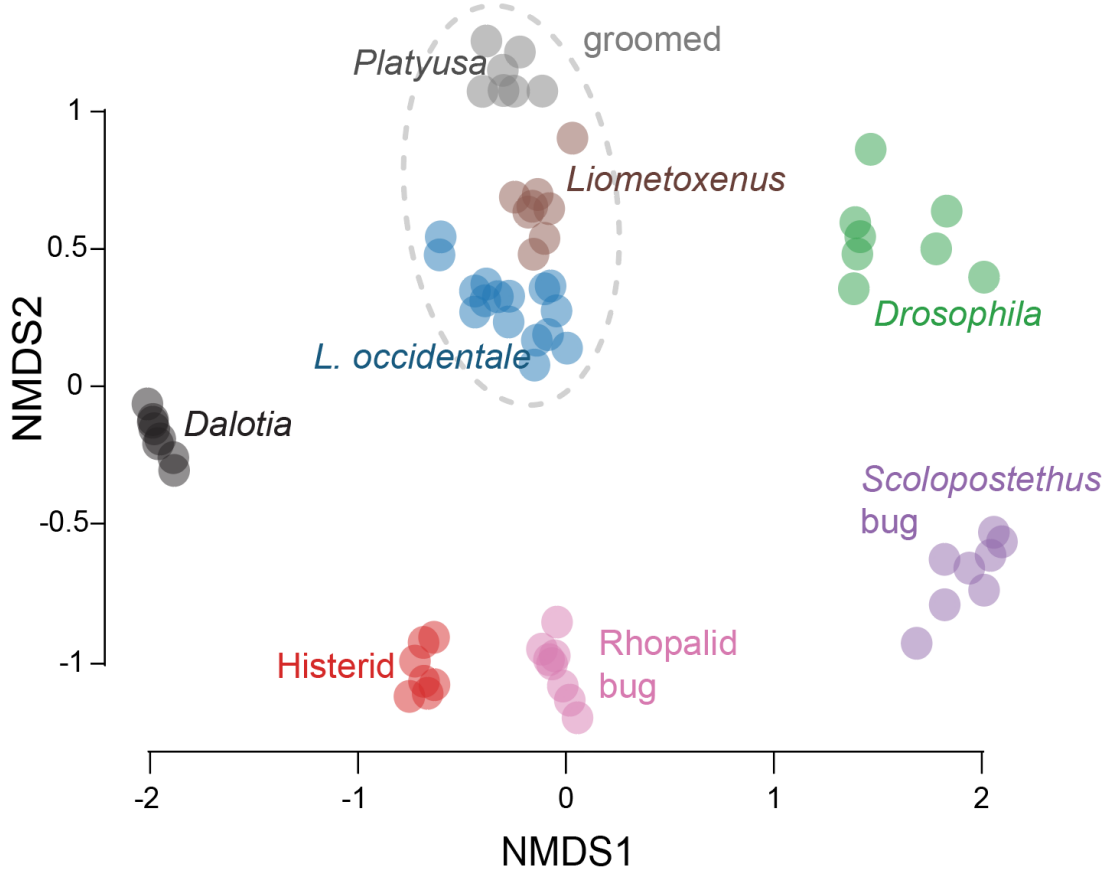

Figure 3.5. *Chemical analysis reveals the myrmecophiles that S. lativentris grooms share much more similar chemical profiles to host than the random other insects that it does not groom.* Shown is a NMDS imbedding of the high dimensional chemical space into two dimensions.

Host ant trail pheromones mediate host finding and dispersal

S. lativentris follows ant trails to maintain close special proximity to its host ants and putatively disburse to new nests, since it is wingless. I built an arena allowing a queen bearing lab colony to lay down trail on filter paper to a food source around a maze object. The maze piece induced ants to lay trail with an otherwise peculiar shape (Fig 3.6A1). After the colony laid trail, we removed the ants and maze piece and placed an *S. lativentris* beetle or a *L.*

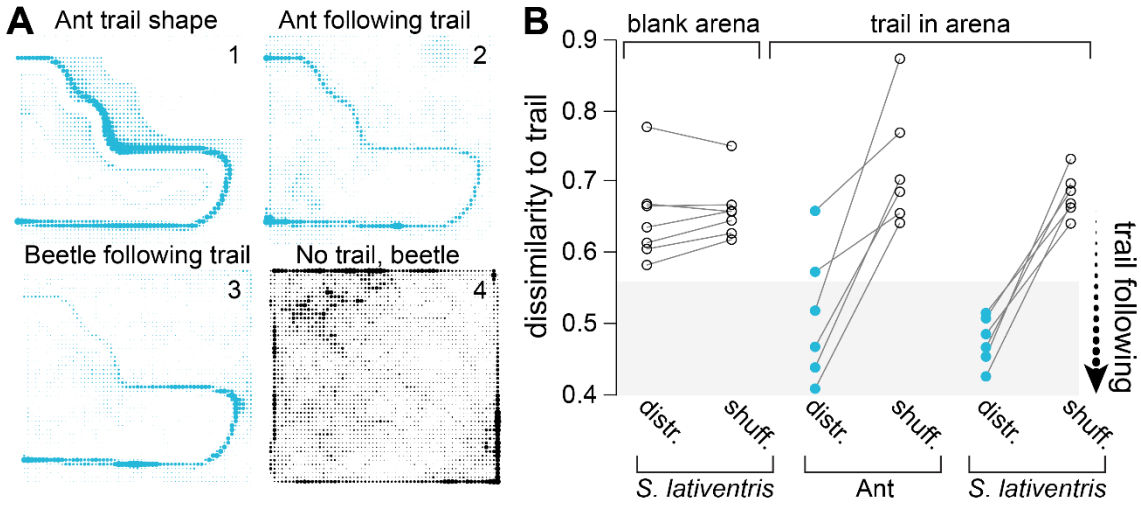

Figure 3.6. *Demonstration of natural trail following by S. lativentris.* After letting an ant nest lay trail in an arena with a maze object to force the ants to forage around an oddly shape block, I calculated ant movement at the end of the foraging time to generate a distribution of ant activity, a proxy for the location of the foraging trail (A1). I then removed the ants and maze object and let single ants or beetles walk in the now-open field with only the foraging trail to guide them and videoed the results. I then tracked the location of movement of the animals, and found extremely clear evidence of trail following, where the beetle's movement precisely followed the oddly shaped trail, with at least as good of acuity as ants (A2,3). Beetles walking in control arenas with no trail had wall following/random distributions in the arena (A4). I treated the movement traces as probability distributions, pooled movement values for 35x35 pixels blocks in the image and calculated the dissimilarity of the single animal movement to the ant trail (B). Numerical analysis confirmed the visual inspection, where beetle movement (distr.) matched the trail. As a computational control, I randomly shuffled the locations in the 2d movement histograms (labeled shuff. in the figure) and the dissimilarity with the trail distribution shot up, matching the degree of dissimilarity of beetles moving in an empty arena, further confirming the coherence of beetle movement with the trail position (B).

occidentale or non-symbiotic rove beetle or the arena to freely move. The single ant's movement corresponds with the location of bulk ant movement during the foraging time, indicating the ants laid robust trail in the lab (Fig 3.6A1, A2). Additionally, the symbiotic beetle location closely followed the ant trail position, showing strong trail following in a lab setting (Fig 3.6A3). By contrast, a non-symbiotic beetle's movement, and a less specialized symbiotic beetle's movement showed no correspondence with the ant trail. Based on a dissimilarity measure derived from the Bhattacharyya distance, I also show quantitatively that movement of *S. lativentris* and the ant correspond closely to the trail distribution whereas a beetle moving in an arena with no trail has no correspondence with the trail (Fig 3.6B). As a further control, I randomly shuffled the position of the beetle movement computationally to test how dissimilar a spatially random distribution with the same intensity values would be. For all trail following cases, the dissimilarity shot up, whereas for the blank arena the beetles movement distribution similarly failed to match the trail as a random distribution, as expected (Fig 3.6B). This shows that *S. lativentris* exhibits a specialized trail behavior absent from non-symbiotic beetles to follow its host.

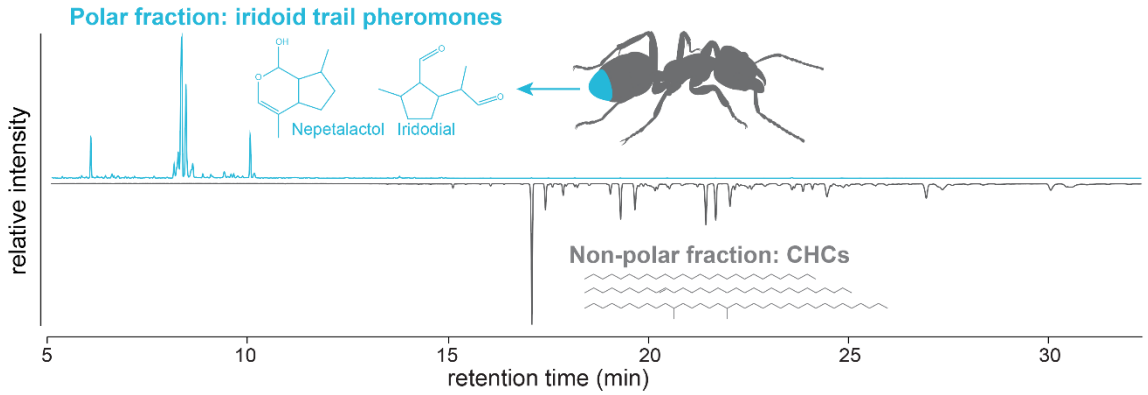

Figure 3.7. *We performed fractionation on bulk ant extract to separate the polar and non-polar pheromones to test in bioassay.* The polar fraction contains primarily iridoids, and the non-polar fraction the CHCs of the ant.

To probe the exact chemical set used as trail, we performed a fractionation of the bulk extract of many ants into polar compounds (for the ants, mostly iridoids) and nonpolar compounds (the cuticular body surface pheromones) (Fig 3.7). I brushed chemicals onto a ground glass sheet in a circular pattern and placed a *S. lativentris* beetle, *L. occidentale* ant, or *D. coriaria* non-symbiotic control beetle into the arena and allowed them to walk freely. The symbiont and ant strongly responded to the iridoid fraction, walking around in circles for sometimes over a hundred meters during a two-hour trial (Fig 3.8AB). The non symbiont showed no circle following behavior (Fig 3.8AB). None of the animals responded to the cuticular hydrocarbon fraction when applied as a circle (Fig 3.8AB). These experiments provide strong evidence that the iridoids represent the trail chemical for the ant and that the symbiotic beetle eavesdrops on this signal to also trail follow.

