Social Recognition in Ants and Trematodes

by

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Abstract

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Life can be hierarchically organized into units of reproduction that function as "individuals". In evolutionary theory, individuals are typically cells or multicellular bodies, but the boundaries or definitions of individuals are ambiguous. A working definition for biological individuals will likely always be circumstantial and debated, but biologists are not the only ones constructing boundaries around the organizations of living things. Cells, bodies, societies, and even species construct their own boundaries to their identities through recognition systems that filter what components are allowed to exist in these levels of organization. My dissertation broadly investigates social recognition in eusocial animals, specifically ants and trematodes. Ants are the model system for "superorganisms", or social groups that collectively reproduce using a reproductive division of labor. Trematodes (i.e. flatworms, blood flukes) are parasitic worms that are recently argued to possess a eusocial life stage while living in snail intermediate hosts. I begin with a review arguing why trematodes are comparable to other eusocial taxa, also explaining why the definitions and evolutionary theories of eusociality struggle to include polyembryonic parasites, such as the trematodes. Next, I report my experiments on colony recognition in my trematode species of interest, *Himasthla rhigedana*. I demonstrate that *H. rhigedana* are capable of distinguishing between conspecific colonies from different coastal marshes, directing more aggression towards trematodes from geographically distant subpopulations. I also suggest that this species is "facultatively" eusocial, as its soldier caste is not strictly composed of non-reproducing trematodes. Finally, I investigate the functions of the chemical cues used in social recognition in the invasive Argentine ant, *Linepithema humile*. Argentine ants use cuticular hydrocarbons to recognize members of their invasive "supercolonies", but these compounds also influence their desiccation resistance. Our experiments show that the ratio of compound classes from an ant's hydrocarbon profile determines their survival in desiccation assays. Virtually every level of life's hierarchical organization employs recognition systems, but we are far from universal theories explaining the function and evolution of recognition from cells to societies. This dissertation is intended to contribute to a comparative biology of recognition systems across taxa, and also validate the inclusion of parasite sociality as comparable to other forms of animal sociality.

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Acknowledgements

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Chapter 1. Introduction

THE ROLE OF RECOGNITION SYSTEMS IN BIOLOGICAL INDIVIDUALITY

"I have struggled with this issue all my professional life, and have often wondered why the questions raised seem so much more recalcitrant, and so much more cascading in implications, than for any other major problem in Darwinian theory."

Steven J. Gould, on the definition of "individuals" in biology

The most familiar organization across the hierarchical levels of life is the "individual". It is the Darwinian vehicle that unites collections of molecules and cells into cohesive units of selection (Hull 1980; Godfrey-Smith 2019). For Darwin, almost all natural selection could be explained by one level of selection (organisms) interacting with the environment. It was individual animals, for instance, that would struggle for survival and reproduction, not the cells or genes within the animal, nor the entire animal species. However, just as the Origin of Species challenged the idea of species as true categories, or static "essences", life's evolution from single cells to multicellular organisms implies that what we perceive as organisms or individuals are also not static or binary properties.

Darwinian individuals, or units of selection, appear to have been reinvented multiple times in a series of hierarchical transitions in individuality (Buss 1987; Maynard Smith & Szathmary 1997). Genes became organized into genomes, cells became multicellular bodies, and multicellular animals became superorganisms (e.g., eusocial insect societies, such as ant colonies). For Maynard Smith & Szathmary (1997), these are transformations on how heritable information is reproduced, as previously separately reproducing individuals evolved into groups that reproduced as one unit. For example, within a human body, our somatic cells multiply and propagate genetic information across asexual generations, but germinal cells (sperm and eggs) enable the entire human body to be replicated, and for heritable information to be transmitted at the scale of the human population.

A multicellular body is an easy target to witness scales of operation that appear united in one entity but, in the eye of natural selection, what scale is being selected on? Evolution by natural selection only "sees" phenotypes, but these can only persist and evolve throughout deep time if they are the product of heritable information (genotypes). Because all heritable information reduces down to genes in genomes, genes are arguably the ultimate unit of selection (Dawkins 1976), with organisms as the vehicle used by genes to interact with the environment (Dawkins 1982). However, units of reproduction and levels of interaction are inseparable in the logic of natural selection. Arguing against genes as the only unit of selection, Hull (1980) takes perhaps the most opposing position, declaring there are no units of selection because:

> "…evolution through natural selection requires an interplay between replication and interaction. Both processes are necessary. Neither process by itself is sufficient. Omitting reference to replication leaves out the mechanism by which structure is passed from one generation to the next. Omitting reference to the causal mechanisms that bias the distribution of replicators reduces the evolutionary process to the 'gavotte of the chromosomes'..."

How to decide amongst these opposing perspectives is confusing, especially since theories of evolution by natural selection withstand scientific scrutiny regardless of a consensus on *what* is actually being selected. Outside my window is a squirrel (*Sciurus niger*), and by commonsense it appears to be an individual, and many of its characteristics can be explained by natural selection whether its genes, or cells, or body, or family, or population, are the true units of selection. The commonsense "individual-ness" of this squirrel can be scientifically framed by many debated perspectives of individuality in biology (Buss 1987; Okasha 2006; Calcott & Sterelny 2011; Bouchard & Huneman 2013). To highlight one in particular, Godfrey-Smith (2019) would consider this squirrel to be an individual not only because it is capable of holistically reproducing itself (due to an organized germ-soma separation (Weismann 1893)), but the squirrel also has a clear "beginning", separating it from the previous squirrel generation from which it came because it developed from a single zygote. Additionally, the squirrel is also highly "integrated", composed of material organized into mutually dependent parts (tissue types, organs, homeostatic systems, etc.) with a boundary between what is and is not part of its body. In other words, we do not confuse the bark of the tree the squirrel sits upon as part of the squirrel individual.

None of the above criteria for individuality can be taken for granted. All of them only exist as perceived demarcations for individuality because of operations of the components of these collectives. Multicellular reproduction only exists because cells coordinate a germ-soma separation, or are at least organize both modular and sexual reproduction, like in plants (Clarke 2013). Additionally, a "bottleneck" origin of an individual, developing from a single zygote, is maintained through mechanisms guiding cells on an ontological pathway preventing cancerous or alternative forms of growth, and the integrated whole that a successful zygote eventually develops into is inevitably occupied by bacterial or viral symbionts. For multicellular bodies, the boundary that separates self from non-self is determined by their immune systems (Pradeu 2019), and the development towards a cohesive, predictable ontogeny and life history is upheld by the communication and cooperation of cells. Essentially, the boundaries of each individual are not strict or true, but temporary and functional, emergent from the actions of the components of the system.

This is the central inspiration for my dissertation on recognition systems in animal societies. We scientists and philosophers can debate how to demarcate self from non-self, guided by reductionist theories on the ultimate units that evolution by natural selection acts upon, but organisms make their own demarcations, using immune systems or recognition systems to decide what components constitute themselves. Cells use RNA interference to prevent foreign double stranded DNA from replicating inside of them (Obbard *et al.* 2009). Prokaryotes use CRISPR-Cas systems as an acquired immunity against viruses (Barrangou 2015). Multicellular bodies use innate and/or adaptive immune systems (Pradeu 2019; Buckley & Dooley 2022), and eusocial animals use recognition systems to control group membership (Tsutsui 2004; Penn & Frommen 2010). My interest in all of this theoretical biology came from an early appreciation for insect societies, specifically ants. Therefore, my research career begins with a focus on the recognition systems of the debatable "individuals" known as superorganisms, or eusocial groups with a reproductive division of labor analogous to the germ-soma separation of multicellular bodies.

In theory, recognition systems evolve to ensure the benefits of group living are directed towards kin (Hamilton 1964, 1987), to preserve their germ line (Grosberg *et al.* 1988; Buckley & Dooley 2022), or to reject threats to the group (or individual) such as parasites, pathogens, and

predators. Many theories explain why this evolves, but *how* organisms make these decisions is unknown in most systems. I chose a comparative biology approach and investigated how recognition works in two eusocial systems: one system that is a model of colony recognition in eusocial animals (ants) and one system that is a recently discovered eusocial animal but with virtually nothing known about its social behaviors (trematodes).

OVERVIEW OF DISSERTATION

My dissertation is composed of three research chapters. In Chapter 2, "The weird eusociality of polyembryonic parasites", I discuss how newly discovered trematodes fit into the history of comparative social evolutionary theories. Trematodes arguably fit in the definition of eusociality because of characteristics they share with other eusocial animals, specifically: overlapping generations, cooperative brood care, and reproductive division of labor. However, there is another parasite very similar to trematodes (polyembryonic wasps) that does not fit this definition because of a lack of overlapping generations. Being categorized differently despite their similarities invites one to challenge how useful these categorizations really are. I explain why both trematodes and polyembryonic wasps could be considered eusocial, while also highlighting how there are likely many parasitic taxa that could be invited into social evolutionary theories.

With the sociality of trematodes summarized, I then move into my goal of investigating trematode social recognition. In Chapter 3, "Colony recognition and competition in the eusocial trematode, *Himasthla rhigedana",* I tackle fundamental questions about what trematodes are capable of recognizing in their enemies. We know trematodes are capable of attacking heterospecifics establishing in their same snail host, but aggression towards conspecifics is only observed in one species, and in no species is it shown that trematodes recognize differences between conspecific colonies. I show that *H. rhigedana* treat some colonies with more aggression than others, that this discrimination varies by geography, and that attack rates between competing colonies are predominantly symmetrical (suggesting the recognition of enemies is mutual in these interactions). These results suggest that trematodes not only recognize other species as threats, but also recognize a gradient of differences between conspecific colonymates (clones) versus non-colonymates.

Our knowledge of colony recognition is greater in ants. We know that cuticular hydrocarbons are the cues used in nestmate recognition, we know which glands produce these cues, how they are acquired during development, and even which compound classes appear more useful for recognition. In Chapter 4, "Body size and cuticular hydrocarbon composition determine desiccation resistance in the invasive Argentine ant (*Linepithema humile*)", I investigate the possible trade-off between using hydrocarbons simultaneously for colony recognition and desiccation resistance (since hydrocarbon wax also prevents water loss). This is a project started by my lab that uses the invasive Argentine ant, whose cuticular hydrocarbons played a major role in their success as ecological invaders of California. In California, they have assembled into massive supercolonies (because of a shared colony odor across nests) and survive in more xeric conditions than their native habitats (so desiccation resistance is vital). Our results suggest that the proportion of hydrocarbon classes on an ant's exoskeleton (specifically linear vs. methyl-branched alkanes) does determine survival during desiccation, but that each compound within those classes does not have the same effect on survival.

Finally, in my concluding remarks (Chapter 5), I return these topics back to the overarching themes of comparative social evolution, the proximate mechanisms of recognition systems, and the role of recognition in the broader questions of biological individuality and major evolutionary transitions. In this, I emphasize yet again my future inspirations for a sub-discipline of parasite sociality, and my perspectives on the ultimate goals of researching how organisms decide who is “friend” and who is “foe”.

Chapter 2. The Weird Eusociality of Polyembryonic Parasites

A version of this chapter has previously been published and is reproduced here with my permission as the sole author.

Whyte, Brian A. 2021. "The Weird Eusociality of Polyembryonic Parasites." Biology Letters 17 (4): 20210026.

ABSTRACT

Some parasitoid wasps possess soldier castes during their parasitic larval stage, but are often neglected from our evolutionary theories explaining caste systems in animal societies. This is primarily due to the polyembryonic origin of their societies. However, recent discoveries of polyembryonic trematodes (i.e., flatworms) possessing soldier castes require us to reconsider this reasoning. I argue we can benefit from including these polyembryonic parasites in eusocial discussions, for polyembryony and parasitism are taxonomically vast and influence the evolution of social behaviors and caste systems in various circumstances. Despite their polyembryony, their social evolution can be explained by theories of eusociality designed for parent-offspring groups, which are the subjects of most social evolution research. Including polyembryonic parasites in these theories follows the trend of major evolutionary transitions theory expanding social evolution research into all levels of biological organization. In addition, these continued discoveries of caste systems in parasites suggests social evolution may be more relevant to parasitology than currently acknowledged.

I: The new eusocial systems

Eusociality is one of the most substantial guiding paradigms for social evolutionary research. Since its popularization in the mid-20th century (Wilson 1971), researchers across study taxa and discipline have engaged in a shared evolutionary theory for how animals evolve overlapping generations, cooperative brood care, and reproductive division of labor. Originally referring to certain species of Hymenoptera (e.g. ants, bees, wasps) and Isoptera (termites), the eusocial category has expanded to include species of aphids (Aoki 1977), thrips (Crespi 1992), shrimps (Duffy 1996), beetles (Kent & Simpson 1992), and naked mole rats (Jarvis 1981). Recently, however, larval colonies of trematodes (i.e. flatworms, blood flukes) are argued to be eusocial, following the discovery of morphologically distinct soldier castes (Hechinger *et al.* 2011), and this claim has received growing support (Newey & Keller 2010; Miura 2012; Garcia-Vedrenne *et al.* 2016, 2017; Poulin *et al.* 2019; Resetarits *et al.* 2020). This discovery is unexpected and exciting, extending our social evolutionary theories into a phylum (Playhelminthes) that seemingly had no relevance to social evolution research. It is very confusing, therefore, that this phenomenon of soldier larvae in a parasitic colony has been known in polyembryonic wasps since 1981 (Cruz 1981), but is still often rejected as an example of eusociality (Cruz 1981; Tian & Zhou 2014; Boomsma & Gawne 2018; Ode *et al.* 2018; Iwabuchi 2019), and even neglected from otherwise broad discussions of social evolution in wasps and Hymenoptera (Queller & Strassmann 1998; Rehan & Toth 2015; Rubenstein & Abbot 2017; Taylor *et al.* 2018; Linksvayer & Johnson 2019).

Polyembryonic wasps have been rejected from the category of eusociality because of their lack of overlapping parent-offspring generations, which is a requirement of the still popular

sensu-Wilson definition (Wilson 1971) . New definitions for eusociality have been proposed by multiple authors since the 1990s, and virtually all of them pull importance away from overlapping generations, focusing more attention on comparing taxa by their reproductive divisions of labor (Gadagkar 1994; Crespi & Yanega 1995; Sherman *et al.* 1995; Rubenstein *et al.* 2016). Unfortunately, updating the deep terminology of social evolution research has been slow and controversial (Costa & Fitzgerald 1996; Wcislo 1997), and arguably no new consensus has been reached (Costa 2018). The eusocial status of polyembryonic wasps remained unique and uncertain, until recently. Trematodes are also polyembryonic parasites with soldier castes in their larval colonies, but they technically do possess overlapping generations (Maule & Marks 2006). To consider only one of these systems as eusocial is confusing and contradictory; a case of semantics clouding comparative biology.

Overcoming this comparative confusion requires more than a terminological debate. Overlapping parent-offspring generations remains important because parental care (i.e. subsociality) is a firmly established prerequisite in evolutionary theories of sterile castes in animal societies (Downing *et al.* 2017; Toth & Rehan 2017). Beyond semantics, how do we account for larval colonies of parasites which have converged upon sterile helper castes absent of the family living context we observe is all other eusocial systems? Answering this is important, as polyembryonic parasites are useful exceptions to the eusocial norms receiving much attention from research on reproductive divisions of labor. Indeed, parasites are potentially full of undiscovered systems possessing behaviors convergent to social and eusocial taxa (Wickler 1976; Crespi 2001; Lopez *et al.* 2011; Secor & Dandekar 2020), and if we are going to include this incredibly common lifestyle in our social evolutionary theories, we can start by understanding the weird eusociality found in polyembryonic parasites.

II: Polyembryonic soldier castes do not require overlapping generations

Trematode and polyembryonic wasp species with soldier castes share similar life histories (Fig. 1) and selective pressures. In each case, an endoparasitic population originates from polyembryony, where a single egg splits into multiple embryos (Esch 2002; Strand 2009) and some of these embryos become morphological and behaviorally distinct soldiers, improving the fitness of their colony by attacking competitors developing in the same host (Giron *et al.* 2007; Lloyd & Poulin 2012). The single difference leading trematodes to be called eusocial, but not polyembryonic wasps, is that the first generation of trematode larvae descending from polyembryony continue to asexually produce new generations of larvae, while the polyembryonic wasp larvae do not (Fig 1B, D). For these parasites, these overlapping generations only highlight differences in polyembryonic development. Unlike the bees in which eusociality was first described (Batra 1966), the presence or absence of overlapping generations in these parasites does not determine what brood care behaviors or reproductive divisions are capable of evolving. Importantly, overlapping generations in most contexts refers to sexually mature stages of a life cycle spatially associated with offspring in earlier life stages, but this is never the case for polyembryonic parasites.

Trematodes are similar to the gall-forming aphids which can exhibit all the criteria of eusociality only during their asexual multiplication stage inside galls (Aoki 1977; Rubenstein & Abbot 2017). In trematodes and aphids, a multigenerational population can occur during these asexual life stages, allowing kin to specialize in caring for their developing siblings. In polyembryonic wasps, a “multi-developmental” population occurs, where the polymorulae descending from the same zygote develop at different rates on different pathways (Cruz *et al.*

1990; Strand 2009) with soldiers developing before their siblings become larvae or pupae. This allows a caste system and brood caring relationship to form within one short-lived generation, absent of parents, as brood care for each other.

This quality of non-helpless-brood is expected for parasite life cycles that use multiple hosts, or only use hosts for certain life stages, since each life stage might develop isolated from the previous, effectively preventing direct care across generations. It is also the norm for some termites, aphids, and other hemimetabolous social insects where the brood are precocial and perform tasks as juveniles (Crespi 1994; Queller & Strassmann 1998; Costa 2006; Howard & Thorne 2011). Despite their differences, polyembryonic parasites have converged upon a hallmark of other eusocial taxa (soldier castes), and they do not represent an alternative explanation for eusociality. If we look past their lack of overlapping generations or traditional parental care, these polyembryonic groups fit surprisingly well into modern theories explaining eusociality in animal groups with overlapping generations.

