A COMPARATIVE APPROACH TO UNDERSTANDING THE EVOLUTION OF SOCIAL BEHAVIOR USING *PEMPHIGUS* APHIDS AS A MODEL SYSTEM

By

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

In partial fulfillment of the requirements

For the degree of

DOCTOR OF PHILOSOPHY

in

Biological Sciences

December, 2014

Nashville, Tennessee

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To those who inspired my passion for science and
encouraged me to continue when I had given up

ACKNOWLEDGEMENTS

I have many people to thank for helping me to succeed as a scientist, mentor and person. First and foremost, I must thank my advisor, Patrick. While in his lab, I was never limited by resources, just by the extent of my ideas. He encouraged my independence, but at the same time he was always there with a helpful analogy whenever I had reached an obstacle. We have a Venn diagram in the Abbot lab that shows the small overlap between good scientist and good people, and I can truly say that Patrick is in the middle of those two circles.

In thanking great scientists, I must also thank my committee members. First, Antonis Rokas, who taught me how to ask a thought-provoking question on any topic; Julian Hillyer for his expertise on qPCR and survivorship statistics; Jay Evans for volunteering to serve on a committee from half-way across the United States and inspiring me with his work on a diversity of social insects; And, finally, Ken Catania, who served as my chair, and always reminded of all the amazing animal adaptations.

I have been incredibly lucky to have worked with some great people while in the Abbot lab. Cassidy Cobbs was always there to help with my basic stoichiometry and all of my spelling or writing needs. Eva Sawyer, an honorary member of the Abbot lab, is a role model for me on how science should be done. I have also had many great undergraduate researchers work with me in the lab. Andrew Legan reminds me how fun science can be. Caroline Graham was one of the most inspiringly hard-working people I have had the pleasure of working with. Natalie Christian, Heather Etsby and Hayden Hill put up with me at the beginning when I was still learning how to mentor. Catie Mitchell and Emily Neil counted thousands of aphids without one complaint. Jibril Hibro, Stephen Ferguson and Stephanie Chiang joined the lab later in my career, but I know they will keep the tradition of greatness alive in the Abbot lab.

This work spans multiple disciplines and that was only possible due to the help of some great collaborators. John Tooker let me spend a week in his lab learning about GC mass

spectrometry. Don Miller and Holly Caravan spend a summer in the lab and taught me about the other social insects. I had a seemingly endless supply of *Drosophila* and *Drosophila* experts in the Bordenstein lab, Daniel LePage, and Brodie labs, Mary-Lynn Dear and Neil Dani. Thank you to the Hillyer lab members, Jonas King, Tania Esteves-Lao and Leah Sigle, who helped with qPCR and injections. Travis Clark at the Vanderbilt sequencing core helped with the RNA-seq project. Jonathan Ertelt helped me grow and take care of my cottonwoods. I am grateful to many Rokas lab members, John Gibbons, Jen Wisecaver, Abigail Lind, Leo Salichos, and Jason Slot for their help analyzing data and their fungal expertise. Also, I want to thank Dave McCauley for teaching me how to perform the correct statistics on my data. I must also thank all the people in the BSCI front office, especially Leslie Maxwell, for helping me sort out an endless amount of questions.

This work could not have been accomplished without funding sources. Most of the lab and field work was supported by a National Science Foundation grant # IOS-1147033 and a Vanderbilt University Discovery grant to PA. My travel to conferences, collaborators labs and field courses was funded by the Gisela Mosig graduate student travel fund.

Finally, I must thank my supportive family and my husband Gary. Growing up, my parents stressed the importance of education and, thanks to them, I have always assumed that graduate school was the path I would take. My brother is one of the most interested in science non-scientist I have ever met. He continues to ask me question that push the boundaries of my scientific knowledge and encourages me to learn. Last, but certainly not least, I must thank Gary. His help in the field and lab is unparalleled. He literally overcame his greatest fear, snakes, to help me in the field. In addition to an excellent field assistant, his support and love helped me through some of the most challenging obstacles. I know I could not have done it alone.

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ABBREVIATIONS

Act5c	Actin 5c
AMP	antimicrobial peptides
ANOVA	analysis of variance
BLAST	Basic Local Alignment Search Tool
bp	base pairs
CA	California
cact	cactus
Cat	Catalase
cDNA	complementary DNA
CecA1	Cecropin A1
CF	colonization frequency
cm	centimeter
Ddc	Dopa decarboxylase
Def	Defensin
df	degrees of freedom
Dhpr	Dihydropteridine reductase
DNA	deoxyribonucleic acid
EF	endophytic fungi
g	grams
GLM	General linear models
h	hours
HDBS	honeydew balls
H_2O	water
Imd	immune deficiency
IR	isolation rate
ITS	internal transcribed spacer
KCl	potassium chloride
km	kilometers
L_3	third instar larvae
L-DOPA	L-3,4-dihydroxyphenylalanine
m	meters
M	molar
min	minute
mL	milliliters
mm	millimeter
µL	microliter
N	sample number
NaCl	sodium chloride
$Na_2 SO_4$	sodium sulphate
NC	North Carolina
NCBI	National Center for Biotechnology Information
ng	nanograms

OTU	operational taxonomic unit
pale	tyrosine hydroxylase
PCR	polymerase chain reaction
PD	Potato Dextrose
PDA	Potato Dextrose Agar
PGRP	peptidoglycan recognition proteins
PO	phenoloxidase
Punch	GTP cyclohydrolase
qPCR	Real-Time PCR
RF	relative frequency
RNA	ribonucleic acid
ROS	reactive oxygen species
RPKM	reads per kilobase of exon per million mapped reads
SC	similarity coefficient
SE	standard error
sec	seconds
Sod	Superoxide dismutase
SPE	Spatzle-Processing Enzyme
spp.	species
spz	spatzle
Tep1	Thioester-containing protein 1
Tep2	Thioester-containing protein 2
TN	Tennessee
v	version
ºC	degrees Celsius

CHAPTER I

INTRODUCTION

"I…will confine myself to one special difficulty, which at first appeared to me insuperable, and actually fatal to my whole theory. I allude to the neuters or sterile females in insect communities..." (Charles Darwin, 1859)

The cooperation conundrum

Cooperation is inherently vulnerable to cheaters. This weakness of cooperation is highlighted in human economics with the famous example of the "tragedy of the commons" (Hardin 1968). On a shared pasture, each shepherd is deciding on the number of sheep to graze. There is a cost and benefit to each sheep added to the shared pasture. The benefit is to the owner of the sheep, while the cost is shared between all the shepherds in the potential of overgrazing the pasture. This discrepancy between cost and benefit results in each shepherd wanting to add more sheep at a shared cost. The tragedy is that all the shepherds would benefit from less grazing, and yet, most will act selfishly by adding as many sheep as possible.

Despite this vulnerability, cooperation is common and found in all groups from microbes to plants and animals. Additionally, cooperation has been crucial in the major transitions in the history of life (Szathmáry & Maynard Smith 1995) from the formation of eukaryotic cells from multiple prokaryotic cells (Margulis 1970) to the origins of multicellularity from single-celled ancestors (Buss 1987; Niklas & Newman 2013) to genes cooperating in the genome, to cooperatively breeding animal groups and, finally, societies. Some individuals within these cooperating society have foregone reproduction completely and instead specialize on specific tasks. These examples of caste differentiation found mainly in eusocial insects posed such a problem to evolutionary theory that Darwin referred to them as the "one special difficulty" (Darwin 1895; Herbers 2009; Ratnieks et al. 2011). These altruistic behaviors would pose a problem to

evolutionary theory if we did not consider both the direct and indirect fitness benefit to the actor of the behavior (West et al. 2007).

Kin selection and inclusive fitness

Decades after Darwin first puzzled over the evolution of sterile castes, scientists began to develop the mathematical framework to begin to explain the evolution of altruistic behaviors. According to legend, it began at a bar, where J. B. S Haldane, famously, remarked that, "I would jump into a river to save two brothers, but not one, or to save eight cousins but not seven." With this statement, he captured the idea that years later W.D. Hamilton would explain mathematically. In 1963, Hamilton developed an equation to explain how natural selection could favor cooperation if $rb > c$, where c is the cost to the altruist, b is the fitness to the beneficiary, and r is their genetic relatedness. This rule illustrates the fine balance between benefit and cost, cooperation and conflict and how increased relatedness can lower the fitness cost of altruistic behaviors (Hamilton 1963). Hamilton's rule takes into account the fact that social behaviors have a fitness effect on both the actor and the recipient. The sum of the direct fitness, the fitness of the actor, and the indirect fitness, the fitness of the recipient by the relatedness to the actor, is the inclusive fitness to the actor of the behavior.

Ecological pressures of group formation

Hamilton's rule helps explain how cooperative behaviors would be selected for, but does not answer how groups form initially. The ecological constraint hypothesis predicts that family groups begin to form when either (1) breeding opportunities are limited because of a lack of suitable habits or (2) rearing young is costly because of harsh variable environments (Emlen 1982). A specific ecological pressure that can select for social groups is increased predation rates. It has been proposed that there are two ways social insect groups have dealt with increased predation (1) remaining at the nest to help defend from predators or (2) forming groups as a life insurance against the consequences of mortality from predators (Queller & Strassmann 1998). Those that remain at the nest, or

the fortress defenders, live and feed within a protected site. Examples of fortress defenders include termites living in deadwood, and many of the newly discovered social taxa, like ambrosia beetles (Kent & Simpson 1992), thrips (Crespi 1992), aphids (Aoki 1980; Stern & Foster 1996) and snapping shrimp (Duffy 1996). In contrast to the fortress defenders, the life insurers must forage for food, which is a risky and often very costly behavior. Another important life history trait of life insurers is that most species are holometabolous, and go through complete metamorphosis. This means that the young must be cared for. If a solitary individual does not return from foraging, the individual along with all of the brood dies. If the individual is a member of a group and does not return from foraging, other group members are present to care for the brood. Bees, wasps and ants all use the life insurer strategy (Queller & Strassmann 1998; Strassmann & Queller 2007).

The other social insects

The study of social evolution in insects remains in many respects synonymous with the study of life insurers, like ants, bees, and wasps. There have been efforts in recent years to broaden the scope, by including such 'non-traditional insects' as aphids and thrips (Choe & Crespi 1997; Costa 2006). However, this has only been met with limited success. The argument for studying these groups is that they directly address the issue of generality in our conceptual understanding of a major thematic problem in evolutionary biology (how do groups suppress conflict such that cooperative integration emerges?), while offering unique opportunities for novel insights. For example, until we look, we don't know what properties of sociality are shared across the furthest reaches of insect diversity. These groups also offer possibilities for empirical research not always present in more traditional life insurer taxa. For example, in many, only a fraction of species are social. Additionally, different ecological pressures lead to sociality in the fortress defenders than the life insurers, like increased predation pressures and lack of suitable nest sites. Experimental work can thus focus precisely on comparing the traits that vary across "major transitions" in evolution (Strassmann & Queller 2010).

Sociality in aphids (Hemiptera)

Aphids are soft-bodied hemipterans that do not undergo complete metamorphosis. All aphids feed on plant phloem. Sociality is a rare phenomenon within aphids, occurring in an estimated 1% of species. Social aphids are often characterized as "primitively eusocial." These social species are characterized by the presence of 1st instars nymphs who act as "soldiers" that exhibit defensive and hygienic behaviors. Sociality has evolved at least 17 times in aphids, which gives ample opportunity for comparative studies across multiple closely related lineages (Stern & Foster 1996; Pike & Foster 2008). There are three major criteria are often cited in the literature as the main factors in the transition to sociality: 1) clonality allows for the selection of group-living even in situations of increased conflict 2) increased pressure from predation or microbes 3) extended portion of life cycle in gall or poor host plant quality leads to a decrease in clonal fecundity and an increased need for defense. Aphid species lacking these 1st instar "soldiers" presumably experience less pressure from these factors.

Social aphid species are confined to only two subfamilies within Aphididae: Pemphiginae and Hormaphidinae (Stern & Foster 1996). Most are gall-forming aphids, which have a complex lifecycle including primary and secondary host plants and separate sexual and asexual life stages. Galls are tumor-like growth on the primary plant initiated by an aphid stem mother. The gall acts as a sink and steals nutrients from the plant in order to grow. Abe (1991) and Crespi (1994) have noted that nest-like shelters, such as galls, provide a combination of food and shelter and thus, are ideal for the development of eusociality. Also, galls physically constrain the actual clonal growth rate and limits available nutrients, which allows for conflict (Abbot 2009).

Study organisms: *Pemphigus* (Hemiptera: Aphididae) aphids

Specifically, this thesis focuses on three closely related species in genus *Pemphigus* (Homoptera: Aphidoidea: Pemphigidae): *Pemphigus obesinymphae* (*P. obesinymphae*),

Pemphigus populitransversus (*P. populitransversus*) and *Pemphigus populicaulis* (*P. populicaulis*). All three are common in the Cumberland River Basin and form galls on primary host plants in the genus *Populus*, cottonwood (Blackman and Eastop 1994). Although the aphids are all closely related and appear on the same host plant, the three species vary in their degree of social behavior. *P. obesinymphae* is considered eusocial, while *P. populitransversus* is considered weakly social and *P. populicaulis* weakly to nonsocial. Aphids sociality is defined by the presence of a caste of "soldiers" that exhibit aggressive, self-sacrificial defense and various housekeeping duties (e.g., expelling waste and cadavers). I will further explore these definitions of aphid sociality in chapter II.

Significance

The evolutionary themes of sociality that span the social insects, from aphids to ants, remain an area of open inquiry. Moreover, there have been no comprehensive studies that have experimentally compared the ecological and molecular correlates of sociality across aphid species that vary in social traits (Pike & Foster 2008). **This research utilizes aphids to address three emerging themes in social evolution.** First, if the canonical expression of altruistic behavior is self-sacrifice for the family group, then characterizing the adaptations for defense means identifying the very traits that have evolved because of selection for altruism. This may be obvious in the caste systems of ants or termites, but it is not the case in the vast majority of social insects. What are the altruistic traits in aphids? In thrips? In ambrosia beetles? Second, new discoveries are suggesting that understanding the control of microbial pathogens by groups may reshape how we think of social life. Finally, the importance of kin selection in the evolution of sociality has recently become a hotly debated topic (Abbot et al. 2011; Boomsma et al 2011; Strassmann et al. 2011; Ferriere & Michod 2011; Herre & Wcislo 2011; Nowak et al. 2010). Aphids' unique natural history, including clonal reproduction which results in high relatedness, offers a new perspective on the role of kin selection in the evolution of sociality in this disparate group.

Outline of chapters

The goal of the proposed research is to capitalize on unique experimental opportunities to broadly test general themes in social evolution, using galling aphids as a model system. I begin my comparing two major features of sociality, housekeeping and defense, across three aphid species. Next, I focus on defense, which has traditionally been the hallmark of sociality in aphids. I, then, return to discuss the impacts of the aphid housekeeping behavior on fungal endophyte growth within the nest. Finally, I explore the role increased relatedness has played in the evolution of social behavior in this group.

In chapter II, I characterized our three aphid species along two axes of social behaviour: housekeeping and defense. Previous evidence suggested that these three species differ in the presence or absence of social traits. I found that for the ecological and behavioural traits tested, there were quantifiable differences between social and nonsocial species. However, there was no clear threshold that differentiated social from nonsocial species, meaning that definitions of sociality in aphids depend in part on the traits that are measured. If sociality is measured by defense, for example, the eusocial species, *P. obesinymphae* clearly expressed the greatest degree of aggressive and effective defense. However, some defensive behaviour was also present in the species traditionally defined as nonsocial. Conversely, if sociality in aphids is measured by traits related to homeostasis and housekeeping, then the species traditionally considered nonsocial expressed nearly the same behaviours as the eusocial species. These results imply that sociality in aphids evolves as a collection of uncorrelated traits. Clear analogues or antecedents of more derived social characters can be identified in species that are nominally nonsocial.

In chapter III, I examine in depth how social aphids defend themselves. The social aphid *P. obesinymphae* has nymphal soldiers that defend the colony from invaders by piercing them with their stylets. The mechanism by which is poorly understood. In the lab, soldiers will attack a surrogate invader (*Drosophila* larva), resulting in death of the larva. We found that attack by *P. obesinymphae* activated immune regulatory genes in the

melanization pathway. This suggests that the soldier attacks are eliciting a melanization immune response in *Drosophila* larvae. Of the transcripts overexpressed in larvae attacked by aphids, some were significantly overexpressed compared to pierced controls. These data indicate that in addition to the physical injury of piercing, *P. obesinymphae* soldiers may be introducing an effector molecule that leads to an upregulation of genes in the melanization pathway. Using mutant *Drosophila* lines with knock-outs in the melanization pathway, we were able to partially recover survivorship of *Drosophila* larvae after attack by aphid soldiers. This implies that overactivation of the melanization pathway is partially responsible for the death of the *Drosophila* larvae.

In, chapter IV, I examine the fitness consequences of the housekeeping behaviors described in chapter II by characterizing the fungal endophyte community within the gall. Insect-induced galls are abnormal plant growths that can provide food and shelter to their inhabitants, resulting in stressed plant tissue that may alter the conditions for the colonization or proliferation of endophytic fungi. I investigated the effect gall formation has on fungal endophyte communities and diversity. Using three closely-related gall-forming aphid species that specialize on poplars, I characterized fungal endophyte diversity in galls and surrounding petiole and leaf lamina tissue. Despite sharing a common host plant and often forming spatially contiguous galls, the endophyte profiles within the galls of each aphid species were distinct, not only from the galls of the other species, but also from surrounding plant tissue. These results suggest that insect galls can affect the composition of fungal endophyte species in plant tissues, by altering either the colonization or proliferation of their endophytic mycobiota. Likewise, fungal endophytes may be important in the ecology and evolution of insect galls.

In the final data chapter, chapter V, I explore the cost of conflict within a eusocial community. Previous work has shown that, as kin selection theory predicts, cheaters bring about a 'tragedy of the commons' within galls (Rankin et al. 2007), incurring competitive clonal interactions within galls likely mediated by resource exploitation . However, this result was correlational, leaving open a critical set of questions only manipulative experiments can answer. An important one is: what are the consequences of

conflict for the social benefits of cooperation? An array of arthropod predators and microbial parasites attack *Pemphigus* galls. If cheaters do not defend galls or perform the hygienic duties that soldiers commonly perform (such as removing cadavers and waste that potentially foul galls; worse, cheaters may even vector microbial pathogens; Stern & Foster 1996), then conflict should lead to higher rates of morbidity due to parasitic infection or predation. Moreover, previous work could not disentangle whether costs of conflict are simply a function of having any unrelated aphids within galls (even if all cheaters derive from a single clone), or if costs are a positive function of the number of clonal genotypes competing within groups (i.e, whether relatedness among cheaters is important). In this chapter, I aim to measure the fitness effect of the relatedness among cheaters and then continue on to explore the cost of conflict in other *Pemphigus* aphids species that are traditionally considered to be nonsocial and weakly social.