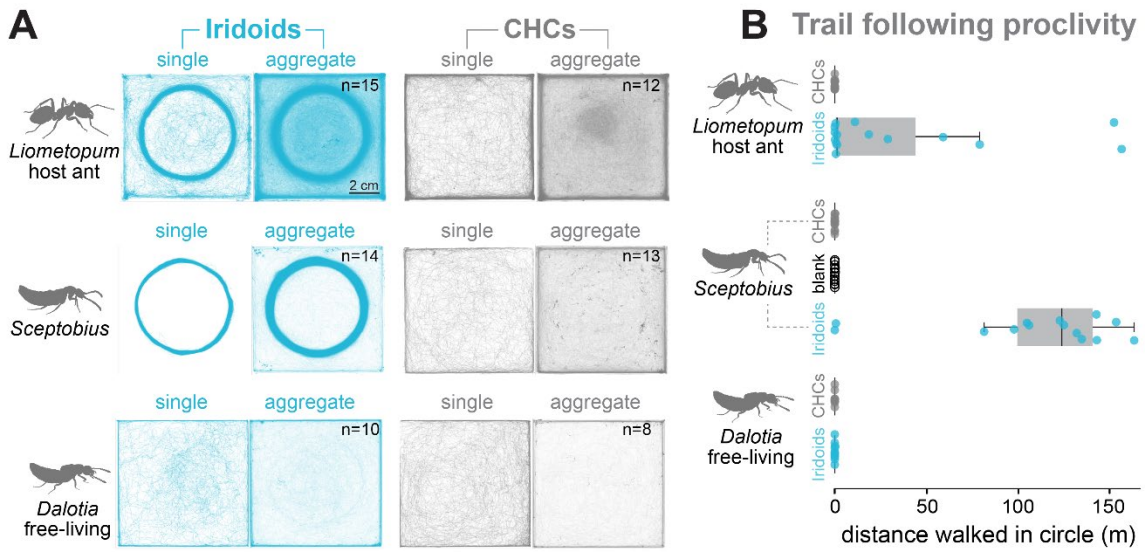

Figure 3.8. *Analysis of circle following in response to the iridoid vs CHC pheromones from ant extracts.* Ants and beetles both follow the iridoid fraction, exhibiting long bouts of circle-following during two-hour trials (A,B), whereas a free-living beetle shows no trail following. This shows that iridoids release trail following behavior in both ants and beetles. Beetles show extreme fidelity to host trails, sometimes walking over a hundred meters in circles on the trail during a two-hour trial.

Our findings demonstrate that dispersal and host finding depend on following ant trail pheromones. *S. lativentris* thus eavesdrops on two major components of ant communication—CHCs and trail pheromones—and interprets them in a manner analogous to that of its host ant. CHCs for host (as opposed to nestmate) recognition, and iridoids for probable dispersal and host finding (as opposed to foraging).

Chapter 4

CONTROLS OF HOST SPECIFICITY OF *S. LATIVENTRIS*

"[O]ne's opinion may be biased by dogma and one's judgment clouded by a favorite hypothesis, how impartial curiosity and worldly ambition are inextricably intertwined, how months or years of apparently futile effort may be more than balanced by a few seconds of joyful discovery, how an idea must underlie such efforts even though it may eventually prove to be wrong. Perhaps the point is that the act of gathering knowledge depends on a complex and partially subconscious process that includes large elements of chance, and that this aspect of scientific research is not manifest in the coldly logical prose of most scientific papers and reports." - Kenneth D. Roeder

Latent surprises appear in careful study

In the previous chapter, I made it sound like the host cues that *S. lativentris* picks up on are a perfect mechanism to explain its host specificity. The beetle ignores other insects and grooms host ants. The beetle diligently follows the exact blend of chemicals its host uses as for its foraging trails. This was, however, and I hope you will forgive me, a feint of storytelling. I knew from one of the earliest experiments that the story was more complicated. Once I had set up my experimental rig to study grooming, I was interested very early in how *S. lativentris* would react to a divergent species of ant. I placed one of these aggressive, and nearly 100 million-year divergent, ants with the beetles, and, to my great surprise at the time, the beetle groomed it. A lot. This fact has long since become old hat in my thinking, but it was not at all obvious at the time and severely contradicted our initial hypothesis, namely that host-specific equaled host specific recognition/behavior. I continued to push the boundaries of the specificity and found that *S. lativentris* groomed all sorts of highly divergent ants. It also shows little to no preference for its host in a two-choice assay. It is known that ants share a large number of similar compounds that they use for nestmate recognition, but they manage to differentiate conspecifics from other ants without issue. In any event, the latent promiscuity of the beetle's close-range recognition system made it clear that some sort of host-specific releaser of grooming couldn't explain the beetle's single host specificity. Much later, I also found that *S. lativentris* follows trails of the sister ant that it never lives with, and prefers whatever trail is higher in concentration, regardless of whether it's from the host or sister ant. I remember in my candidacy meeting that one of my committee members, Betty Hong, immediately wanted to know how the ants themselves responded to the beetle grooming when I mentioned the promiscuity. She strongly encouraged me to

consider the critical ant-side of the symbiosis. At the time, I nearly dismissed this point, and emphasized that, for now, I would stick with parsing the beetle side of things; I had yet to develop my trail assay, and thought, incorrectly, that the trail cue would explain the specificity. When trail chemicals also failed to explain specificity, I realized that I really did need to take the ant-side of the symbiosis into account to understand the beetle. I noticed that even as the beetle worked to groom the wrong ants, the ants themselves attacked the beetle. Ants are notoriously aggressive, so perhaps they enforced the specificity by rejecting symbionts that would otherwise be compatible with them. I also wondered how the distance between nests might influence a beetle's ability to host switch. Maybe nests unconnected by trails were effectively infinitely distant from each other and the beetle had no chance to wander into wrong-ant-nests. With some collaborators, we built an in-silico model of beetles interacting with two ant species to generate some hypothesis about the strength of the spatial-aggressive enforcement barrier. I then constructed an arena to test the model predictions and found great accord between the model and experiments. They demonstrated that even small spatial distances between colonies were insurmountable for a beetle, and that highly aggressive ants would unilaterally eviscerate beetles attempting to join their ranks. However, I also found that when faced with less aggressive ants, the sister species to *S. lativentris*' host, the beetle was able to host switch, and integrate long term with the ants. Together, I demonstrated that the latent promiscuity of the beetles sensory tuning and social-behavioral programs afford a possibility for it to host switch, even on ecological time scales. We have yet to observe *S. lativentris* successfully switching to new ants in their sympatric zones, but it may be just a matter of time. The story of this beetle turned out to be much more interesting than our initial all-neural-tuning hypothesis, and in this I experienced the joy of discovery.

Promiscuity of grooming cue/behavior

Having established that host ant cues mediate host recognition and dispersal, I asked if they also mediate host specificity. To investigate host specificity, I employed our established behavioral assays to assess whether cues derived from the host ant were sole releasers of symbiotic behaviors. To our surprise, despite the extreme specificity of the *S. lativentris-L. occidentale* association, *S. lativentris* robustly performed its grooming behavior with its host's sister species of ant, *L. luctuosum* (Fig 4.1). I further found that we push the breaking of partner fidelity to extremes, showing that that the beetle would even groom several species of ants more than 95 million years divergent from its host. Not only would the beetle groom these highly divergent ants, but its grooming also effectively steals the pheromones of whatever it grooms (Fig 4.2, 4.3). I placed beetles with their sister ant for a varied amount of

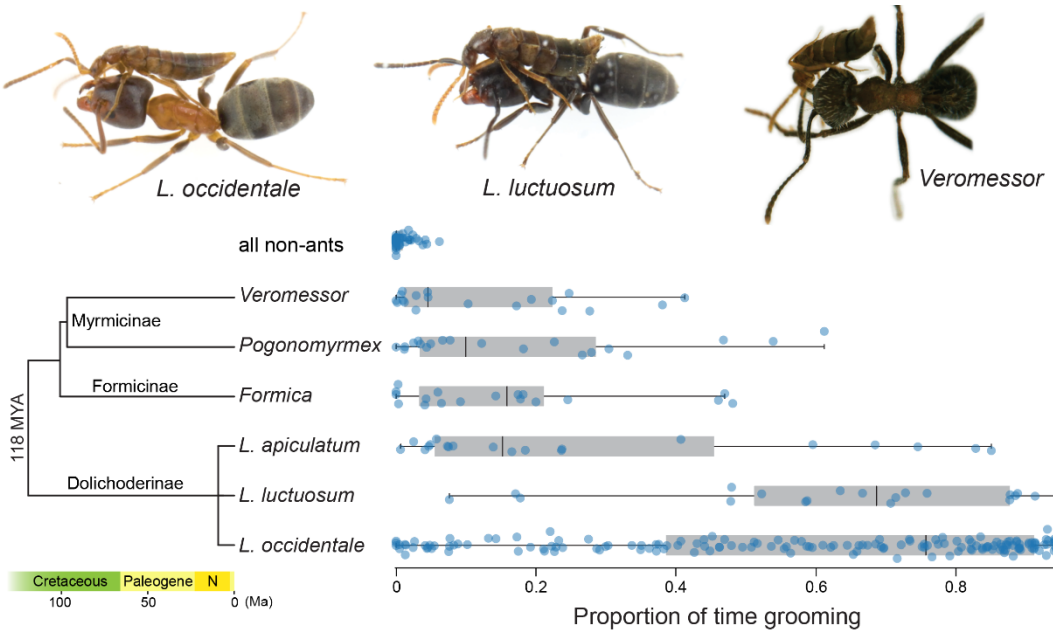

Figure 4.1. *Promiscuity of grooming behavior of S. lativentris.* Though *S. lativentris* ignores non-ants, it grooms highly phylogenetically divergent non-host ants from multiple subfamilies, as well as both sister species to its host ant. Ants share very similar CHCs, and the beetles contact recognition system is compatible with multiple alternate ant hosts.