III: Family living facilitates social evolution, but so does polyembryony

Why are overlapping generations important for the evolution of eusocial groups? While convincing arguments have been made for why we can ignore this trait in our terminological categories (Crespi & Yanega 1995), parent-offspring grouping plays a fundamental role in the evolution of social behaviors via kin selection (Hamilton 1964), and is incorporated into many theoretical frameworks of social evolution (Alexander 1974; Maynard Smith & Szathmary 1997; Boomsma 2009; Bourke 2011; Socias-Martínez & Kappeler 2019). However, family living is not intrinsically important. It is the benefits and the consequences of family living that make it relevant to our evolutionary theories (Kramer & Meunier 2019). Family living is a proxy for more specific traits that facilitate social evolution, and polyembryonic groups achieve many of these same qualities (Table 1). For instance, family groups and clonal groups achieve high relatedness facilitating the evolution of cooperative or altruistic behaviors following Hamilton's rule (Hamilton 1964). The resilience and relevance of Hamilton's theories has contributed to the popularity of the subsocial (i.e. fraternal) hypothesis for evolving eusociality (Queller 2000; Rehan & Toth 2015; Linksvayer 2019), countering the alternative semi-social (i.e. egalitarian) hypothesis (Bourke 2011). Interestingly, even controversial alternatives rejecting Hamilton's rule (Nowak *et al.* 2010) suggest group living inside a shared food source can substitute a family living or kinship requirement. All endoparasites live inside their food source, and most, if not all, polyembryonic parasites are endoparasitic (Iwabuchi 2019).

An extension of the subsocial route to eusociality is the "lifetime monogamy" hypothesis, which predicts that the ancestral state of all eusocial groups with obligate sterile helpers was once both subsocial and monogamous (Boomsma 2009). A central argument for the importance of this bottleneck origin is that the offspring of a monogamous pair are the closest a sexually-produced group of animals can come to having a shared singular origin analogous to the zygotes of multicellular eukaryotic organisms. A polyembryonic colony is even more similar to this, being a group of animals literally descending from a single egg.

While these theories emphasize the role of kinship, others emphasize ecological conditions favoring sterile helper evolution. The "completely overlapping generations rule" (Downing *et al.* 2017) suggests that obligately sterile helpers can only evolve if they can commit their entire lifetime to raising their parent's brood. This commitment is made possible by living with a mother who lives longer than her offspring. In theory, a sterile helper can continue this

commitment even if the mother is absent, as long as her brood persist and need care (Downing *et al.* 2017). This is precisely the situation of polyembryonic parasites. For both trematodes and polyembryonic wasps, the mother of the polyembryonic egg is absent, but soldier morphs can spend their entire lives defending her offspring, never dispersing from their host. However, while polyembryonic wasps soldiers are sterile (Strand 2009), the totipotency of trematode soldiers is not yet ruled out.

Polyembryonic parasites are consistent with core principals meant for explaining eusocial groups of parents living with adult offspring. They join other parasite taxa (aphids, thrips) as examples of "fortress-defender" or "soldier-first" eusociality, in which a primary function of the non-reproducing caste is nest defense, rather than foraging, feeding, or housekeeping (Crespi 1994; Queller & Strassmann 1998; Tian & Zhou 2014). For this reason, it makes sense that authors don't include polyembryonic wasps in reviews of Hymenopteran sociality (Rehan & Toth 2015; Rubenstein & Abbot 2017; Taylor *et al.* 2018; Linksvayer & Johnson 2019), as virtually all ants, bees, and wasps fit a "life-insurer" or "worker-first" pathway to eusociality (Queller & Strassmann 1998; Tian & Zhou 2014), and parasitoid wasps are phylogenetically distinct from other social wasps.

IV: Where do the polyembryonic parasites fit in?

Fortunately, researchers of polyembryonic wasps are aware of their similarities to eusocial taxa, and study topics such as caste determination (Grbic *et al.* 1997; Strand 2009), caste allocation (Harvey *et al.* 2000; Watanabe *et al.* 2012), nestmate recognition (Giron & Strand 2004; Giron *et al.* 2004), and even sex-ratio conflict like in other Hymenoptera (Grbić *et al.* 1992; Gardner *et al.* 2007; Rautiala & Gardner 2016). While some authors claim they are eusocial (Crespi & Yanega 1995; Nishide *et al.* 2013; Carmel & Shavit 2020), others avoid explicit attribution of eusociality to polyembryonic wasps (Grbic *et al.* 1997; Harvey *et al.* 2000; Zhurov *et al.* 2004; Giron *et al.* 2007; Newey & Keller 2010; Segoli *et al.* 2010; Watanabe *et al.* 2012; Rautiala & Gardner 2016; Otsuki *et al.* 2019), or clearly state they are not eusocial (Cruz 1981; Tian & Zhou 2014; Ode *et al.* 2018; Iwabuchi 2019)[Supplemental Table 1]. I urge authors to not feel pressured to fit their system into the *sensu*-Wilson definition (Wilson 1971), for they can cite the *sensu*-Crespi definition (Crespi & Yanega 1995), and/or refer to them as fortress defenders (Crespi 1994; Queller & Strassmann 1998), as these terms were inspired by the discovery of parasites with soldier castes. The contributions from polyembryonic wasps on the evolution of reproductive division of labor are not invalidated by their lack of overlapping generations, and this character requirement was only popularized as an initial demarcation to guide, not blind, our search of eusociality across taxa (Wilson 1971).

How we categorize and compare eusociality in polyembryonic wasps and trematodes will change as we learn more about each system, and incorporate other instances of polyembryony and parasitism featuring divisions of labor (Craig *et al.* 1997). Cnidarias and bryozoans are both polyembryonic, but can exhibit division of labor among their polyps and zooids separate from a parasitic context (Ayre & Grosberg 2005; Lidgard *et al.* 2012). Additionally, while defense against competitors or predators is a common function of polyembryonic castes, it is not their only function (e.g., nutrient transfer in bryozoans (Jenkins *et al.* 2017); sex ratio optimization in polyembryonic wasps (Grbić *et al.* 1992; Gardner *et al.* 2007)). At the moment, trematodes might be the only taxon without a clear alternative function for their soldier castes. Hypotheses on their caste evolution can be informed with continued research on sociality in trematodes, and a better understanding of their phylogenetic relationships. For instance, trematode species

exhibiting a specialized role of the first reproductive larva (Sapp *et al.* 1998) could be viewed as a eusocial precursor, analogous to the dwarf eldest daughter in carpenter bees (Rehan *et al.* 2014), depending on how we build our phylogenies.

Parasitism, regardless of polyembryony, can facilitate the coincidence of food, shelter, and group living (Crespi 1994; Lopez *et al.* 2011), which are factors relevant to social evolution in all taxa. Unique to parasitism and other host-symbiont relationships, though, is the potential influence of the host on social evolution. In aphids and thrips, soldiers are associated with host plants that prolong gall formation (Pike *et al.* 2007; Rubenstein & Abbot 2017), but comparable metrics are not yet supported in polyembryonic wasps or trematodes. In theory, when parasite niches overlap, the fitness benefits of aggressive interference (and thus soldier morphs) should positively correlate with host characteristics that increase susceptibility (or exposure (Resetarits *et al.* 2020)) to parasite co-infections. We will learn more about the selective conditions favoring soldier castes with further understanding of parasite competitive ecology, which, serendipitously, is also a potentially useful avenue of research for medically relevant parasitology (Mideo 2009; Laidemitt *et al.* 2019).

Beyond comparing competitive contexts, the developmental biology of these parasites is also necessary for understanding soldier caste evolution. The detailed ontogeny of polyembryonic wasps shows how the sterility of soldier morphs is determined early in development (Strand 2009), but a similar depth of caste determination has yet to be described in trematode species with soldier castes.

V: Conclusion

Both trematodes and polyembryonic wasps possessing soldier castes can be considered eusocial, regardless of an overlapping generations criterion. Polyembryonic parasites have many differences from parent-offspring groups, but also possess many similarities that facilitate the convergent evolution of social behaviors and sterile castes. Polyembryonic parasites support the bottleneck origin of the lifetime monogamy hypothesis (Boomsma 2009), meet the special exceptions to the completely overlapping generations rule for evolving sterile castes (Downing *et al.* 2017), and are more comparable to a subsocial than semi-social route to eusociality (Bourke 2011). This highlights how important relatedness and ecological conditions are for social evolutionary explanations in any system. While trematodes possess overlapping generations of larvae, and polyembryonic wasps do not, at the moment this only amounts to a difference in polyembryonic development and caste determination.

Acknowledging the eusociality of polyembryonic parasites will build a constructive conversation around the special case of polyembryony for major evolutionary transitions theories (Maynard Smith & Szathmary 1997; West *et al.* 2015). An egg developing on a path towards one multicellular body, eventually splitting into multiple multicellular bodies, provides unique challenges to our concepts of biological individuality. For instance, a group of polyembryonic parasites could be considered a “modular organism” (Boomsma & Gawne 2018), like clonal plants or siphonophores. However, polyembryonic wasps separate germ and soma early on during embryogenesis, and the soma never exhibits modular reproduction, as occurs in other modular organisms (Strand 2009). In fact, the caste determination mechanism of polyembryonic wasps is perhaps their most fascinating contribution. Soldiers are polymorulae that never receive germinal cells (Strand 2009; Iwabuchi 2019). Their separation of castes is not functionally similar to a multicellular germ/soma separation: it *is literally the same mechanism* of

embryogenic cell differentiation. For this reason, polyembryonic wasps represent one of the greatest empirical confirmations of major evolutionary transitions theory, and the universal nature of social evolutionary principles across levels of biological organization.

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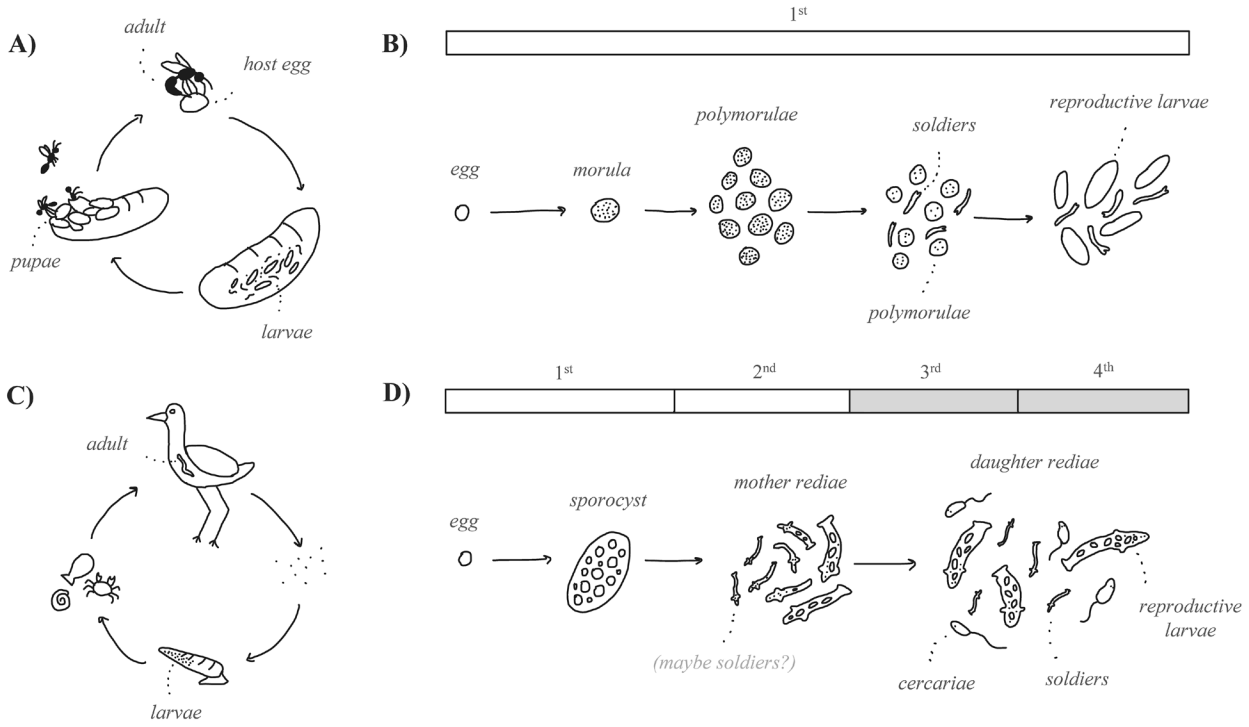

Figure 1: Life cycle and larval development in polyembryonic wasps and trematodes. A) Polyembryonic wasps (e.g., *Copidosoma floridanum* (Grbic *et al.* 1997; Strand 2009)) lay one or more eggs into hosts, which develop into larvae and pupae while in this host. **B)** The egg becomes a morula, splitting into polymorulae, which develop into sterile soldiers or regular larvae (i.e. "reproductive larvae") which pupate and become sexual mature. All of these developmental stages are technically one generation (white bar). **C)** Trematode (e.g., *Himasthla rhigedana* (Adams & Martin 1963; Erasmus 1972; Hechinger 2019)) adults lay eggs released from their vertebrate hosts, which find snails and multiply into a population of larvae. **D)** Trematode eggs develop into a single sporocyst larva, which produces the first generation of rediae (i.e., larvae with mouths). It is unknown if soldier morphs are also produced in this first generation, but soldiers are certainly present in the daughter rediae generation, as well as cercariae - the dispersive morph. Multiple generations overlap during the daughter generations (gray shaded bar).

Table 1: Similarities and differences between social groups featuring parent-offspring overlap (i.e., family living) versus larval colonies descending from polyembryony.

CHARACTERISTICS	FAMILY LIVING	POLYEMBRYONY
Spatial and temporal overlap of individuals	✓ Yes, living in the same nest	✓ Yes, living in the same host
High genetic relatedness	✓ 50, 75, or 100% related	✓ 100% related
Variety of developmental stages	✓ Yes, due to production of multiple generations	✓ Yes, due to embryos developing at different rates (polyembryonic wasps) or larvae asexually reproducing (trematodes)
Offspring help other offspring	✓ Foraging for non-self, nest defense, reproductive sacrifice	✓ Nest defense, reproductive sacrifice
Offspring help developing young	✓ Adults care for and/or defend brood	✓ Brood defend brood, even within the same generation.
Offspring help parents	✓ Adult offspring care for and/or defend mother.	× Mother is absent. Soldiers defend brood in her absence.

Supplemental Table 1: Ambiguity of polyembryonic wasps eusociality among authors comparing polyembryonic wasps to other eusocial taxa. These quotes are from select publications where authors are mentioning polyembryonic wasps in a discussion of eusociality, and either explicitly describe them as eusocial ("✓"), as not eusocial ("×"), or don't make an explicit decision on whether they fit a eusocial definition ("~"). It is important to note that this table does not include papers where soldier castes are broadly reviewed but polyembryonic wasps are not mentioned, or papers where polyembryonic castes are discussed, but eusociality is not mentioned. These conditions, too, shed light on how the literature treats polyembryonic wasps in comparison to other eusocial taxa. All of the mentioned sources can be found in the reference section of this dissertation.

SOURCE	QUOTE	INTERPRETATION
Cruz (1981)	"The presence of castes and intergenerational cooperation characterizes eusocial insects[8]. Although the polyembryonic wasps are not eusocial…"	× *Described as not eusocial.*
Crespi & Yanega (1995)	"Examples of taxa exhibiting facultative eusociality include some encyrtid wasps…"	✓*Described as eusocial.*
Grbic et al (1997)	"The dimorphic larvae produced by polyembryonic wasps clearly have different functions, just as the morphological castes produced by many eusocial species do."	~ *Comparable, but not called eusocial.*
Harvey et al. (2000)	"Morphologically specialized castes are well known in eusocial species like ants and termites[1], but castes have also evolved in less-studied groups like thrips, aphids and polyembryonic wasps."	~ *Comparable, but not called eusocial.*
Zhurov et al. (2004)	"In contrast with monoembryonic eusocial Hymenoptera, *Copidosoma* castes form clonally from the same zygote in the same host environment."	~ *Implies polyembryonic wasps are a "polyembryonic eusocial Hymenoptera", but does not explicitly state this.*
Giron et al. (2007)	"A more general constraint against male soldier development may thus underlie the tendency of mated C. floridanum females to produce mixed-sex broods and only infrequently oviposit single male eggs into hosts (Hardy et al., 1993; Ode & Strand, 1995). We also note that soldier/worker castes are exclusively female in other eusocial Hymenoptera."	~ *Implies polyembryonic wasps are eusocial, contrasting them against "other eusocial Hymenoptera".*
Segoli et al. (2010)	"Soldier larvae benefit their clone-mates by eliminating competitors inside the host, and die prematurely. This extreme form of altruism, comparable to that found among eusocial insects, might have evolved because of the high relatedness between clone-mates."	~ *Comparable, but not called eusocial.*

Newey & Keller (2010)	"High relatedness as a result of inbreeding is also at the origin of the evolution of eusociality among gall-forming thrips [2]. Finally, the evolution of a soldier caste in gall-forming aphids and polyembryonic wasps both occurred in groups of clonal individuals [10] … The discovery of eusociality in trematodes is interesting because the group is taxonomically very rich…"	*~ Thrips and trematodes are eusocial, while polyembryonic wasps are similar.*
Watanabe et al. (2012)	"In non-parasitic eusocial hymenopterans one of the difficulties is that genetic factors are expressed in at least two decision makers, the queens and workers[5,6]… A polyembryonic parasitoid wasp with sterile solder larval morph is highly suitable for the study of caste structure, because the genetic control is only via the single egg that produces many embryonic clones[9]."	*~ Implies polyembryonic wasps are "parasitic eusocial hymenopterans", but does not explicitly state this.*
Nishide et al. (2013)	"Eusociality, a level of social organization that is characterized by having a nonreproductive caste, has been observed in ants, bees, termites, aphids, thrips, and polyembryonic wasps.	✓*Polyembryonic wasps are eusocial.*
Tian & Zhou (2014)	"Interestingly, a physical soldier caste is also documented in non-eusocial animals. A trematode species (*Himasthla* sp.), which infects the California horn snail, *Cerithidea californica*, produces two morphologically distinct forms, a soldier morph and a reproductive morph, within the host… A physical soldier caste is also documented in a polyembryonic wasp, *Copidosoma floridanum* (Encyrtidae), during its larval stage inside the host.	× *Considers both trematodes and polyembryonic wasps as not eusocial.*
Rautialia & Gardner (2016)	"Our analysis concerns the function of the sterile-soldier caste of polyembryonic parasitoid wasps… we expect that these predictions will apply widely to female soldiers in many haplodiploid taxa (e.g., eusocial thrips; Crespi 1992) … the scope for genomic imprinting in relation to soldiering in diploids (e.g., eusocial trematodes; Hechinger et al. 2011) represents an avenue for future study."	*~ Thrips and trematodes are eusocial, while polyembryonic wasps are similar.*
Ode et al. (2018)	"Accordingly, the evolution of the soldier caste is often considered as equivalent to the evolution of non-reproductive castes in eusocial insects [39,42,46]. However, it should be cautioned that polyembryonic wasps do not share many of the basic characteristics that are thought of as pre-adaptations to eusociality such as parental care or cooperative breeding, suggesting a different evolutionary path."	× *Comparable, but not eusocial.*
Boomsma & Gawne (2018)	"…we have listed polyembryonic wasps (Giron et al., 2004) and trematodes (Hechinger, Wood & Kuris, 2011) with altruistic soldier castes that have been called	× *Acknowledges their claim to eusociality,*

	'eusocial', but are not fully comparable with the animals in the middle section because they live within compartmentalized hosts rather than in their own nests or burrows; their parasitism has also induced complexity reductions analogous to those in parasitic myxozoan Cnidaria which are reduced multicellular eukaryotes…"	*but explains why they are not comparable.*
Otsuki et al. (2019)	"Here, we report another unique case of adaptive self-sacrifice based on kin selection in C. floridanum… This phenomenon is related to kin selection, polyembryony, cloning, eusociality, after-birth sex ratio control, extraordinary sex ratios, adaptive suicidal behaviour and self-sacrifice."	*~ Comparable, but not called eusocial.*
Iwabuchi (2019)	"Although Encyrtidae are not eusocial in the strict sense of the term because they do not form intergenerational colonies, they develop precocious larvae as a sterile soldier caste, which is one of the basic features of eusocial insects." "5.2 Eusociality in Polyembryonic Encyrtids"	*~ Described as strictly not eusocial, but continues to use the term when discussing their sociality.*
Carmel & Shavit (2020)	"Plenty of formations that correspond to intermediate stages of the transition to eusociality currently exist. We selected seven different taxa as case studies for the transition to eusociality (table 1d)." [*The table includes polyembryonic wasps as an example*].	✓*Described as eusocial.*

Chapter 3. Colony recognition and competition in the eusocial trematode, *Himasthla rhigedana*

ABSTRACT

Some species of trematodes competitively exclude conspecifics and heterospecifics through the use of a soldier caste. How these trematodes can distinguish colonymates from competitors is not known. Here I examine patterns of colony discrimination in *Himasthla rhigedana,* a marsh-dwelling species of trematode that possesses a soldier caste. Aggression assays pairing colonies against multiple opponents demonstrate that *H. rhigedana* distinguish between conspecific colonies, consistently directing more attacks towards colonies collected from a distant marsh. I demonstrate that conspecific interactions between colonies are symmetrical (both colonies attack during encounters), and that the likelihood of aggression is the same whether the attacker soldier is "sterile" (soldier redia with no germinal balls) or an "intermediate" (soldier redia with developing germinal balls). These results provide a critical foundation for understanding colony discrimination in *H. rhigedana* and should help to inform studies of colony discrimination in other trematode species as well as other parasite taxa with similar competitive interactions.