CHAPTER II

COMPARATIVE PHENOTYPING ACROSS A SOCIAL TRANSITION IN APHIDS

Abstract

In some insects, eusociality has evolved independently more than once, such that closely related species differ in the presence or absence of altruistic traits. Such groups offer opportunities to study the ecological and evolutionary drivers of transitions to sociality. In *Pemphigus* aphids, for example, eusociality has evolved independently multiple times, but most species are assumed to be nonsocial. Eusocial aphids thus typically have close relatives that are nonsocial, indicating a rapid and distinct transition to sociality. However, there has been only limited study of the behaviour of nonsocial species that permit direct comparisons with eusocial species. In this study, we characterized three aphid species along two axes of social behaviour: housekeeping and defence. Previous evidence suggested that these three species differ in the presence or absence of social traits. We found that for the ecological and behavioural traits we tested, there were quantifiable differences between social and nonsocial species. However, there was no clear threshold that differentiated social from nonsocial species, meaning that definitions of sociality in aphids depend in part on the traits that are measured. If sociality is measured by defence, for example, the eusocial species, *Pemphigus obesinymphae* clearly expressed the greatest degree of aggressive and effective defence. However, some defensive behaviour was also present in the species traditionally defined as nonsocial. Conversely, if sociality in aphids is measured by traits related to homeostasis and housekeeping, then the species traditionally considered nonsocial expressed nearly the same behaviours as the eusocial species. These results imply that sociality in aphids evolves as a collection of uncorrelated traits. Clear analogues or antecedents of more derived social characters can be identified in species that are nominally nonsocial.

Introduction

One of the major transitions in animal evolution is the shift from solitary to social lifestyles (Queller 2000; Szathmary & Smith 1995). The goal of comparative studies of social evolution is to understand the factors involved in this transition and to answer questions such as (1) what are the commonalities of social behaviour across socially living taxa and (2) what are the ecological/ demographic/ life history predictors of these commonalities (Bourke 1999; Danforth 2002; Ratnieks, Foster & Wenseleers 2006; Ross 2001)? Such questions are addressed with phylogenetic comparative approaches and ideally, detailed ecological analyses of related nonsocial and social species. However, ecological studies that bridge social transitions are often not possible in many species with advanced eusociality either because nonsocial species are absent, or because the evolutionary change between taxa is so great that the interpretation of ecological features is difficult. Consequently, species that express sociality facultatively or in which sociality is phylogenetically labile are valuable in comparative studies of social evolution (Gunnels, Dubrovskiy & Avalos 2008; Soro, Field, Bridge, Cardinal & Paxton 2010; Wcislo 1997). Social aphids offer unique opportunities for comparative studies, because sociality exhibits a remarkable degree of evolutionary lability and has been independently gained and lost multiple times among closely related species (Abbot 2009; Pike, Whitfield & Foster 2007; Stern 1994).

Aphids are small, soft-bodied herbivorous hemipterans that feed exclusively on plant phloem. There are about 5000 species, and many have complex life cycles that span two or more host plants and that alternate between sexual and asexual generations. Sociality is rare in aphids, and nearly all social species are found in two subfamilies in the Aphididae: Hormaphinae and Eriosomatinae (Stern & Foster 1996). Unlike better-studied eusocial taxa, such as bees and ants, social aphids do not express cooperative brood care. Rather, the defining feature of aphid sociality is aggressive, self-sacrificial defence against natural enemies by wingless subadult females, often involving specialized morphology or other weaponry (Stern & Foster 1996, 1997). These females are ‘soldiers’, and what they defend is their kin groups, which aggregate in dense clusters of clonally produced

females often within tumor-like plant growths known as galls. Gall-forming has evolved independently several times in aphids, and while the complexity of aphid life cycles makes it difficult to generalize about what exactly causes groups to form (in some species, soldiers are present during nongalling points in the life cycle), at the species level, sociality has rarely been observed in species that do not form galls at some point. Because of fierce defence of the nest-like gall by nymphal soldiers, a habit they share with other species that defend nests and refuges, such as termites, thrips, snapping shrimp and Damaraland mole-rats, *Cryptomys damarensis*, aphids are considered 'fortress defenders' (Cooney 2002; Crespi, Carmean & Chapman 1997; Duffy 1996; Queller & Strassmann 1998; Sobotnik, Jirosova & Hanus 2010). Note that this is somewhat overly simplified when it comes to aphids, because of the presence of soldiers in some species when no gall or 'fortress' is present.

One of the most valuable contributions of primitively eusocial insects like aphids is the potential they offer for comparative studies of social evolution, because closely related species, often sharing the similar habitats or overlapping distributions, seem to differ in the presence or absence of sociality. However, an unresolved issue is that what precisely constitutes a social aphid species is not obvious, limiting the practical use of comparative studies of social and nonsocial species. In some species, the presence of morphologically specialized or even reproductively sterile soldiers is unmistakable. But in others, the threshold for sociality has traditionally been defined by a combination of life history traits and behaviours. Most species have not been formally examined at all. Pike and Foster (2008) pointed out that many more aphid species live in groups than are nominally defined as social, and in those, social behaviours may be cryptically expressed, suggesting unappreciated complexity in aphid sociality. Moreover, aphids can express a range of behaviours that some authors describe as social. The primary social behaviour not related to defence in aphids is 'housekeeping', in which some group members actively remove waste and cadavers to prevent fouling of the gall, in a manner similar to that of other eusocial insects (Sun & Zhou 2013). According to Benton and Foster (1992), this behaviour in aphids is likely an act of kin-selected altruism, because the energetically expensive or even dangerous act of cleaning confers a group-level benefit. The presence

of housekeeping behaviours may indicate rudimentary forms of cooperative group living in aphids that, from a comparative perspective, represent precursors or evolutionary routes to more advanced sociality in aphids. No study has yet attempted to study evolutionary correlations between defence and other social behaviours in aphids in a phylogenetic framework. It is not known whether, across different aphid species, sociality is expressed as syndromes of covarying cooperative behaviours, or whether these behaviours vary independently, with species that express various combinations of social traits. In short, it is unclear what a social transition is in aphids.

What, then, is sociality in aphids, and how is it measured? Most studies of social behaviour in aphids have focused on single species. There have been relatively few comparative studies of social aphid evolution (Stern 1994) or that have placed single species studies into comparative frameworks (Shibao, Shimada & Fukatsu 2010), and only one that has systematically evaluated the behaviours of congeneric aphids that vary in the expression or degree of social behaviour (Rhoden & Foster 2002). In this study, we evaluated two axes of social behaviour in aphids (housekeeping and defence) in a group of congeneric North American aphid species. The three closely related species we studied each form galls on *Populus* spp. but vary in the degree to which they express social defence behaviours. Two species, *Pemphigus populitransversus* and *Pemphigus obesinymphae* are sister species, while the third species, *Pemphigus populicaulis* is more distantly related in a monophyletic group that includes all North American *Pemphigus* species (Abbot & Withgott 2004). Our goal was to define objectively a social transition in aphids. Below, we compare the ecology and life history of these three species. Next, we use behavioural assays to compare another feature of sociality in aphids, altruistic housekeeping. Finally, we use an objective measure of aphid sociality, natural predator morbidity, to provide a common scale on which to rank the species in terms of social behaviours. Our results indicate that there is no evidence of sharply defined social thresholds in *Pemphigus* aphids, much as Pike and Foster predicted (2008), and the foundations of the most derived social behaviours can be gleaned in species that are not traditionally defined as social. However, our results also indicate that clear and objective

demarcations of social behaviour in aphids can be identified. The most unambiguous of these is the ability to kill much larger insects than themselves.

Methods

Some background on the basic biology of *Pemphigus* aphids is helpful in understanding their social biology. Aphids are hemimetabolous insects, meaning that unlike ants and bees, which are holometabolous, they do not undergo complete metamorphosis from juveniles to adults. Rather, the juveniles are typically morphologically similar to adults and undergo a serious of moults from an initial or first instar, through progressively larger instars before reaching adulthood. Thus, whereas holometabolous social insects have relatively immobile larva that may be provisioned by workers, aphids and their allies have mobile larvae that are capable of feeding themselves. In *Pemphigus,* social behaviour occurs within galls and is expressed primarily by the first-born (first instars). Aphids express viviparous parthenogenesis, which means that an 'army' of clonal nymphal soldiers can rapidly accrue during colony development.

Ecology of Pemphigus populicaulis: *an Aphid without Social Behaviour*

Pemphigus is a genus of aphids in the holarctic subfamily Eriosomatinae (Aphidoidea: Aphididae; formerly Pemphiginae). *Pemphigus populicaulis* (Fitch) is a gall-forming species that is widely distributed across North America (Blackman & Eastop 1994). This species has a typical heteroecious life cycle, involving annual alteration between primary hosts in the genus *Populus* (primarily *Populus deltoides* or *Populus tremuloides*), on which sexual generation and gall formation occur, and an undetermined secondary host plant (Blackman & Eastop 1994). Galls are initiated in the spring on the petiole at the base of the leaf blade. Within the gall, and as with the species described below, the foundress reproduces parthenogenetically. Four wingless instars occur in rapid succession before a final moult into winged alates, which migrate in late spring (April–May) to the secondary hosts, ultimately returning to poplars, where a sexually produced, overwintering egg is laid beneath the bark (Table 1). With only a brief galling phase on

Table 1. A comparison of the life history and ecological traits of *P. populicaulis*, *P. populitransversus* and *P. obesinymphae.*

Trait	***P. populicaulis***	***P. populitransversus***	***P. obesinymphae***
Gall			
Distribution	North America	Eastern USA	USA and northern Mexico
Primary host plant	*P. deltoides* and *P. tremuloides*	*P. deltoides* and *Populus* spp.	*P. deltoides* and *P. fremontii*
Secondary host plant	Compositae	Brassicaceae	Brassicaceae
Location of gall	On petiole near leaf lamina	Petiole	On petiole near leaf lamina
Month of gall induction	March	April	May
Gall length	2-3 months	3-4 months	5-6 months

poplars, *P. populicaulis* does not appear to express defence and, thus, has been described as 'nonsocial' (Abbot 2009).

Ecology of Pemphigus populitransversus: *an Aphid with Intermediate Social Behaviour*

Pemphigus populitransversus Riley distribution is limited to the eastern United States (Blackman & Eastop 1994). Although closely related to *P. obesinymphae* (see below), the life cycle of this species is similar to the life cycle of *P. populicaulis*. *Pemphigus populitransversus* has been reported on many *Populus* spp., but most frequently forms galls on *P. deltoides*, alternating between these and a secondary host in the Brassicaceae (Table 1; Blackman & Eastop 1994). Galls are initiated later in the spring than are those of *P. populicaulis* (Abbot & Withgott 2004). Both species, however, form galls on the petioles of the first or spring flush of leaves that sprout from the pre-formed overwintering poplar buds. Previous work has suggested that first-instar nymphs express some degree of defensive behaviours and, thus, *P. populitransversus* is 'weakly social' and represents a transitional phase in social behaviour in aphids (Pike, Whitfield & Foster 2007; Rhoden & Foster 2002).

Ecology of Pemphigus obesinymphae: *an Aphid with Highly Social Behaviour*

Pemphigus obesinymphae is distributed across the United States and into northern Mexico (Blackman & Eastop 1994). Although closely related to *P. populitransversus*, the life cycle of *P. obesinymphae* is unusual, differing from the traditional life cycle of many *Pemphigus* aphids (Abbot & Withgott 2004). Its life cycle incorporates a different overwintering strategy in which it has omitted diapause all together. The life cycle is temporally rotated forward, overwintering on the secondary plant (Brassiceae), rather than returning to the primary host (*Populus fremontii* or *P. deltoides*) in the autumn. Unlike *Pemphigus populicaulis* or *P. populitransversus*, the galls of *P. obesinymphae* are initiated on the second or summer flush of leaves, which sprout from newly formed buds

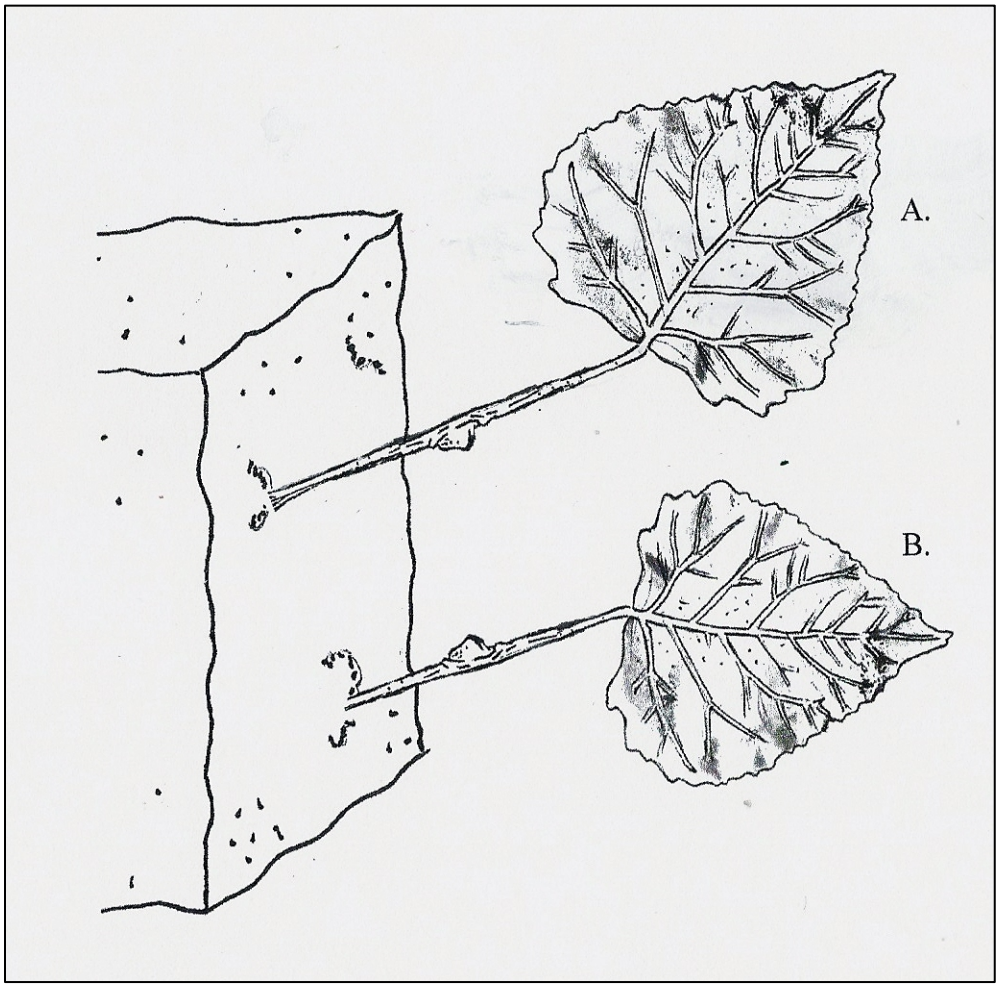

Fig. 1. (A) Illustration of *P. populitransversus* gall in floral foam with ostiole in its natural direction (B) or manipulated in an upwards direction.

(Diptera, Drosophilidae) to mimic dipteran larval predators (Abbot, Withgott & Moran 2001). We monitored the predator or *D. melanogaster* larvae every 20 min and noted survivorship. Controls involved placing these insects in empty galls without aphids to establish each predator's baseline survival. The interior surfaces of *Pemphigus* aphid galls are covered in a waxy substance that the aphids exude and which likely acts to deter predators (Pope 1983). Experiments with *Orius* spp. ended after 8 h and those with *C. rufilabris* ended after 12 h, because the *Orius* spp. were only tested against *P. obesinymphae* soldiers. *Orius* spp. were only tested against *P. obesinymphae* soldiers because they are frequently found in galls of *P. obesinymphae* but not as frequently found in the galls of the other species (S. P. Lawson & P. Abbot personal observation). The sample size for each group was at least 20 galls. We recorded survival of *D. melanogaster* larvae every 20 min and performed statistical analyses using the log-rank survival test. Differences were considered significant at $P < 0.05$. To further characterize defence, we introduced third-instar *D. melanogaster* larvae to the gall and counted the number of aphids actively attacking the larvae every 15 min for 180 min ($N = 20$). We compared the average number of attackers for each species using a Wilcoxon Lifetest. All statistical analyses were performed in JMP v. 7.01 (SAS Institute).