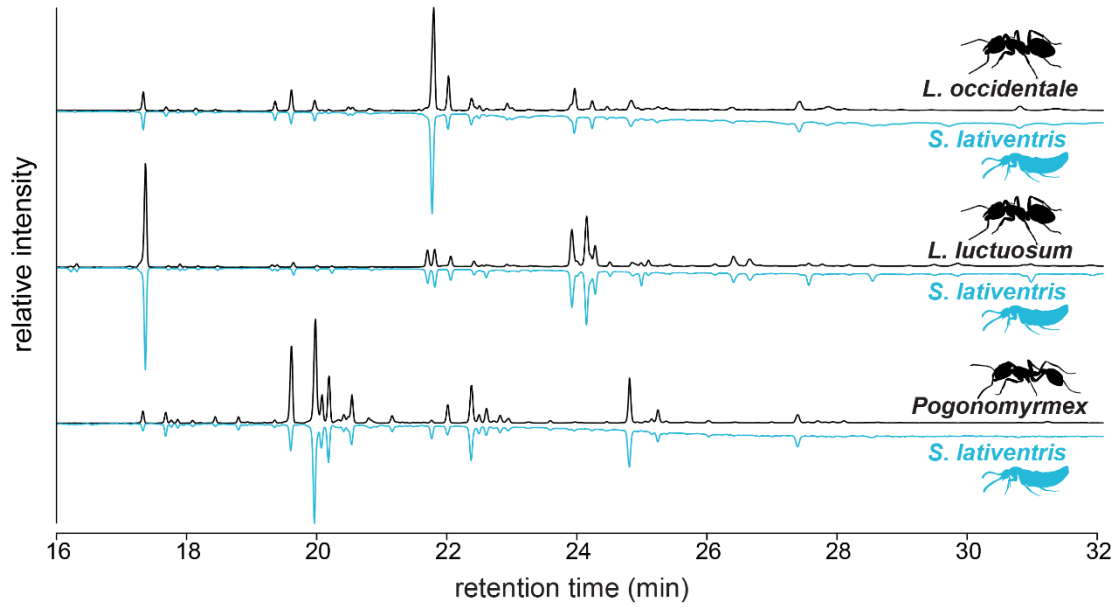

Figure 4.2. *S. lativentris grooming behavior allows stealing of a novel pheromone profile to match non-host ants.* Shown are GCMS traces for the beetle after spending 48+ hours grooming a non-host ant. The pheromones on the body surface of the beetle shift to match the non-hosts, though the fidelity of the match is slightly reduced for the highly divergent *Pogonomyrmex* as compared to the match to the sister ant species.

time, and found that, within 24 hours the beetles hydrocarbon profile fully turned over to match the new ants. At six hours, the beetles' profiles were chemically intermediate between host and non-host ants (Fig 4.3). Not only did greater time with novel ants lead to greater turnover in beetle chemical profiles, but these shifts correlated with the amount of time spent in annotated grooming bouts during a six-hour trial (Fig 4.4). This indicates that not only does the beetle recognize non-hosts as potential symbiotic partners, but also its symbiotic behavior works similarly with these non-hosts as with the host.

I was then interested in whether the beetle would prefer its own host over these other ants, as this might also give rise to host specificity. I found, however, that the beetle only showed a very small preference for its host over the sister ant species and performed long grooming bouts with the wrong ant (Fig 4.5A). Further, I found that this preference disappeared when I killed the ants (Fig 4.5B), suggesting that the ants' own response to attempted grooming bouts might explain the difference in response. I pushed this assay further, and found that the beetle still showed only a minor preference for its host over the highly divergent *V. andrei* ant, and performed long grooming bouts on the divergent ant even when its host was available to groom instead (Fig 4.5B).

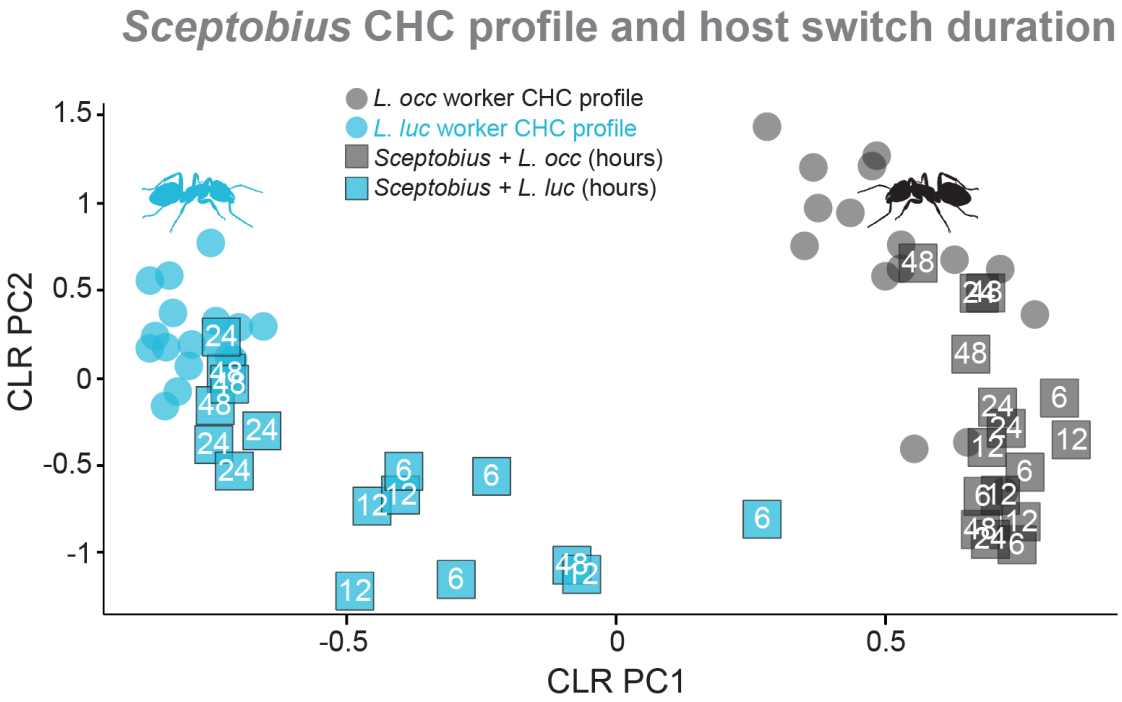

Figure 4.3. *Time-dependent turnover of beetle surface CHC profile.* Depicted here is a two-dimensional representation of the location in chemical space of host, non-host, and beetles, based on a centered-log-ratio transformed vectors of chemical composition. Boxes represent beetles, the number inside shows the amount of time the beetle was housed with the given ants. Within six hours, profiles showed substantial shifts towards non-hosts when housed with them, and near perfect chemical matching achieved within a day of interaction time.

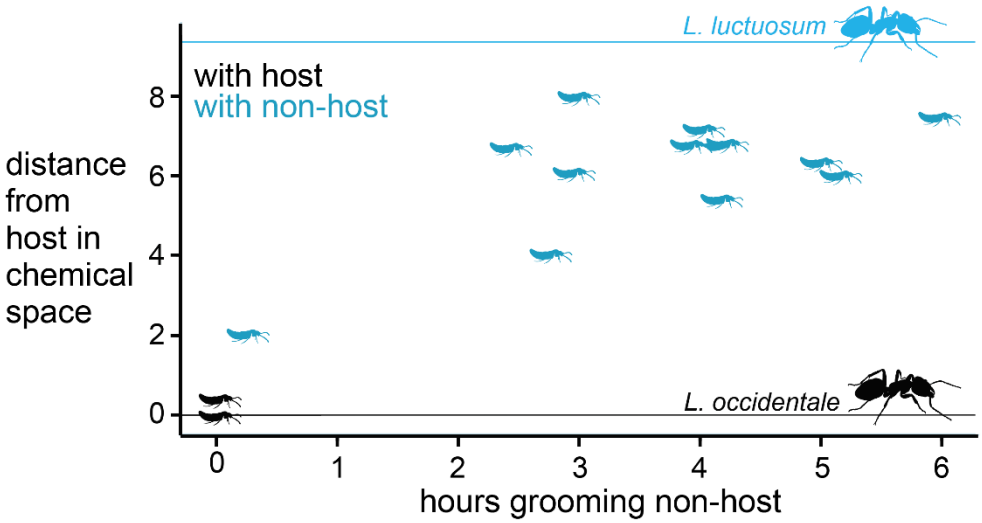

Figure 4.4. *Correlation of grooming time with CHC profile turnover.* When paired one-on-one with host ants, the amount of time in grooming bouts correlates with a movement in chemical space away from host ants towards non-hosts.

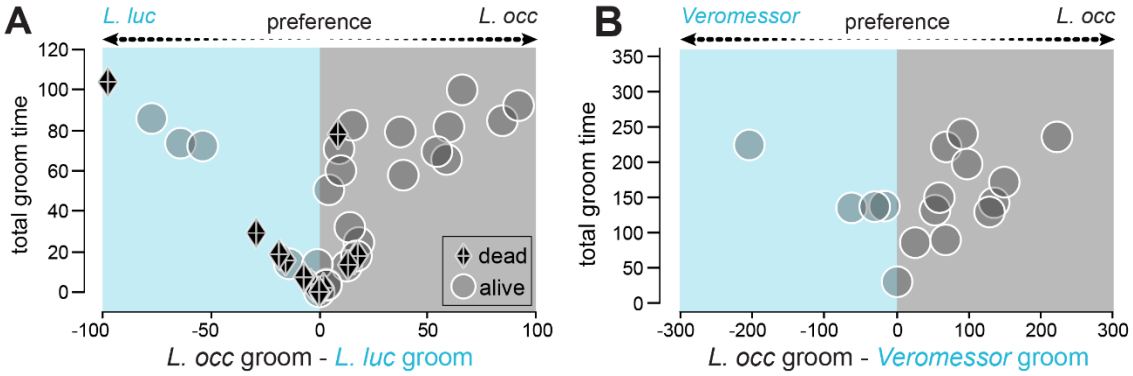

Figure 4.5. *Little-to-no preference for host in two-choice grooming assay.* The beetles show a small preference for host in a two-choice assay with both the sister and species and the highly divergent *Veromessor*, grooming hosts marginally more than non-hosts; however, ant behavioral response to grooming attempts likely explains this as the preference disappears when giving dead ants to the beetle (A, B). Multiple of the experimental wells showed beetles performing long grooming bouts of the non-host ant, even when the host was available to groom.