I: Introduction

Parasites are typically aggregated, with a large fraction of the population clumped in a subset of the host population, not evenly distributed among them (Poulin 2011). This can be due to multiple factors, such as individuals multiplying upon successful host invasion, or specific hosts being more susceptible to multiple infections. When co-infections occur, competition can arise between conspecifics or heterospecifics, altering aspects of the parasites (decreased body size or colony size (Poulin 2011)) and their impact on the host (increased virulence and impaired host immunity (Choisy & de Roode 2010; Alizon *et al.* 2013)). Competition has long been considered as a selective force explaining parasite diversity and the niche partitioning of parasite communities (Holmes & Price 1986; Bashey 2015), but empirical or experimental studies of the parasite competitive interactions are lacking (Mideo 2009), and the impact of co-infections on host virulence is still complex and unpredictable (Alizon *et al.* 2013).

Just like interactions among free-living species, parasites can competitively exclude each other by consuming resources from the same host, sometimes occupying different infection sites during co-infection (Poulin 2011), or directly interfere with competitors through aggression (Sapp *et al.* 1998; Giron *et al.* 2007; Hechinger *et al.* 2011; Kapranas *et al.* 2016) or the release of toxins (allelopathy) such as bacteriocins (Martínez 2008; Ruhe *et al.* 2013). Allelopathy is likely only beneficial in the presence of competitors (Mideo 2009; Bashey 2015), and physical attacks as exhibited between parasitic helminths (Sapp *et al.* 1998; Hechinger *et al.* 2011; Kapranas *et al.* 2016) and polyembryonic wasp larvae (Giron & Strand 2004) require the parasites to recognize the presence and identity of their competitor. It is important to understand how parasites recognize and find their hosts (Haas 2003), and how host immune systems recognize and reject parasites (Hambrook & Hanington 2020), but if/how do parasites recognize other parasites?

Some communities of parasitic trematodes exhibit interference competition within their intermediate snail hosts, and this competition likely requires self/non-self-recognition (Coombe

et al. 1984; Tsutsui 2004). The human-infecting *Schistosoma mansoni* shares the same intermediate host as *Echinostoma paraensei*, and *E. paraensei* produces a specialized "precocious mother redia" which can recognize and consume Schistosome sporocysts (Basch & DiConza 1975; Sapp *et al.* 1998). Other Echinostomids and Philopthalmids using the California horn snail (*Cerithideopsis californiensis*) as their intermediate host produce soldier castes, where a portion of the redial infrapopulation has a relatively smaller body size and appear to not reproduce asexually, as they lack developing germinal balls (Hechinger *et al.* 2011; Leung & Poulin 2011; Miura 2012; Garcia-Vedrenne *et al.* 2016, 2017). These arguably eusocial colonies (Whyte 2021) live in dense populations of hundreds or thousands of clones, but recognize and attack up to 18 competing trematode species, often completely excluding each other, as co-infections are rarely observed (Sousa 1993; Mordecai *et al.* 2016). This distribution of host use could be explained by both "isolationist" and "interactive" dynamics (Holmes & Price 1986) but *in-vitro* observations of aggressive interactions and morphological adaptations for aggression suggest that competitive interactions could be effectively isolating colonies from co-infection.

If individuals in a trematode colony are capable of recognizing and rejecting multiple species of competitors, a number of questions can be raised. Are all species equally "recognizable", or are some species more difficult to recognize than others? Does a colony express the same aggressive effort towards all competitors, or does aggressiveness vary? Studies on trematodes with soldier castes have confirmed the soldier function by pairing trematodes against competitors in assays, and observing soldiers attacking more frequently than their larger "reproductive" colonymates, who almost never engage in aggression (Hechinger *et al.* 2011; Garcia-Vedrenne *et al.* 2016). When colonies of *Himasthla sp. B* are paired against *Euhaplorchis californiensis*, considerable variation is observed, with 10-60% of the soldiers showing aggression (Hechinger *et al.* 2011). A similar pattern is seen for *Acanthotrema hancocki* (previously *Stictodora hancocki*), where 0-50% of the soldiers fight *E. californiensis* (Garcia-Vedrenne *et al.* 2017). This variation could be driven by differences in the attackers (i.e. some colonies are more aggressive, aggressiveness may vary with colony development) or differences in the enemies (i.e. some are easier to consume, some may possess cues that stimulate greater aggressive responses). For instance, in ants, the decision to attack is typically determined by the perception of chemical cues from their enemy which are different from their own colony cues (Lenoir *et al.* 1999; Thomas *et al.* 2005; Guerrieri *et al.* 2009).

In trematodes, the cues used to recognize and attack competitors are unknown, but behavior assays can help determine if variation in aggression is due to intrinsic colony differences or perceived differences from a trematode's opponent. Here I use unidirectional assays pairing colonies of a dominant trematode competitor (*Himasthla rhigedana)* against multiple enemies to test hypotheses about inter- and intra-specific aggression. If aggression levels are the product of genetic predispositions for aggressiveness, I expect attack rates to correlate with the colony identity of the attacker, regardless of which enemies they are paired against. If aggression levels are determined by perceived differences in their enemies, I expect attack rates to correlate with the colony identity of their opponent. Simultaneously, my assay design tests the symmetry of aggression between *H. rhigedana* colonies (i.e., if one colony is usually more aggressive). These experiments establish fundamental properties of a colony recognition mechanism between parasites that influences the within-host competitive dynamics of trematodes, and potentially other parasites with similar competitive interactions.

II: Methods

Snail collection and maintenance. A total of 523 California horn snails (*Cerithideopsis californiensis*) were collected from mud flats in Bolinas lagoon (37.919966, -122.687907) and Morro Bay (35.345648, -120.836115), California, between July and September of 2020. At each site, California horn snails around 3 cm or more in length were collected from the mud surface. Previous research has shown that the parasite of interest (*Himasthla rhigedana*) is often found in adults in this size range (Sousa 1983). Permissions for collection of marine invertebrates was granted by the California Department of Fish & Wildlife and the Department of Parks and Recreation (SC-13858).

Snail hosts were brought back to the University of California, Berkeley and maintained in lab aquariums. These aquariums were tilted so half of the floor of the container was submerged in a salt water mixture (Instant Ocean®, 1.020-1.025 specific gravity), while the upper half was exposed to air. This allowed snails to move in or out of the water. The water was replaced every day from a stock solution constantly aerated using a small aquarium pump. Snails were maintained in ambient temperature and artificial lighting for a maximum of 2 weeks, otherwise they were not used in assay. This is because snail mortality in these lab conditions would begin after around two weeks.

Parasite identification and snail dissection. Snail hosts were screened for trematode infection by isolating snails in small containers of salt water and exposing them to sunlight for 1-2 hours, stimulating the release (aka "shedding") of cercariae; the trematode dispersing morph that finds the next host. These cercariae were morphologically identified to species using the most recently published key on the trematode parasites of California horn snails (Hechinger 2019). All cercariae were documented using pictures and/or videos (see supplemental material) to provide visual vouchers for future analyses. Snail hosts harboring colonies of *H. rhigedana* or *A. hancocki* were marked on their shells with nail polish and returned to their aquariums until dissection. No co-infections of these two species were observed. Co-infections of *H. rhigedana* were unobservable as it could only be tested with genetic markers. Therefore, all single infections of *H. rhigedana* are assumed to be one clonal strain.

A total of 157 snails were dissected, and 27 of these contained the colonies used in the experimental assays described in this paper. Snails were dissected by first fracturing their shell with a ball peen hammer to crack the shell, after which shell fragments were removed with tweezers. The gonads were partitioned from the mantle of the snail using a scalpel, and both body sections were submerged in salt water in separate small Petri dishes. The reproductive rediae of *H. rhigedana* were collected from the gonads, while the soldier rediae were collected from the mantle (specifically, the hemosinuses near the rectum), as this is where soldier morphs congregate, and is a likely nexus of host defense by these parasites (Hechinger *et al.* 2011). Rediae of *A. hancocki* were also collected from the gonads of their snail hosts. All trematodes were isolated from their hosts by pulling apart host tissue with forceps such that trematodes would be released into the salt water medium. Trematodes were individually moved to a separate petri dish with Ringer's solution (ThermoFisher Scientific®, Cat. No: J67572) using pipettes with 20 μl plastic tips.

The soldiers and reproductives of *H. rhigedana* are distinguished by their body sizes (Garcia-Vedrenne *et al.* 2016; Hechinger 2019), which was estimated for each individual during host dissection (each worm was not measured). Another defining feature of soldiers is their lack of offspring or germinal balls developing in their body cavity. Individuals were only identified as

soldiers if they also met this requirement. Some trematodes initially identified as soldiers did possess germinal balls once examined in the assay, raising the possibility that they were intermediate morphs transitioning to a reproductive stage, behaviorally or developmentally distinct from non-reproductive soldiers (Garcia-Vedrenne *et al.* 2016). Accordingly, the number of germinal balls in each soldier was recorded and included as a variable in subsequent analyses to assess if the number of germinal balls influenced behavioral outcomes (see "Statistical analyses). For this manuscript, I will refer to all of the worms selected for aggression assays as "soldiers", referring to soldiers possessing zero germinal balls as "sterile" and soldiers possessing greater than zero germinal balls as "intermediates".

Assay design. Behavioral assays were conducted by placing trematodes in 1-on-1 pairings in a well of a 96-well PCR plate filled with 200 μl of Ringer's solution. Two sets of assays were conducted:

1. Conspecific (ABC) assays. *H. rhigedana* from three source snails (two from Bolinas Lagoon, one from Morro Bay) were tested against each other (Fig. 1). Bolinas lagoon colonies were paired against each other to observe the symmetry of conspecific aggression. Specifically, 10 soldiers from one Bolinas colony were paired with 10 reproductive rediae from the other Bolinas colony and vice versa (A to B and B to A; Fig 1). Soldiers from both Bolinas colonies were also paired against reproductive rediae from Morro Bay (A to C and B to C; Fig. 1) to test if they could discriminate conspecifics from other marshes, and treat them with different levels of aggression. These trials were repeated 4 times with different colonies (n = 160 soldiers, from 8 colonies). All colonies used in these assays were collected from Bolinas Lagoon and Morro Bay in the same week.
2. Heterospecific (ABX) assays. *H. rhigedana* from two source snails (both from Bolinas) were tested against *A. hancocki* from one source snail (also from Bolinas) to test if trematodes show more aggression towards heterospecifics than conspecifics. These trials shared the same design as the ABC assays, but with *A. hancocki* replacing the Morro Bay *H. rhigedana* as the third colony. Soldiers from both Bolinas colonies were tested against each other as well, to provide more data points on aggression symmetry between conspecifics. These trials were replicated 5 times (n = 200 soldiers, from 10 colonies).

For all trials, I observed each pair of individuals (each well) for 30 seconds twice a day (morning and evening) for 3 days. Observations were conducted under 10x magnification and were filmed using an Amscope® microscope camera. An aggressive encounter was recorded if the soldier was seen using its pharynx to bite the surface (i.e. tegument) of the other animal enough to pinch and consume flesh, or if evidence of a previous attack was detected (e.g., the other animal was missing from the well or was partially mangled and the soldier possessed new material in its translucent and previously-empty pseudo-stomach).

To maximize the number of soldier replicates in each treatment, soldiers were not paired against their own colonymates as a control. This is because each snail host would only reliably yield around 20 viable soldier candidates upon dissection. My preliminary experiments, however found colonymates to never attack each other (n = 72 soldiers, from 15 colonies), so it is assumed that the number of attacks towards colonymates, would be zero.

Statistical analyses. Data for each assay were analyzed separately as they included different numbers of replicates and the assays were conducted roughly one month apart during the summer of 2020. Aggression was recorded as a binary character indicating whether a soldier

showed evidence of aggression over the three days it was observed. To test if a colony was more aggressive towards one opponent than another, I would sum the number of soldiers showing evidence of aggression (per colony). Because each colony was sampled twice for this experimental design, I used paired t-tests to compare the mean number of attacks from each colony in each treatment (e.g. A vs. B and A vs. C, Fig 1).

To further describe the magnitude of the differences between these means, I calculated an effect size, specifically Cohen's d with a Hedges correction, as the mean comparisons analyzed here were from a paired sampling design. These effect sizes were used to estimate power values, to also assess if Type I errors can be confidently avoided despite the small colony replicate number (ABC: n = 8 colonies, ABX: n = 10 colonies). Finally, to incorporate the possible effects of germinal ball development on soldier attack chance, I used a logistic regression model with opponent type and germinal ball count as the independent variables (covariates), and the binary character of aggression (1) or no aggression (0) per soldier as the dependent variable. All soldiers came from a few colonies, and colonies are clones descending from the same polyembryonic miracidium, so I included versions of each model with and without a random effect for colony identity. Comparisons between these models inform if the chance of soldiers engaging in aggression might be influenced by their colony identity, not just their opponent type or number of germinal balls.

III: Results

Soldiers in the ABC assay attacked conspecifics from Morro Bay significantly more than conspecifics from their own marsh, Bolinas Lagoon (Fig. 2, Table 1, p = .0069). All colonies showed equal or greater aggression towards conspecifics from Morro Bay ("C") than conspecifics from Bolinas Lagoon ("A", "B") (Fig. 3). In the ABX assay, soldiers attacked heterospecifics significantly more often than conspecifics (Fig. 2, Table 1, p = 1.467e-06), and all colonies showed greater aggression towards heterospecific enemies ("X") than conspecific enemies ("A", "B") (Fig. 3). In at least one colony from the ABX assay, all soldiers attacked all *A. hancocki* opponents, and there were no colonies where zero soldiers attacked their *A. hancocki* enemies. The effect sizes for each of these mean comparisons were large (Table 1, Cohen's d > 0.8), corresponding to high statistical power values (Table 1), despite the low colony replicate sizes.

Examining the symmetry of aggression in both assays, there were two instances of "complete asymmetry" – where one colony engaged in aggression, and the other did not. In every other pairing, at least one or more soldiers showed aggression. There were three instances of "complete symmetry" – where both colonies had the same number of soldiers engage in aggression. The difference in the number of attacking soldiers between the two colonies in each symmetry test was averaged across all tests, providing a mean aggression differential of 1.2 (range = 0-2). This means on average, one colony had 1.2 more soldiers biting than the other (i.e., 12% more worms engaged in aggression). This average was not statistically compared to any null hypotheses.

In 44% of all trials (across both ABC and ABX), the colony that showed greater aggression towards their conspecific competitor from Bolinas Lagoon ("A", "B") also showed greater aggression towards their conspecific competitor from Morro Bay ("C") and their heterospecific competitor ("X") (Fig. 3). In all logistic regression models the opponent type was a significant covariate of the chance for each soldier to attack, while the number of germinal

balls in each soldier did not have any significance as a covariate (Table 2). According to odd's ratios (exponents of coefficients) from the non-mixed effects models, soldiers in the ABC assay were 4.08-times more likely to attack Morro Bay conspecifics than Bolinas Lagoon conspecifics (Table 2, Model 1), while soldiers in the ABX assay were 9.84-times more likely to attack heterospecifics than conspecifics (Table 2, Model 3).

IV: Discussion

My experiments suggest that the eusocial trematode *Himasthla rhigedana* is capable of distinguishing colony-level differences among conspecifics, and that discrimination varies with geography. *H. rhigedana* from Bolinas lagoon consistently attacked conspecifics from Morro Bay more than conspecifics from their own marsh (Fig 2, Table 1). Assuming the cues used in discrimination are chemicals present on the trematode surface (i.e., the tegument, or the glycocalyx of the tegument), this pattern could be explained by chemical and/or genetic differences between the trematode sub-populations in each marsh. However, it is equally plausible that differences between snail hosts could influence colony recognition cues. Trematode populations in the New Zealand snail *Zeacumantus subcarinatus* are genetically diverse and are homogenous across marshes, while their snail host possess low diversity and appear geographically isolated (Keeney *et al.* 2009). This is likely due to trematodes being dispersed in their definitive shore bird hosts, while the snails only disperse across mudflats in their lifetime, and this is likely the case for *H. rhigedana* and the California horn snail host.