RESULTS

Altruistic Housekeeping Behaviours

Housekeeping behaviour was observed in the putatively nonsocial *P. populicaulis* as well as in the weakly social and eusocial species. However, the individuals that performed this behaviour differed between species. In both *P. populicaulis* and *P. populitransversus,* housekeeping was mostly performed by first-instar nymphs, whereas in the eusocial species, all larvae performed housekeeping duties. Groups with first instars ejected more honeydew balls over the 7-day period than groups without first instars (Wilcoxon Lifetest: *P. populicaulis*: $\chi^2_1 = 6.7583$, $P = 0.0093$; *P. populitransversus*: $\chi^2_1 = 8.6628$, $P = 0.0032$; Fig. 2a). Surprisingly, there was no significant difference in the number of

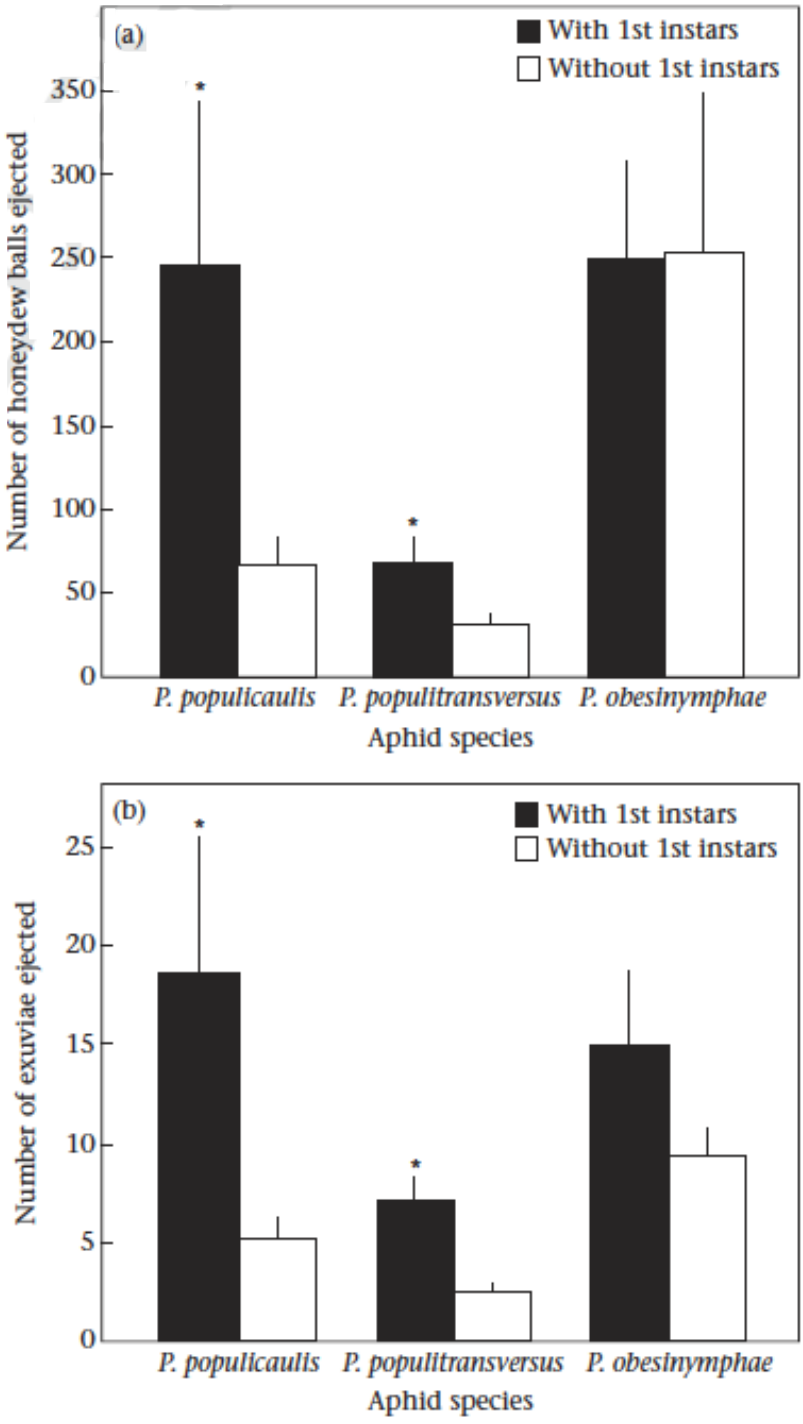

Fig. 2. (a) Average number (± SE) of honeydew ball waste ejected from the gall of three aphid species. (b) Average number of exuviae, or aphid exoskeletons, ejected from the gall of three aphid species. Each gall contains either 100 total aphids (50 1st instar and 50 late instar; $N = 10$ galls; black bars) or 90 total aphids (30 2nd instar and 60 late instar; $N = 10$; white bars). Asterisks indicate a significant difference at ($P < 0.05$).

honeydew balls ejected with or without first instars in *P. obesinymphae* galls (ANOVA log transformation: $F_{1,16} = 1.6038$, $P = 0.2247$; Fig. 2a). Overall, more honeydew balls were ejected from groups of eusocial *P. obesinymphae* and nonsocial *P. populicaulis* than from weakly social *P. populitransversus* groups (ANOVA log transformation: $F_{5,47} = 13.3842$, $P < 0.0001$; Fig. 2a). Similar trends were seen in the ejection of exuviae from the gall over the 7-day period. Groups with first instars ejected more exuviae over the 7-day period than groups without first instars in both *P. populicaulis* (Wilcoxon Lifetest: $\chi^2_1 = 6.0814$, $P = 0.0137$) and *P. populitransversus* ($\chi^2_1 = 5.3343$, $P = 0.0209$), while in *P. obesinymphae* groups, the presence or absence of first instars made no difference in the amount of exuviae ejected (ANOVA with log transformation: $F_{1,16} = 0.5773$, $P = 0.4584$; Fig. 2b). We found no significant difference between the number of winged alates or the number of dead aphids in galls with and without soldiers for any species (Supplementary Table S1).

There was a significant difference in the number of aphids in the gall of each species (Fig. 3; Wilcoxon Lifetest: $\chi^2_2 = 86.7542$, $P < 0.0001$; *P. populicaulis* versus *P. populitransversus*: $P = 0.4191$; *P. populicaulis* versus *P. obesinymphae*: $P < 0.0001$; *P. populitransversus* versus *P. obesinymphae*: $P < 0.0001$;). Because aphid densities within galls varied across species, we normalized the number of honeydew balls ejected against the average number of aphids per gall for that species. First, changing the normal position of the ostiole (from angled towards the ground to pointing directly upward) clearly reduced the ability of each aphid species to eject waste (Fig. 4; Wilcoxon Lifetest: *P. populicaulis*: $\chi^2_1 = 21.5628$, $P < 0.0001$; *P. populitransversus*: $\chi^2_1 = 10.8609$, $P < 0.001$; *P. obesinymphae*: $\chi^2_1 = 12.5374$, $P = 0.0005$). Second, comparing across species, *P. populitransversus*, the species with weakly expressed defence, had significantly more honeydew balls remaining in the gall when the ostiole was pointing upwards relative to the other two species (Fig. 4; Wilcoxon Lifetest: $\chi^2_5 = 52.0618$, $P < 0.0001$), and *P. populicaulis* had significantly more honeydew balls remaining in the gall when the ostiole was pointing down in its natural orientation (Fig. 4; $\chi^2_5 = 52.0618$, $P < 0.0001$)

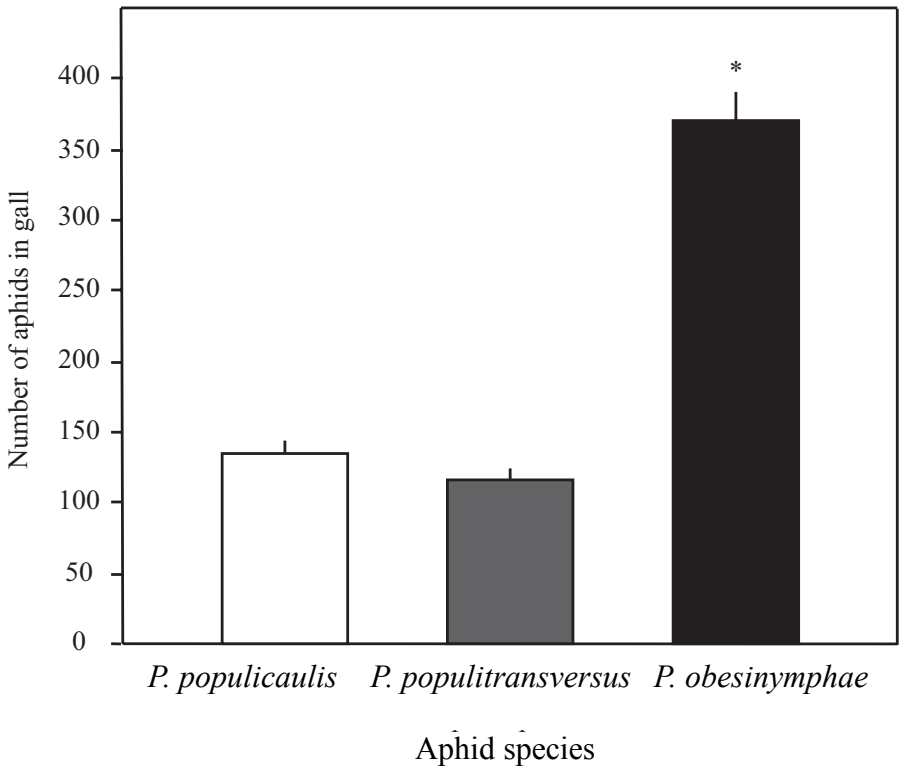

Fig. 3. Average (±SE) number of aphids in galls of each aphid species: *Pemphigus populicaulis* (*N* = 40); *P. populitransversus* (*N* = 50); *P. obesinymphae* (*N* = 49). Asterisks indicate a significant difference ($P < 0.05$).

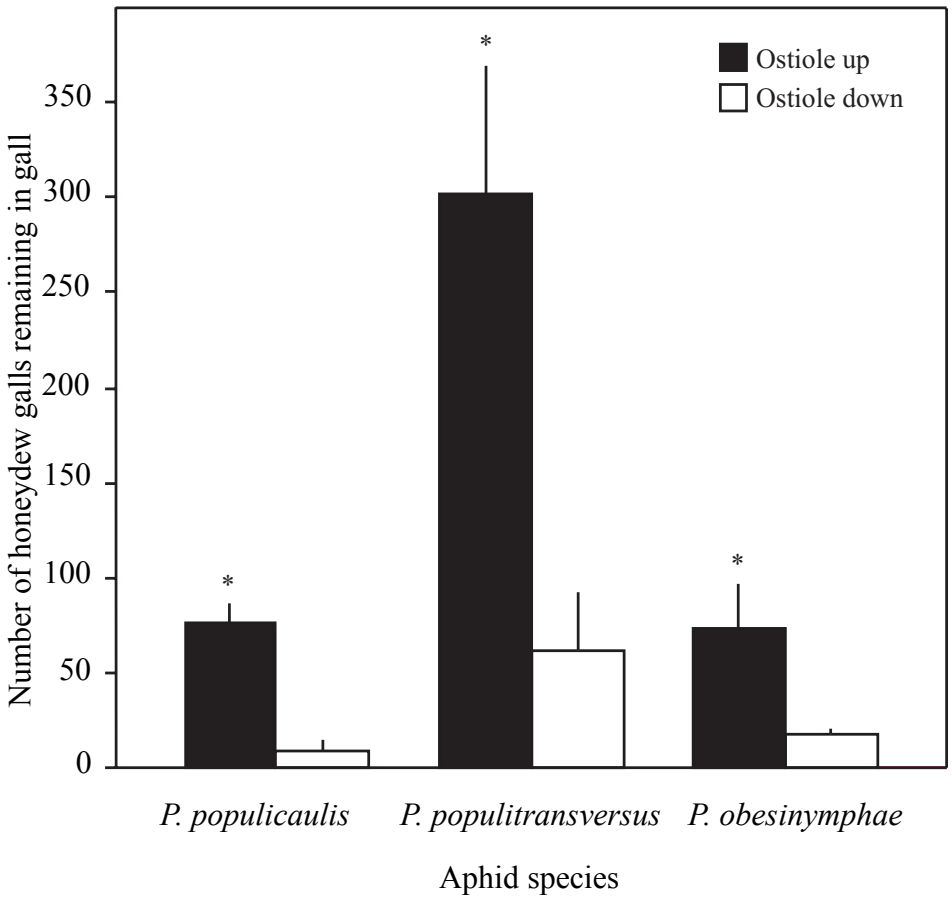

Fig. 4. Average (± SE) number of honeydew ball waste ejected by aphids from galls with the ostiole in the natural position (white bars; $N = 12$ for each species) or manipulated to point upwards (black bars; $N = 12$ for each species). Asterisks indicate a significant difference ($P < 0.05$).

Fortress Defence

We found that the average time to death of the *Drosophila melanogaster* larvae was negatively correlated with the nominal characterization of sociality in each species (i.e. nonsocial, weakly social or eusocial). *D. melanogaster* larvae that were introduced to the gall of the nonsocial aphid species, *P. populicaulis,* showed an average survival of 198.8 min (range 75–450 min), while larvae introduced to the galls of the weakly social species, *P. populitransversus,* showed an average survival of 175.6 min to death (range 60–540 min), and larvae in the gall of the eusocial species, *P. obesinymphae,* had an average survival of 83.6 min to death (range 45–195 min; log-rank test: *P. populicaulis* versus *P. populitransversus:* $\chi^2_1 = 0.8158$, $P = 0.3664$; *P. populicaulis* versus *P. obesinymphae*: $\chi^2_1 = 56.4564$, $P < 0.0001$; *P. populitransversus* versus *P. obesinymphae*: $\chi^2_1 = 35.6498$, $P < 0.0001$; Fig. 5a). Overall, regardless of species, the presence of aphids reduced survivorship of *Drosophila* larvae relative to controls placed in empty galls (log-rank test: $\chi^2_3 = 84.0620$, $P < 0.0001$; Fig. 5a).

We found similar trends using a natural predator, *C. rufilabris*. The eusocial species had the most effective soldiers (average survival of *C. rufilabris*: 45 min, range 20–80 min), while the weakly social aphid species' soldiers were the second most effective (average survival of *C. rufilabris*: 64 min, range 20–180 min), although not significantly so. The nonsocial aphid species' soldiers were the least effective (average survival of *C. rufilabris*: 266.3 min, range 20–580 min; log rank test: *P. populicaulis* versus *P. populitransversus:* $\chi^2_1 = 12.5042$, $P = 0.0004$; *P. populicaulis* versus *P. obesinymphae*: $\chi^2_1 = 15.9044$, $P < 0.0001$; *P. populitransversus* versus *P. obesinymphae*: $\chi^2_1 = 2.5934$, $P = 0.1073$; Fig. 5b). Again, *C. rufilabris* that were introduced into a gall with aphids died earlier than the controls in an empty gall (log rank test: $\chi^2_3 = 74.9785$, $P < 0.0001$; Fig. 5b). We tested another natural predator, *Orius* spp., against the social aphid, *P. obesinymphae*, and obtained similar results. *Orius* spp. placed in a gall with aphids had a significantly earlier time to death (average: 109.5 min, range 40–220 min) compared with

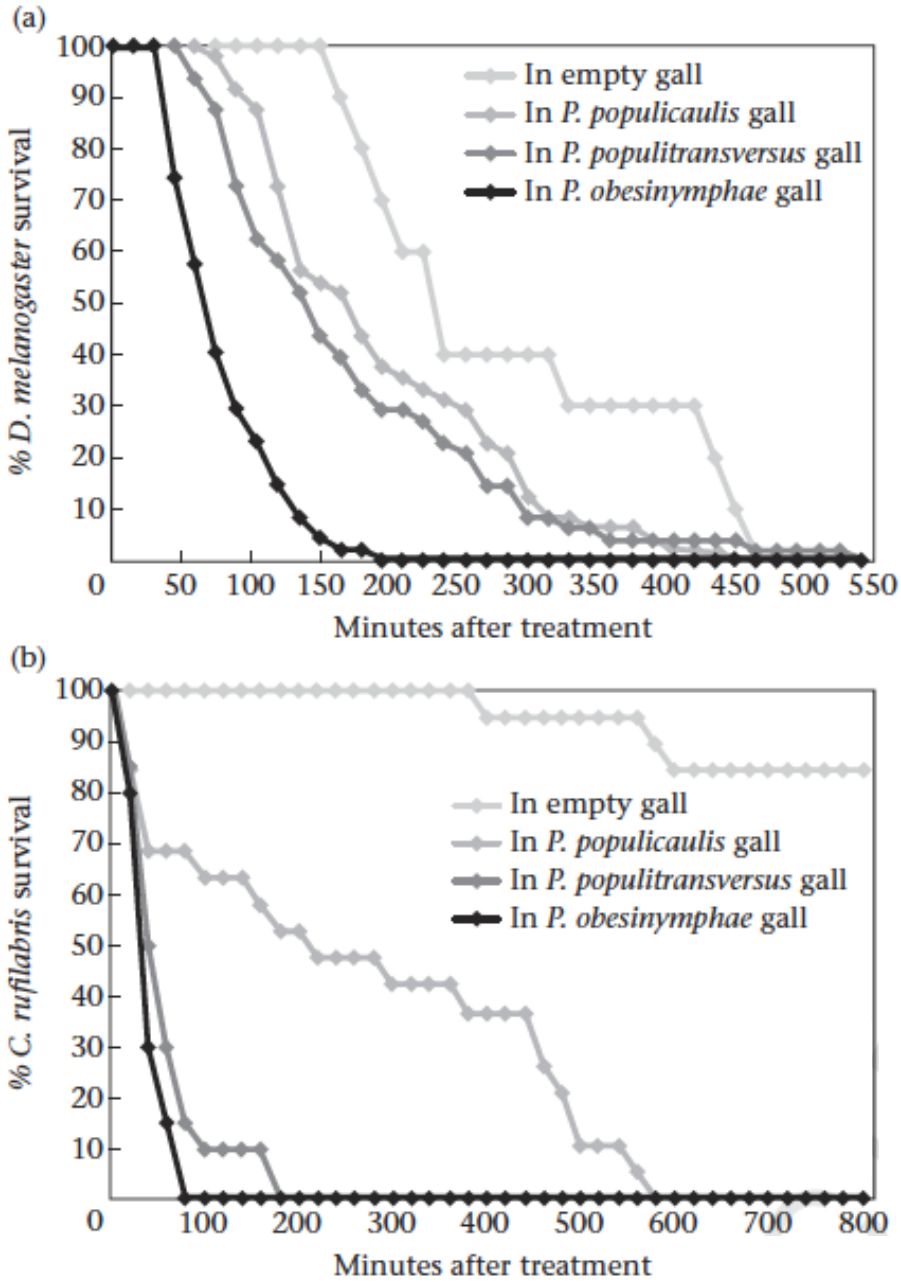

Fig. 5. (A) Survival of 'invading' *Drosophila melanogaster* larvae placed in an empty gall (control, $N = 20$), in gall containing *Pemphigus populicaulis* ($N = 48$), *P. populitransversus* ($N = 48$) or *P. obesinymphae* ($N = 48$) aphids. (B) Survival of predatory *Chrysoperla rufilabris* larvae in an empty gall ($N = 19$), in a gall containing *P. populicaulis* ($N = 20$), *P. populitransversus* ($N = 20$) or *P. obesinymphae* ($N = 20$) aphids.

controls (average: 1098.9 min, range 360–1440 min; log-rank test: $\chi^2_1 = 43.5414$, $P < 0.0001$; Supplementary Fig. S1). To quantify defence behaviour of *Pemphigus* further, we compared the raw number of soldiers recruited by each species to attack an invader of the gall. We found that, on average, *P. obesinymphae* had significantly more soldiers per invader (18.8 soldiers) than *P. populitransversus* (8.6 soldiers) or *P. populicaulis* (3.1 soldiers) (Wilcoxon Lifetest: $\chi^2_2 = 160.7328$, $P < 0.0001$; *P. populitransversus* versus *P. populicaulis*: $\chi^2_2 = 49.7268$, $P < 0.0001$; Fig. 6).

Discussion

Among their various experimental advantages, aphids offer opportunities to compare closely related species that differ in their expression of social behaviours (Stern & Foster 1996). However, there are few comparative studies of social behaviour in aphids that characterize the behaviours of both social and nonsocial aphids, and as yet, it is not clear how to categorize the spectrum of sociality in aphids. To describe the behaviours involved in the transition from nonsocial to social lifestyles in aphids empirically, we compared three closely related species of *Pemphigus.* We found that the two behaviours most often used to characterize sociality in aphids (defence and housekeeping) varied between eusocial and nonsocial species, but in surprising ways. An objective measure of defence (e.g. how fast do predators die?) clearly differentiated species previously described as social and those not formally recognized as social. However, nonsocial species expressed housekeeping behaviours on par with that of the eusocial species and, thus, another measure of social behaviour (the extent to which they performed maintenance of their nests) conflicts with the fortress defence standard of aphid sociality. Thus, there is not a single syndrome of social behaviour in aphids. Rather, while more thorough studies of social and nonsocial species are needed, it appears likely that the social behaviours that aphids express vary independently across species.