Promiscuity of trail chemical/behavior

I pushed the assessment of sensory-cue specificity even further and tested whether the beetle discriminates between trails of its host or other ant species. I allowed a field collected group of workers of the sister ant species *L. luctuosum* to lay foraging trails down in an arena in lab before removing the ants from the arena. As with its host, the beetle followed naturally laid trails of this non-host ant species, whereas a non-symbiont showed no correspondence with the trail (Fig 4.6A). I also painted bulk extracts of the sister ant species in circles and found the beetle extremely robustly followed these trails (Fig 4.6B). Note that the higher degree of trail following for sister-ant extracts is likely because this trail was at higher concentration as compared to host extract. When painted in abutting semi-circles, *S.*

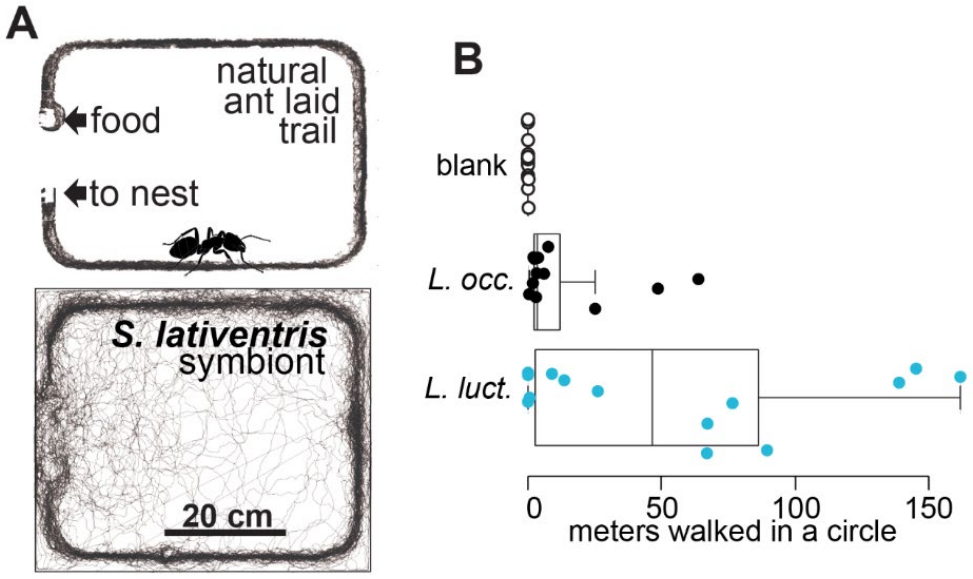

Figure 4.6. *Beetles follow both naturally laid and applied extracts of the siter ant species, L. luctuosum.* I allowed field-collected foragers of *L. luctuosum* to lay foraging trails to a food object in an arena with an enforced beveled-square shape, I then removed the barrier and ants, allowing the beetle to walk in an open arena with only the chemical cues. As with host trails, the beetles followed the naturally laid trail of the non-host sister ant species (A). I also made a bulk extract of the sister ant pheromones and applied the chemicals in a circle in the arena. I saw strong circular trail following of the bulk extract, as with host extracts (B).

lativentris preferred its host trail extract when it was at higher concentration than extracts from the sister ant species (Fig 4.7AB). However, as soon as I switched the sister ant extract to higher concentration, the preference flipped (Fig 4 GH). This indicates that the trail concentration drives preference, and the beetle simply follow the higher concentration trail regardless of whether it was laid by its host or sister ant. As with the grooming assay, I pushed further to test the phylogenetic range for the cues that *S. lativentris* would follow. I made bulk extractions of Argentine ant, which share no compounds with the host ant trail, but do have a similar class of trail compounds; the beetle did not follow these extracts (Fig 4.8) This indicates that the beetles specialize on the *Liometopum* trail chemicals but cannot distinguish

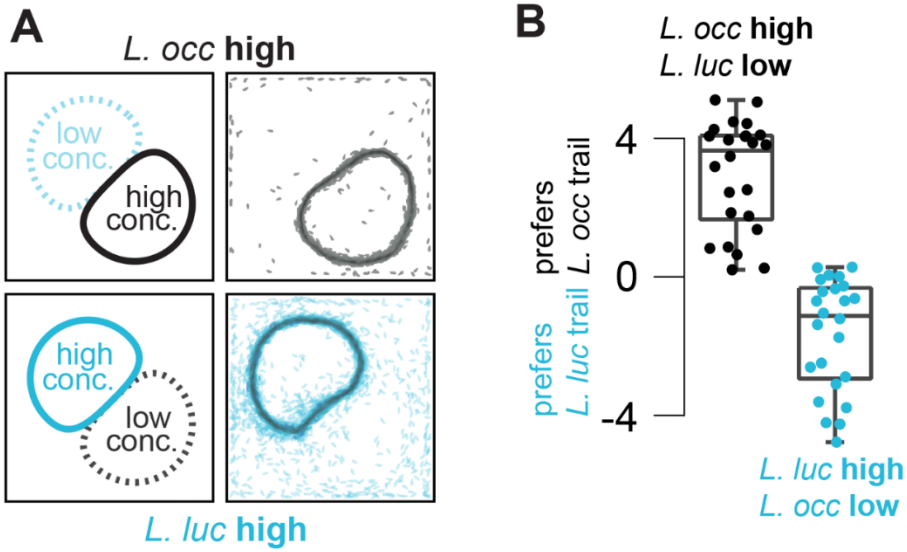

Figure 4.7. *Concentration, not species identity, drives trail preference.* I tested beetle responses to abutting semi-circular tails of different concentrations from the two species and found that the beetle followed the higher concentration trail, regardless of whether the extract came from the host or sister ant. (A) shows a representative trace of beetle position during a couple of runs, and (B) shows a measure (with arbitrary units) of the difference in total beetle movement in ROIs associated with host and non-host ant applied extracts.

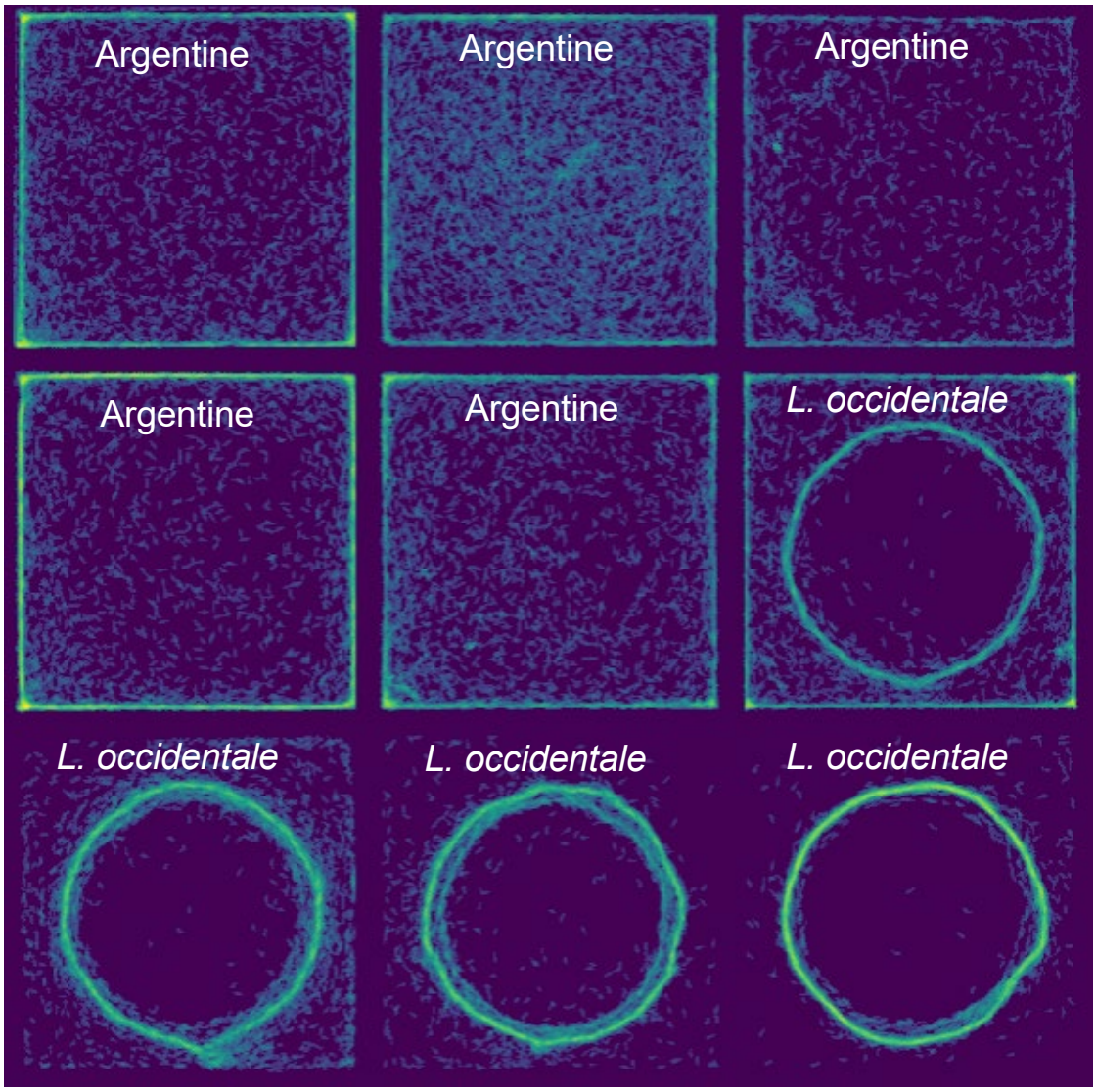

Figure 4.8. *Trails from another Dolichoderine do not release grooming behavior.* Though *S. lativentris* is promiscuous with trail following for host and sister ant trails, it does not follow bulk extract from the Argentine ant, another dolichoderine with a similar class (though they have no shared compounds) of trail chemicals. Shown is the movement between frames in the behavioral trials, indicating strong correspondence of beetle movement to applied host extracts, and no such accord with Argentine ant extracts.

between the trails of the sister ant species. Together, these experiments demonstrate that, despite its extremely restricted host usage, pheromones from non-host ant species release the symbiotic behaviors from *S. lativentris,* and the beetle shows no preference for its host when

confronted with a choice. This runs counter to the logic from literature on extreme host-specialists: despite its extreme host specialization, the cue space which releases symbiotic behavior from the beetle cannot explain its host specificity. Why, then, is the beetle host specific?