Trematode colony recognition cues, and the mechanisms for their production or acquisition, are completely unknown, so speculation on how snail hosts influence a trematode colony identity is very limited. Parasitic helminths are known to employ "molecular mimicry" to evade gastropod host immune systems, and the human infecting *Schistosomiasis mansoni* expresses host antibodies on its tegument to avoid snail innate immune systems (Hambrook & Hanington 2020). In theory, modification of surface compounds for immune evasion may also modify compounds used in colony identities. If snail hosts do influence colony recognition cues, then the variation in aggression observed in my experiments does not explain aggression variation between *H. rhigedana* in nature, as two different colonies only ever interact if their miracidia infect the same snail host. It would be useful to develop an *in-vivo* assay for observing colony interactions within a snail host, perhaps a method to sustain snail hosts in lab after their shells have been removed.

When one trematode distinguishes another as a non-colony-mate, can we expect the other trematode to be capable of the same discrimination? Essentially, can we assume trematode recognition and rejection is symmetrical, and not a biased affair of one colony acting as aggressors, while the other acts as recipients? My symmetry tests are descriptive, and do not test a threshold for "symmetrical" vs. "asymmetrical" aggression, but my interpretation is that aggression is predominantly symmetrical. Of all *H. rhigedana* colonies used in symmetry tests, only one pairing of colonies showed no aggression between them (Fig. 3), and in every other interaction, one colony would on average have 1.2 more worms showing aggression than their rival colony. Extrapolating this result into the hypothetical scenario of colony competition, this close-to-zero difference in aggressive effort between colonies is unlikely to be the deciding factor in a competition for the same host. At the very least, we can be confident that when two colonies of *H. rhigedana* interact, each colony typically perceives the other as a non-colonymate. Interestingly, if intraspecific aggression occurs in nature but is symmetrical and weak (with many soldiers not engaged in aggression), it could lead to each colony co-existing at low

infrapopulation sizes. In theory, this is identical to what would be observed if two colonies co-infected without being capable of recognizing or rejecting one another.

Despite their predominantly symmetrical participation in aggression, it is possible that some colonies are potentially innately more aggressive than others. In almost half of all assays (44%) one *H. rhigedana* colony ("A") showed greater aggression to both its enemies than the other *H. rhigedana* colony ("B") did. This pattern could not be explained by differences in recognition cues amongst the opponents, as each colony was paired against the same opponent ("C" or "X"). However, this pattern could also be explained by chance, and my regression models showed that models with or without a random effect for colony identity were almost identical in their coefficients, AIC scores, and the significance of each covariate was unchanged (Table 2). If soldiers from the same colony had consistently lower or higher aggression compared to other colonies, we would expect the colony identity to have a greater effect on the models, potentially decreasing the significance of the opponent type covariates (Table 2, "vs. Morro-HIRH", "vs. Bolinas-ACHA), or leading to relatively lower AIC scores.

The propensity to participate in aggression could have genetic correlates, or could vary with an individual's (or colony's) age, or experiences. Additionally, it could vary depending on if the soldier in question has invested in reproduction by developing germinal balls, which ultimately grow considerably in size and become daughter rediae or cercariae. The reproductive rediae of *H. rhigedana* are large and swollen with developing offspring, and their movement appears dramatically limited compared to soldiers with no germinal development visible. This is an obvious reason why a trade-off between reproductive investment and soldier function might exist. Initiating investment into germinal development may also be enough to stimulate a behavioral change in these "intermediate" *H. rhigedana* morphs, or intermediates may be behaviorally distinct because they are a different developmental pathway from seemingly sterile soldier morphs. The results of my regression models, however, suggest that the germinal ball count of a soldier did not influence the probability of engaging in aggression (Table 2). Only the type of opponent they were paired against explained aggressive outcomes (Table 2, "vs. Morro-HIRH", "vs. Bolinas-ACHA). This is an important result for assessing the comparability of *H. rhigedana* to other eusocial systems, for their sterile soldiers do not appear behaviorally distinct from the intermediate soldiers, and these intermediates could be transitioning to a reproductive stage, which would distinguish "facultative" as opposed to "obligate" eusociality (Crespi & Yanega 1995).

Since the discovery of a small "secondary" caste in trematodes, most species possessing these castes show aggression towards heterospecific competitors (Hechinger *et al.* 2011; Leung & Poulin 2011; Miura 2012; Garcia-Vedrenne *et al.* 2016, 2017), but have yet to show evidence of attacking conspecifics. For the trematode guild parasitizing California horn snails, competitive exclusion is a likely selective force driving the evolution of the 10 eusocial species (Hechinger 2019) in that guild, but for almost all species, it is unknown if discrimination of conspecifics also provides a fitness benefit, or if it even occurs. My experiments show that *H. rhigedana* are capable of distinguishing conspecifics, and therefore, this mechanism may play a role in intraspecific competition.

The intraspecific aggression investigated here could play a significant role in sustaining the coexistence of the diverse trematode guild of California horn snails, but needs to be investigated further. Genetic markers such as microsatellites, single nucleotide polymorphisms (SNPs), or ultra-conserved elements (UCEs) could identify whether single-species infections are actually co-infections of two conspecific strains. If conspecific coinfections can be identified this

way, then behavioral assays could assess if aggression is occurring within these co-infections. Currently, this is untested, but aggression between *H. rhigedana* found in the same snail host has yet to be observed, outside of the apparent consumption of dead cercariae birthed from rediae from the same snail host (personal observation).

Recognition of competitors is necessary for these direct interactions between competing trematodes, and is likely beneficial for within-host parasite competition in other parasite taxa (Mideo 2009; Bashey 2015). For parasites generally, how the host body recognizes and attack their parasites, and how the parasites recognize and find their hosts, are studied for their practical applications in improving host immunity, or preventing parasite transmission. How parasites recognize other parasite competitors, however, is virtually unstudied, but also has potential medical applications. Co-infections of competing parasites and pathogens drastically change the complexity, predictability, and severity of infection in some cases (Alizon *et al.* 2013). Understanding the dynamics of within-host parasite competition requires us to consider the perceptual world of parasites (Sukhdeo & Sukhdeo 2004), especially for systems which form colonies with social identities.

Acknowledgements.

I would like to thank Dr. Ana Garcia-Vedrenne and Dr. Ryan Hechinger for introducing me to the social trematodes of California, and showing me their methods for trematode behavioral assays. I also thank Benjamin Malit for his help in conducting the many preliminary experiments for this project.

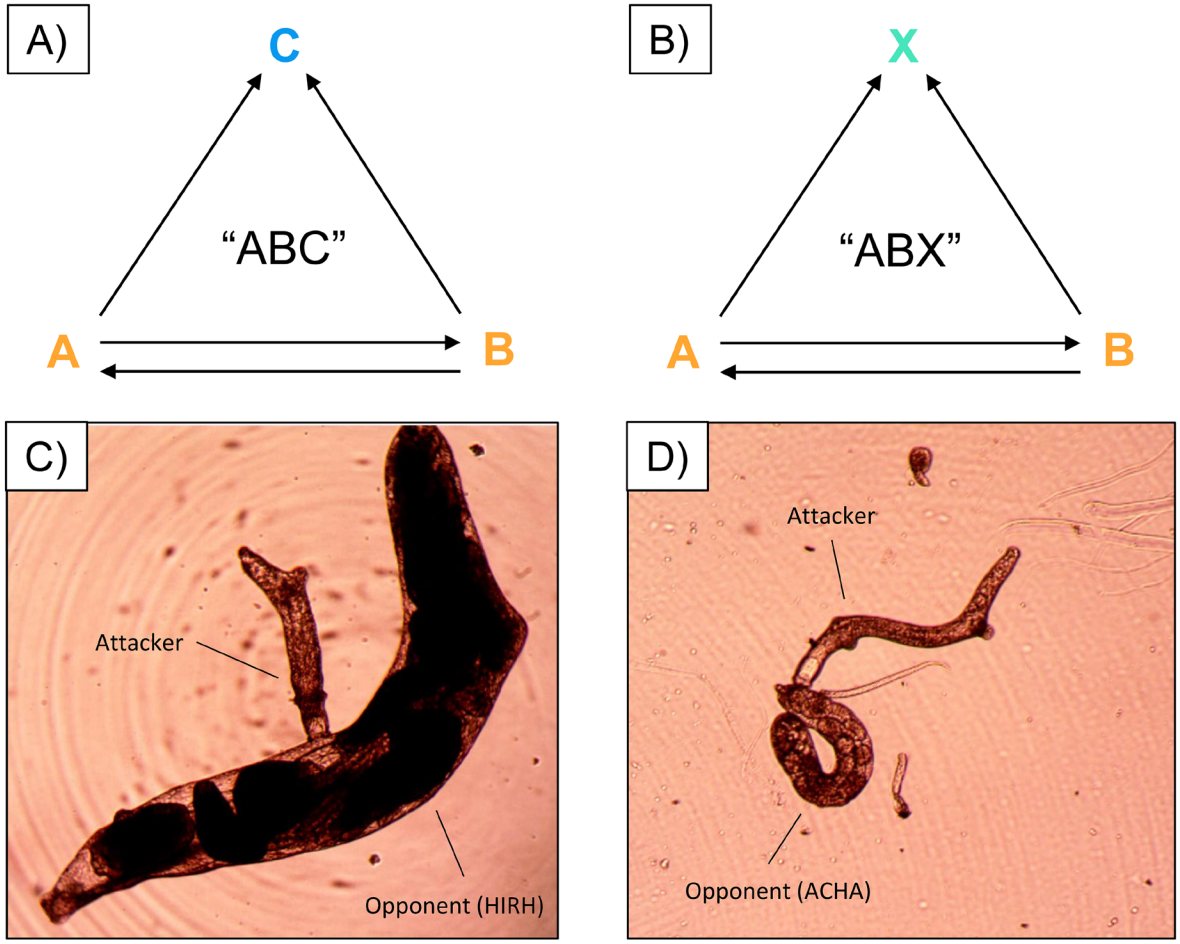

Figure 1. Assay designs. "A" and "B" (orange) refer to colony replicates of *Himasthla rhigedana* (HIRH) assayed against each other, and sampled from Bolinas lagoon. "C" (blue) symbolizes colonies of HIRH sampled from Morro Bay. "X" (green) symbolizes colonies of *Acanthotrema hancocki* (ACHA) sampled from Bolinas Lagoon. Arrows indicate the unidirectional aggression assayed in pairs of soldiers vs. reproductive redia. A) "ABC assay, designed to test if Bolinas HIRH show more aggression towards conspecifics from Morro Bay. B) "ABX" assay, design to test if HIRH show more aggression towards heterospecifics. C) Example of HIRH vs. HIRH interactions. D) Example of HIRH vs. ACHA interactions.

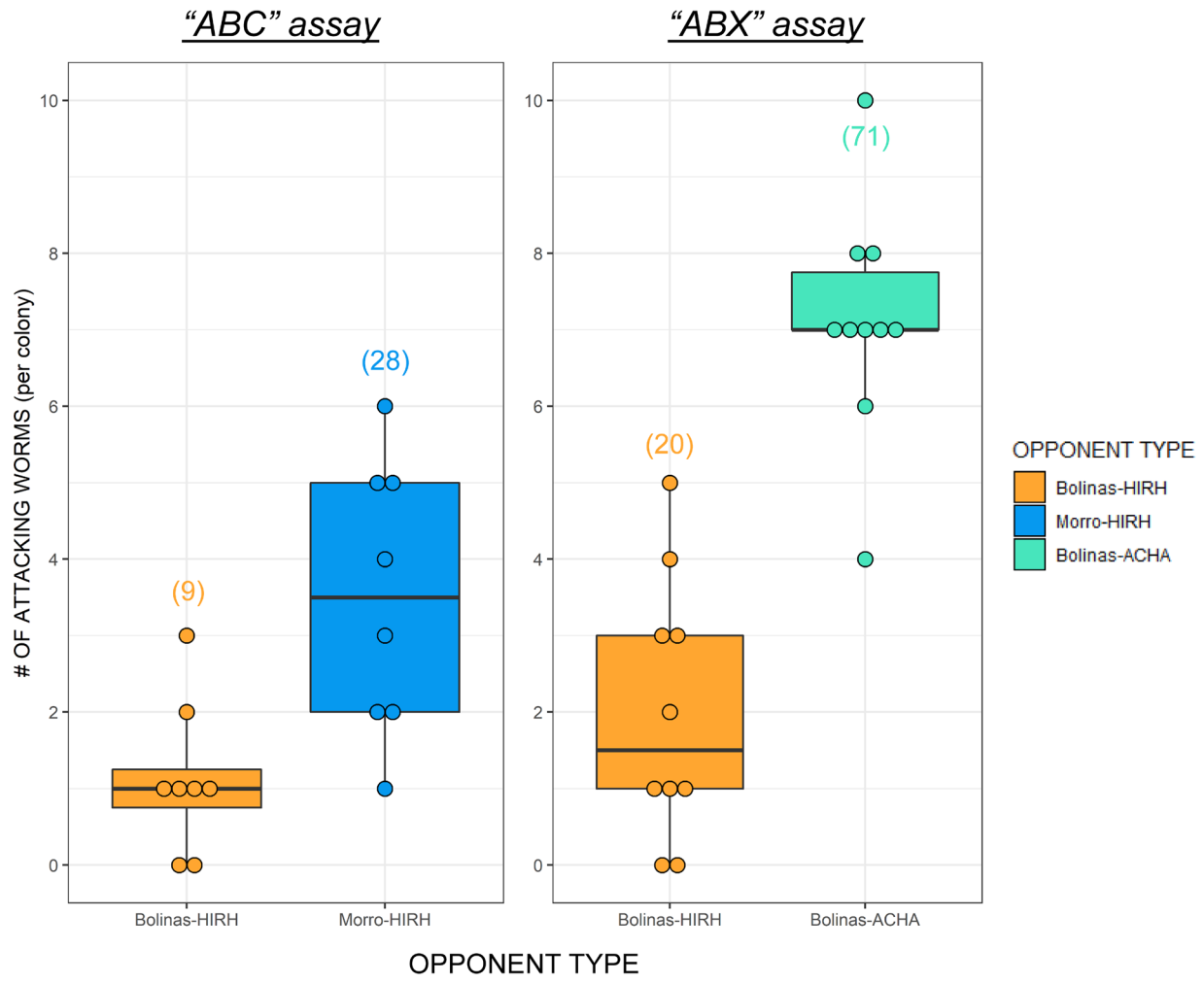

Figure 2. Aggression levels of *H. rhigedana* colonies toward a variety of enemies. Each dot is the sum of attacking worms from a colony paired against that opponent type labelled on the x axis (ABC n = 8 colonies, ABX n = 10 colonies). The colored number above each boxplot shows the total number of attacking worms across all colony replicates in that assay. "Bolinas-HIRH" = opponent *H. rhigedana* from Bolinas Lagoon. "Morro-HIRH" = opponent *H. rhigedana* from Morro Bay. "Bolinas-ACHA" = opponent *A. hancocki* from Bolinas Lagoon.

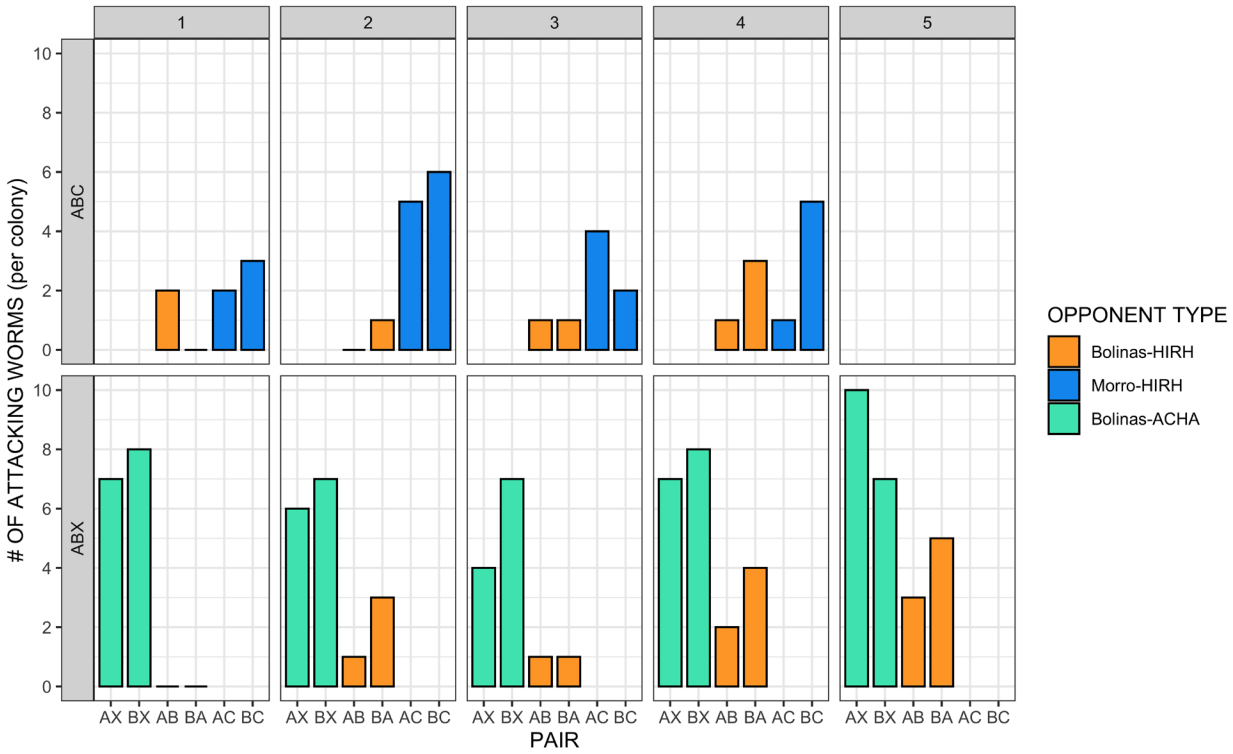

Figure 3. Aggression separated by trial. This is the same data shown in Figure 2, but expanded so the behavior of each colony in each trial can be observed. Each of the five columns is a trial (i.e. a replicate of an assay, using three colonies). Each of the two rows is an assay type (ABC or ABX). The pair abbreviations on the x-axis indicate which colony is attacking and which is receiving (e.g. "AB" = A soldiers paired against B reproductives).