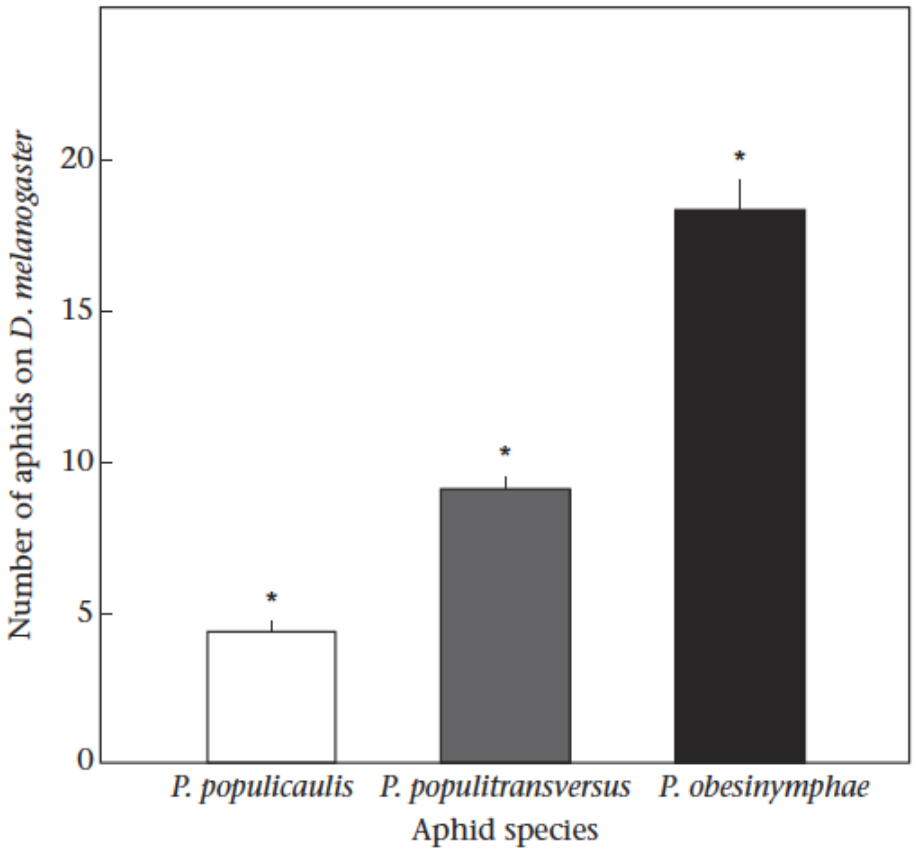

Fig. 6. Average (±SE) number of *Pemphigus populicaulis* ($N = 48$), *P. populitransversus* ($N = 48$) and *P. obesinymphae* ($N = 47$) aphids attacking an invading *D. melanogaster* during a 3 h period. Asterisks indicate a significant difference ($P < 0.05$).

Life History Traits

Most Pemphigus aphids have two distinct host plants during the life cycle: a galling phase on the primary host plant in the genus *Populus* and a herbaceous secondary host plant. Interestingly, compared to *P. populitransversus* and *P. populicaulis, P. obesinymphae* has shifted its life cycle to overwinter in the roots of its secondary host plant and spend more time within the gall (Abbot & Withgott 2004). It has been hypothesized that predation during the galling phase is one of the major ecological drivers of soldier evolution in aphids (Foster & Northcott 1994). *Pemphigus obesinymphae* spends more of its life cycle in the gall and has the most effective soldiers (Fig. 5). The question then arises: did the shift in life cycle occur because *P. obesinymphae* evolved effective soldiers, which allowed them to stay in the gall longer, or did the shift in life cycle occur first and thus put intense selective pressure on the development of soldiers? The role of host plants in driving social evolution in aphids has been an open question for some time (Stern & Foster 1996). To date, there are still no comprehensive studies that have investigated social evolution in aphids from the perspective of plant–insect interactions.

Altruistic Housekeeping Behaviours

Group living is inherently vulnerable to the increased risk of pathogens and disease transmission. To combat this, eusocial organisms have evolved cooperative immune responses known as social immunity (Cremer, Armitage, & Schmid-Hempel 2007). Social immune behaviours can be prophylactic, like resin collection by ants to reduce microbial growth (Christe, Oppliger, Bancala, Castella & Chapuisat 2003), antimicrobial secretions by parents (Arce, Smiseth & Rozen 2013), or corpse removal by workers (Diez, Le Borgne, Lejeune & Detrain 2013) or activated in response to a pathogen, like social fever in honeybees (Starks, Blackie & Seeley 2000). Identifying these cooperative immune responses is key to understanding how groups reduce the risks of living together and transmitting pathogens. Aphids excrete a large volume of sugary waste or honeydew. Honeydew poses a threat to groups of aphids because they can become entrapped and

drown in the watery substance (Denny 1993). Honeydew also provides the perfect environment for microbial growth (Fokkema, Riphagen, Poot & Dejong,1983; Lawson, Christian & Abbot 2014). Free-living aphids can avoid these problems by changing feeding sites, flicking honeydew waste from the leaf, or being tended by aphids. Gall-forming aphids must find other ways to avoid these challenges (Benton & Foster 1992; Pike, Richard, Foster & Mahadevan 2002). Gall cleaning has been noted in some species, and there is even evidence of gall repair (Aoki 1980; Aoki & Kurosu 1989; Kurosu & Aoki 1991; Kutsukake, Shibao, Uematsu & Fukatsu 2009; Pike & Foster,2004). More experimental approaches to measure altruistic housekeeping behaviours have only been thoroughly explored in *P. spyrothecae*, *P. dorocola* and *Hormaphis betulae* (Aoki 1980; Benton & Foster 1992; Kurosu & Aoki,1991). To our knowledge, there have been no studies of housekeeping in species that lack soldiers. To explore how altruistic housekeeping behaviours vary across species with and without soldiers, we compared the ability of first-instar soldiers to remove honeydew balls and exuviae waste from the gall. Past research in other species has shown that first or second instars are the predominate housekeepers (Aoki 1980; Benton & Foster 1992; Kurosu & Aoki 1991). We found that all aphid species tested ejected waste from the gall. In both *P. populicaulis* and *P. populitransversus*, galls with first instars ejected significantly more honeydew balls and exuviae than galls without first instars (Fig. 2). This implies that the first instars in these species perform a majority of the housekeeping behaviours. Although there was no significant difference in fitness measurements between groups with and without first instars, our measures of fitness were rough ones, taken from laboratory populations over a short duration (Table S1). Considering that the galling length of these species varies from 3 to 9 months, this experimental period is likely not enough time to see a significant effect of the increase in honeydew balls caused by the absence of the first instars. In the highly social species, the first instars are important in the maintenance of the gall, but later instar individuals also participate in housekeeping behaviours. Interestingly, unlike *P. obesinymphae*, in *P. populicaulis* and *P. populitransversus,* first instars do not delay development, but moult rapidly after larviposition, and their clonal groups are typically composed of individuals at various stages of development. Given that cooperative housecleaning behaviours are only expressed by the first instars in these species, whereas

all instars express these behaviours in the eusocial species, the implication is that there has been an elaboration of behaviours associated with group homeostasis in the eusocial species that is absent from other, less social species. Moreover, the presence of housekeeping in the nonsocial *P. populicaulis* indicates that selection can favour homeostatic behaviours in the absence of selection for defence, a result that is mirrored by the persistent expression of housecleaning behaviours by *P. obesinymphae* juveniles as they age, despite the fact that only the first instars express defence behaviours. Thus, in *P. obesinymphae,* defence and homeostasis can vary independently through development, and is a form of temporal polyethism not unlike that seen in other eusocial taxa like honeybees, stingless bees and ants (Mersch, Crespi, & Keller 2013; Seeley 1982; Sommeije 1984).

Another interesting finding was the significantly lower amount of honeydew ejected by *P. populitransversus* galls compared to *P. populicaulis* or *P. obesinymphae* galls (Fig. 2a). To test whether this was caused by less housekeeping activity or by less honeydew production, we manipulated the direction of the ostiole to examine the amount of honeydew produced by each species. We found that changing the direction of the ostiole led to an increase in the number of honeydew balls in the gall for all three species, and *P. populitransversus* produced significantly more honeydew balls (Fig. 4). *Pemphigus populitransversus* galls have a very different shape compared to the other two species and occur on the petiole of the poplar leaf, rather than at the base of the leaf lamina. The gall is much longer and the ostiole is not as pronounced. It is possible that there is no functional advantage to cleaning behaviour in *P. populitransversus* because the ostiole is so small; instead, they allow the honeydew to build-up within in gall. Another possibility is that the petiole is a nutritionally poor niche relative to the leaf lamina, requiring petiole-gallers to feed more than species in more nutritionally rich sections of a host plant. There is some evidence that aphid galls are resource sinks, and there may be interspecific differences in the degree to which galls concentrate plant metabolites (Larson 1991). Finally, unusual adaptations have been described in some aphid species whose galls are closed, preventing the removal of honeydew (Kutsukake et al. 2012). Possibly, *P.*

populitransversus expresses behaviours for managing honeydew that we did not account for.

Fortress Defence

Fortress defence is the defining characteristic of sociality in aphids and has been described in multiple aphid species (Aoki, Kurosu, Shin & Choe 1999; Aoki, Kurosu & Sirikajornjaru 2007; Aoki, Kurosu & von Dohlen 2001; Kurosu, Buranapanichpan & Aoki 2006; Moran 1993; Rhoden & Foster 2002). Surprisingly, however, there is no single means to identify fortress defence in aphids, and different studies typically use one or several life history, morphological and behavioural indices to define the presence of altruistic defence in aphids. Past measures of defence behaviours have been largely qualitative, typically noting only whether aphids placed in an arena with a predator will show aggressive behaviours. We used an objective and quantifiable measure of defence (the ability and rate at which aphids kill natural predators) that, theoretically at least, could be applied to any aphid species. Although this measure of defence quantifies the effective defence of the entire group and, thus, does not capture individual differences in the effectiveness of attack, it does allow us to measure the effective consequences of defence as an emergent property of eusocial aphid groups, which tend to be larger and composed of more aggressive soldiers than weakly social or nonsocial species, as described below.

It has been previously argued that galling aphid species display a continuously varying spectrum of altruistic defensive behaviours, from weak to highly aggressive. This is what we observed. *Pemphigus populicaulis* is anecdotally a nonsocial species, but we found that its soldiers were capable of killing a natural predator, *C. rufilabris*. However, soldiers of *P. populicaulis* were less effective than those of the weakly social *P. populitransversus*, which is intermediate to the eusocial *P. obesinymphae* (Fig. 5). *Pemphigus obesinymphae* invests almost four times as many soldiers during attacks of invaders as the nonsocial *P. populicaulis* (Fig. 6). More research is needed to understand

whether *P. obesinymphae* soldiers are more efficient recruiters, or whether *P. obesinymphae* galls have more soldiers available.

Another question is how these aphid soldiers are able to kill natural predators much larger than themselves. Unlike some social aphid species, the species we studied have monomorphic larvae that lack obvious morphological adaptations for defence. However, little is known about the chemical defences of aphids and whether morphological adaptations for defence predict or correlate with defensive chemistry. It has been demonstrated that soldiers in one aphid species, *Tuberaphis styraci*, use a secreted venomous protease for colony defence (Kutsukake et al. 2004).

Conclusions

What is sociality in aphids? Traditionally, the presence of a soldier caste is the defining feature of sociality in aphids. Based on this definition, *P. populicaulis* is considered a nonsocial species, but our results indicate that *P. populicaulis* does indeed have workers with modest defensive behaviours. These workers are not as effective as the weakly social *P. populitransversus* or the eusocial *P. obesinymphae* (Fig. 5). However, if housekeeping were the hallmark of sociality in aphids, much as brood care is in some other social insects, then *P. populicaulis* and *P. obesinymphae* would be considered highly social species, while *P. populitransversus* would be considered a nonsocial to weakly social species (Table 1). Our work has demonstrated that defining sociality in aphids depends critically on what trait is being measured. In addition, these data contribute to evidence suggesting that most galling aphid species, including those not traditionally described as nonsocial, may express some form of social behaviour. Most social insects outside of the Hymenoptera remain poorly studied, and even basic natural history information is often lacking, or largely anecdotal (Costa 2006). As studies of these 'other social insects' are undertaken, the lesson from aphids is that the nonsocial species tend to be particularly poorly studied, if at all. If the goal is to understand social transitions, however, studies of nonsocial species are as necessary to comparative studies as those with social behaviour . Second, aphids illustrate the degree to which the

antecedents to advanced sociality may be identified in nonsocial species. It will be revealing as studies of sociality begin to identify both the ecological as well as the more mechanistic factors that amplify or tune the latent expression of advanced social behaviours in aphids.

Acknowledgements

This work would not have been as successful without the assistance of the co-authors, Andrew Legan, Caroline Graham and Patrick Abbot. A.L. and C.G. assisted with much of the data collection and P.A. helped develop the ideas and write the manuscript. This work was supported by a National Science Foundation grant (number IOS-1147033) and a Vanderbilt University Central Discovery Grant awarded to P.A. We are grateful to Cassidy Cobbs and Jibril Hirbo for their feedback on the manuscript and to the anonymous referees for their helpful and insightful comments. We also thank Gary Lawson his fabulous illustration of galls in floral foam, which was used in Fig. 1.

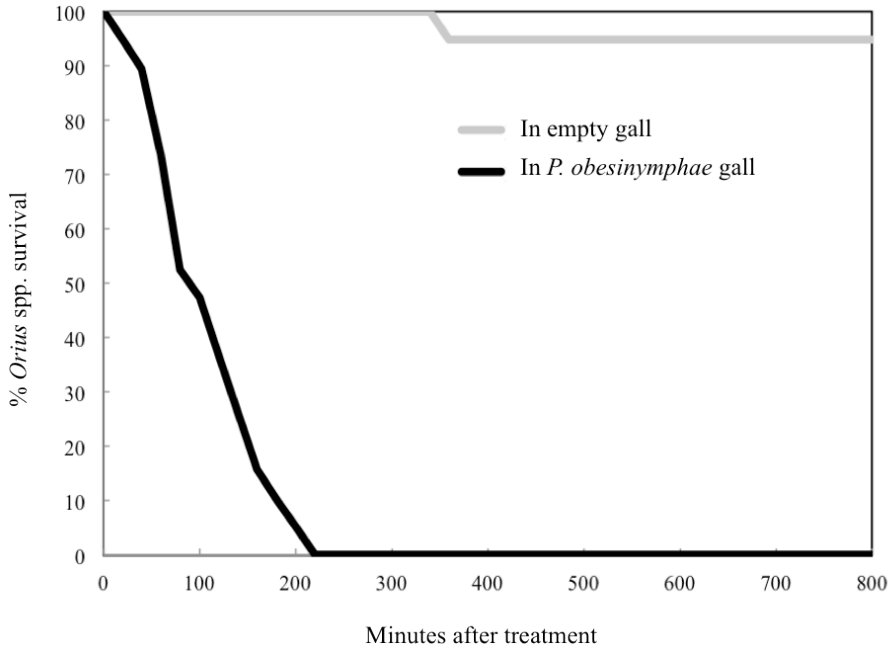

Fig. S1. Survival of *Orius* spp. placed in either an empty gall (control; $N = 19$; grey line) or a gall containing *Pemphigus obesinymphae* ($N = 19$; black line).

Table S1. Average number (±SD) of total winged alates produced during the experiment and the average number of dead aphids remaining in the gall at the conclusion of the experiment across the three aphid species

	Total number of alates produced [a]		Average no. of dead aphids [b]	
	Without 1st instars	With 1st instars	Without 1st instars	With 1st instars
P. populicaulis	4.78±3.83	11.6±11.92	23.77±15.20	25.30±12.25
P. populitransversus	1.20±2.20	2.60±2.55	18.5±6.57	37.8±13.25
P. obesinymphae	17.2±6.43	13.6±4.88	15.6±5.68	14.9±5.72

Each gall contained either 100 total aphids (with first instars: 50 first instars and 50 late instars; $N = 10$ galls), or 90 total aphids (without first instars: 30 second instars and 60 late instars; $N = 10$ galls).

[a] Total number of winged alates produced over a 7-day period and remaining in the gall when the experiment was terminated.

[b] Number of dead aphids removed from the gall and remaining in the gall when the experiment was terminated.

CHAPTER III

THE MOLECULAR BASIS OF AN EFFECTIVE DEFENSE IN APHID SOLDIERS

Abstract

Defense is key to the formation and maintenance of groups, but most small sap-feeding insects are not well-equipped to defend themselves. The social aphid *Pemphigus obesinymphae*, however, has nymphal soldiers that defend the colony from invaders by piercing them with their stylets. The mechanism by which is poorly understood. In the lab, soldiers will attack a surrogate invader (*Drosophila* larva), resulting in death of the larva. After attack by *P. obesinymphae*, systemic melanization of the *Drosophila* larva is observed. This response is likely due to the overactivation of immune-regulatory pathways. To begin to quantify this phenotype, we used RNA-seq to compare *Drosophila* larva before and after attack. We found a high percentage of immune-related genes overexpressed after attack. To further quantify this observation, we used real-time PCR to focus on the expression of specific immune genes before and after attack by aphid soldiers. We selected genes from the major immune pathways, including Toll, Imd, and melanization signaling pathways. We found that attack by *P. obesinymphae* activated immune regulatory genes in the melanization pathway. This suggests that soldier attacks are eliciting a melanization immune response in *Drosophila* larvae. Of the transcripts overexpressed in larvae attacked by aphids, some were significantly overexpressed compared to pierced controls. These data suggest that in addition to the physical injury of piercing, *P. obesinymphae* soldiers may be introducing an effector molecule that leads to an upregulation of genes in the melanization pathway. Using mutant *Drosophila* lines with knock-outs in the melanization pathway, we were able to partially recover survivorship of *Drosophila* larvae after attack by aphid soldiers. This implies that overactivation of the melanization pathway is partly responsible for the death of the *Drosophila* larvae.

Introduction

Social insects exhibit complex societies with reproductive division of labor, brood care, and morphological and behavioral specializations for colony tasks, including defense, foraging, and hygiene. These insects are typically described as exhibiting advanced eusociality, because of both their complex social organization and their evident ecological success. In recent years, the catalog of insects and other arthropods that show various forms of rudimentary, or even complex sociality, has grown rapidly (Crespi, Carmean & Chapman 1997; Duffy 1996; Sobotnik, Jirosova & Hanus 2010). For most of these, however, relatively little is known about the functional and evolutionary aspects of their sociality. For many of the newly-discovered social organisms, collective defense is the primary expression of cooperative behaviors (Crespi 1992; Duffy 1996; Stern and Foster 1996; Tian & Zhou 2014; Yamamura 1993). Social organisms that live within protected nests face threats of usurpation and predation from an array of predators and pathogens. In some, selection has favored reproductive division of labor and specialized castes whose sole function is defense of nestmates. These social organisms have been described as "fortress defenders" (Costa 2006; Queller & Strassmann 1998).