Spatial-aggressive enforced specificity underlies stringent host association

Sensory specialization fails to explain the host specificity we see between *S. lativentris* and its host. To identify the source of host specificity and generate testable hypotheses, we built an agent-based in-silico model of beetle-ant interactions and asked what conditions promote versus repress host switching between nests of different ant species. We based the model on three core observations of their life history (demonstrated also in Figures 4.9, 4.10):

1) *Ants attack insects when they have different CHCs from their own.* Ants are notoriously aggressive, and I had to glue the mandibles of many of the ants I presented to *S.*

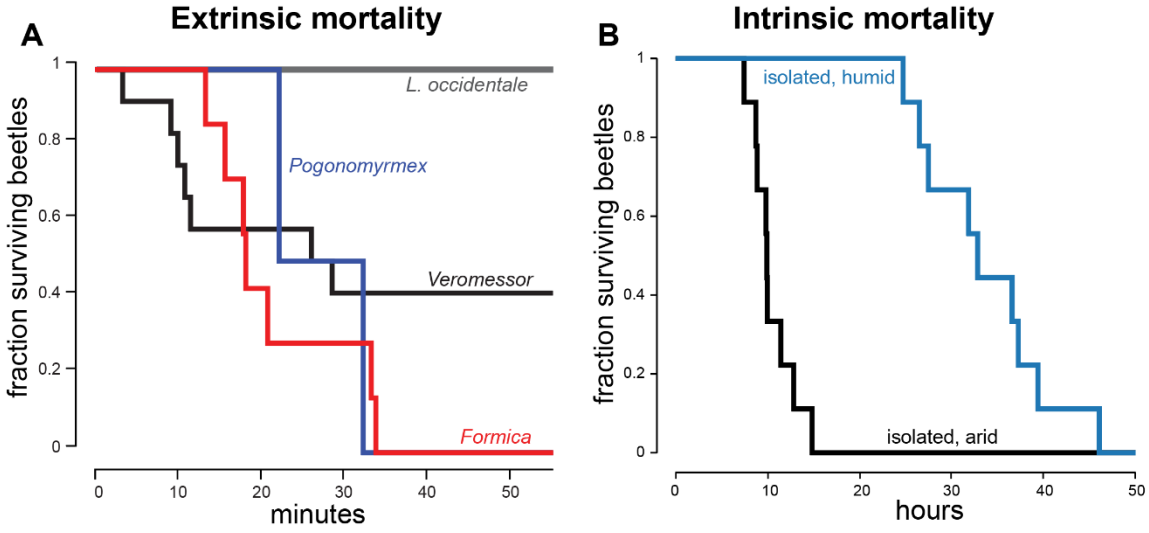

Figure 4.9. *Extrinsic and intrinsic mortality of beetles.* When placed in a confined arena with ants, divergent non-hosts immediately recognize beetles as non-nestmates and attack them. Within an hour, the ants have killed most of the beetles. When I isolated beetles from their host ant, they all died within a couple of days. They are at extreme hazard from desiccation, likely because they have highly reduced CHC (important for waterproofing) and lose CHCs when separated from ants (see Figure 4.10).

lativentris in the experiments in Fig 4.2 to prevent them from killing the beetles. Extrinsic mortality in the face of divergent non-host ants is nearly total when the mandibles of the ants are not glued (Fig 4.9). We encoded this information in a parameter ΔCHC_{ID}. The more different the CHCs between the beetle and an ant, the more likely the ant will kill the beetle in an encounter before the beetle can groom it to replenish CHCs.

2) *Beetles rapidly die when away from ants*. I have shown that beetles lose CHCs when away from ants (since they steal CHCs instead of making them) (Fig 4.10), leaving them susceptible to dying from desiccation (Fig. 4.9). Experimental evidence shows that arid conditions are a particular hazard to beetles, which die in less than a day when isolated in low humidity chambers (Fig. 4.9). Higher moisture levels rescue survival, with beetles lasting in isolation around three times as long with moisture, though all beetles die within a couple of days without hosts (Fig 4.9). I found that the longer beetles are away from ants, the

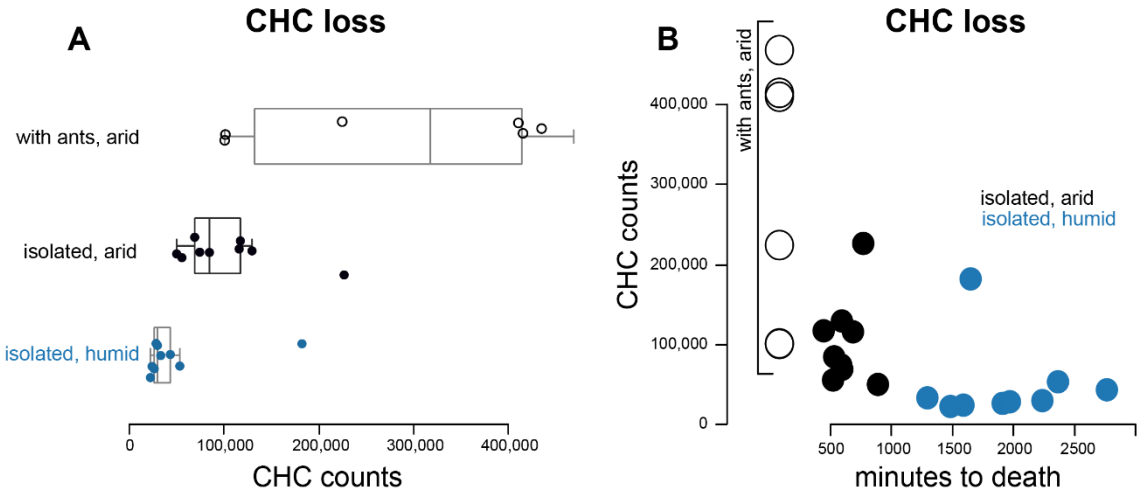

Figure 4.10. *CHC loss and desiccation as one mechanism of intrinsic beetle mortality.* When separated from ants, beetles lose their CHC and cannot replenish them. Humid conditions rescue some beetle survival, and these beetles also end with lower CHC levels at time of death, suggesting they better tolerate CHC loss when in less arid conditions. Loss of CHCs away from ants puts beetles at extreme risk and contributes to an intrinsic death timer on any dispersal away from ants.

lower their CHC level, leading to ever higher risk of desiccation. We encoded this information in a parameter ΔCHC_{loss}. Simulated beetles lose CHC away from ants and die when CHCs run out. When they encounter ants, they steal the CHCs and replenish their supply, if they survive the encounter.

3) *Ant nests are spatially separated*. Ant nests are separated by topographically complex natural substrate. We encoded the distance between nests the beetle was attempting to switch between in the parameter Δdistance. As linear distance between nests increases, the area the beetle need explore to locate ants goes as at a minimum of a power of two if the environment is flat, and area increases faster when natural environments are topographically complex.

Fig 4.11 summarizes these parameters and how they influence behavior of the model. With these assumptions, we instantiate an NxN grid of 'forest floor' tiles which the beetles and ants move in at each step, attack each other, groom to gain CHCs, and attempt to host switch (Fig 4.11). We ran the model for a thousand steps and recorded the number and reason for beetles' deaths, and whether they successfully switched to neighboring non-host ant colonies. This model predicted three regimes of interest that we experimentally verified: 1) beetles die from CHC loss death when ant nests are far apart, blocking host switching; 2) high aggression ants kill off beetles when nests are close together, preventing host switching; and 3) beetles can successfully host switch when ant nests are close together and non-hosts have similar CHCs as the host (Fig 4.11, panels 1-3).

I first tested the prediction that beetles would die when isolated and attempting to cross a navigation-cue-free-space to a new ant nest. I built a maze and tracked whether beetles