Table 1. Statistical tests. All tests were separated by assay (ABC, ABX). Within each assay, colonies fought against two opponent types in a paired design (i.e., each colony was sampled from twice, one for each opponent to be paired against). Number of attacking worms was the dependent variable. Results for these paired t-tests are shown, as well as effect size estimates, and the statistical power estimated from those effect sizes. Conf. int. = confidence intervals.

Statistic	**Value**	**ABC**	**ABX**
Welch's paired t-test	*Num. pairs*	8	10
	t	-3.3075	-7.0649
	df	10.986	17.79
	p-value	0.0069	1.467e-06
	conf. int.	[-3.9557, -0.7943]	[-6.6179, -3.5821]
Cohen's d, with Hedges correction	*effect size*	-1.4654	-2.8851
	conf. int.	[-2.8099, -0.1209]	[-4.5176, -1.2525]
Power analysis	*power*	0.9220	0.9999

Table 2. Regression model comparisons. (1) Logistic regression using ABC data to test the effect of opponent type and germinal ball count on whether soldiers would attack or not. (2) Model 1 but including a random effect for colony identity, as multiple individuals would come from the same colony. (3) Logistic regression using ABX data to test the effect of opponent type and germinal ball count on whether soldiers would attack or not. (4) Model 3 but including a random effect for colony identity. Table created using stargazer R package (Hlavac 2015).

	Dependent variable: "Attacked? (0/1)"			
	ABC	*ABC (mixed effects)*	*ABX*	*ABX (mixed effects)*
	(1)	(2)	(3)	(4)
ABC opponent type	1.407***	1.458***		
	(0.442)	(0.431)		
ABX opponent type			2.287***	2.348***
			(0.328)	(0.342)
Germinal balls	−0.026	0.046	0.105	0.102
	(0.129)	(0.137)	(0.112)	(0.114)
Observations	169	169	211	211
Log Likelihood	−83.729	−83.386	−116.618	−116.209
Akaike Inf. Crit.	173.458	174.772	239.237	240.417
Bayesian Inf. Crit.		187.292		253.825
Note:				*p<0.05; **p<0.01; ***p<0.001

Chapter 4. Body size and cuticular hydrocarbon composition determine desiccation resistance in the invasive Argentine ant (Linepithema humile)

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This chapter has been written with co-authors and is included here with their permission.

ABSTRACT: An insect's cuticle is typically covered in a waxy layer of hydrocarbons that are involved in desiccation prevention and chemical communication. In Argentine ants (*Linepithema humile*), cuticular hydrocarbons (CHCs) communicate colony identity, but also provide waterproofing necessary to survive dry conditions. Different classes of CHC compounds are thought to create functional trade-offs, such that selection for compounds used in communication decreases waterproofing and vice versa. We collected supercolonies of Argentine ants from across California to test if CHC differences between them can explain differences in their survival against desiccation. We found that a larger ratio of methyl-branched to straight-chain alkanes was associated with decreased survival under increased desiccation stress. However, when only a subset of compounds potentially involved in profile acclimation to xeric conditions was considered, the opposite pattern was observed. Our results suggest that selection on or upregulation of individual compounds – rather than entire classes of compounds – may allow for optimization of CHC profiles with multiple functions.

I: Introduction

The Argentine ant *Linepithema humile* (Mayr) is a globally widespread and damaging invasive species (Holway *et al.* 2002a) that was first introduced to North America in 1891 and established in California by 1907 (Suarez *et al.* 2001). In its introduced range, Argentine ants displace native ant species, which can lead to cascading impacts on other organisms (Sockman 1997; Suarez *et al.* 2000; Christian 2001; Laakkonen *et al.* 2001; Fisher *et al.* 2002; Holway *et al.* 2002a; Suarez & Case 2002). Argentine ants are also significant structural pests (Klotz *et al.* 2008) and cause both direct and indirect agricultural damage (Newell & Barber 1913; Horton 1918; Way 1963; Markin 1970; Buys 1987, 1990; Visser' *et al.* 1996; Holway *et al.* 2002a; Lach 2005). The invasive success of Argentine ants results, in large part, from the unusual colony structure of introduced populations (Tsutsui *et al.* 2000; Tsutsui & Case 2001), which form geographically vast (>1000 km long) supercolonies that lack territory boundaries, allowing these ants to dominate invaded ecosystems through sheer numerical superiority (Holway *et al.* 1998). In most introduced populations, widespread genetic homogeneity correlates with uniformity in the cuticular hydrocarbon (CHC) profiles that are used for nestmate recognition (Torres *et al.* 2007; Brandt *et al.* 2009a; van Wilgenburg *et al.* 2010). Although a single supercolony occupies nearly the entire introduced range in California, several small, genetically and chemically distinct colonies also occur in southern California, likely originating from separate introductions (Suarez *et al.* 1999; Tsutsui *et al.* 2000; Tsutsui & Case 2001).

In general, as major components of the waxy lipid layer secreted by the epicuticle of insects, CHCs constitute a barrier that protects against desiccation and microbial infections (Blomquist *et al.* 1987, 1998; Gibbs 2002, 2011; Blomquist & Bagnères 2010). Small-bodied animals such as ants have a high surface area to volume ratio, making them particularly

susceptible to desiccation. Argentine ants in particular show lower survival rates in xeric habitats compared to other ant species (Holway *et al.* 2002b; Schilman *et al.* 2005). CHCs of Argentine ants are comprised primarily of straight-chain *n*-alkanes and straight chain *n*-alkenes, as well as mono-, di- and tri-methyl-branched alkanes (Brandt *et al.* 2009b; Buellesbach *et al.* 2018). Each of these CHC classes possess different physical characteristics that determine their efficacy in preventing water loss through the cuticle.

For example, lower melting temperatures (T_m) correspond to higher cuticular permeability and lower efficacy in preventing desiccation (Gibbs 2002, 2011; Blomquist & Ginzel 2021). Within CHC classes, T_m increases with longer carbon chains (Blomquist & Ginzel 2021). Across classes, for CHCs of equal carbon chain length, *n*-alkanes have 20 to 50 °C higher T_m than CHCs that possess double bonds or methyl branches (Gibbs & Pomonis 1995; Gibbs 2002). Thus, selection for higher desiccation resistance in xeric habitats should favor the evolution of CHC profiles containing a larger proportion of *n*-alkanes and less *n*-alkenes and methyl-branched alkanes. In several ant genera, including *Pogonomyrmex*, *Temnothorax,* and *Myrmica* more desiccation resistant profiles with proportionally upregulated *n*-alkane quantities and downregulated methyl-branched alkane quantities have been observed in more xeric conditions (Wagner *et al.* 2001; Menzel *et al.* 2018; Sprenger *et al.* 2018).

We previously (Buellesbach *et al.* 2018) found correlative evidence of profile acclimation in Argentine ants, where the abundance of some *n*-alkanes negatively correlated with precipitation (while also positively correlating to temperature), and some methyl alkanes positively correlated with precipitation (while also negatively correlated with temperature). This suggests that CHCs of Argentine ants which likely improve waterproofing (n-alkanes) are increased in xeric habitats, while classes less likely to influence waterproofing (methyl-branched alkanes) are increased in habitats with less desiccation stress (higher precipitation, lower temperatures). The composition of their CHC profiles, however, are also necessary for their supercoloniality, but each function relies on compound classes with opposing chemical properties. Methyl-branched alkane (not *n*-alkanes) variations appear to be predominantly responsible for nestmate recognition and mediating aggression between different supercolonies (Torres *et al.* 2007; Brandt *et al.* 2009b). This raises an interesting question: does optimizing desiccation protection conflict with signaling colony affiliation, since both are mainly mediated by different CHC compound classes?

To begin answering questions like this, we must first test if there is a connection between these two compound classes (methyl-branched vs. *n*-alkanes) and desiccation resistance in Argentine ants. We expect the ratio of methyl-branched alkanes vs. n-alkanes abundance to correlate with survival under increased desiccation stress. Moreover, we must consider how variation in body size influences survival under desiccation stress, as smaller body sizes generally translate to higher surface area to volume ratios, and thus higher susceptibility to desiccation (Kühsel *et al.* 2017). Additionally, we investigate how dietary differences between ant colonies correlate with body sizes, CHC compositions, and desiccation survival. We hypothesized that 1) different supercolonies will have different capacities to survive desiccation, and 2) covariates such as body size, diet, and CHC composition would correlate with desiccation survival. These experiments test how the multiple functions of a CHC profile contribute to the observed success of a globally invasive species, and could help explain the invasive biology of other ant species.

II: Methods

Ant nest collection. We collected Argentine ant nests from eight sites across California during January-May 2017 (Fig.1). Five of these sites (Ukiah [UK], Davis [DA], Albany Bulb [AB], Los Peñasquitos [LP], and Mission Trails [MT]) are behaviorally members of the "large" supercolony that dominates nearly all the Argentine ant's introduced range (Tsutsui *et al.* 2003; Buellesbach *et al.* 2018). We also collected Argentine ants from three of the separate "small" supercolonies in southern California (Lake Skinner [LS], Lake Hodges [LH], and Sweetwater [SW]) (Tsutsui *et al.* 2003; Buellesbach *et al.* 2018). Fragments of each colony (hereafter, "nests") were collected by excavating substrate (soil, leaf litter, and/or decomposing wood) with a shovel or trowel and placing it into a 5-gallon plastic bucket. When the substrate was sandy or dusty soil, crumpled paper towels were added to provide structure and air gaps. In the laboratory, the collections were distributed in a ~2-4 inches deep layer across the bottom of large (58.4 cm × 41.3 cm × 15.2 cm) plastic tubs. The ants were then provided with food (Bhatkar & Whitcomb 1970) and water and left undisturbed for 24-48 hours to allow workers to collect scattered brood and consolidate colony members into cavities. The nests are hidden while in substrate, so entire nests were extracted from the substrate over the course of several hours by slowly flooding the tubs with water and providing a narrow heavyweight paper bridge for relocation into an adjacent tub. The ants were contained in the new tubs by painting Insect-A-Slip (BioQuip, USA) along the inner walls and placing the tub on bricks in a second tub that functioned as a moat of soapy water. Within the tubs were nesting containers, consisting of Petri dishes with a layer of plaster of Paris on the bottom, a small entrance hole drilled in the side, and a lid to block light. The ants were maintained at room temperature in the lab and used for desiccation assays during their second week after collection.

Desiccation resistance assays. Survival of Argentine ant workers was quantified under three humidity treatments designed to impose different levels of desiccation stress: negligible desiccation ("water", relative humidity (RH) = ~1), moderate desiccation ("air", RH = ~0.55), and severe desiccation ("Drierite", RH = ~0) (Figure 2A). Preliminary experiments using iButtons (iButtonLink©, Innovation Drive Whitewater, WI, United States) confirmed these RH estimates in each humidity treatment (Supplemental Figure 1). Water tubes were constructed by filling 15 mL Corning® conical vials with ~2 mL distilled water and pushing a cotton ball down to the top of the water (Figure 2A). A second cotton ball was inserted above, creating an air gap of ~4 mm between the two cotton balls. This acted as a barrier to prevent the ants from accessing drinking water or excavating into the water and flooding the tube. The remaining empty volume (~7-8 mL) housed the worker ants during the desiccation assay. Air and Drierite tubes were constructed in the same manner, except air tubes had no water beneath the cotton balls, and Drierite tubes had ~2 mL of desiccant (Drierite CO. LTD, Xenia, OH, USA) at the bottom instead of water. All tubes were prepared the day before initiation of the desiccation assays, to allow RH to stabilize before the ants were introduced.

To perform desiccation resistance assays, we used aspirators to arbitrarily select 23 worker ants from each of the 8 collected nests and placed them in separate small, temporary holding dishes (Petri dishes (35 mm diameter × 10 mm height) with a small cotton ball (7 mm) saturated with 200 µL of water) for 24 hours before initiation of desiccation assays. This was repeated 10 times for each of the three humidity treatments for each colony, leading to a total of 240 assay tubes. Only 20 ants from each replicate were transferred to their respective assay tube; the extra 3 ants were served as backups in case of deaths or escapes during the 24 hours after

removal from their laboratory homes. Occasional miscounts led to instances of 19 (n = 4) and 21 (n = 1) ants occurring out of the total 240 assay tubes. We recorded the mortality of ants in each tube every two hours for the first 12 hours, then every four hours until there were no surviving ants remaining in the air and Drierite tubes (water tubes experienced little to no mortality during assay). We assessed mortality by lightly tapping the sides of tubes and rolling them horizontally to observe the ants. All ants that were moving, even if not standing, were counted as alive. Ants that did not move during this assessment were scored as dead.

Body mass and stable isotope analysis. After all ants in our dessication trials had died, indivdiduals from the Drierite treatment were used for analsyes of body mass and stable isotope composition. Only ants from the Drierite tubes were included in these analyses because they were less likely to have been degraded by moisture. Samples were stored at -20°C immediately after the desiccation assays ended and remained frozen until processing. For measuring body mass, desiccated worker ants from the Drierite tubes were removed, counted, and placed in clean 2 mL microcentrifuge tubes with snap tops. These tubes, with lids open, were placed in an airtight container on a 4 cm layer of Drierite for three days at room temperature. The dry mass of each sample was measured and divided by the number of individual ants in the sample to provide an average mass for the ants of each sample tube.

To infer if there were dietary differences between the nests before they were collected (which might influence their body size or survival in assays), we recorded stable isotopes from ants from each nest. Desiccated ants from the Drierite tubes were placed under a dissecting microscope and the gaster of each ant was removed with forceps, leaving only the head, mesosoma, and legs of each individual for stable isotope measurement. Each sample (i.e. all ants in a desiccation tube) was weighed, placed in a tin capsule, and analyzed on a CHNOS Elemental Analyzer in combination with an IsoPrime100 mass spectrometer. Pee Dee Belemnite (PDB) was be used as a standard for comparison. Simultaneous analysis of carbon (δ13C) and nitrogen (δ15N) isotopes was performed by continuous flow (CF) dual isotope analysis. Precision for C isotope measures on this instrument is ± 0.10‰ and for N isotope measures is ± 0.20‰.

GC-MS Analysis of Cuticular Hydrocarbons. Cuticular hydrocarbons (CHCs) were extracted and analyzed by gas chromatography coupled with mass spectroscopy (GC-MS) as described previously (Buellesbach *et al.* 2018). We used workers collected directly from the same nests as the ants used in our desiccation assays to determine representative CHC profiles for the ants used in experiment. All ants from the same nest were therefore attributed with the same representative CHC profile in our data set.

Individual workers were placed into 2 mL screw-top GC vials (Agilent Technologies, Santa Clara, California, USA), 100 µl of hexane (HPLC grade, Fisher Scientific, Fair Lawn, New Jersey, USA) was added, and the vials were swirled for 10 minutes on a Thermolyne Roto Mix (Marshall Scientific, Hampton, New Hampshire, USA). These extracts were transferred to a conical 250 µl GC insert (Agilent Technologies, Santa Clara, California, USA), and subsequently evaporated under a flow of nitrogen gas (Praxair, Inc., Danbury, Connecticut, USA). The dried extract was then resuspended in 10 µl hexane with 7.5 ng/µl of n-dodecane (EMD Millipore Corp., Billerica, Massachusetts, USA) as an internal standard.

Half of the re-suspended CHC extract (5 µl) was injected into a gas chromatograph coupled with a mass selective detector (GC: 7890A; MS: 5975C; Agilent Technologies, Santa Clara, California, USA) which was operated in electron impact ionization mode. The injection was performed in a split/splitless injector in the splitless mode with an inlet temperature of 250°C.

Compounds were separated on a fused silica capillary column (DB-5MS, 30 m x 0.32 mm x 0.25 µm, Agilent J&W GC columns, Santa Clara, California, USA) using a temperature program starting at 80°C for 5 min and increasing by 80°C per min to 200°C, followed by an increase of 5°C per min to 325°C, which was then held for 3 min. Helium was used as carrier gas at a constant flow rate of 1.8 mL/min. Peak area integration and calculation were performed using the software "Enhanced Chemstation", G1701EA, Version E.02.02 (Agilent Technologies, Santa Clara, California, USA). Peaks were automatically integrated with an initial area reject of 0, an initial peak width of 0.017, and an initial threshold of 13, with shoulder detection turned off. CHC compounds were identified according to their retention indices, diagnostic ions, and mass spectra. Absolute quantities (in ng) for a total of 72 individual CHC compounds were determined based on the internal *n*-dodecane standard.

Survival analysis (LT50, Cox proportional hazards model). All data were analyzed with the statistics programming language R (version 4.1.2, R Development Core Team 2018). To assess the effect of our humidity treatments, we compared survival curves of all ants from all colonies grouped by humidity treatment (Fig. 2B). Since mortality was nearly absent in the water treatments, we only analyzed the air and Drierite groups (separately) when comparing nest survival rates. We measured survival of each nest by calculating median lethal time (LT50) for each sample tube (command dose.p, package MASS), which measures the time at which half of the ants in each tube were expected to be dead. The LT50s of all sample tubes from a nest in a treatment (n = 10 sample tube replicates) were averaged to be compared to other nests in the same treatment. Significant differences between the nests were assessed with a pairwise permutations analysis of the variance (command pairwise.adonis, package pairwiseAdonis), using Euclidian distances and significane levels were Holm-Bonferroni corrected.

We used a Cox proportional hazards model (command coxph, package survival) to assess which factors influenced the survival of ants during desiccation, with time-to-death of individual ants as the response (dependent) variable. We combined all parameters into a single model, based on our hypothesis that CHC properties, body size, and stable isotopes would all influence survival under desiccation. We could not include abundance estimates for individual CHC compounds in our models, as all these masses are correlated, and correlation prevents reliable estimation of regression coefficients (Mela & Kopalle 2002; Xue *et al.* 2007; P. Vatcheva & Lee 2016). Therefore, in addition to average body mass and stable isotope ratio (δ15N: δ13C), our predictor variables were two different measurements of the ratio of total methyl-branched alkane amounts to *n*-alkane amounts (M:N). Total M:N refers to the ratio including all methyl-branched and *n*-alkanes found in our CHC extracts, while Selected M:L includes only the methyl-branched and *n*-alkanes previously found to be significantly correlated with temperature and precipitation (Buellesbach *et al.* 2018) (compounds listed in Supplemental Table 1). According to this previous study, the selected methyl-branched alkanes correlated positively with precipitation and negatively with temperature, while the n-alkanes correlated negatively with precipitation and positively with temperature. Average weighted chain lengths were not included in our models because they were correlated with these M:N ratios.