Eusocial Hymenoptera and Isoptera exhibit an array of venoms, toxins and other biochemical and morphological weaponry for defense (Brand et al. 1972; Kubelka et al. 1993; Prestwich 1979; Sobotnik et al. 2010), but much less is known about how these fortress defenders deter predators. Fortress defenders, like gall-forming aphids and thrips, have soldier-like morphs specialized for defense (Crespi et al. 1997; Stern & Foster 1996). Both aphids and thrips are herbivorous insects, and despite tantalizing evidence that they possess toxic secretions that deter or kill natural enemies (Kutsukake et al. 2004; 2008), it remains a mystery how these herbivores, often orders of magnitude smaller than their predators, successfully defend their "fortresses". This is in stark contrast to our growing understanding of the biochemistry and functional targets of ant and bee venoms (Haberman 1972, Kuhn-Nentwig 2003, Ozdemir et al. 2011).

Here, we describe a novel mechanism for defense in social aphids. Social aphids form tumor-like galls on their host plants, inside which they feed and reproduce parthenogenetically. All social aphids possess specialized defenders or soldiers that aggressively attack other insects that threaten their groups. Sociality is rare in aphids, occurring in less than 1% of species and only occurring in two families, Hormaphididae and Eriosomatinae (Stern & Foster 1996). Many species in Hormaphididae have specialized morphological adaptations for attack, like enlarged forearms for grasping invaders (Stern 1998). The soldiers of most species attack with their mouthparts, known as stylets, in a manner that appears as if they are feeding on their natural enemies (Abbot et al. 2001; Moran 1993). Kutsukake et al. (2004) showed that in at least one aphid species in Hormaphididae, *Tuberaphis styraci*, soldiers secrete a midgut-expressed cysteine protease through their stylets, which can immobilize or even kill their victims. However, subsequent work failed to show that other social aphids utilize the same cysteine protease, suggesting that there are lineage-specific and possibly diverse biochemical adaptations in aphids for defense (Kutsukake et al., 2008).

We describe how a species from a divergent social aphid lineage*, Pemphigus obesinymphae* (Hemiptera: Aphididae: Eriosomatinae), which has no morphological adaptations for defense, can successfully defend its gall from predators. Previous work in the lab has shown that *P. obesinymphae* soldiers are capable of effectively killing various larval and adult insects, which they attack indiscriminately (Lawson et al. 2014). *Drosophila* larvae, for example, are not aphid predators, but induce attack by aphid soldiers, and rapidly die thereafter (Abbot et al. 2001; Lawson et al. 2014). *Drosophila* provides a clue to the how *P. obesinymphae* soldiers deter and even kill much larger insects. *Drosophila* larvae exhibit a marked pattern of systemic melanization upon attack. Melanization is the product of an immune response to injury known as the phenoloxidase cascade. Because toxic and reactive quinones are produced during melanin biosynthesis, the phenoloxidase cascade is tightly-regulated in insects (Bolton et al. 2000; Cerenius & Soderhall 2004). We predicted that aphid soldiers overwhelm a tightly-regulated immunological response in their victims, resulting in deterrence and morbidity in their enemies, and, ultimately, successful defense of their groups. We tested this hypothesis by

first using RNA-seq to compare genes overexpressed before and after attack by aphid soldiers. We found a significant number of immune related genes overexpressed after attack. To further explore this phenotype, we used qPCR to focus on specific genes involved in major components of the *Drosophila* larvae immune response, in the Toll, the immune deficiency (Imd), phagocytosis and melanization signaling pathways. We found evidence that attack by aphid soldiers results in overexpression of genes involved in melanization. *Drosophila* melanization mutants lived longer than controls after attack by aphid soldiers, implying that overactivation of the melanization pathway is partially responsible for the death of the *Drosophila* larvae. Given that components of the invertebrate innate immune system can be targets of the venoms of insects, these results suggest that herbivorous social aphids have converged on a set of biochemical adaptations that function in analogous ways to those found in other insects.

Methods

Specimen collection

Pemphigus obesinymphae galls were collected from cottonwood trees (*Populus deltoides*) in Dyersburg, Tennessee. Wild-type *Drosophila melanogaster* lines were provided by the Bordenstein and Brodie labs at Vanderbilt University. All experimental manipulations used *Drosophila* third instar larvae (L_3). The larvae of various fly species are natural predators of gall-forming aphids, and given the resources provided by *Drosophila,* they serve as useful models of the response of dipterans attacked by social aphids (Abbot et al. 2001).

Degree of Drosophila *melanization*

To quantify the melanization phenotype before and after attack by aphid soldiers, the degree of melanization 1 hr following attack was assessed using previously described methods (Infanger et al. 2004; Shiao et al. 2001). Briefly, a score was assigned to each sample based on the proportion of the body melanized: 1 (no observable melanization) to

4 (surface completely covered by melanin) (Fig. 1). Each *Drosophila* larvae was photographed before attack, immediately following attack, after removing the attacking soldiers, and 1 hr following attack. Only *Drosophila* larvae being attacked by at least 10 aphid soldiers were included in the study. As a control, *Drosophila* larvae were left in an empty gall for 1 hr and then melanization was scored. The sample size for each group was at least 10. The data was analyzed as nonparametric equivalents of two-way ANOVAs (Sokal & Rohlf 1995). All statistical analyzes were performed in JMP v. 7.01 (SAS Institute). All reported *P*-values are two-tailed.

High-throughput data generation and analysis

Total genomic RNA was extracted from two samples of 9 *Drosophila* larva, one group, which were in an empty gall for 1 hr and one group, which were in a gall with *P. obesinymphae* aphids for 1 h. Each sample was extracted using the Qiagen RNeasy Mini Kit® for cells, tissues and yeast, including the DNase digestion protocol. Purified RNA was quantified with a NanoDrop ND-1000 spectrophotometer. Two libraries were constructed and sequenced through a single-end read protocol on the Illumina Genome Analyzer at the Vantage core at Vanderbilt University (http://vantage.vanderbilt.edu). Illumina instrument software performed data analysis and base calling.

Low-quality bases and Illumina adapter sequences were trimmed from reads using Trimmomatic (version 0.27; Bolger et al. 2014). After adapter and quality trimming, reads were aligned to the *Drosophila melanogaster* reference genome (FlyBase r5.57) with TopHat2 using default parameters (Kim et al. 2013). Reads aligning to each gene were counted with htseq-count using the intersection-strict mode (Anders et al. 2014). For each sample, gene expression was quantified using the reads per kilobase of exon per million mapped reads (RPKM) metric. Differential expression between genes in the unattacked and attacked samples was calculated using two cutoffs. The first cutoff compared the fold change between genes by calculating the relative RPKM (rRPKM = $RPKM_{attacked}$ / $RPKM_{unattacked}$) for each gene. The second cutoff compared the proportion

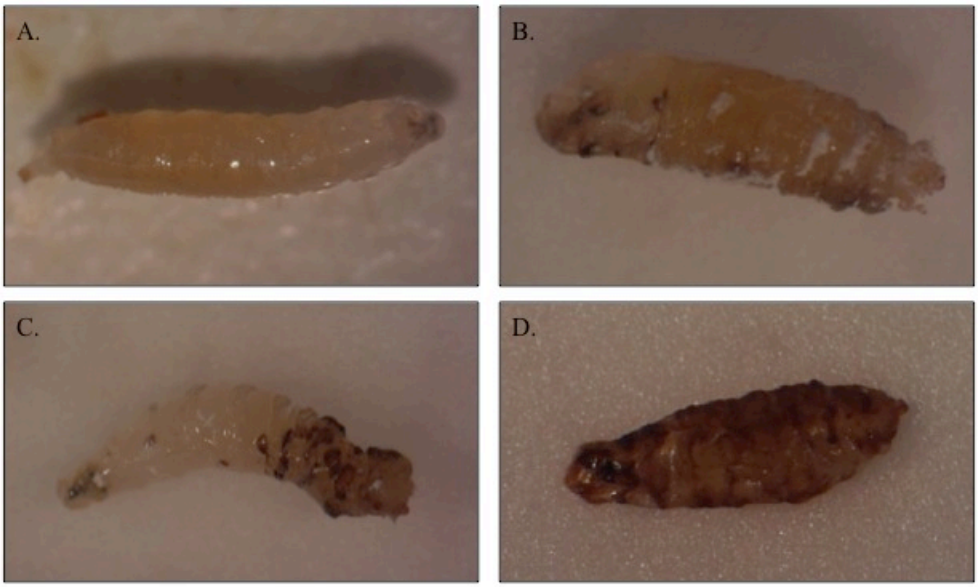

Fig. 1: Categories of melanization in *Drosophila* larvae. (A) Larvae with no evidence of melanotic capsule material on their surface were given a score of 1. (B) Those with few melanotic capsules were given a score of 2. (C) Those with half the surface melanized were given a score of 3. (D) Individuals that were completely melanized were given a score of 4.

of reads mapping to a gene in both samples using Fisher's exact test with Bonferonni's correction for multiple comparisons. A gene was considered differentially expressed if the $\log_2$ rRPKM value was equal or greater than 2 and the Bonferonni-corrected Fisher's exact *p*-value was less than 0.05.

Real-time PCR protocol

Expression levels were analyzed in larvae, which were attacked by *P. obesinymphae* for 1 hr, placed in an empty gall for 1 hr or pierced with a sterile needle and placed back in fly medium for 1 hr. One hour was chosen because it was prior to the average observed time of death caused by aphid soldiers (Lawson et al. 2014), but enough time to observe a melanization response. Total RNA was extracted from whole body individual larvae of each treatment using the RNeasy Mini Kit (Qiagen, Valencia, CA) and eluted in 40 µl of RNase-free water. RNA samples from at least six individuals were obtained for each treatment. Extracted RNA was treated with DNase to eliminate any residual genomic DNA. For first strand cDNA synthesis, 2 µl of RNA was added to 4 µL of 5x iScript reaction mix, 1 µl of iScript reverse transcriptase and RNase-free water to a total volume of 20 µl (BioRad, Hercules, CA). Samples were incubated at 25 ºC for 5 min, 42 ºC for 30 min and 85 ºC for 5 min. All cDNA was stored at -20 ºC.

Primers for genes in multiple immune pathways were used (Supplementary Table S1). Real-Time PCR (qPCR) was carried out on a BioRad Real-Time PCR Detection System in 25 µl reactions and based off from Coggins et al. (2012). Forward and reverse 0.3 µM primers were mixed with 12.5 µl Power SYBR Green Master Mix (Applied Biosystems) and added to 300 ng cDNA and molecular grade water. All qPCR reactions were repeated three times on different plates. Reactions were carried out: 40 cycles at 90 ºC for 15 sec, 60 ºC for 1 min, followed by a *melt* curve up to 95ºC. CT values from the BioRad software were used for expression analysis. Expression levels of mRNA were calculated with the comparative CT method. CT values were normalized to the expression of a non-regulated internal control gene, Actin 5C (Act5c), and calibrated to mean expression of larvae from empty galls (Ling & Salvaterra 2011). Comparative CT method: $\Delta\Delta CT$ =

[ΔCT Treatment X Sample 1] – [Average (ΔCT Calibrator Sample)], where treatment X represents the different manipulations to larvae. Fold change was calculated by $2^{-\Delta\Delta CT}$ (Livak & Schmittgen 2001). Mean fold change from at least six individual samples was graphed for each treatment. For validation of primer efficiencies, ΔCT values were calculated with serial dilutions of template cDNA. The data were fit using linear regression. Absolute values of the slope less than one were assumed to have similar efficiencies (Livak & Schmittgen 2001).

Time to death of Drosophila *knock-outs*

To examine if the overactivation of the melanization pathway affected the survivorship of *Drosophila* following attack by aphid soldiers, we compared the survivorship of multiple *Drosophila* mutants in the melanization pathway to wild-type *Drosophila* following attack by aphid soldiers. *Drosophila* mutant lines were ordered from the Bloomington *Drosophila* Stock Center (Indiana University) with the following FlyBase IDs and genotypes: FBst0003279, ple^4 st^1 e^1/TM3, Sb^1 (tyrosine hydroxylase mutant), FBst0000173, Df(2R)min, Pu^1/T(2;3)ap^{Xa}, ap^{Xa} (GTP cyclohydrolase mutant), FBst0000360, Dp(1;2;1)AT/+; hk^1 Ddc^7 (Dopa decarboxylase mutant), and FBst0027207, y^1 w^*; P{EP}$Dhpr^{G6439}$ (Dihydropteridine reductase mutant). $Mgat1^1$/CyO-GFP *Drosophila* larvae were used as mutant controls (Sarkar et al. 2006). To measure survivorship, we introduced *Drosophila* larvae to the gall, monitored the larvae every 20 minutes and noted the time to death of the larvae. Distribution of the time to death data was tested with the Shapiro-Wilk test for normality and Levene's test for equality of variance. All statistical analyzes were performed in JMP v. 7.01 (SAS, Cary, NC, USA). All comparisons were analyzed using a two-way ANOVA or nonparametric equivalent. Where necessary, data was normalized via log transformation (Sokal & Rohlf 1995). All reported *p*-values are two-tailed.

Results

Attack by aphid soldiers induces melanization

Past research has demonstrated that social aphid soldiers are capable of defending the gall from invaders (Lawson et al. 2014). Aphid soldiers grasp and probe the invader with their piercing mouthparts. After attack, *Drosophila* larvae typically exhibit a systemic and sometimes massive melanization response. Using double-blind observational assays, we visually quantified the melanization phenotype in *Drosophila* larvae by recording the degree of melanization based on a scale 1 (no melanization) to 4 (completely melanized; Fig. 1). Our results show that attack by aphid soldiers leads to a significant increase in the degree of melanization relative to unattacked controls (Fig. 2; Wilcoxon test, df = 1, $P < 0.0001$). The melanization of these individuals ranged from 1 to 4 and the average was a score of 2 ($N = 11$; Fig. 1, Fig. 2).

Whole-body RNA-sequencing

To begin to quantify the observed phenotype, RNA-seq was used to compare the overall composition of genes expressed before and after attack by aphid soldiers. Of the 13,624 genes, only 218 genes were significantly overexpressed in the *Drosophila* larva that had been attacked and 19 genes were significantly underexpressed. Genes with biological functions involved in immunity, like phagocytosis, defense response to bacterium and autophagic cell death, made up many of the overexpressed genes. The only other group with as many genes overexpressed in the attacked larva were genes associated with metabolism (Table 1).

Transcriptional induction of genes in melanization pathway

To further quantify the observed overexpression of immune response of *Drosophila* larvae following challenge by aphid soldiers, we used qPCR to examine the expression of multiple genes, which comprise various parts of the immune response. First, we examined genes involved in the Toll pathway. The Toll pathway is activated during infection by gram-positive bacteria or fungal infection through spatzle activation of Toll. Cleavage of the Toll ligand spatzle (spz) by Spatzle-Processing Enzyme (SPE) causes the binding of spatzle to Toll, which activates the heterodimeric protein complex Cactus–

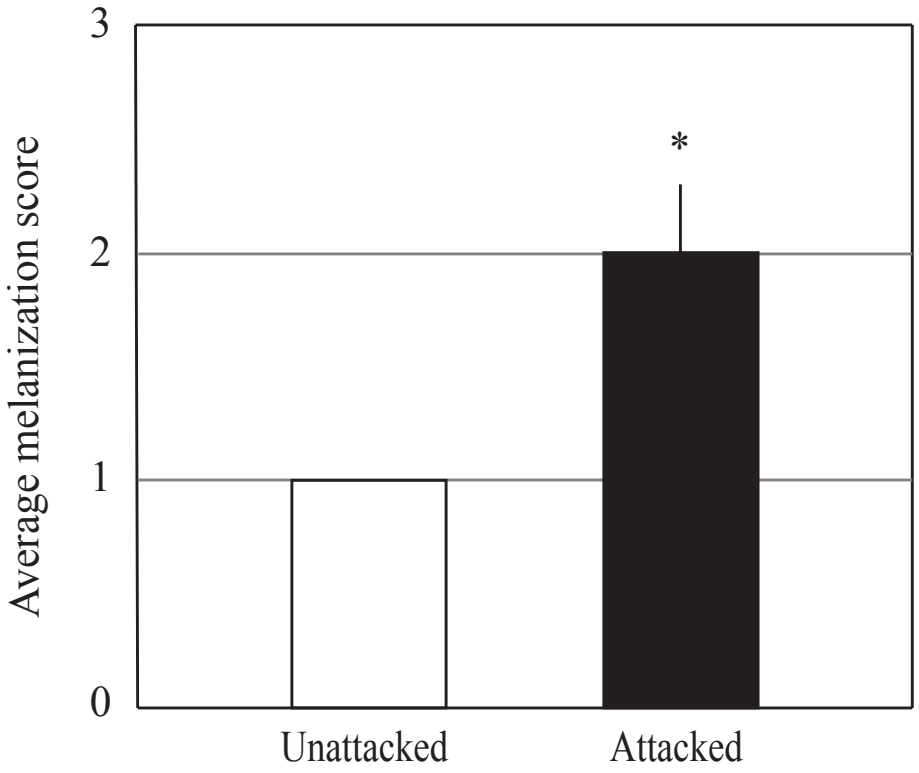

Fig. 2: The average (± SE) degree of melanization score of *Drosophila* larvae in an empty gall for 1 hr (white bar) or attacked by aphid soldiers for 1 hr (black bar). The average was taken for at least 10 replicates in each group. Asterisk indicates a significant difference ($P < 0.05$).

Table 1. Biological processes of genes overexpressed in *Drosophila* larva attacked by aphid solders.

Biological Process	# of genes overexpressed	% of genes overexpressed
Unknown	111	47.03
Transport	17	7.20
Immune function	15	6.36
Other	15	6.36
Metabolism	14	5.93
Morphogenesis	14	5.93
Post-transcriptional modifications	12	5.08
Reproduction	10	4.24
Ion binding	5	2.12
Proteolysis	5	2.12
Transcription	5	2.12
Translation	5	2.12
Structure	5	2.12
Catabolic process	3	1.27

Dorsal, ultimately leading to the release of antimicrobial peptides (AMPs), like Defensin (Def) (Brennan & Anderson 2004; Hoffmann 2003; Janeway & Medzhitov 2002; Jang et al. 2006; Romeo & Lemaitre 2008). At 1 hr post-treatment, we found no significant expression differences of cactus (cact), SPE, or Defensin (Fig. 3A-C). However, there was a greater than 5-fold increase in the expression of spatzle following attack by *P. obesinymphae* aphids and a 4-fold increase of *Drosophila* larvae pierced with a sterile needle compared to control (Fig. 3D).