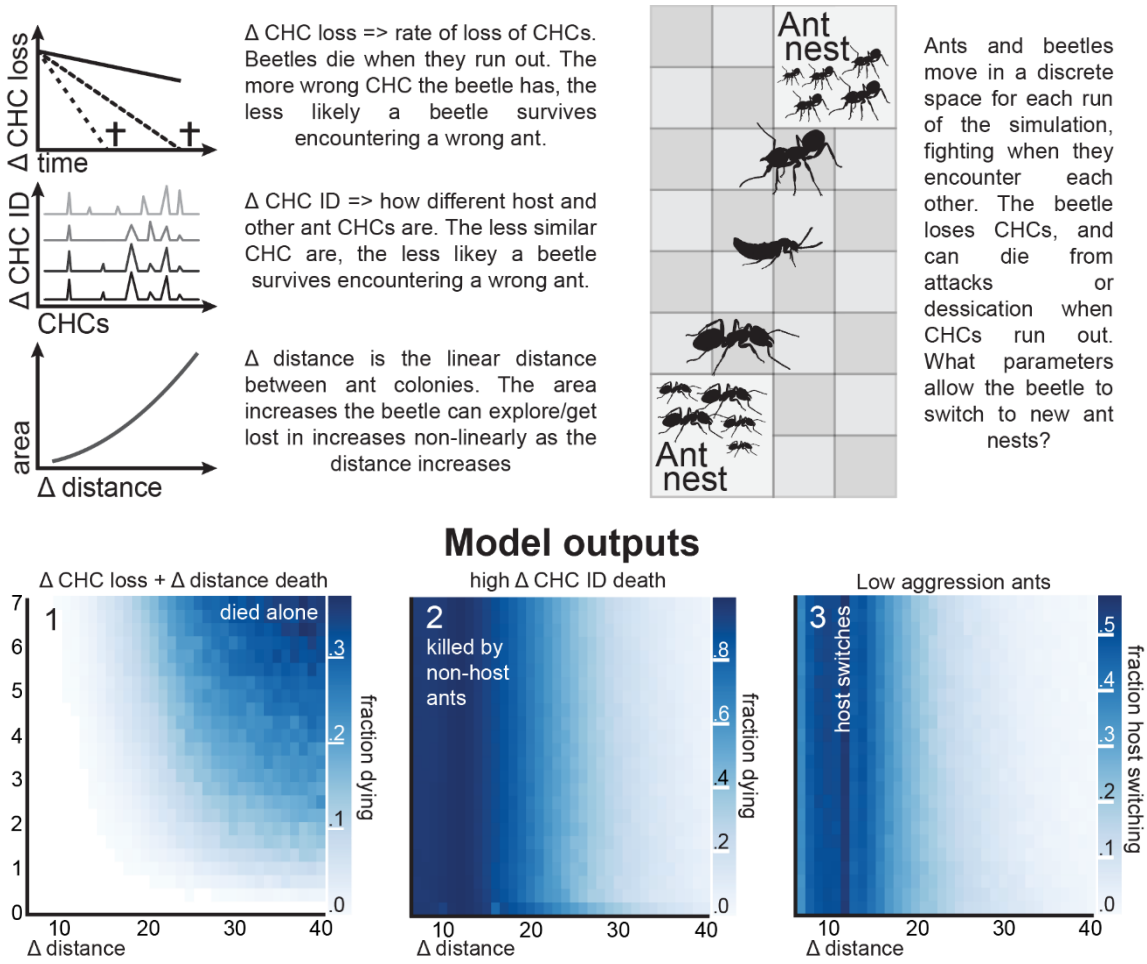

Figure 4.11. *In-silico model probing the conditions limiting and promoting host switching.* On top is a description of the model. The model made testable predictions (shown on the bottom): 1) beetles would die alone from desiccation as distance between ant colonies increased; 2) aggressive ants would kill beetles, preventing host switching; and 3) low aggression ants would often permit host switching when colonies were close together.

crossed to an ant nest (Fig. 4.12). The optimal path to cross the maze was ~4 meters. No beetles successfully crossed the maze, despites wandering for well over 100 meters in some cases (Fig 4.13A). Most beetles made it less than 2 meters through the maze and ended up dying less than a meter from their starting place (Fig 4.12, 4.13A). I measured the CHC levels of the beetles in the arena and saw a massive drop off in CHCs for beetles isolated from ants, matching exactly with our biological assumption the model (Fig 4.13B). Together, these data

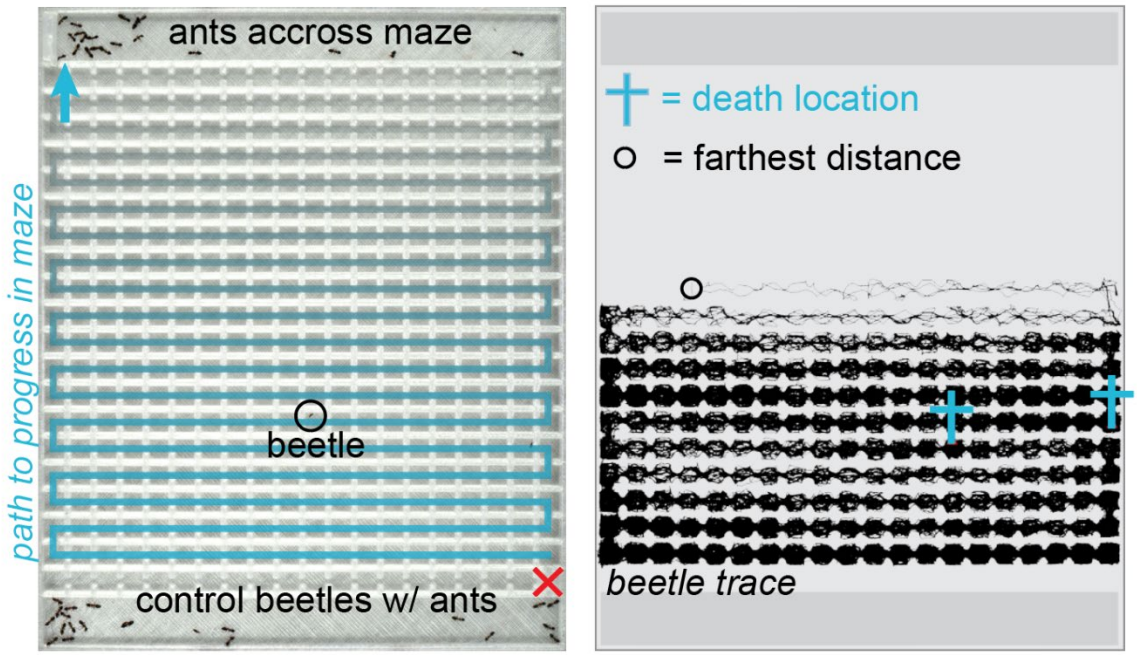

Figure 4.12. *We built a maze-style arena to see how far the beetle could walk while away from ants and whether if could cross to a new colony through tricky geometry.* Control beetles were housed with ants on one side of the arena but were inaccessible to the beetles in the maze. On the opposite side of the arena was a door large enough for beetles to enter, but not for ants to exit. None of the twelve beetles successfully crossed the maze (shortest traversal path ~4 meters), though they wandered for hundreds of meters in the arena. Even if they traveled far in the arena at some point during the trial, they ended up dying far from making it across. See also Figure 4.13.

support that even small linear distances between ant nests may be nearly insurmountable physical barriers for the beetles to navigate, strongly preventing them from switching ant nests sans navigational cues (e.g. ant trail). As linear distance between nests increases, the area of a topographically complex space the beetle need explore balloons, necessitating a nearly infinite walking distance before the beetle could find its host.

I next tested the prediction that, when in close enough spatial proximity to feasibly host switch, ants with a high ΔCHC_{ID} with the beetles would kill beetles, thus aggressively enforcing specificity. To do this, I built another arena variant with spatial structure but no

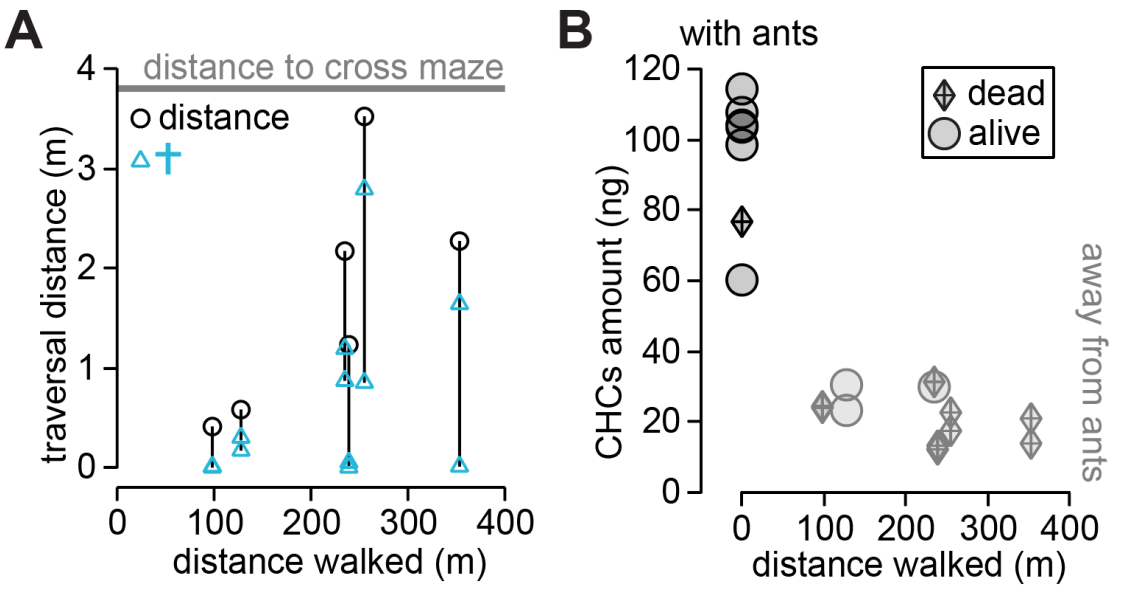

Figure 4.13. *Experimental confirmation of model predictions: dispersal.* Even if beetles made it a significant way across the arena during the trial, they often turned around at some point and ended the trial very far from making it to new ants (A). The beetles wandered for astounding distances in the arena, wandering for hundreds of meters without making it to the host ants. The beetles rapidly lost their surface pheromones when away from ants also, matching the model assumption, and most died in less than a day away from ants (B). Together, these support dispersal constraints and death-partially-mediated by CHC loss as mechanisms preventing host switching in *S. lativentris*.

maze (Fig 2.17). I placed various numbers of ants of different species on either side of the arena as well as beetles. Within hours, the CHC divergent ants killed the beetles (Fig 4.14). The lone beetle that survived one of these experiments was a trial with 20 hosts and 20 non-host ants, and the beetle survived by staying with its host ants and did not host switch. I saw similar results with three species of CHC divergent ants. This demonstrates that, even if the beetle does cross the gap between nests, any wrong CHCs it bears that differ substantially from a new ant nests profile trigger aggression and death at the mandibles of the ants.