Robust standard errors were generated for our model coefficients using a cluster term, given all ants used in the experiment were clustered into only eight nests. In addition, due to our covariates being measured on different scales, all model covariates were z-transformed to be more comparable. The data, code, and outputs from our Cox proportional hazard model are presented in Supplemental Data 3, Supplemental Code file, Supplemental Table 2. Differences in

body mass and stable isotope ratios between nests and supercolonies are in Supplemental Figures 2 and 3.

III: Results

Desiccation resistance varies by supercolony and nest. Survival was high in the negligible desiccation treatment ("water", RH = 1), lower in the moderate desiccation treatment ("air", RH = 0.55), and lowest in the severe desiccation treatment ("Drierite", RH = 0) (Fig. 2B). The negligible desiccation treatment never approached a point where all ants died (Fig 2B). In the moderate desiccation treatment, all ants died by 48 hours (Fig. 2B), while in the severe desiccation treatment, all ants died within 30 hours (Fig. 2B). Focusing on the moderate and severe treatments, we found significant variation among nests in their capacity to resist desiccation (Fig. 3, Supplemental Data 1). In the moderate treatment, while nests within the large supercolony (specifically DA) had significantly different mean LT50s from each other, all the large supercolony nests had significantly higher LT50s than the nests from the small supercolonies (LS, LH, SW). These differences between the large supercolony nests were less pronounced in the severe desiccation treatment, where only two large supercolony nests with significantly different LT50s (Fig. 3B, AB and LP). All large supercolony nests still had significantly higher LT50s than the small supercolony nests.

Body mass and CHC profiles determine desiccation resistance. The largest body sizes on average were in the large supercolony (specifically UK = 0.17 mg per ant), though some of the large supercolony nests were not significantly different than the small supercolonies (Supplemental Fig. 2; AB and MT compared to LS and SW). In the Cox proportional hazard models, the magnitude of the effect of each covariate on survival can be interpreted from the exponents of the associated coefficients, known as the "hazard ratios" (Fig. 4). For instance, body mass has a hazard ratio of 0.772, meaning a one unit (milligram) increase in body mass led to a 0.772-fold change in death probability (i.e., a 23% decrease in probability of death) (Fig. 4). All covariates except for the stable isotope ratio (Supplemental Fig. 2) had significant impacts on the hazardousness of desiccation (Fig. 4). Specifically, Total M:N (Fig. 4, Fig. 5A) had a strong positive effect, associated with a 65% per-unit increase in death probability (i.e. hazard ratio of 1.659). Selected M:N (Fig. 4, Fig. 5B), however, had a negative effect, associated with a 37% per-unit decrease in death probability. Further information on the CHCs composing these M:N ratios can be found in Supplemental Figure 4 and Supplemental Table 1.

M:N ratios and stable isotopes. Within the large supercolony, southern nests (Los Peñasquitos and Mission Trails) were characterized by more *n*-alkanes in their cuticular profiles compared to northern nests (Fig. 5A, Ukiah, Davis, Albany Bulb). This *n*-alkane bias in their profiles increased when considering only the the Selected M:N compounds (Fig 5B, compounds listed in Supplemental Table 1). For both total and selected M:N ratios, the southern large supercolony nests had the highest *n*-alkane proportions (i.e. lowest M:N) out of all nests from all supercolonies sampled. Ukiah had the highest total M:N and Davis has the highest selected M:N (Fig. 5). There were some patterns of stable isotope differences between nests; the large supercolony nests varied on the carbon axis while the small supercolony nests varied on the nitrogen axis (Supplemental Figure 3). We found no correlation between stable isotope profiles and body size.

IV: Discussion

We found consistent differences among introduced Argentine ant supercolonies in their ability to resist desiccation. Specifically, in both moderate (RH = 50%) and severe (RH = 0%) desiccation treatments, ants from the large supercolony survived the longest, having significantly higher LT50 values than ants from the three smaller colonies sampled. Resistance to desiccation also varied among different nests within the large supercolony. Because this colony is geographically widespread (~1000 km long), comparisons of populations from distant sites provide insights into how genetically similar ants – all with the same supercolony identity – perform in different abiotic environments (Buellesbach *et al.* 2018). Our data suggest that greater resistance to desiccation may contribute to the relative success of this supercolony in xeric habitats, such as the Mediterranean climate of coastal California.

Previous research has shown that body size accounts for much of the difference in water loss rates across several different ant species (Schilman *et al.* 2007). Moreover, when compared to native ants in southern California, Argentine ants generally have higher cuticular permeability and are significantly more susceptible to water loss and lethal desiccation (Holway *et al.* 2002b; Schilman *et al.* 2007). This susceptibility to desiccation likely limits Argentine ant invasion into habitats with lower humidity levels. Irrigation experiments in the field, have shown that water subsidies allow Argentine ants to spread into formerly inhospitable chaparral habitat and, conversely, cessation of irrigation leads to their withdrawal (Menke & Holway 2006; Menke *et al.* 2007).

Multiple factors may contribute to the greater desiccation resistance observed in the large versus the smaller supercolonies sampled. One of the most influential factors was the higher proportional *n*-alkane amounts, in both of our accessed metrics (Total M:N, Selected M:N). Within the large supercolony, southern nests (Los Peñasquitos and Mission Trails) had lower Total M:N than northern nests, which agrees with acclimation patterns seen in other ant species (Menzel *et al.* 2018; Sprenger *et al.* 2018), although this difference is not evident within the smaller supercolonies, as their *n*-alkane proportions are lower than both Los Peñasquitos and Mission Trails.

As mentioned before, we previously (Buellesbach *et al.* 2018) found correlative evidence of profile acclimation, where the abundance of some *n*-alkanes negatively correlated with precipitation (while also positively correlating to temperature), and some methyl alkanes positively correlated with precipitation (while also negatively correlated with temperature). The results of our current analyses partially agree with this outcome. Profiles with a higher Total M:N are expected to have lower melting points and increased water permeability (Gibbs 2011; Blomquist & Ginzel 2021); in this study, they were associated with more rapid mortality due to desiccation. Surprisingly, however, Selected M:N showed the opposite effect, with higher amounts of this subset of methyl-branched alkanes improving survival against desiccation relative to lower quantities.

Several factors may have contributed to this contrast in outcomes. First, our experimental design differed from other studies in which ants acclimated to low humidity for 3 weeks before testing; with acclimation, survival is higher under desiccation and individuals increase proportions of *n*-alkanes (Menzel *et al.* 2018; Sprenger *et al.* 2018). In contrast, we allowed ants to acclimate for just one week under ambient conditions (21-26°C, ~50% humidity) before testing began. It is possible that this acclimation period in ambient conditions may have reversed or weakened the acclimation they might have from the harsher conditions of their respective

habitats. Additionally, the northern nests (UK, DA, AB), which all live in environments with higher precipitation than the southern nests (LP, MT, LS, LH, SW) (Fick & Hijmans 2017), showed the greatest survival against desiccation. This disagrees with the hypothesis that ants from xeric habitats have improved resistance against desiccation (Menzel *et al.* 2018; Sprenger *et al.* 2018). Another explanation, however, could be that these selected methyl-branched alkanes do have a role in assisting survival against desiccation.

Because methyl-branched alkanes have lower melting points than *n*- alkanes of equivalent carbon chain length, CHC profiles with increased levels of methyl-branched alkanes often show decreased viscosity as opposed to more *n*-alkane rich profiles (Menzel *et al.* 2019; Blomquist & Ginzel 2021). Profiles with a mixture of compound classes should be more optimal for waterproofing, as a profile of purely linear alkanes be solid and cracking, and would be difficult to maintain and repair a complete seal around the entire body (Menzel *et al.* 2019). A study on invasive *Solenopsis* species using differential scanning calorimetry to measure melting points of overall CHC profiles found that profiles with increased methyl-branched alkane proportion still exhibited comparably high overall melting points (Xu *et al.* 2018). Grooming might be a potential mechanism for maintaining a complete seal of CHC wax around the exoskeleton, spreading CHCs stored in the post-pharyngeal gland, which are predominantly methyl-branched alkanes (Sprenger *et al.* 2021). This could be tested by analyzing CHC compositions from the post-pharyngeal gland, comparing them to the composition of whole-body CHC profiles, and including the presence of grooming as a covariate in future survival analyses.

Importantly, we have observed that CHCs of certain compound classes (*i.e. n*-alkanes and methyl-branched alkanes) can differ in their respective effects on desiccation resistance, depending on which of these compounds are assessed and their specific ratios (Selected M:N vs. Total M:N). This finding is in line with results from previous studies assessing differential effects of particular CHC compounds in different combinations (primarily methyl-branched alkanes) on colony recognition and aggression (Brandt *et al.* 2009b; Van Wilgenburg *et al.* 2012).

We know that CHCs are not the only factors driving survival under desiccation, and our results show that body mass also has a significant role. The ants with the highest average body mass were found in the large supercolony (Ukiah, Davis, and Los Peñasquitos), whereas the ants with the lowest average body masses were from one of the small supercolonies (Lake Hodges) (Supplemental Figure 2). This is consistent with their desiccation performance, which may reflect a smaller surface-area-to-body-volume ratio in ants with larger bodies (Kühsel *et al.* 2017), with a smaller ratio predicted to reduce water loss through the cuticle. In our Cox proportional hazard model, the body mass coefficient was close to 0, but still significantly decreased the hazardousness of desiccation. Although the direction of causality remains unclear, it is possible that in a warming environment, direct selection for increased desiccation resistance could produce correlated increases in body size.

In conclusion, it appears the most geographically widespread Argentine ant supercolony in California is better able to resist desiccation than smaller supercolonies, reflecting the larger body sizes and more *n*-alkane rich CHC profiles for members of the large supercolony. A higher ratio of methyl-branched to *n*-alkanes decreased survival, suggesting a functional trade-off between compounds potentially more relevant for nestmate recognition (methyl-branched alkanes) and compounds more attuned to water proofing (*n*-alkanes). The opposite effect, however, was observed for the subset of CHCs including only methyl-branched alkanes correlated with low temperature and high precipitation of northern California climates, and n-

alkanes that correlated to the with the high temperature and low precipitation of xeric southern California climates (Buellesbach *et al.* 2018). This opposite result suggests profile acclimation may not be as simple as increasing one compound class in relatively higher proportions to the other. Perhaps the upregulation of or selection on individual CHCs, instead of entire compound classes, may serve to optimize CHC profiles for a variety of different functions related to differential ecological conditions.

Acknowledgements

This work was supported by the US National Science Foundation (IOS-1557934/1557961), USDA National Institute of Food and Agriculture (2016-67013-24749), USDA Hatch Project (CA-B-INS-0087-H), and the UC Berkeley Bakar Fellows program. We thank Katie Sanko, Ali Setayesh, and Jessica Maccaro for their assistance with the desiccation resistance experiments.

Author contributions

This study was conceived by NDT and designed with input from all authors. All authors performed field work and contributed to the desiccation resistance experiments. RS collected the body size and stable isotope data and JB collected and organized the CHC data. BAW performed the statistical analyses. BAW and EIC created the data visualizations. RS, BAW and NDT wrote the first drafts of the manuscript and all authors subsequently provided input.

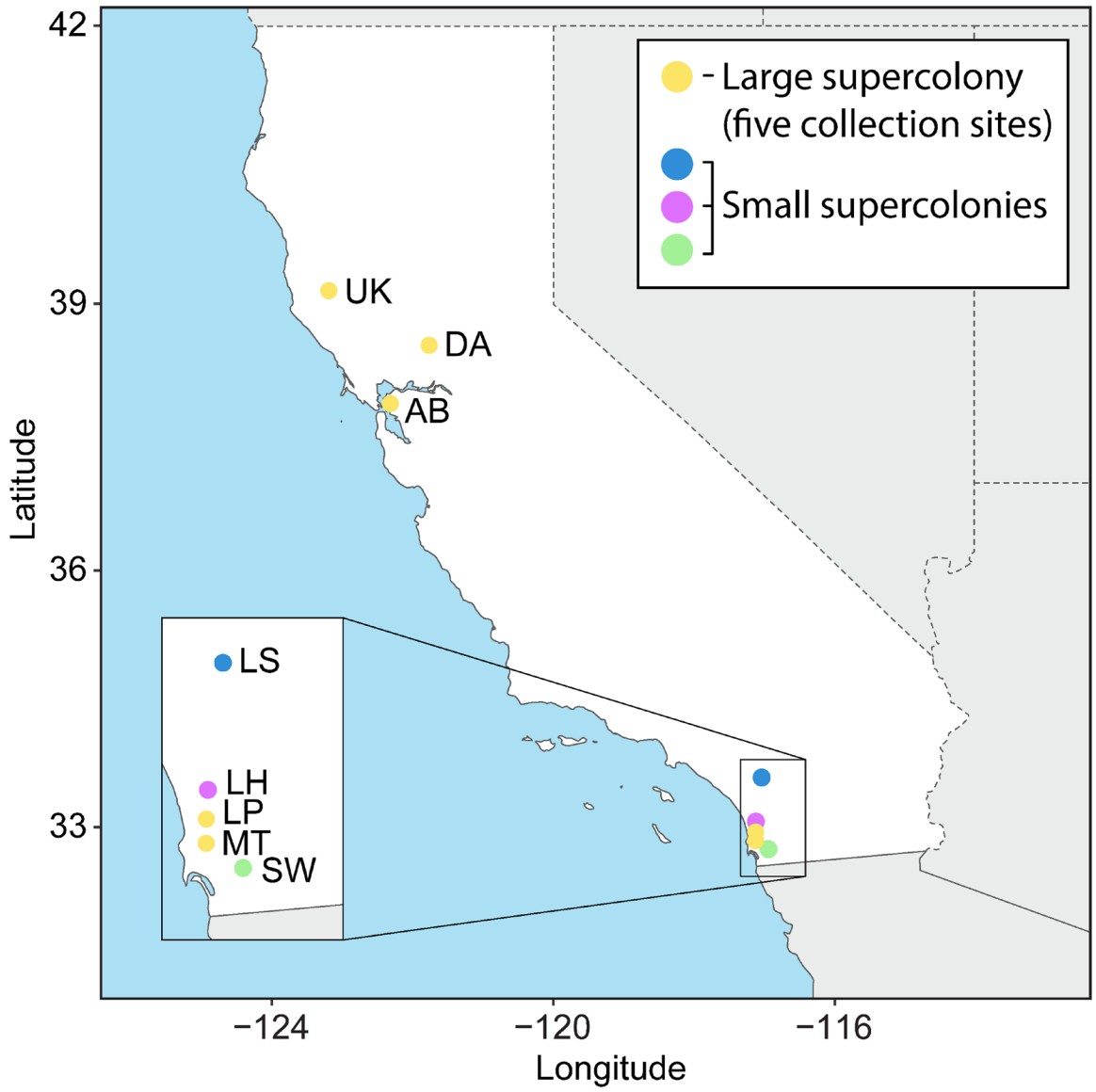


Figure 1. Map of Argentine ant nest locations. Letter abbreviations indicate collection site locations (UK = Ukiah, DA = Davis, AB = Albany bulb, LS = Lake Skinner, LH = Lake Hodges, LP = Los Peñasquitos, MT = Mission Trails, SW = Sweetwater). Color indicates supercolony identity. Yellow = large supercolony, blue = Lake Skinner supercolony, purple = Lake Hodges supercolony, and green = Sweetwater supercolony.

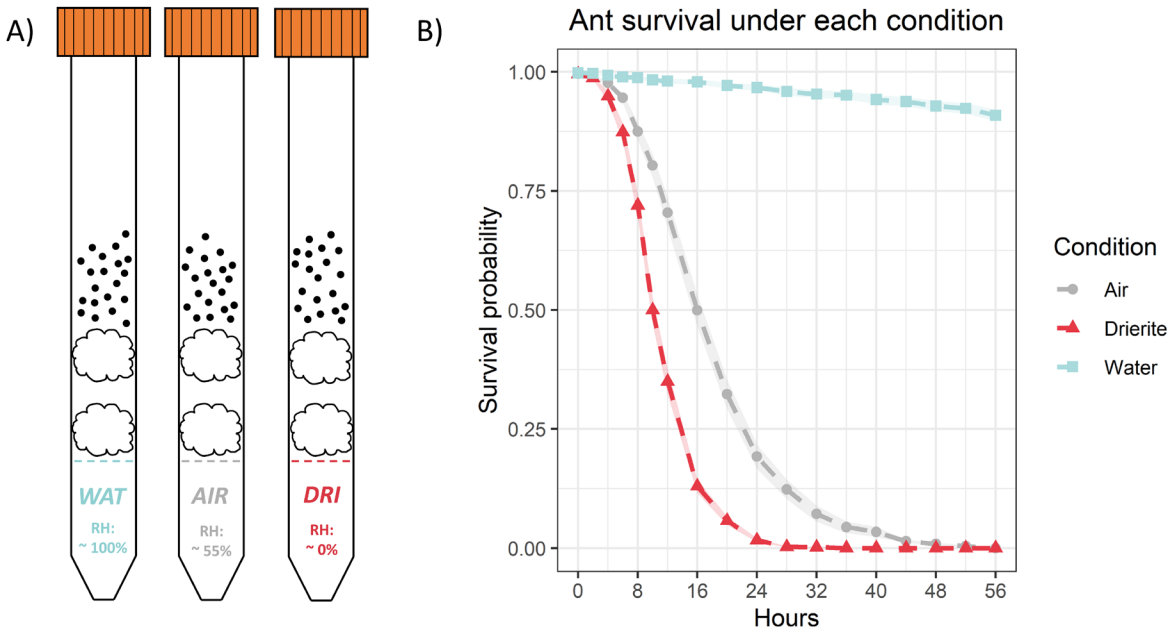


Figure 2. Desiccation assay design and survivorship. **A)** Illustration of 15 ml conical tubes with ants (black dots) inside, resting on top of cotton balls which separate them from the contents underneath. The contents influence the relative humidity (RH) of each tube. Left to right: water (relative humidity = 1), air (relative humidity = 0.55), and Drierite (relative humidity = 0). **B)** Survival probability of Argentine ants over time were combined over all assayed ants per treatment to show general survival patterns in each treatment. Standard errors are indicated in narrow ribbons around each survival curve.