The Imd signaling pathway is induced during infection of gram-negative bacteria. Peptidoglycan components of gram-negative bacteria are recognized through peptidoglycan recognition proteins (PGRPs) that activate the Imd pathway (Brennan & Anderson 2004; Hoffmann 2003; Romeo & Lemaitre 2008). PGRP-LC is a recognition receptor for the IMD pathway and Cecropin A1 (CecA1) is an AMP-activated in response to gram-negative infections (Lemaitre et al. 1997; Samakovlis et al. 1990). There was no significant difference in PGRP-LC expression between any of the groups (A). Cecropin A1 was overexpressed by about 3-fold in *Drosophila* larvae pierced with a sterile needle and attacked by aphid soldiers (Fig. 4B).

Thioester-containing protein 1 (Tep1) is expressed in hemocytes and is involved in immune responses in the epithelial cells in the JAK/STAT pathway (Agaisse & Perrimon 2004; Brennan & Anderson 2004). While Tep1 is involved in induction of phagocytosis, it has also been implicated in early melanization responses in the epithelia (Blandin et al. 2004). Tep1 expression was increased by almost 4-fold in larvae attacked by *P. obesinymphae* and the larvae pierced with a sterile needle compared to controls (Fig. 5A). Thioester-containing protein 2 (Tep2) has peptidase inhibitor activity (Stroschein-Stevenson et al. 2006). There is also experimental evidence that it is involved in responding to gram-negative bacterium, phagocytosis, and engulfment (St. Pierre et al. 2014). Tep2 expression was not significantly different in any treatment group (Fig. 5B).

Superoxide dismutase (Sod) and Catalase (Cat) prevent the damaging effects of superoxide and hydrogen peroxide, respectively, by converting these compounds

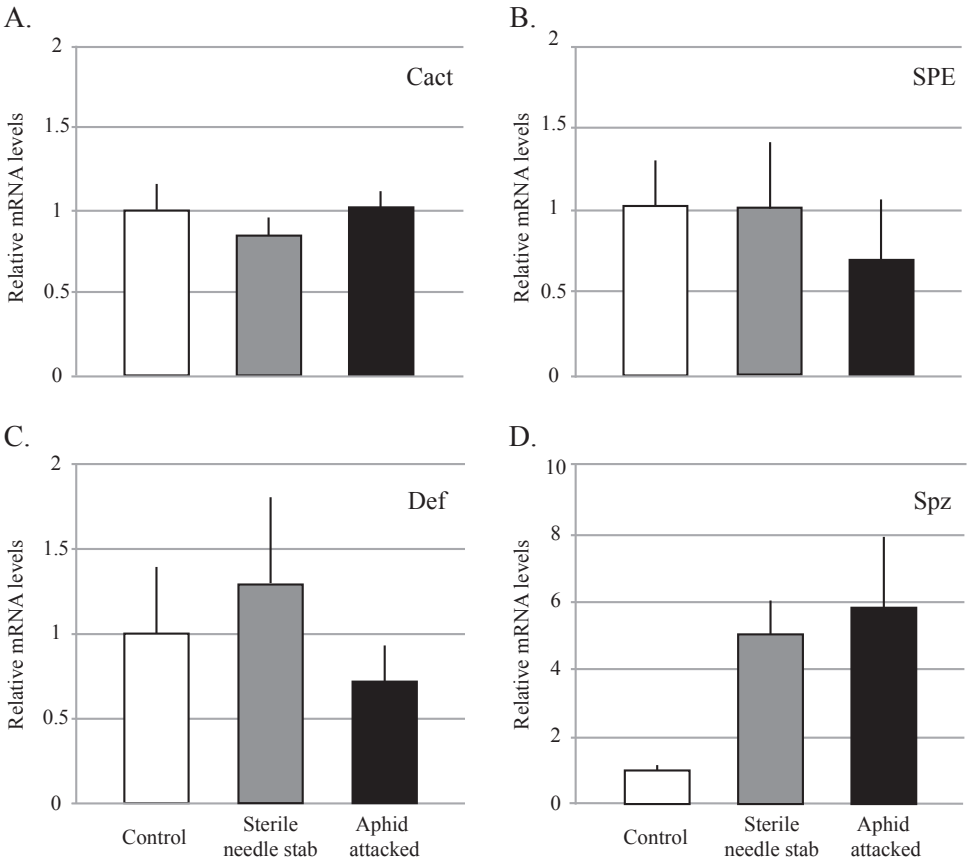

Fig. 3: Relative expression of immune gene transcripts involved in the Toll pathway in *Drosophila* larvae either in an empty gall (white bar), stabbed with a sterile needle (grey bar) or attacked by aphid soldiers (black bar). (A) At 1 hr post-treatment, Cactus (Cact) was not transcriptionally induced in any group. (B) At 1 hr post-treatment, Spaetzle processing enzyme (SPE) was not overexpressed in any group. (C) At 1 hr post-treatment, Defensin (Def) was similarly expressed across all groups. (D) At 1 hr post-treatment, Spaetzle (Spz) was overexpressed by 4-fold in the stab control group and 6-fold in the group attacked by aphid soldiers relative to controls.

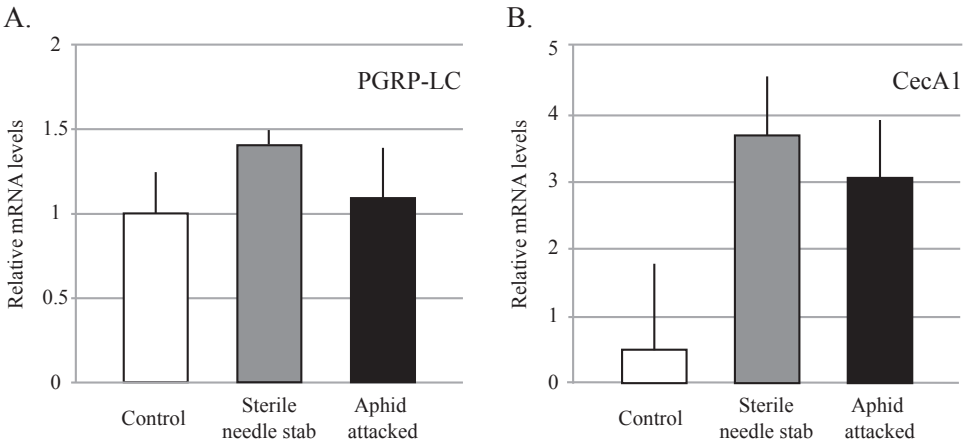

Fig. 4: Relative expression of immune gene transcripts involved in the Imd pathway in *Drosophila* larvae either in an empty gall (white bar), stabbed with a sterile needle (grey bar) or attacked by aphid soldiers (black bar). (A) At 1 hr post-treatment, peptidoglycan recognition protein LC (PGRP-LC) was not overexpressed in any group. (B) At 1 hr post-treatment, CecropinA1 (CecA1) was induced greater than 3-fold in both the stab control group and the group attacked by aphid soldiers relative to controls.

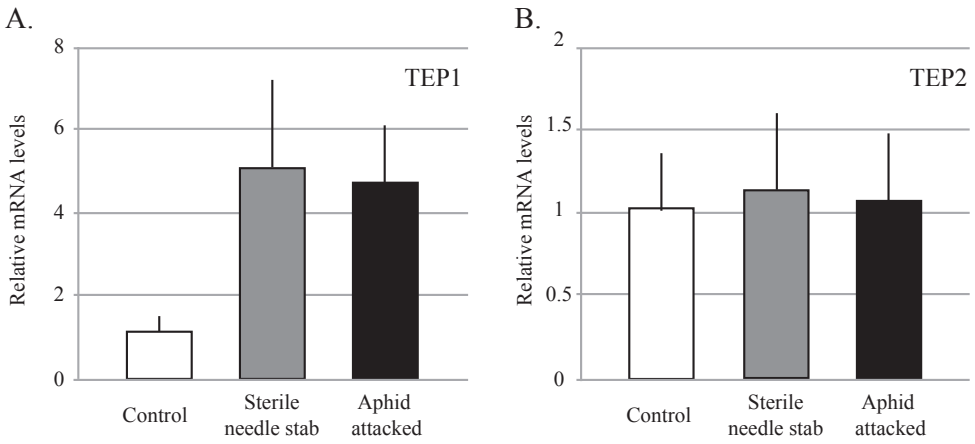

Fig. 5: Relative expression of thioester-containing proteins transcripts in *Drosophila* larvae either in an empty gall (white bar), stabbed with a sterile needle (grey bar) or attacked by aphid soldiers (black bar). (A) At 1 hr post-treatment, thioester-containing protein 1 (TEP1) was overexpressed by at least 4-fold in both stab controls and those attacked by soldiers compared to controls. (B) At 1 hr post-treatment, thioester-containing protein 2 (TEP2) was not transcriptionally induced in any group.

into oxygen and water (Mackay et al. 1989; Phillips et al. 1995; Sun and Tower 1999). It has also been shown that catalase can induce expression of AMPs (Nappi & Vass 1998). Superoxide dismutase was not differentially expressed in any of the treatment groups (Fig. 6A). Catalase showed a 2-fold increase in expression in *Drosophila* larvae attacked by *P. obesinymphae* soldiers (Fig. 6B).

Finally, we quantified the expression of four genes involved in *Drosophila* melanization pathway. For melanization to occur, dopamine must be converted to melanin, which is catalyzed when prophenoloxidase is cleaved to phenoloxidase (PO) (Ashida et al. 1995; De Gregorio et al. 2002). Dopamine in the pathway is derived from the conversion of tyrosine to dopa, and then dopamine. Tyrosine to dopa conversion is catalyzed by tyrosine hydroxylase (pale) (Tang et al. 2006). GTP cyclohydrolase (Punch) and Dihydropteridine reductase (Dhpr) are involved in the biosynthesis of cofactors involved in the conversion of tyrosine to dopa (Funderburk et al. 2006; Reynolds & O'Donnell 1988; Weisberg & O'Donnell 1986). The final step prior to melanin production is the conversion of dopa to dopamine, which is catalyzed by Dopa decarboxylase (Ddc) (Livingstone & Tempel 1983). These genes were chosen from the cascade because prior experiments had identified them as induced during septic injury as analyzed by microarray (De Gregorio et al. 2002). All the genes tested in the melanization pathway were overexpressed by more than 2-fold in all treatment groups (Fig. 7). Dopa decarboxylase and Dihydropteridine reductase showed over a 2-fold increase in the group pierced with a sterile needle and about a 3-fold increase in larvae attacked by aphid soldiers (Fig. 7A-B, respectively). We found a 2-fold increase in GTP cyclohydrolase and tyrosine hydroxylase after piercing with a sterile needle and a greater than 5-fold increase in expression following aphid attack (Fig. 7C-D). In summary, we found a trend in which all genes tested in the melanization pathway and genes implicated in the melanization response were overexpressed after attack by aphid soldiers compared to unattacked controls.

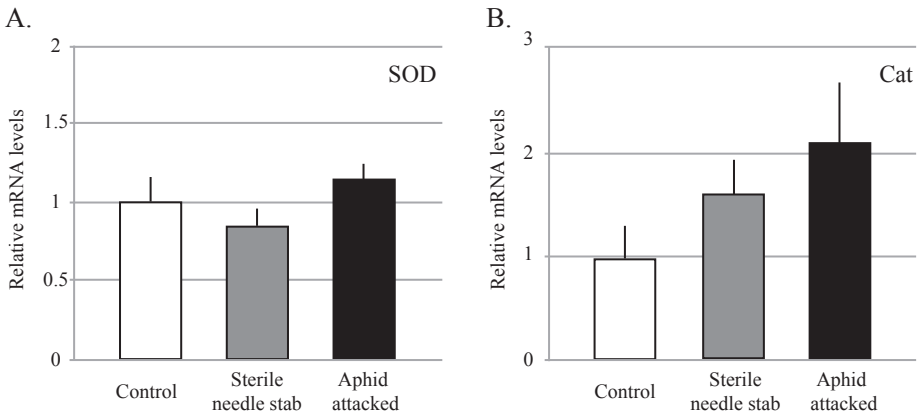

Fig. 6: Relative expression of reactive oxygen species transcripts in *Drosophila* larvae either in an empty gall (white bar), stabbed with a sterile needle (grey bar) or attacked by aphid soldiers (black bar). (A) At 1 hr post-treatment, superoxide dismutase (SOD) was not overexpressed in any group. (B) At 1 hr post-treatment, catalase (Cat) was induced 2-fold in larvae attacked by aphid soldiers relative to controls.

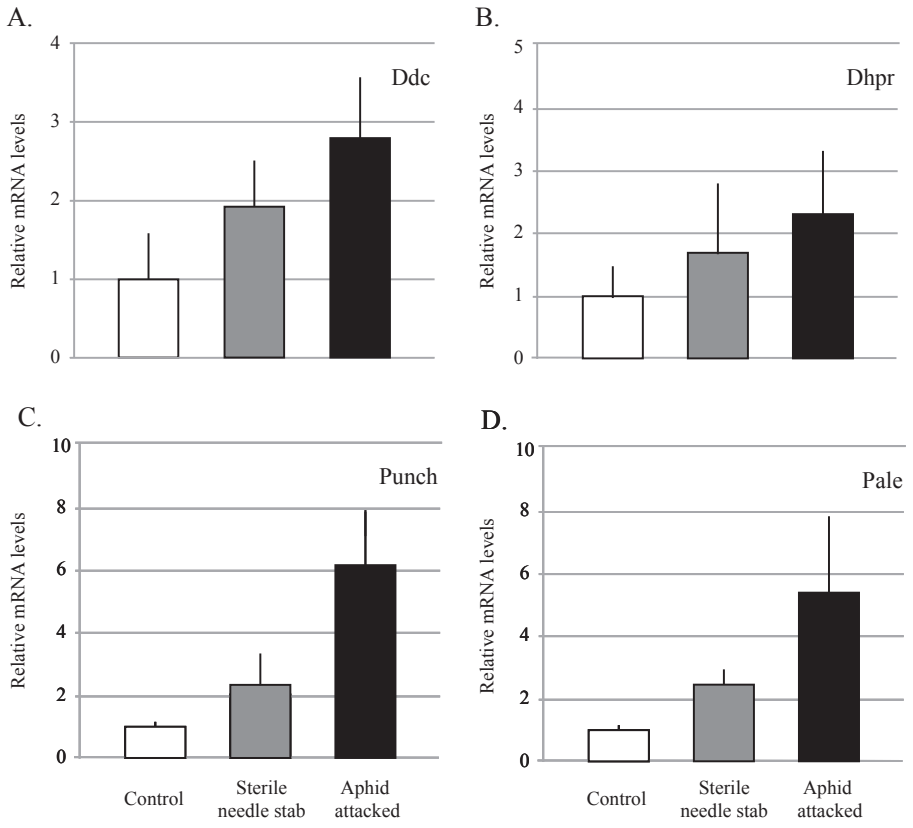

Fig. 7: Relative expression of immune gene transcripts involved in the melanization cascade in *Drosophila* larvae either in an empty gall (white bar), stabbed with a sterile needle (grey bar) or attacked by aphid soldiers (black bar). (A) At 1 hr post-treatment, Dopa decarboxylase (Ddc) was overexpressed by at least 2-fold in the stab control group and 3-fold in the attacked by aphid soldier group compared to controls. (B) At 1 hr post-treatment, Dihydropteridine reductase (Dhpr) was induced greater than 2-fold in the group attacked by aphid soldiers relative to controls. (C) At 1 hr post-treatment, GTP cyclohydrolase (Punch) was expressed 2-fold higher in larvae stabbed with a sterile needle and 6-fold higher in larvae attacked by aphid soldiers (D) At 1 hr post-treatment, Tyrosine hydroxylase (Pale) was transcriptionally induced by greater than 2-fold in stab controls and by greater than 5-fold in the treatment group attacked by aphid solders relative to controls.

Partial recovery of survivorship using Drosophila *knockout*

To determine if induction of a melanization response could play a role in the survivorship of *Drosophila* larvae following attack by aphid soldiers, we compared the survival of four different *Drosophila* mutant lines in genes in the melanization biosynthetic pathway to that of wild-type *Drosophila* larvae. We found a significant difference in time to death of larvae attacked by aphid soldiers compared to unattacked larvae in each mutant line tested (Fig. 8; Wilcoxon test, df = 11, $P < 0.0001$). When comparing attacked larvae of each mutant line to wild-type *Drosophila* larvae, we found that *Drosophila* tyrosine hydroxylase mutants survived significantly longer than wild-type when attacked by aphid soldiers (Wilcoxon each pair test, df = 1, $P = 0.0483$). Survival was not significantly different between the wild-type larvae and any of the other mutant *Drosophila* lines tested, including Dopa decarboxylase mutant ((Wilcoxon each pair, df = 1, $P = 0.0855$), GTP cyclohydrolase mutant ($P = 0.6840$), and Dihydropteridine reductase mutant ($P = 0.0709$). We found no significant difference in survival between the glycan mutant (glycan) and wild-type *Drosophila* larvae (Wilcoxon each pair test, df = 1, $P = 0.6686$).

Discussion

Increased predation is a major risk of group-living. To decrease this cost, socially living organisms have evolved numerous defense mechanisms from morphological adaptations, like the large mandibles and stingers seen in the Hymenoptera to chemical defenses, like apitoxins or autothysis (Breed et al. 2004, Shorter & Rueppell 2012). The ability to effectively defend the nest from invaders is key to group-living. And yet, very little is known about how phytophagous, eusocial insects, such as aphids or thrips, are able to effectively defend their colonies from natural enemies and pathogens.