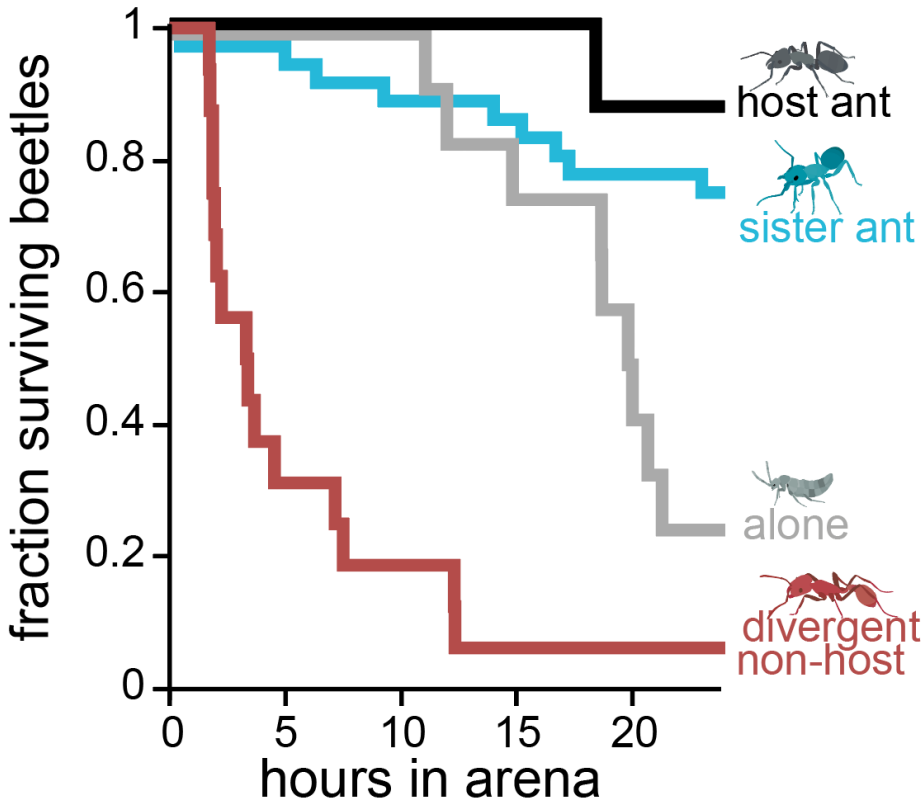

Figure 4.14. *Further experimental confirmation of model predictions: aggressive exclusion.* Beetles die when isolated in the cross arena. When faced with a highly aggressive, divergent non-host ant, the beetles faced extreme mortality, often dying in their first encounters with non-host ants. With the sister ant that had similar CHCs to beetle host, the aggression was much lower, and beetle survived at high rates.

Finally, I tested the prediction that a low ΔCHC_{ID} with a potential new host would allow the beetles to host switch. For these experiments, I used the sister ant *L. luctuosum* since it shares nearly all the CHC compounds with *L. occidentale*. I found high survival rates for the beetles placed with the sister ant species, across ratios of host and non-host ant number (Fig 4.14). Markedly, even when we ran 250 non-host ants against 20 host ants, all the host ants were killed by the sister ant species, but all the beetles survived. When confronted by a high number of non-host ants, the beetles not only survived, but also groomed the non-hosts and

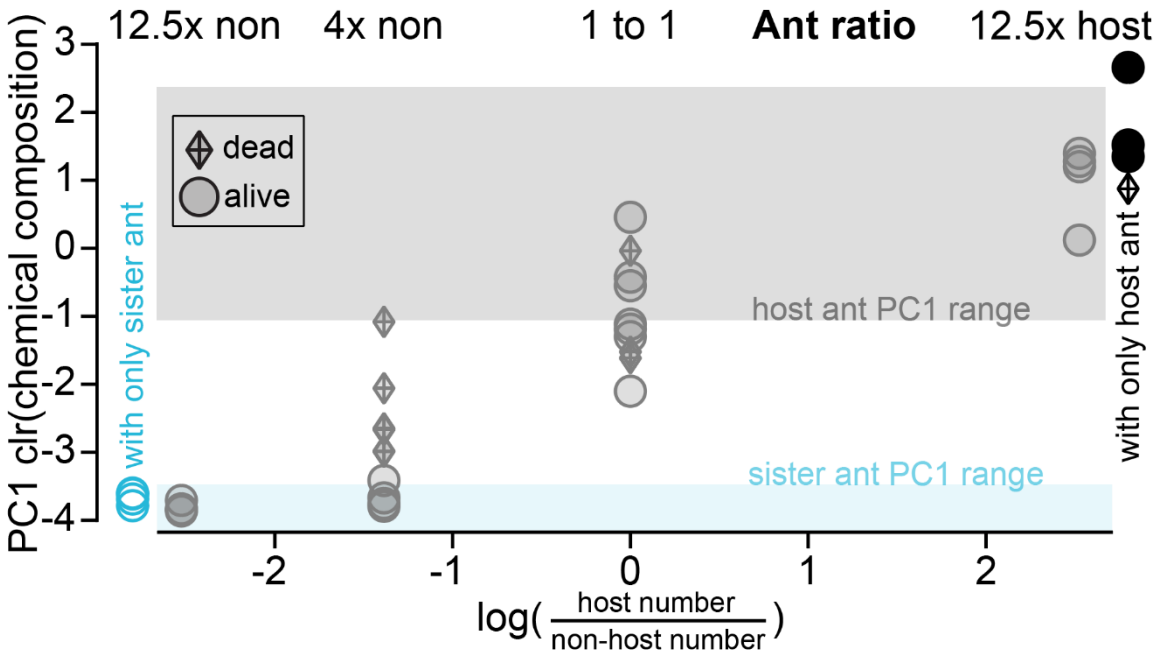

Figure 4.15. *Chemical analysis confirms host switching to sister ant, with chemical integration to novel ant related to proportion of novel hosts in arena.* Principle component analysis of the clr transformed vectors of chemical composition showed that host ants and sister ant species were fully separated by PC1. Plotted here is the PC1 coordinate of beetles that interacted with a varying ratio of host and non-host ants in the cross arena. Points on the left were at a high ratio of non-hosts to hosts, and show non-host like chemical profiles. Points on the right have a high ratio of host ants compared to non-hosts. Bands of color represent the minimal and maximal PC1 coordinates for ants in chemical space. When beetles survive the onslaught of non-host ants at higher numbers than hosts, they highly effectively acquire non-host profiles, thereby realizing a host switch.

acquired their pheromone profiles (Fig 4.15). The higher the ratio of hosts to non-hosts, the closer the pheromone profiles of the beetles to the non-host became (Fig 4.15). Beetles with intermediate pheromone profiles were animals that died during the run, having failed to host switch (Fig 4.15). This demonstrates that, given a low aggression ant in close proximity, the beetle successfully host switches in lab with high probability. The beetle survives encounters, grooms the non-host, fully acquires its pheromone profile, and integrates with the new ants,

surviving the run. After grooming the non-host, we have observed the beetle survive two weeks or more with the new ant in lab.

Together, our experimental and computational approaches demonstrate the strength of the spatial-aggressive enforcement barrier to host switching, as well as conditions that allow host switching. Beetles survive very poorly away from ants and can only explore a few meters of a complex environment before death. Ants with dissimilar CHCs completely annihilate the beetles if they do encounter them. Ants with similar CHCs offer little enough resistance to beetles that they can perform their symbiotic behavior and chemically integrate into their nests, though beetles would rarely encounter them given the spatial barrier.

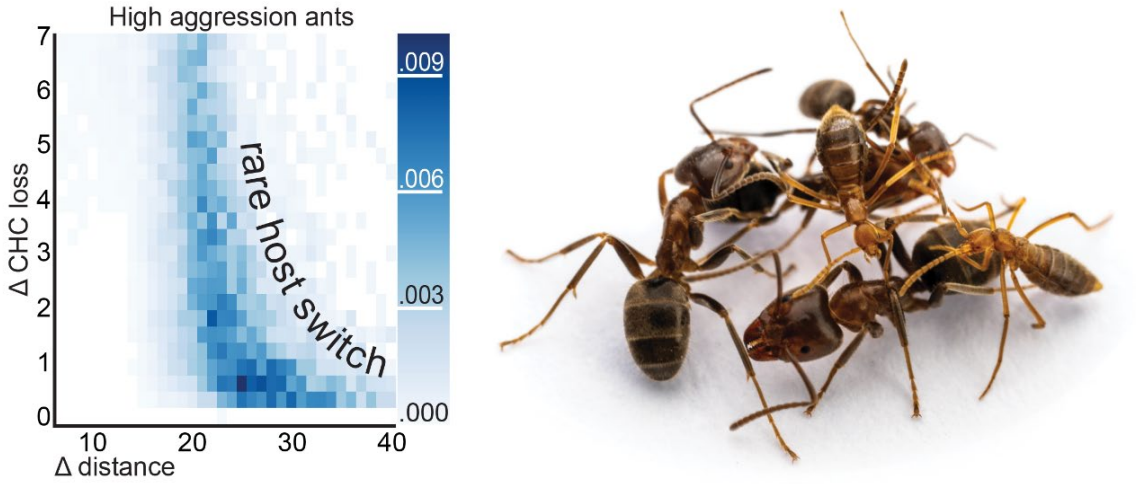

Figure 4.16. *Final predictions.* The model made one final prediction: even against a highly aggressive ant, beetles might be able to host switch at very low probability if they first lost a large amount of their CHCs by wandering away from ants and then encountered a non-host in this less-detectable state. We suspect that a similar mechanism may have underpinned the realized host switch event of a sister species to *S. lativentris,* namely *S. schmitti*, which uses at least two *Liometopum* species as host. We suspect also that *S. lativentris* may eventually host switch as well in ecosystems where its host and sister species are sympatric. Only time will tell if the beetles will realize such a host switch.

Our in-silico model predicts one final possibility: even against aggressive ants, if beetles wander enough to reduce their CHCs to near zero before encountering a non-host, they have a very small chance of host switching (Fig 4.16). Our own collecting efforts have uncovered a case of the sister species (*S. schmitti*) to our study organism host-switching from its documented host *L. luctuosum* to *L. apiculatum* (Fig 4.16), strongly suggesting that the latent host switching potential that we uncovered here has been actualized within this beetle genus, and additionally supporting the predictions of our model. One of our field sites in the San Bernardino mountains has a recent sympatry of *L. luctuosum* and *L. occidentale*. We have collected *S. lativentris* with *L. occidentale* but never *L. luctuosum* even when the ants were in close neighboring trees, suggesting the enforcement barriers we propose here have so far repressed host switching. Given enough time, though, I predict that the beetle might express its latent host-switching capacity and eventually expand its range to use the sister ant as a novel host. I summarize the spatial-aggressive enforcement model we propose (Fig 4.17). We also survey the proposed model of host specificity preventing *S. lativentris* from associating from the groups of organisms that we tested the beetle grooming behavior against (Fig 4.18).