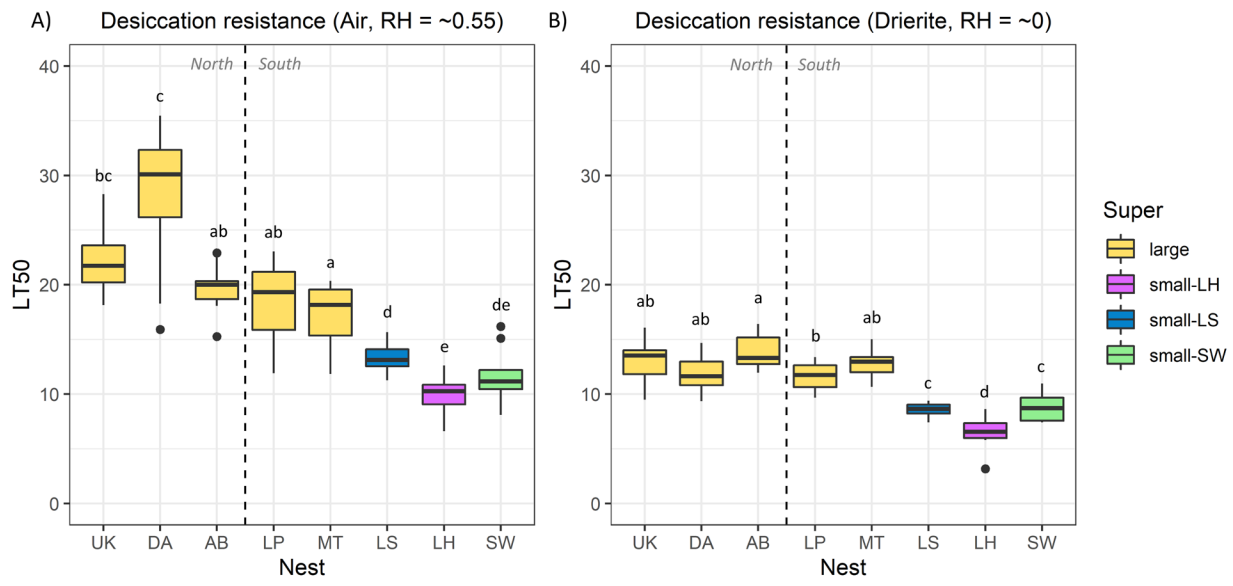


Figure 3. Desiccation resistance of ants from each nest site. Median lethal time ("LT50", in hours), averaged across desiccation tubes, separated for the **A)** air and **B)** Drierite treatments. The vertical dotted lines separate the geographic regions of the nests (north on the left, south on the right, see Figure 1 for more detail). Letters indicate significance groups determined by a pairwise permanova test (full test results are indicated in Supplemental Data 1). Note that these letters only explain within-graph comparisons – they do not correspond across graphs A and B.

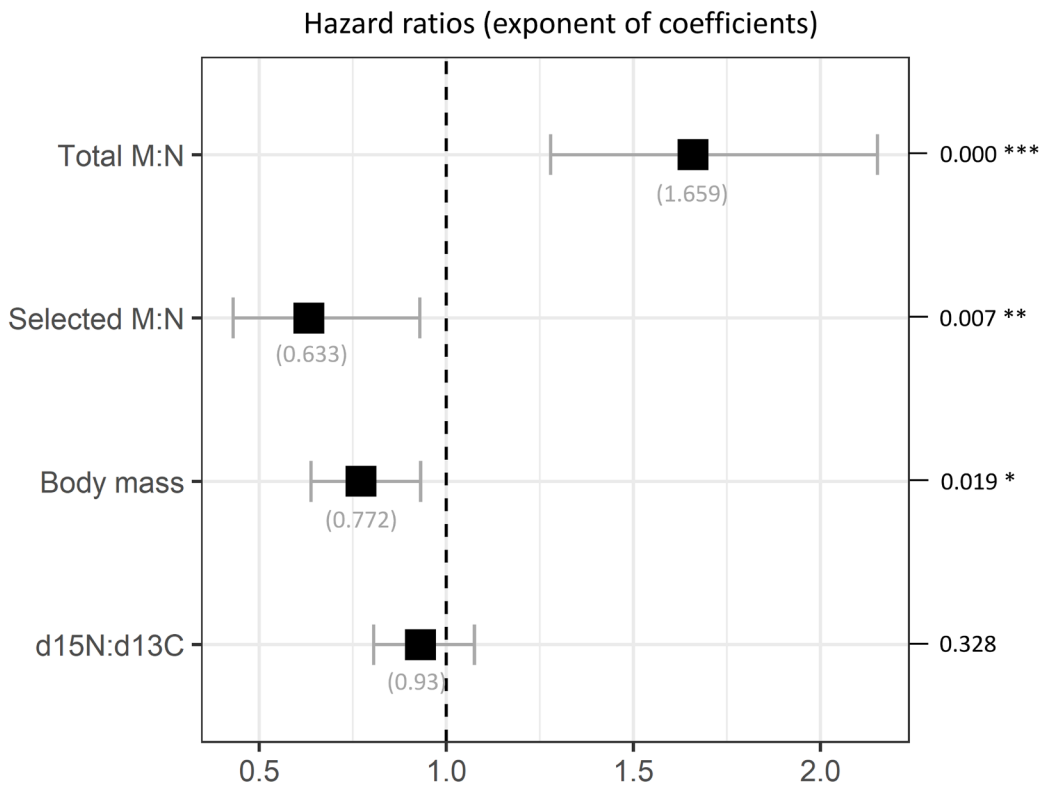


Figure 4. Cox proportional hazard ratios. A forest plot translating the coefficients from our Cox regression model into hazard ratios (i.e. exponents of each coefficient). Blocks indicate hazard ratios of each covariate (reported exactly in parentheses), surrounded by their confidence intervals. Hazard ratios are significant if their confidence intervals do not overlap with 1 (i.e., the vertical dashed line). A positive value indicates increased hazardousness per-unit increase of this variable. A negative value indicates decreased hazardousness per-unit increase of this variable. P-values from our Cox regression model can be found on the right ($p<1,<0.05$*,$<.005$**,$<.000$***). More details on this model can be found in Supplemental Table 2, and the Supplemental R code.

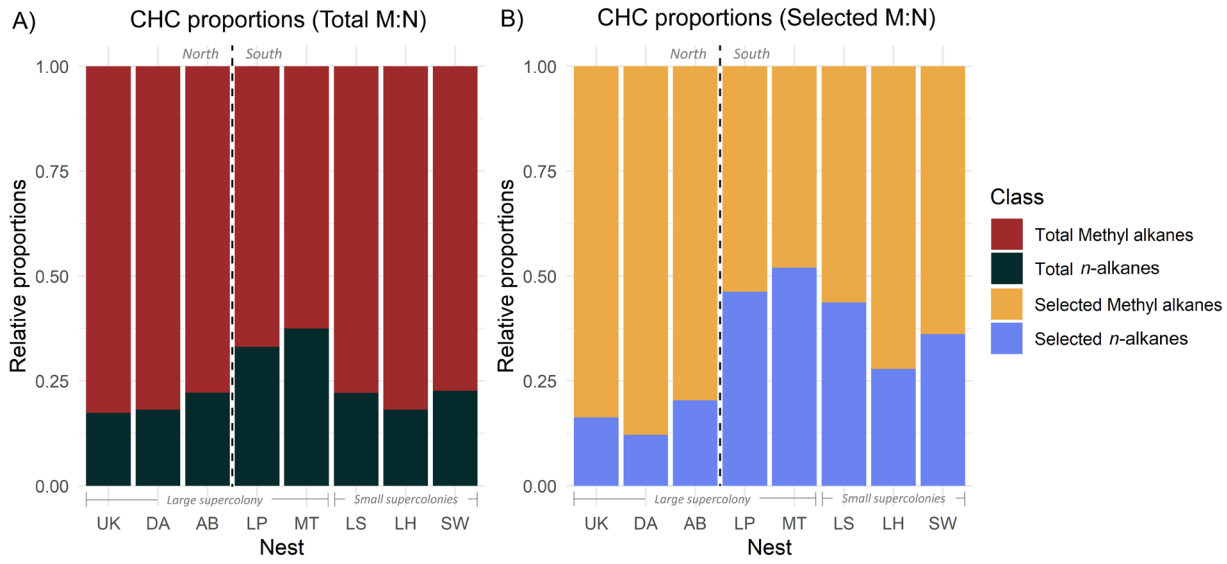
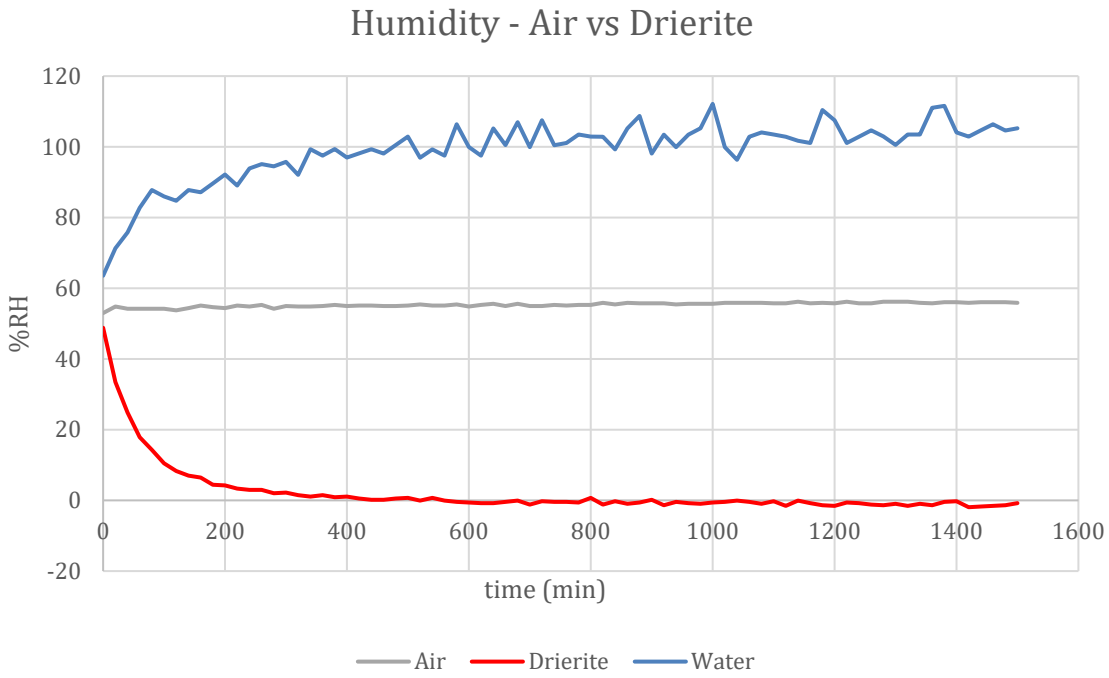
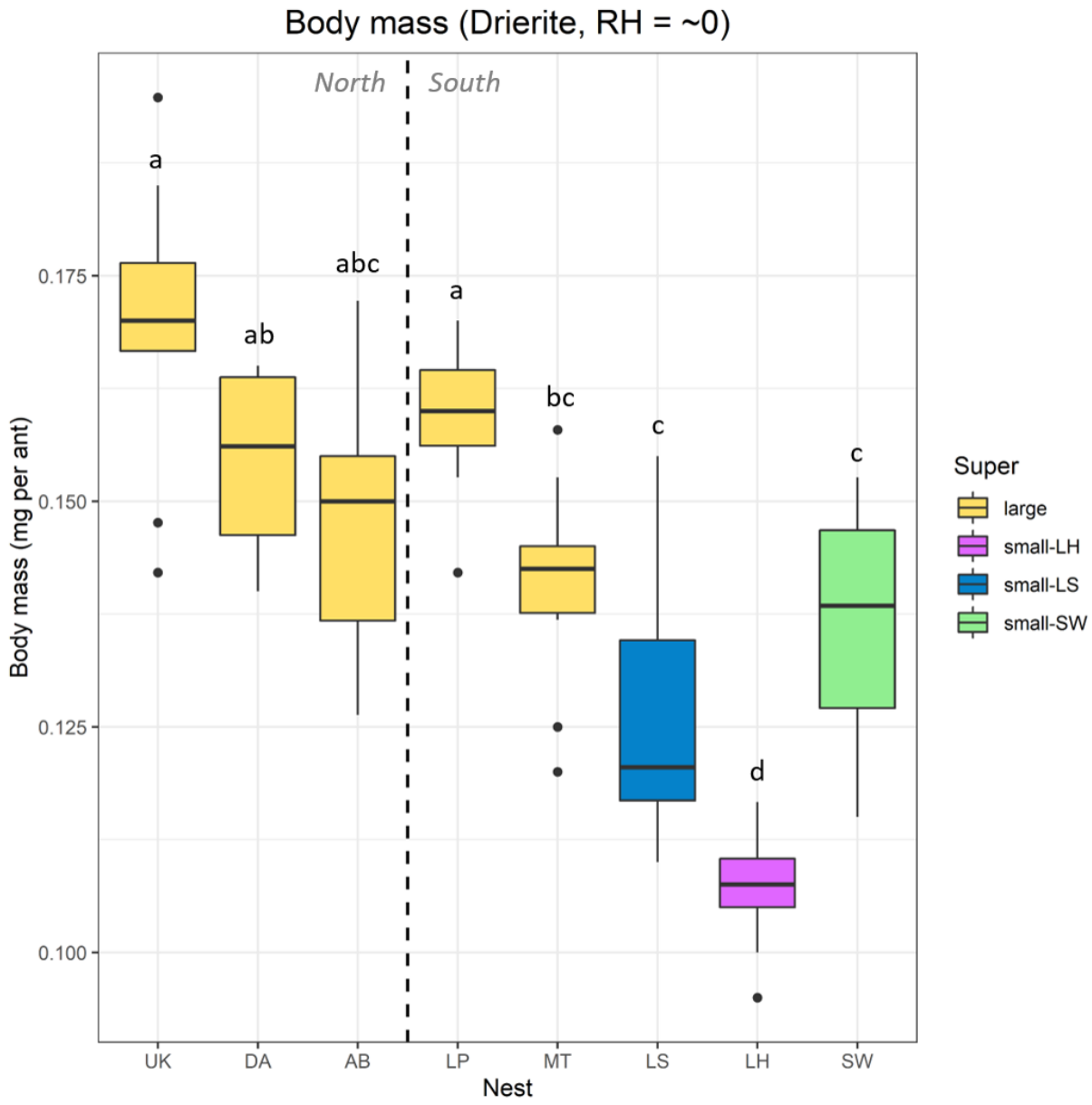


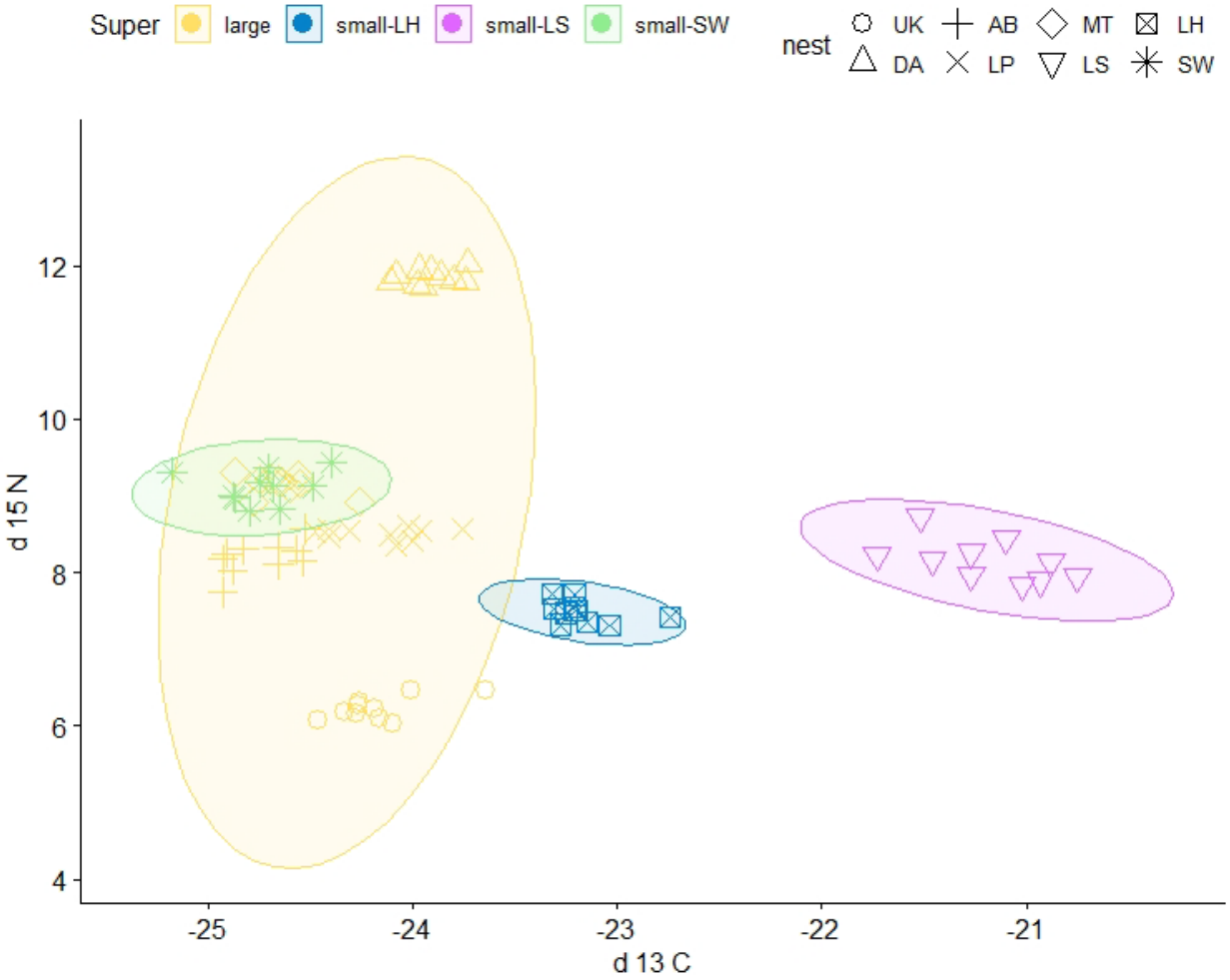
Figure 5. *n*-Alkane and methyl-branched alkane abundances in the CHC profiles of our studied Argentine ant supercolonies. The stacked bar plots show the relative abundances of *n*-alkanes and methyl-branched alkanes, the most abundant compound classes in the CHC profiles of all of our studied supercolonies. A) Total identified *n*-alkanes and methyl-branched alkanes are included in the compound class comparisons. B) Selected *n*-alkanes and methyl-branched alkanes previously found to be significantly correlated with precipitation and temperature (Buellesbach *et al.* 2018) are included in the compound class comparison. The compounds included in these classes, as well as the subset used in Selected M:N, are listed in Supplemental Table 1.