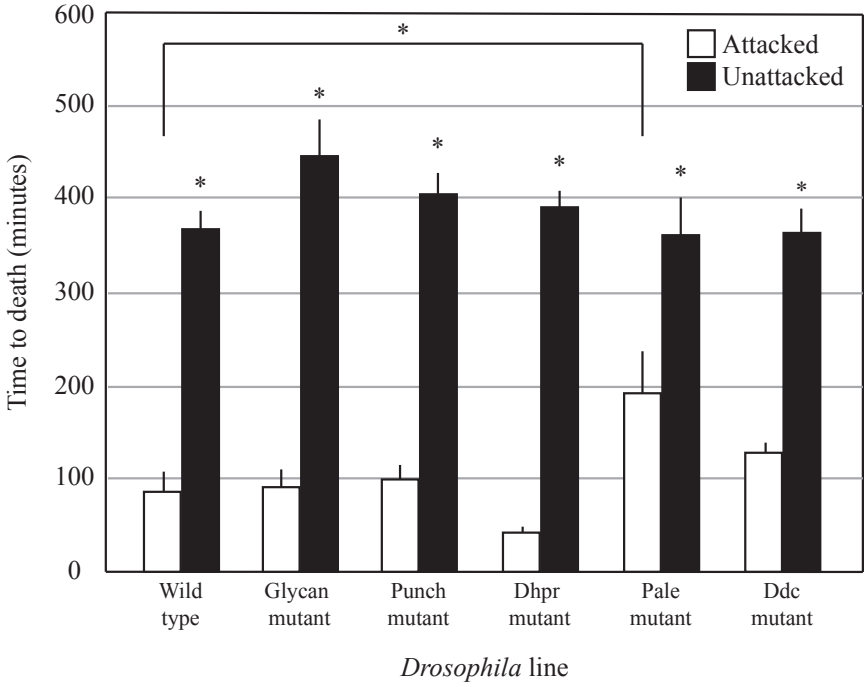

Fig. 8: The average (± SE) time to death in minutes of *Drosophila* larvae attacked by aphid soldiers (white bar) or unattacked in an empty gall (black bar). Each *Drosophila* line has a mutation in one of the melanization genes shown to be overexpressed after attack by aphid soldiers (Punch mutant, Dhpr mutant, Pale mutant and Ddc mutant). A control of wild type *Drosophila* larvae and a mutant line with a knockdown of a gene not involved in an immune response (Glycan mutant) was used. Asterisks indicate a significant difference ($P < 0.05$).

Recent work on thrips has shown that soldiers harbor cuticular agents that exhibit anti-fungal activity (Turnbull et al. 2012). What these results suggest is greater complexity in defensive traits of species like thrips than previously realized. In this vein, we have shown that aphid soldiers generate an overexpression of melanization, which overwhelms the tightly-regulated immunological response in their victims, resulting in morbidity and ultimately successful defense of their groups.

Pemphigus obesinymphae aphid soldiers have no morphological adaptations for defense. In fact, it is the smallest and youngest 1st instar individuals who serve as defenders of the nest or gall. To defend their gall from predators much larger than themselves, the aphid soldiers swarm in a manner similar to that of bees or ants and use their needle-like mouthparts to attack the predator. This attack by *P. obesinymphae* soldiers leads to systemic melanization in the victim (Fig. 2). Melanization is the first line of defense in the immune response against pathogens, which is visible at the site of cuticular wounding (Nappi & Christensen 2005; Tang et al. 2006). To begin to explore this melanization response, we used RNA-seq to compare the expression of genes in *Drosophila* before and after attack by aphid soldiers. Because we found a significant portion of the overexpression in immune related genes, we focused on immune pathways (Table 1).

To further quantify this observation, we used real-time PCR to compare expression of immune genes before and after attack by aphid soldiers. After attack, we found that all genes tested in the melanization pathway were overexpressed by at least 2-fold in the victim, while no genes solely involved in the Toll or Imd signaling pathways were significantly overexpressed (Fig. 3-7). In addition to the upregulation of genes in the melanization pathway, genes which are normally associated with other signaling pathways, but have been implicated in the melanization response were also overexpressed: spatzle, Tep1 and Catalase (Fig. 3D, 5A, and 6B). Past research of spatzle has shown that the melanization signal passes through the membrane to the hemolymph, where it activates persephone, a protease involved in cleaving spatzle, generating the ligand for the Toll receptor (Tang et al. 2008). Tep1 has also been implicated in the melanization response as a first responder to pathogens penetrating the cuticle (Blandin &

Levashina 2004) and Catalase is a ROS, which degrades free radicals that are released in the melanization response (Phillips et al. 1995; Sun & Tower 1999). Altogether, the expression data supports our visual observation that aphid soldiers are inducing an immune response in their victims. Because no genes associated with only the Toll or Imd signaling pathway were induced by aphid attack we predict that the immune response induced by aphid soldiers is isolated mainly to the melanization cascade (Fig. 3 and 4).

Another interesting result is that Cecropin A1 was overexpressed by 3-fold in both treatment groups, pierced with a sterile needle and attacked by aphid soldiers. Cecropin A1 is an AMP activated in response to gram-negative infections (Lemaitre et al. 1997Samakovlis et al. 1990;). It is possible that when aphids attack other insects with their mouthparts, they cause sepsis, either by virtue of simply piercing the outer cuticle of their victims, or because they translocate microbial species found in or on their stylets or midguts. Additionally, we observed overexpression of Cecropin A1 in the treatment group pierced with a sterile needle. This could be explained by the fact that after being pierced, the larvae were placed back into fly media where they could be exposed to microbes.

The phenoloxidase cascade, which induces melanization, is tightly regulated in insects, because of the toxic quinones produced during melanin biosynthesis (Bolton et al. 2000; Cerenius & Soderhall 2004). Past research on the melanization response in *Drosophila* has demonstrated that mutations in serpin genes, which inhibit melanization, lead to uncontrolled melanization, which generates excessive toxic intermediates and lethality (De Gregorio et al. 2002; Tang et al. 2008; Tang 2009). We hypothesized that aphid soldiers may be hijacking this immune response in their victims leading to a build-up of toxic quinones and ultimately death. We found partial recovery of mortality when tyrosine hydroxylase mutant *Drosophila* larvae were attacked by aphid soldier compared to wild-type *Drosophila* larvae (Fig. 8). Tyrosine hydroxylase catalyzes the rate-limited step of the conversion of tyrosine to dopa in biosynthesis of dopamine (Shi et al. 2014). By knocking down a rate-limiting enzyme, we are reducing the melanization response, which leads to increased survivorship of *Drosophila* larvae following attack. This further

supports the hypothesis that aphid soldiers inducing a melanization response in victims, which overwhelms the immune system, ultimately, leading to death.

The observed physical and molecular responses elicited by *P. obesinymphae* correspond to both physical injury and an induced melanization response. Of the transcripts overexpressed in larvae attacked by aphids, some were significantly overexpressed compared to pierced controls. These data indicate that in addition to the physical injury of piercing, *P. obesinymphae* soldiers may be introducing an effector molecule that leads to an upregulation of genes in the melanization pathway. *Pemphigus obesinymphae* may have evolved effector molecules to aid in defense of the colony to compensate their lack of morphological adaptations for defense.

To further elucidate the effects of *P. obesinymphae* attacks at the molecular level, we would like to identify possible candidate aphid effector molecules. Like many toxins and venoms, the aphid soldiers seem to induce a system wide failure in their victims (Fry et al. 2009). Of particular interest are proteases. Past research has shown that soldiers of one social aphid species, *Tuberaphis styraci*, produce an unusual cysteine protease that they secrete when they attack invaders with their mouthparts (Kutsukake et al. 2004). However, other social aphids apparently do not share this particular proteolytic enzyme (Kutsukake et al. 2008), nor have we found evidence of it in *Pemphigus.* Aphids therefore may employ diverse, lineage-specific biochemical strategies for defense against natural enemies. Another point of interest is that proteases have been shown to induce melanization, which often leads to death (Harrison & Bonning 2010). For example, the entomopathogenic fungi *Metarhizium anisopliae* protease PR1A digests cuticle proteins and is vital in the penetration of host cells. The expression of PR1A in the hemocoel of infected *Manduca sexta* leads to degradation of hemolymph proteins and increased melanization. It has been hypothesized that the protease triggers the melanization cascade by the cleavage and activation of prophenoloxidase, which contributes to the toxicity of PR1A (Harrison & Bonning 2010). Another cathepsin L, ScathL from the flesh fly, *Sarcophaga peregrine*, when expressed in the virus AcMLF9 and introduced to multiple insect orders, including the tobacco budworm, *Heliothis virescens,* the tomato moth,

Lacanobia oleracera, and the pea aphid, *Acyrthosiphon pisum*, causes significant melanization and death in all insects tested (Cerenius et al. 2008; Harrison & Bonning 2010).

Conclusions

Defense is one of the defining feature of sociality in aphids, and yet, very little is known about how aphid soldiers successfully defend their gall from natural predators. Here, we describe and quantify the systemic melanization phenotype observed in victims after aphid soldier attack. Because toxic and reactive quinones are produced during melanin biosynthesis, the phenoloxidase cascade is tightly-regulated in insects (Bolton et al. 2000; Cerenius & Soderhall 2004). Aphid soldiers are likely using a protease to trigger the melanization cascade by activating the proteolytic cascade that leads to the cleavage and activation of prophenoloxidase. This unregulated phenoloxidase activity causes toxicity in victims, leading to death, and successful defense of the nest.

Acknowledgements

I would like to thank the co-authors of this study. First, Leah Sigle for her help with the qPCR experiments, Andrew Legan for his help with the survivorship assays and the video footage, Abigail Lind for her help with the analysis of the RNA-seq data, and Patrick Abbot for his help with the ideas and the writing of the manuscript. The *Drosophila* larvae were generously donated by the Bordenstein and Brodie labs at Vanderbilt University.

Supplementary Table S1. Gene names, FlyBase IDs, primary immune pathway and primer sequences used to amplify *Drosophila* cDNA.

Gene	Flybase Symbol	Pathway	Forward	Reverse	CT Slope
Cactus	Cact	Toll	CAGGCAACTGTCATGGGATTG	GCTTTGGTGATCCTCGCTATTT	0.061
Spaetzle processing enzyme	SPE	Toll	GGCTGGGGACTTACCGAGAAC	ACCGCATGTATCCACGCCCAACTG	-0.41
Spaetzle	Spz	Toll	TGATGACGCCCATGTGGAT	GTGGCAAAGAAGGCGAACA	0.79
Defensin	Def	Toll/AMP	GCCAGAAGCGAGCCACAT	CGGTGTGGTTCCAGTTCCA	0.043
PGRP-LC	PGRP-LC	IMD	AAGATCCGGCGCAAACC	CCTTGCGTCCGACAGTGTT	0.148
CecropinA	CecA1	IMD/AMP	TCTTCGTTTTCGTCGCTCTC	CTTGTTGAGCGATTCCCAGT	0.093
Thioester-containing protein 1	TEP1	JAK/STAT	ACCGCAAGTGCCTGACCC	GCGTTCGGTGGCATAGTA	1.88
Thioester-containing protein 2	TEP2	JAK/STAT	GCCCTTAGAAGACGCCGACA	CCGGGAGTTAAGCTGTCA	0.534
Superoxide dismutase	SOD	ROS	GAACTCGTGCACGTGGAATC	GGTGGTTAAAGCTGTCTGCGTA	0.278
Catalase	Cat	ROS	AGATGCTGATGGTCGTCTGTTGTTCT	TCCATCCCGCTGGAAGTTCTCAAT	0.573
Dopa decarboxylase	Ddc	Melanization	ACACAAATGGATGCTGGTGA	AAGTGGGATTTGCCAGTGAC	-1.7
Dihydropteridine reductase	Dhpr	Melanization	ATTGCAACTTTAAAACTGGC	TTCCACCCAACTAGCATC	-0.392
GTP cyclohydrolase	Punch	Melanization	GTGGTCGTAGAGGGAGTC	TTGGGATCGTCTCGGAAC	-0.319
Tyrosine hydroxylase	Pale	Melanization	TTCCTTGCAGAGACCGAACT	TATGTGCGCCACGTTAACTC	-0.888
Actin 5c	Act5c	Standard	CCGAGCGCGGTTACTCTTT	CTCCTTGATGTCACGGACGAT	-0.046

CHAPTER IV

COMPARATIVE ANALYSIS OF THE BIODIVERSITY OF FUNGAL ENDOPHYTES IN INSECT GALLS

Abstract

Insect-induced galls are abnormal plant growths that can provide food and shelter to their inhabitants, resulting in stressed plant tissue that may alter the conditions for the colonization or proliferation of endophytic fungi. We investigated the effect gall formation has on fungal endophyte communities and diversity. Using three closely-related gall-forming aphid species that specialize on poplars, we characterized fungal endophyte diversity in galls and surrounding petiole and leaf lamina tissue. A total of 516 fungal endophyte samples were isolated from 272 tissue samples (32 leaves, 31 petioles, and 209 galls), resulting in 23 distinct morphotypes. Despite sharing a common host plant and often forming spatially contiguous galls, the endophyte profiles within the galls of each aphid species were distinct, not only from the galls of the other species, but also from surrounding plant tissue. These results suggest that insect galls can affect the composition of fungal endophyte species in plant tissues, by altering either the colonization or proliferation of their endophytic mycobiota. Likewise, fungal endophytes may be important in the ecology and evolution of insect galls.

Introduction

Over 13,000 species of herbivorous insects can induce structures known as galls on their host plants. Galls are tumor-like tissues induced by the insect as it feeds and provide shelter, nutrition, and protection from natural enemies (Stone & Schonrogge 2003). Galls often have conspicuous morphology, and in groups such as the nematine sawflies, gall midges, and cynipid wasps, gall formation is associated with exceptional phenotypic and evolutionary diversity. Much of the research on insect-induced galls has focused on their ecological and evolutionary functions, as well as the biochemical basis of gall induction

(Raman 2011). One aspect of insect-induced galls that has received comparably less attention is how gall-forming insects interact with endophytic fungi embedded in surrounding plant tissues, and the consequences of these interactions for the fungal endophyte community.

Endophytic fungi compose a polyphyletic group of highly diverse fungi that are functionally defined by internal and asymptomatic occurrence in plant tissue (Saikkonen et al. 1998). In recent years, there has been a growing interest in how endophytic fungi affect patterns of insect herbivory, particularly with respect to the endophytic clavicipitalean fungi of grasses (Clay 1988; Clay & Schardl 2002; Rodriquez et al. 2009). Less is known about the diversity or functional roles of fungal endophytes in the foliar tissues of herbaceous plants and trees. Previous research has demonstrated that infection can be highly localized to distinct tissues in woody plants (Saikkonen et al. 1998; Porras-Alfaro & Bayman 2011; Albrectsen et al. 2010; Botella & Diez 2011; Koukol et al. 2012; Li et al. 2012). These fungal endophytes typically remain quiescent within plant tissues until senescence or stress results in proliferation of fungal thalli (Stone et al. 2004; Sieber 2007). In contrast to fungal endophytes in grasses, it is less clear whether those which are dormant and localized in the leaf and vascular tissues of trees and shrubs act as mutualists or antagonists. Some evidence suggests, for example, that non-clavicipitalean species readily shift functional roles, depending on ecological or seasonal conditions (Sieber 2007; Purahong & Hyde 2011).

Galls may represent sites where either endophytic abundance or diversity persistently differs from that of surrounding tissues. Insect-induced galls can act as resource sinks, concentrating nutrients from surrounding plant tissues (Larson & Whitham 1997; Schonrogge et al. 2000). This concentration of nutrients likely affects the composition or proliferation of endophytes or other saprophytic or pathogenic fungi. Moreover, fungal endophytes may affect the performance or patterns of herbivory by gall-forming insects. The galling lifestyle represents an unusually intimate and persistent interaction between insects and plants. If fungal endophytes have effects that inhibit or promote the persistence of herbivores on plants, gall-forming insects may be acutely sensitive to their

distribution in plant tissues or organs. Wilson and Carroll (1997), for example, found that a gall-forming cynipid wasp tends to avoid the area of oak leaves with greater densities of a common fungal endophyte, *Discula quercina* (Sordariomycetes: Diaporthales). Faeth and Hammon (1997) found positive associations between fungal endophyte infections and *Cameraria* sp. (Lepidoptera: Gracillariidae), leafminers, which form small tunnels on the leaves of their host plants while they feed.

Only a small number of studies have characterized the interaction between the fungal endophytes of trees and gall-forming or leafmining insects; a majority of those studies have described interactions on oak trees (Table 1). In this study, we asked how insect-induced galls affect the fungal endophytes of poplars. We characterized fungal endophyte diversity in the galls of three *Pemphigus* (Hemiptera: Pemphigidae). *Pemphigus* consists of 65 described species distributed throughout the northern hemisphere (Blackman and Eastop 1994). All species form galls on the leaves or petioles of poplars (*Populus* spp.). The three species, *P. populicaulis*, *P. populitransversus* and *P. obesinymphae* have overlapping ranges in eastern North America, and form galls on their primary host, *Populus deltoides* (Salicaceae). They differ, however, in the precise locations on the plants where they initiate galls, and in the seasonal timing and duration of the gall (Table 2; Abbot & Withgott 2004). The life history differences between these three species that share a common host plant allow for comparisons of how insect galls differ in fungal endophyte composition across plant tissues and seasons.

Methods

Study system and field site

P. populicaulis and *P. obesinymphae* form galls at the base of the leaf lamina, while *P. populi-transversus* forms galls on the leaf petiole. *P. populicaulis* initiates galls in early spring, while *P. populitransversus* and *P. obesinymphae* initiate galls later in the spring or early summer (Blackman & Eastop 1994). All plant tissues were collected from

Table 1. Summary of studies on the interaction between gall-forming or leafmining insects, fungal endophytes and their host plant. The type of study indicates if the focus was on a specific interaction between one endophytic fungal species and one insect species (pair-wise) or examining the community of fungal endophytes in the gall tissue and subsequently, concentrating on the most common fungal species (community)

Plant	Fungus	Insect	Type of study	Effect of fungus on insect	Citation
Quercus robur	*Kabatiella apocrypta* *Gloeosporium quercinum* *Dichomera saubinetii*	*Trioza remota* (psyllid) *Neuroterus numismalis* (gall wasp) *Polystepha panteli* (gall midge)	Pair-wise	Negative Negative Negative	Butin 1992
Pseudotsuga menziesii	*Rhabdocline parkeri*	*Contarinia* sp. (gall midge)	Pair-wise	Negative	Carroll 1995
Quercus emoryi	*Ophiognomia cryptica* *Asteromella* sp. *Plectophomella* sp.	*Cameraria* sp. (leafminer)	Community	Neutral Neutral Neutral	Faeth and Hammon 1997
Pinus densiflora	*Phialocephala* sp.	*Thecodiplosis japonensis* (midge)	Community	Neutral	Hata and Futai 1994
Picea glauca	*Chladysporium sphaerospermum*	*Adelges abietis* (gall adelgid)	Pair-wise	Negative	Lasota et al. 1983
Tilia cordata *Quercus robur* *Fagus sylvatica*	*Gloeosporium* sp. *Gloeosporium* sp. *Gloeosporium* sp.	Multiple sp. Multiple sp. Multiple sp.	Community	Negative Negative Negative	Pehl and Butin 1994
Acer pseudoplatanus	*Diplodina acerina*	*Dasynerua vitrina*		Negative	
Quercus gambelii	*Gnomonia cerastis*	*Phyllonorycter* sp. (leaf mining moth)	Community	Positive	Preszler et al. 1996
Quercus agrifolia	*Discula quercina* *Cryptosporiopsis quercina* *Auerobasidium* sp. *Phomopsis* sp.	*Dryocosmus dubiosus* (gall wasp)	Community	Unresolved	Wilson 1995
Populus angustifolia	*Verticillium lecanii* *Cladosporium cladosporioides* *Penicillium* sp.	*Pemphigus betae* (gall aphid)	Community	Negative	Wilson 1995
Quercus garryana	*Discula quercina* *Apiognomonia* sp. *Fusarium* sp.	*Besbicus mirabilis* (gall wasp)	Community	Negative	Wilson 1995
Quercus garryana	*Discula quercina*	*Besbicus mirabilis* (gall wasp) *Bassettia ligni* (gall wasp)	Pair-wise	Neutral Neutral	Wilson and Carroll 1997
Quercus emoryi	*Ophiognomonia cryptica* *Plectophomella* sp. *Asteromella* sp.	*Cameraria* sp. (leafminer)	Pair-wise	Negative Negative Negative	Wilson and Faeth 2001

Table 2. Time, duration and location of gall development of the investigated aphid species.