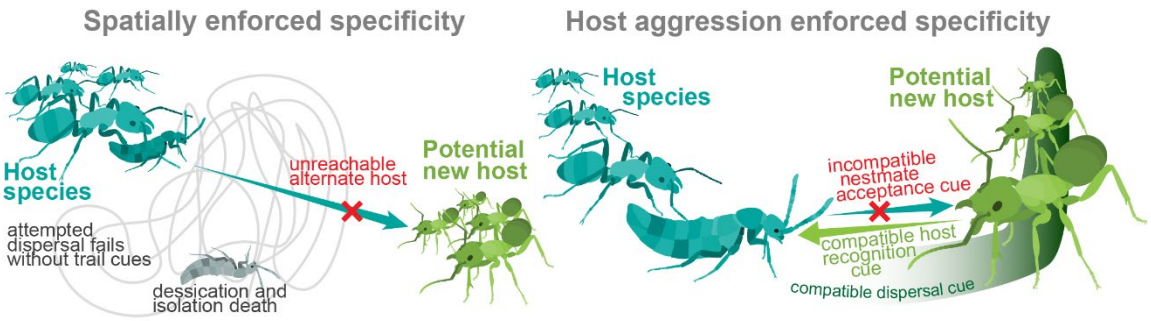

Figure 4.17. *A schematic of the enforced specificity mechanism described here.*

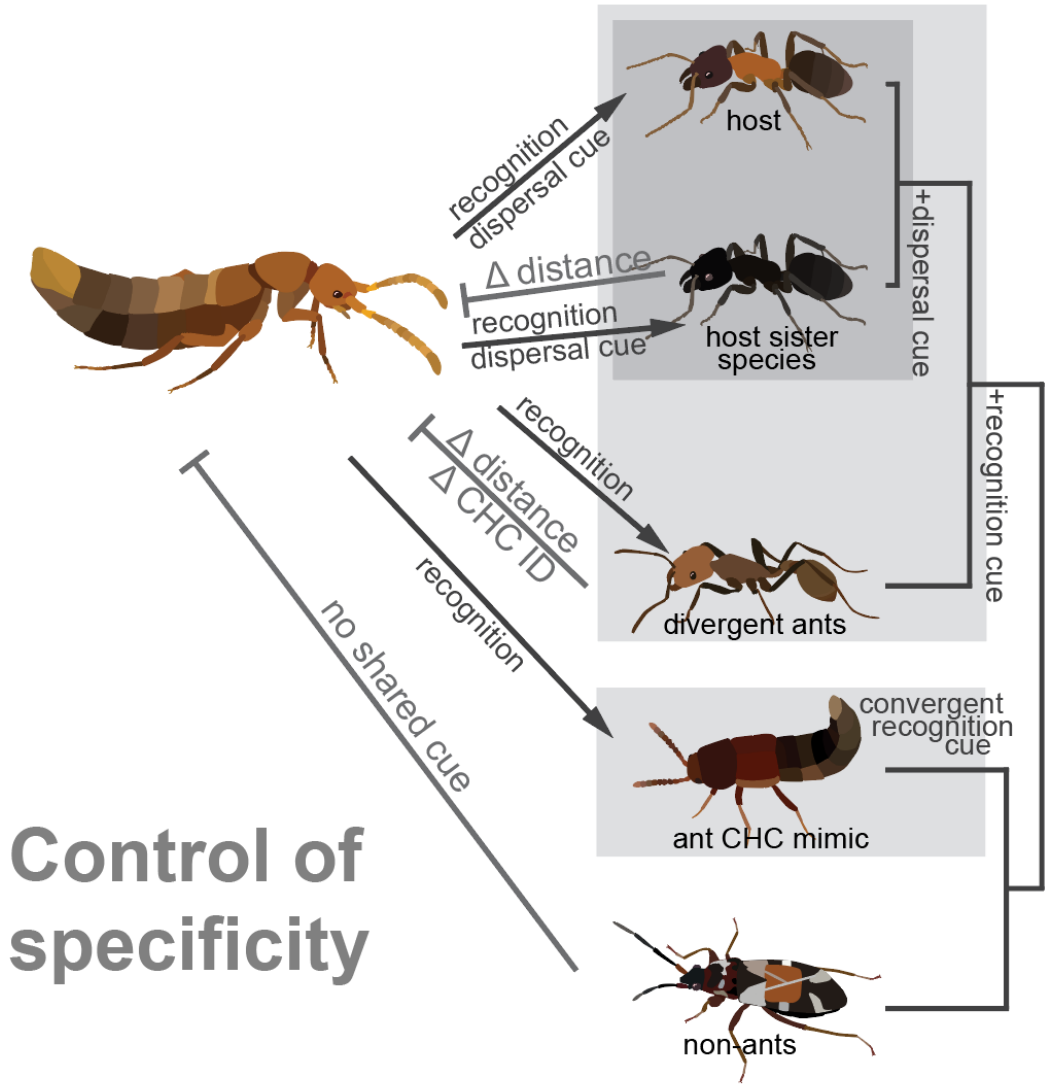

Figure 4.18. *A schematic of the mechanisms maintaining the specificity of S. lativentris with its host ant.*

Bringing it all together: insights from a myrmecophile to our understanding of host specificity

Knowledge of the sensory information that connects symbiotic organisms to their hosts is limited; so too is concrete experimental evidence about the forces that shape the often-strict fidelity of these partnerships. Using a tractable ant-myrmecophile model, I have identified the host-derived cues that govern host recognition and long-range dispersal behavior of the myrmecophile. Surprisingly, I uncovered a pronounced lack of chemosensory preference of the myrmecophile for its host, manifested in the beetle's equivalent ability to use cues from alternative ant species for social recognition and trail following. Hence, despite these host-derived cues possessing many species-specific features, they do not underlie the observed, stringent specificity of the myrmecophile towards its single ant host. Instead, I found that rapid senescence coupled with an inability to disperse to new ant nests without long-range dispersal cues strongly spatially enforces host association. Additionally, I found aggressive behaviors of alternative species towards the beetle that strongly reduce survival, and demonstrated through simulation that this aggression is sufficient to make host switching rare, enforcing the association of the beetle to a single ant species. I then showed experimentally that aggression acts as a barrier to host switching, matching the model.

I cannot rule out that unknown host-derived cues may exist that attract *S. lativentris* to its natural host over alternative ant species (perhaps odors from nest material, so far not studied by us). Nor can I be certain that *S. lativentris*' life history is compatible with alternative ants (though we hypothesize compatibility with at least congeneric ants that are biologically similar to its natural host). Regardless, my findings show that even if such impediments to host switching exist, spatial/external enforcement through aggression is by itself a major

barrier, capable of restricting the range of this symbiont to a single ant species despite the beetle's lack of host preference.

Enforced specificity contrasts with models of host specificity that invoke sensory tuning to host-derived cues. Such models have emerged from studies primarily of host-specific free-living species, such as phytophagous flying insects. For such organisms, an abundance of competing environmental stimuli may drive the evolution of sensory tuning, limiting non-adaptive interactions with off-target plants. Conversely, I propose that enforced specificity may be prevalent in intimate symbiotic partnerships, such as many parasitic relationships, where the potential for non-adaptive interactions with alternative hosts are scarce and impose weak selection for partner discrimination. The mechanisms that I outline here which maintain the specificity (dispersal and host defenses) are often evoked in literature seeking to explain parasite specificity, but rarely with the direct experimental evidence I have brought with our system. The latent attraction to novel hosts is typically non-adaptive when realized, but should such encounters arise sufficiently frequently, I predict that a sporadic host switching event will ultimately occur, just as countless specialists have switched host on ecological and evolutionary timescales. Ecological fit between symbiont and novel host will dictate whether the switched partnership attains evolutionary stability.

Observations of highly successful myrmecophile clades (Pselaphines, Histerids, Lycenids, Paussines) have revealed highly specialized symbiotic strategies as well as rampant and radical host switching events in their lineages (*77–79*, *112*). We suggest that many highly specialized symbiotic species have latent compatibility with different hosts that are rarely realized in nature due to the negative interactions with these alternate hosts or dispersal mechanisms preventing contact. However, in deep time, when coextinction

threatens specialist lineages, they may at low frequency host switch, thus averting their extinction. Latent compatibility with new hosts is likely crucial to deep-time persistence of symbiotic lineages.

Thus concludes my foray into the biology of a little beetle that lives symbiotically inside of ant nests. I hope that the reader can see how this seemingly esoteric microcosm represented by the *S. lativentris*-*L. occidentale* demonstrates many larger trends that shape our natural world in profound ways. How organisms recognize other species, how they decide who to associate with, and how factors wholly out of their control make these decisions for them are all considerations with deep implications for ecosystems, evolution, and all life on earth.

PUBLICATIONS IN ASSOCIATION WITH THESIS WORK

Naragon TH, Wagner JM, Parker J. **Parallel evolutionary paths of rove beetle myrmecophiles: replaying a deep-time tape of life**. Current Opinion in Insect Science. 2022 Jun 1;51:100903. https://doi.org/10.1016/j.cois.2022.100903

JMW participated in idea generation, literature search, figure making, and writing of this review article.

Sun JJ, Marks M, Ulmer A, Chakraborty D, Geuther B, Hayes E, Jia H, Kumar V, Oleszko S, Partridge Z, Peelman M, Robie A, Schretter CE, Sheppard K, Sun C, Uttarwar P, Wagner JM, Werner E, Parker J, Perona P, Yue Y, Branson K, Kennedy A. **The MABe22 benchmarks for representation learning of multi-agent behavior.** International Conference on Machine Learning (ICML) 2023, Article 1368, 32936-32990. https://doi.org/10.48550/arXiv.2207.10553

JMW contributed an annotated dataset of animal interactions.

Kitchen SA, Naragon TH, Brückner A. Ladinsky MS, Quinodoz SA, Badroos JM, Viliunas JW, Kishi Y, Wagner JM, Miller DR, Yousefelahiyeh M, Antoshechkin IA, Eldredge KT, Pirro S, Guttman M, Davis SR, Aardema ML, Parker J. **The Genomic and Cellular Basis of Biosynthetic Innovation in Rove Beetles.** Cell, in press, 2024. https://doi.org/10.1101/2023.05.29.542378

JMW participated in data generation.

Wagner JM, Wong J, Millar JG, Haxhimali E, Brückner A, Naragon TH, Boedicker JQ, Parker J. **Enforced specificity of an animal symbiosis.** In preparation, 2024

JMW helped with project conception, collected all the data, performed all analysis, generated figures, and drafted the manuscript.

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