Supplemental Figure 1: Relative humidity of assay tubes over time. Humidity was recorded with iButton Hygrochron sensors (iButtonLink©, Innovation Drive Whitewater, WI, United States). These sensors could not fit into the 15 ml conical tubes used in experiment, so we placed iButtons into 50 ml conical tubes on top of two cotton balls with our air, drierite, or water treatment below (see Figure 2 in the manuscript).



Supplemental Figure 2: Body mass box plots for each nest. Body mass was an important parameter in our Cox Regression models. Here is the body mass variation we recorded across nests. This data only uses ants from the Drierite assay tubes. Colors indicate supercolony identity. The vertical dotted line indicates nests from northern vs. southern California. Nest abbreviations are explained in the main manuscript. Our methodology for recording body mass was described in the methods sections of the manuscript. Significant differences between the nests were tested with a pairwise permutations analysis of the variance (same as for Figure 3 in the manuscript). Letters indicate significance groups determined by a pairwise permanova test (full test results are indicated in Supplemental Data 1).



Supplemental Figure 3: Stable isotope results. Scatterplot with carbon (δ13C) isotope abundance on the x-axis, and nitrogen (δ15N) isotope abundance on the y-axis. Probability ellipses surround data from each supercolony (color), and each nest is given a unique symbol for their data points. Each data point refers is the averaged isotope value from a group of ants in a Drierite tube in our experiment.

Supplemental Table 1: List of all cuticular hydrocarbons (CHCs) extracted and analyzed. The CHCs used in the "Selected M:N" parameter for our Cox regression models are highlighted in blue (methyl-branched alkane) or yellow (*n*-alkane), as these compounds were found to be significantly correlated with climate conditions in our past study (Buellesbach et al. 2018, see manuscript references). The CHCs used in the "Total M:N" parameter include the entire list, excluding three *n*-alkenes (in grey).

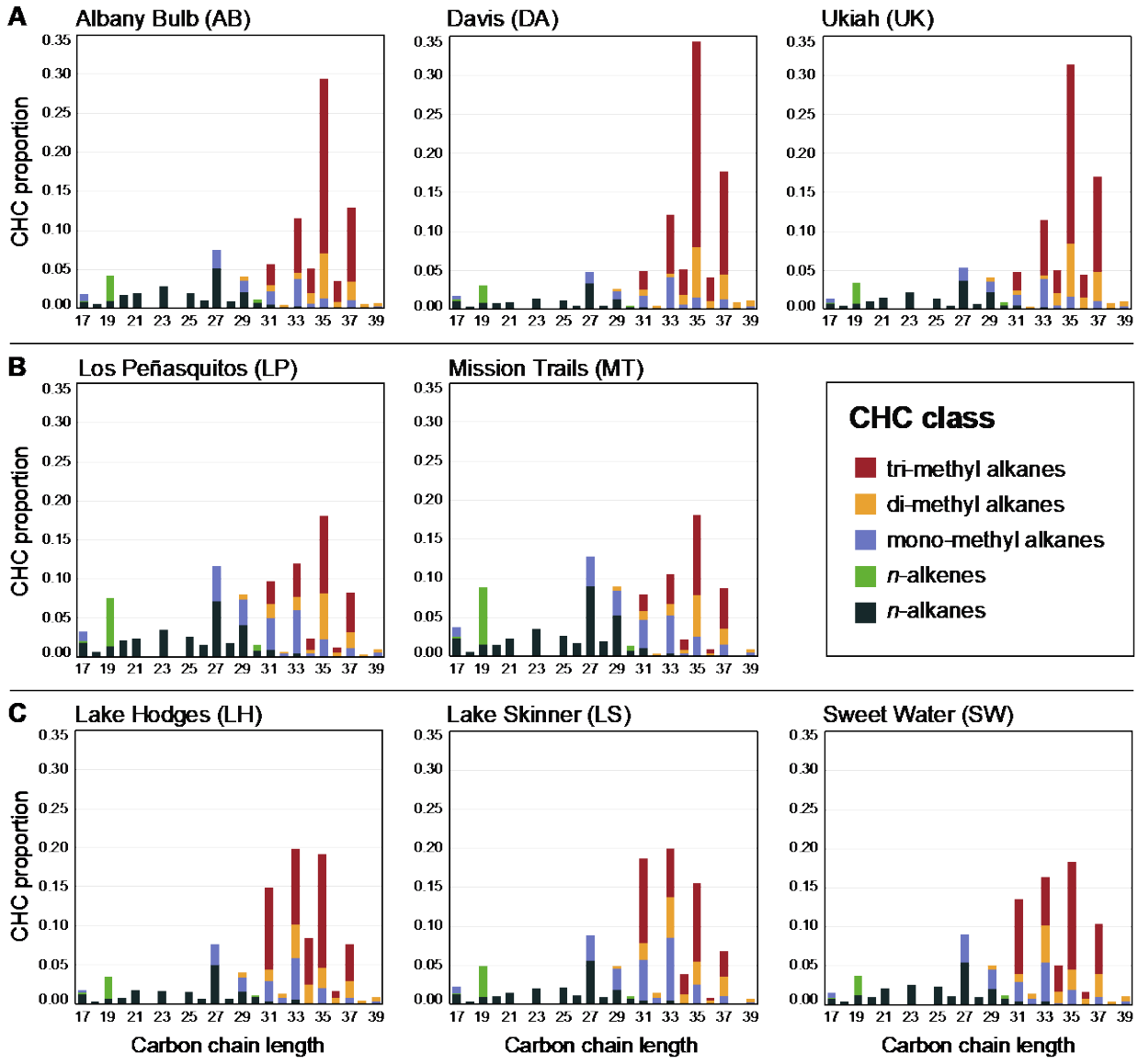
Retention index	*Structure assignment*	*Absent from these nests:*
1675	C17:1 *n*-alkene	
1700	C17 *n*-alkane	
1760	MeC17 alkane	
1780	MeC17 alkane	
1800	C18 *n*-alkane	
1880	C19:1 *n*-alkene	
1900	C19 *n*-alkane	
2000	C20 *n*-alkane	
2100	C21 *n*-alkane	
2300	C23 *n*-alkane	
2500	C25 *n*-alkane	
2600	C26 *n*-alkane	
2700	C27 *n*-alkane	
2731	MeC27 alkanes	
2754	MeC27 alkane	
2777	MeC27 alkane	
2800	C28 *n*-alkane	
2900	C29 *n*-alkane	
2931	MeC29 alkanes	
2938	C30:1 *n*-alkene	
2954	MeC29 alkane	
2969	MeC29 alkane	
2977	DiMeC29 alkane	
3000	n-C30 (+ MeC30 alkane + DiMeC29 alkanes)	
3100	C31 *n*-alkane	
3125	MeC31 alkane	
3149	MeC31 alkane	
3158	DiMeC31 alkanes	
3167	MeC31 alkane	
3175	DiMeC31 alkanes	
3183	TriMeC31 alkane	AB, DA, LP, UK
3192	TriMeC31 alkanes	AB, DA, LP, UK
3200	TriMeC31 alkanes (+ DiMeC31 alkane)	
3225	TriMeC31 alkanes	

3258	MeC32 alkane	
3283	DiMeC32 alkanes	
3300	C33 *n*-alkane	
3309	DiMeC33 alkane	
3327	MeC33 alkane	
3345	MeC33 alkane + DiMeC33 alkane	
3355	DiMeC33 alkanes	AB, DA
3373	MeC33 alkane + DiMeC33 alkanes	
3382	DiMeC33 alkanes	AB, DA, LP, UK
3391	TriMeC33 alkanes	AB, DA, LP, UK
3400	TriMeC33 alkanes (+ DiMeC33 alkanes)	
3409	TriMeC34 alkane	AB, DA, LP, UK
3427	TriMeC34 alkanes	
3455	DiMeC34 alkanes	
3482	DiMeC34 alkanes (+ TriMeC34 alkane	
3491	DiMeC34 alkanes	
3492	MeC34 alkanes	
3509	TriMeC34 alkane	
3527	MeC35 alkane	
3545	DiMeC35 alkanes	
3575	DiMeC35 alkanes	LS, SW
3578	TriMeC35 alkane	AB, DA, UK
3591	TriMeC35 alkanes	AB, DA, LP, MT, UK
3600	TriMeC35 alkanes (+ DiMeC35 alkane)	
3620	TriMeC35 alkanes	
3650	DiMeC36 alkanes	
3690	TriMeC36 alkanes	
3697	TriMeC36 alkane	
3727	MeC37 alkanes	
3745	DiMeC37 alkanes	
3764	DiMeC37 alkanes	
3773	TriMeC37 alkane + DiMeC37 alkanes	
3782	TriMeC37 alkanes	
3800	TriMeC37 alkanes (+ DiMeC37 alkanes)	
3822	TriMeC37 alkanes	
3844	DiMeC38 alkanes	
3927	MeC39 alkanes	
3945	DiMeC39 alkanes	

Supplemental Table 2. Cox regression model results. The response (dependent) variable was the time of death for each individual ant (n = 1593, using only ants from the Drierite tubes). Coefficients for each covariate (independent variables) are shown in each row, with robust standard errors in parentheses, generated by clustering the data by nest. This table was created in R with stargazer (Hlavac 2015). The code used to calculate this cox regression can be found in the Supplemental Code file, and the data can be found in Supplemental Data 2.

	Dependent variable:
	Time-to-Death
Total M:L	0.506^{***}
	(0.043)
Selected M:L	-0.458^{*}
	(0.051)
Body mass	-0.259^{**}
	(0.032)
d15N:d13C	-0.072
	(0.030)
Observations	1,593
R^2	0.189
Max. Possible R^2	1.000
Log Likelihood	$-9{,}990.687$
Wald Test	63.270^{***} (df = 4)
LR Test	333.409^{***} (df = 4)
Score (Logrank) Test	381.014^{***} (df = 4)
Note:	*p<0.05; **p<0.01; ***p<0.001

<u>Supplemental Figure 4: Complete cuticular hydrocarbon (CHC) profile proportions for each colony</u>. Stacked bar plots of *L. humile* CHC proportions including all CHCs detected (i.e., *n*-alkanes, methyl branched alkanes, and *n*-alkenes), with methyl-branched alkanes divided into subcategories based on their number of methyl branches (i.e., one, two, or three). CHC proportions are organized by carbon chain length on the x-axis and CHC profiles are grouped according to supercolony identity and location: (A & B) large supercolony sites, (C) small supercolonies, (A) northern California, and (B & C) southern California.



Chapter 5. Concluding remarks

"How continuous the development, how sharp the beginnings and endings, and how well integrated the entity must be are determined by the processes in which these individuals function, not by the contingencies of human perception."

Individuality and Selection, David L. Hull (1980)

COMPARATIVE SOCIOBIOLOGY AND PARASITE EUSOCIALITY

Life is rife with sociality. It is relevant at every level of biological organization. We may perceive sociality as necessarily advanced and complicated, as it appears to be in our own human lives. But many things big and small live in groups and navigate the costs and benefits of this situation. Ants have been a model system for advanced sociality, or "true" sociality, as its *eu*social title implies. Eusociality is still a popular and meaningful category, and many authors including myself sought to find more systems that fit this category. This is why I so willingly focused my dissertation on newly discovered eusocial trematodes, rather than the deeply researched ants. During this experience, I have come to find more importance in the characteristics of eusociality than the category itself. I do not believe this category to be real, and I believe the most important reason for trying to attach a shared evolutionary explanation across eusocial-labelled systems is to explain how reproductive divisions of labor, specifically, evolve.

I still believe the separation of germinal (reproducing) and somatic (non-reproducing) parts of a collective is critical for fitting our concepts of individuality or organismality to the living world. I do also agree, however, that this characteristic is insufficient is many cases (Buss 1987; Clarke 2013)) and that organismal immunity and recognition systems are perhaps more influential in generating the perceived boundaries of biological individuals (Pradeu 2019).

It has been very eye-opening and fascinating to throw the "wrench" of polyembryonic parasites into these "gears" of evolutionary theory. As mentioned in Chapter 2, an egg developing on a path towards one multicellular body, eventually splitting into multiple multicellular bodies, provides unique challenges to our concepts of biological individuality. If these "individuals" were never physically separated, I think we would just consider them as a multicellular body with specialized parts, no different than every other animal. This is a consequence of polyembryonic origins, but the sociality of trematodes and polyembryonic wasps is also in a parasitic context.

It its perhaps too ambitious to say eusociality in parasites is common, but it is surprising how four disparate taxa (aphids, thrips, trematodes, and polyembryonic wasps) have species converging on a specialized soldier caste during an endoparasitic stage of their life cycle. Parasitism is a much more common lifestyle than currently realized (Lafferty 2008) and I doubt every instance of sociality in parasites comes from clonal groups evolving a secondary morph that performs a function like aggression that can be intuitively observed under a microscope. The expansion of sociobiology into parasites generally will require the discovery of obscure forms of social communication and interaction, many of which might require advancements in the

biological applications of analytical chemistry, for the perceptual world of parasites (Sukhdeo & Sukhdeo 2004) is likely chemosensory.

THE MULTIPLE FUNCTIONS OF SOCIAL RECOGNITION CUES

Much is known about colony recognition is ants (Hölldobler & Wilson 1990; Lenoir *et al.* 1999; Bos & d'Ettorre 2012), so it is exciting to expand into the fringes of the topic by questioning *what else* might the cues used in colony recognition be doing? It makes sense that traits used in recognition have other functions, especially if those functions (such as waterproofing of the exoskeleton) are ancestral to the derived social functions. This is because the simultaneous de-novo evolution of both the signal and the behavioral response is theoretically challenging. To theorize a more step-wise evolutionary pathway, cuticular hydrocarbons (CHCs) exist as an expressed phenotype controlled by insect genotypes, and the insects possess olfactory receptors that can sense CHCs, then later these CHCs acquire sensory meaning in a context like mate choice (Adams & Tsutsui 2020) or colony recognition.

Regardless, it at least appears that semiochemicals are not equally likely to be selected for recognition purposes. Multiple (or redundant) complex cues provide more accurate information for avoiding recognition errors (Tibbetts *et al.* 2020), volatile pheromones should have low molecular weights and vaporize at ambient temperatures and pressures, and in Argentine ants, it appears methyl-branched alkanes are better for nestmate recognition than *n*-alkanes. In Chapter 3, however, we found this functional trade-off to be not so simple. Generally speaking, an exoskeleton covered in relatively higher proportions of *n*-alkanes to methyl-branched alkanes was better at surviving desiccation, but a subset of compounds in each of these classes would show the opposite. Therefore, I believe that selection for desiccation (or selection for recognition) might not just favor the relative proportions of compound classes, but specific compounds within those classes instead.

I wish to investigate a similar trade off in the trematode recognition system. We do not know their cues, but in my Chapter 4 behavior experiments (and the many preliminary experiments in preparation of this chapter), I began to hypothesize that contact cues were used by the trematodes. This is because of a "rubbing" behavior they do upon enemy teguments before biting them that looks very much like "tasting". However, this could just be what searching for a bite looks like, with the decision for biting already made due to information received before contact.

Still, if they do use tegument cues, this would lead to a fascinating trade-off, because the tegumental surface of a trematode is the target of the hosts immune factors. Snail host immune factors (e.g. hemocytes, functioning like white blood cells) respond to peptidoglycan, lectins, mucins, and glycosylation patterns on the surface of trematode parasites (Hambrook & Hanington 2020), and a common trematode mechanism for avoiding host immunity (in both definitive and intermediate hosts) is to manipulate the content of their tegument or glycocalyx (Hurford & Day 2013). We know parasites have ways of finding their hosts, and we know hosts

have ways of finding their parasites, but do we ever consider how parasites recognize other parasites within their host?

THE ROLE OF RECOGNITION SYSTEMS IN BIOLOGICAL INDIVIDUALITY

Why do I find this interesting? I sometimes think my deep interests in social evolution come from not understanding my own society, or social interactions in general. Perhaps the answers to solve my social anxieties are hidden in the billion-year stories of evolution! In all seriousness though, I can at least assume that some swirling of my upbringing, development, culture, education, experiences, etc. attributed me with this niche research obsession.

Just as Darwin was influenced by famous thinkers of his era (e.g., Thomas Malthus), it is possible that his world view was influenced by the enlightenment era and French revolution that preceded him. Gould (2002) suggests that Darwin's natural selection and these movements had something in common: the focus of individuals as the ultimate agents of change within large systems. Darwin's theories even pulled the power of creation away from the supernatural, into the realm of the free actions of individual organisms. Our evolutionary study of sociality takes a similar perspective, typically asking how individuals optimize the costs and benefits of living in and depending on the larger system that is their social group. But as we studied how individuals evolved to form groups, we began to realize how groups become individuals. Even if we can't agree on how to define individuals in biology beyond commonsense understandings, we do know that is evolves, and it appears to be hierarchical, with smaller units collecting into larger ones.

The major evolutionary transitions theory started by Maynard-Smith & Szathmary (1995) defines individuals as reproducing units, and it does appear that for two of these transitions (to multicellularity, and to eusociality), the evolution of a recognition system maintains the boundaries of these reproducing units. But what about the other transitions? Did the predecessor of eukaryotes need to recognize and accept their endosymbionts? Going further back in time, we do not know if replicating molecules grouped into genomes before the advent of cellular membranes, so there is no theory I can offer on how something like "social boundaries" evolve in a genome outside of the context of a cell (though cells certainly do possess several mechanisms for controlling replication within them).

For the multicellular bodies and eusocial groups, the ultimate purpose of recognition is likely the prevention of germ-line "corruption" that leads to the death of the collective, usurping of the group's reproduction, or just reduction in fecundity. What illuminates my skepticism is that even if we are certain of these ultimate purposes, the realized expression of social boundaries seems fraught with cost and error. Immune systems can harm, to a sometimes-fatal degree, their own bodies in a cascade of recognition errors, and animal sisters can brutally attack each other in a rage of mistaken identities. The consequences of social boundaries, the pervasive need to recognize foes amongst friends, and the unreliable use of identity as a proxy of behavior, are tragedies of social living with recognition systems. I have pondered this since before my dissertation, and I will do so afterwards, always. Bazinga.

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