Species	Month of gall initiation	Duration of gall	Location of gall
P. populicaulis	March	2-3 months	Base of leaf lamina
P. populitransversus	April	3-4 months	Middle of petiole
P. obesinymphae	May	5-6 months	Base of leaf lamina

eastern cottonwoods (*Populus deltoides* W. Bartram ex. Marshall) in the greater Nashville, Tennessee area. The sites were in disturbed areas near major roadways. The site coordinates were: Site 1: N 35.967552, W 086.778438; Site 2: N 36.08395, W 086.8882; Site 3: N 36.07786, W 086.91150; Site 4: N 36.13066, W 086.90326; Site 5: N 36.15406, W 086.95084, and Site 6: N 36.20914, W 086.88243. At each sampling date between May and August 2011, we collected between 30 and 40 galls with attached leaves and petioles. Standardized sections of leaves and petioles were obtained by cutting a 1cm long section of the petiole proximal to the gall and a 1cm x 1cm square of the leaf immediately distal to the gall. The leaf section included both midrib and leaf lamina.

Fungal isolations and identification

Within 24h of collection, fungal endophytes were cultured from galls, and from a section of the leaf and petiole of approximately every 10^{th} gall. Prior to plating, attached ungalled tissues were removed from galls using a sterile razor blade. Whole galls and samples of surrounding tissue were then plated and subcultured (described below).

Surface sterilization was performed on all gall, leaf, and petiole samples following a protocol from Deckert et al. (2001). Samples were agitated in 70% ethanol for one minute, then allowed to soak in the ethanol for four minutes. Samples were then soaked in 50% bleach (6% Sodium hypochlorite) for five minutes, sterile distilled H_2O for five minutes, and an additional wash in clean sterile distilled H_2O for five minutes. Under sterile conditions, gall segments were allowed to dry for five minutes before they were plated on Potato Dextrose Agar (PDA) plates with ampicillin at 1 ng/µL. A total of 210 galls, 32 leaves and 31 petioles were plated. There was no replication between tissue samples, because all the tissue was plated together on one plate at one time. All plant samples were then incubated on sealed plates at room temperature and checked daily under a microscope for signs of hyphal growth.

At the end of four weeks, fungi were subcultured from original inoculations. Under a sterile hood, agar plugs with one fungal morphotype were transferred from the original PDA plate to a fresh PDA plate and grown at room temperature for two weeks. Following the successful isolation a fungal morphotype, plugs from the subculture were removed and placed in 15 mL polypropylene tubes containing five mL sterile Potato Dextrose (PD) broth. Liquid cultures were grown for approximately two weeks at room temperature. Each morphotype was archived as a living voucher in 400 µL of an 80% PDA, 20% glycerol solution and stored at -80˚C in screw cap tubes (Hoffman and Arnold 2010).

Total genomic DNA was extracted directly from pure, liquid cultures by grinding fungal samples in liquid nitrogen with a mortar and pestle, followed by application of a Qiagen DNeasy Plant Mini Kit®. The internal transcribed spacer sequence (ITS, using primers described in Bellemain et al. 2010) was amplified in a polymerase chain reaction (PCR) using the following protocol: 94˚C for 2.5 min; 30 cycles of 94˚C for 15s, 56˚C for 30s, 72 for 1.5 min; and 72˚C for 10 min. The PCR product was purified using ExoSAP-IT® (USB corporation, Cleveland, OH, USA). Purified samples were Sanger sequenced at GENEWIZ, Inc. (http://www.genewiz.com). Nearest species were determined using the Basic Local Alignment Search Tool (BLASTn) at the National Center for Biotechnology Information (NCBI). Sequences that showed ≥ 98% similarity to the best BLAST hit were taxonomically assigned to the same operational taxonomic unit (OTU). Those sequences with < 98% similarity were assigned to the genus or family of the best BLASTn hits. The 98% similarity cut-off is a conservative criterion and is based on studies suggesting the variability of the ITS region across Kingdom Fungi is on average 2.51% with a standard deviation of 4.57 (Nilsson et al. 2008). All the nucleotide sequences obtained in the study have been deposited in GenBank under accession numbers KF530731-KF530752.

Data analysis

Analyses were performed on gall, leaf and petiole samples (the total number of galls sampled exceeded that of the leaves and petioles). We assessed fungal endophyte diversity by counting the number of different OTUs isolated from the plate of a single gall, leaf or petiole sample. Based on these counts, colonization frequency (CF), isolation rate (IR), relative frequency (RF), and similarity coefficient (SC) were calculated. CF is the fraction of sampled tissue with at least one fungal endophyte and IR describes the average number of fungal endophytes per sample (Petrini et al. 1982). CF was compared using a contingency analysis. Indices of abundance or composition between the galls of aphid species and surrounding plant tissues were analyzed as nonparametric equivalents of one-way ANOVAs and, in the case of count data, general linear models with Poisson-distributed variances. Whole model tests were followed by pairwise contrasts (Sokal and Rohlf 1995). All statistical analyzes were performed in JMP v. 7.01 (SAS, Cary, NC, USA). All reported p-values are two-tailed. RF is the frequency of a specific fungal morphotype relative to the total number of fungal endophytes (Su et al. 2010; Yuan et al. 2010). We calculated the similarity coefficient (SC) as $2w/(a+b)$ where w equals the sum of the lowest RF of species in common between samples, a is the CF of the first sample, and b is the CF of the second sample (Carroll and Carroll 1978). The similarity coefficient measures the overall resemblance of the fungal endophyte communities between two samples.

Results

Fungal endophyte communities in galls

A total of 423 fungal endophytes were isolated from 209 gall samples (69 *P. populicaulis* galls, 69 *P. populitransversus* galls, and 70 *P. obesinymphae* galls). Colonization frequencies of galls were uniformly high, ranging from 95.7-98.6% (Table 3). However, the average number of fungal endophytes isolated from each gall (IR) differed across

Table 3. Colonization frequency (CF) and isolation rate (IR) of endophytic fungi (EF) in galls of three different aphid species. Asterisks indicate significant differences ($P < 0.05$).

Species	Tissue	No. of samples plated	No. colonized by EF	CF (%)	Total # of EF	IR
P. populicaulis	Gall	69	68	98.6	153	2.22
P. populitransversus	Gall	70	67	95.7	119	1.70*
P. obesinymphae	Gall	70	69	98.6	151	2.16

aphid species (Wilcoxon test, df = 2, $P < 0.001$). The galls of the petiole-galler *P. populitransversus* had a significantly lower isolation rate (1.70) than that of *P. populicaulis* and *P. obesinymphae* galls (2.22 and 2.16, respectively; Wilcoxon tests on pairwise contrasts of IR, *P. obesinymphae* vs. *P. populicaulis*, $P = 0.69$; *P. obesinymphae* vs. *P. populitransversus*, $P < 0.002$; *P. populicaulis* vs. *P. populitransversus*, $P < 0.0001$).

A total of 19 distinct morphotypes were isolated from all galls. Given the 98% similarity threshold for name assignment using ITS, 16 of 19 morphotypes were successfully grouped to OTUs based on sequence similarity (Table 4). More fungal endophyte OTUs were identified in *P. obesinymphae* galls (18 OTUs) than in *P. populicaulis* or *P. populitransversus* galls (both with 13 OTUs). The frequency of particular OTUs also differed between the galls of the three aphid species. For example, *Cladosporium* (Dothideomycetes: Capnodiales) was the most commonly isolated fungal endophyte in *P. obesinymphae* galls, but detected only at low rates in the other two species. While further sampling may uncover more fungal diversity in the galls of each aphid species, some OTUs in our survey were unique to the galls of a particular aphid species. For example, OTUs with affiliation to *Alternaria* (Dothideomycetes: Pleosporales)*, Neofusicoccum parvum* (Dothideomycetes: Botryosphaeriales)*, Nigrospora* (Sordariomycetes: Trichosphaeriales), and *Xylaria* (Sordariomycetes: Xylariales) were only found in *P. obesinymphae* galls, while *Collectotrichum gloeosporioides* (Sordariomycetes: Glomerellales) was isolated only from *P. populitransversus* galls. No unique OTUs were cultured from *P. populicaulis* galls. Overall, the species composition was most similar between *P. populicaulis* and *P. populitransversus* galls (81.02%), while the species composition of *P. obesinymphae* galls was markedly distinct from the other two species (60.14% and 64.93%, respectively).

Fungal endophyte communities in poplars

Next, we asked how fungal endophytes vary across tissue (gall, petiole and leaf) in the three aphid species. In all, we isolated 155 fungal endophytes from 95 samples across the three aphid species (31 petioles, 32 galls, and 32 leaves; Table 5). First, we considered

Table 4. Relative frequency of fungal OTUs in galls of three aphid species. OTUs are named on the basis of the names under which the best matching sequences were deposited.

		Relative frequency (%)			
Class	**Fungal OTU**	***P. populicaulis***	***P. populitransversus***	***P. obesinymphae***	**Total**
Dothideomycetes	*Phoma putaminum*	25.49	19.33	12.58	19.15
Sordariomycetes	*Glomerella acutata*	18.95	9.24	11.92	13.71
Agaricomycetes	*Peniophora cinerea*	14.38	20.17	16.56	16.79
Agaricomycetes	*Phlebia*	11.76	15.97	5.30	10.64
Dothideomycetes	*Cladosporium cladosporioides*	6.54	4.20	3.31	4.73
Dothideomycetes	*Cladosporium*	5.88	6.72	19.88	11.11
	Unknown morphotype 1	5.23	8.40	0.66	4.49
Dothideomycetes	*Aureobasidium pullulans*	5.23	4.20	0.66	3.31
Dothideomycetes	*Epicoccum nigrum*	2.61	2.52	0.66	1.89
Sordariomycetes	*Plectosphaerella*	1.31	1.68	5.30	2.84
Exobasidiomycetes	*Malassezia restricta*	1.31	0.00	2.65	1.42
Agaricomycetes	*Schizophyllum commune*	0.65	5.04	6.62	4.02
	Unknown morphotype 2	0.65	0.00	0.66	0.47
Sordariomycetes	*Colletotrichum gloeosporioides*	0.00	1.68	0.00	0.47
Sordariomycetes	*Xylariaceae*	0.00	0.84	0.66	0.47
Sordariomycetes	*Xylaria*	0.00	0.00	5.96	2.13
Dothideomycetes	*Alternaria*	0.00	0.00	5.30	1.89
Dothideomycetes	*Neofusicoccum parvum*	0.00	0.00	0.66	0.24
Sordariomycetes	*Nigrospora*	0.00	0.00	0.66	0.24
	Total no. of distinct morphotypes	13	13	18	19

Table 5. Colonization frequency (CF) and isolation rate (IR) of endophytic fungi (EF) in different plant tissue types; combined for all aphid species. Asterisks indicate significant differences ($P < 0.05$).

Species	Tissue	No. of samples plated	No. colonized by EF	CF (%)	Total # of EF	IR
Combined	Leaf	32	32	100.0	56	1.75
Combined	Gall	32	31	96.9	62	1.94
Combined	Petiole	31	25	80.6*	37	1.19*

the joint effects of aphid species and tissue type on fungal endophyte numbers. No transformation of the fungal endophyte count data satisfied the requirements for a two-way ANOVA, thus we analyzed the data with a general linear model and Poisson-distributed variances. The overall model was not significant ($\chi^2 = 9.87$; df = 8; $P = 0.28$) and there was no significant interaction between aphid species and tissue type on fungal endophyte numbers. However, although the overall model was not significant, there was a significant effect of tissue type on fungal endophyte numbers ($\chi^2 = 6.89$; df = 2; $P = 0.032$). We, therefore, analyzed the relationship between tissue type and fungal endophyte numbers for each aphid species separately. For two of the three species, there were significant differences across tissues in the number of isolated fungal endophyte OTUs (*P. populicaulis:* Wilcoxon test, $\chi^2 = 7.0$; df = 2; $P = 0.03$; *P. populitransversus,* $\chi^2 = 11.02$; df = 2; $P < 0.01$). As above, the overall pattern seems to be driven by relatively impoverished state of fungal endophytes in the petiole tissue compared to the leaf or gall. In petiole tissue, not only was the number of unique endophytic OTUs recovered significantly lower, but the CF index was also significantly lower. The CF index measures the percentage of plant tissue colonized by fungal endophytes, and ranged from 80.6% in the petiole, to 96.9% in the gall and in the 100% in the leaf (Table 5; $\chi^2 = 10.62$, df = 2, $P < 0.001$). When all tissues were analyzed together, regardless of the aphid species, the number of fungal endophytes in the petiole was significantly lower than in the gall or leaf (Wilcoxon multiple comparisons test; leaf vs. gall, Z score = -1.06, $P = 0.29$; petiole vs. leaf, Z score = -2.92, $P = 0.004$; petiole vs. gall, Z score = - 3.53, $P = 0.0004$). Independent contrasts of the number of fungal endophytes between tissues in each species revealed the same pattern: petiole tissue in poplars harbors fewer fungal endophytes than leaf tissue or gall tissue.

However, while petioles may be impoverished, it is the gall that is most distinctive in terms of fungal endophyte composition. In particular, there is a notable contrast between the gall and the plant tissue from which it was formed. For each aphid species, the similarity coefficients were quite small: *P. populicaulis* gall vs. leaf 47.09%; *P. obesinymphae* gall vs. leaf 46.76%; *P. populitransversus* gall vs. petiole 35.29%. However, regardless of species, leaf and gall tissues were the most different, with a

similarity coefficient of 66.35% (Table 6). Moreover, while the most common OTUs in all plant tissue types were *Peniophora cinerea* (Agaricomycetes: Russulales), *Phoma putaminum* (Dothideomycetes: Pleosporales), *Phlebia* (Agaricomycetes: Corticiales) and *Glomerella acutata* (Sordariomycetes: Glomerellales), many OTUs were found to be unique to a specific tissue type. *Coniochaetaceae* (Sordariomycetes: Coniochaetales) and *Neofusicoccum parvum* (Dothideomycetes: Botryosphaeriales) were only isolated from petiole tissue. *Xylaria* and a not namable OTU were found exclusively in the gall, and *Blakeslea trispora* (Mucorales), *Colletotrichum gloeosporioides*, *Nigrospora*, *Phomopsis* (Sordariomycetes: Diaporthales) and another not namable OTU were present only in leaf tissue (Table 7). However, the fungal endophytes isolated exclusively from a specific tissue type were isolated at a low frequency and could represent an artifact of sampling.

Discussion

Plant galls are abnormal growths that may represent sites of altered proliferation or colonization of fungal endophytes. Some endophytes can provide various beneficial services to woody plants. In particular, foliar fungal endophytes have been shown to have adverse effects on insect herbivores, either by deterring herbivory, slowing larval development, or reducing survivorship and fecundity of adults (Hartley & Gange 2009; Saikkonen et al. 2010). We surveyed the fungal endophyte diversity associated with the galls of three species of gall-forming aphids on poplars. We found that the fungal endophyte composition differed between the galls of aphid species, and that the site of gall induction is important in determining the IR and composition of fungal endophytes.

Fungal endophyte communities in galls

It is not known to what degree, if any, fungal endophytes alter the success of gall formation by aphids, or whether these aphids choose galling sites based on the fungal endophyte composition in leaves. Sedentary insects like gall-formers or leafminers may avoid high fungal endophyte space if endophytes negatively affect fitness. Wilson and Carroll (1997) found that the cynipid gall wasp, *Besbicus mirabilis* (Hymenoptera:

Table 6. Similarity of the endophyte community among different plant tissues for all aphids and separated by aphid species. Highlighted is the similarity between communities in the galls and the corresponding uninfected tissue.

Tissue comparison	**SF (%)**
Leaf vs. gall	66.35
Leaf vs. petiole	77.66
Gall vs. petiole	74.66

Tissues divided by species	**SF (%)**
P. populicaulis leaf vs. petiole	67.23
P. populicaulis leaf vs. gall	47.09
P. populicaulis gall vs. petiole	55.27
P. populitransversus leaf vs. petiole	39.22
P. populitransversus gall vs. petiole	35.39
P. populitransversus leaf vs. gall	69.29
P. obesinymphae leaf vs. petiole	55.73
P. obesinymphae gall vs. leaf	46.76
P. obesinymphae gall vs. petiole	60.68

Table 7. Relative frequency of fungal OTUs in the three tissue types. OTUs are named on the basis of the names of the best BLAST hits.

		Relative frequency (%)		
Class	**Fungal OTU**	**Leaf**	**Gall**	**Petiole**
Agaricomycetes	*Peniophora cinerea*	26.79	16.13	21.62
Dothideomycetes	*Phoma putaminum*	19.64	24.19	13.51
Sordariomycetes	*Glomerella acutata*	12.50	9.68	10.81
Dothideomycetes	*Cladosporium*	8.93	4.84	2.70
Dothideomycetes	*Alternaria*	7.14	3.23	5.41
Dothideomycetes	*Epicoccum nigrum*	5.36	1.61	5.41
Agaricomycetes	*Phlebia*	3.57	16.13	5.41
Sordariomycetes	*Plectosphaerella*	3.57	3.23	5.41
Agaricomycetes	*Schizophyllum commune*	1.79	6.45	13.51
	Unknown morphotype 1	1.79	1.61	5.41
Dothideomycetes	*Aureobasidium pullulans*	1.79	0.00	0.00
Sordariomycetes	*Nigrospora*	1.79	0.00	0.00
Sordariomycetes	*Colletotrichum gloeosporioides*	1.79	0.00	0.00
Sordariomycetes	*Phomopsis*	1.79	0.00	0.00
	Unknown morphotype 3	1.79	0.00	0.00
Dothideomycetes	*Cladosporium cladosporioides*	0.00	8.06	2.70
Sordariomycetes	*Xylaria*	0.00	3.23	0.00
	Unknown morphotype 2	0.00	1.61	0.00
Dothideomycetes	*Neofusicoccum parvum*	0.00	0.00	2.70
Not assigned	*Blakeslea trispora*	0.00	0.00	2.70
Sordariomycetes	*Coniochaetaceae*	0.00	0.00	2.70
	Total no. of distinct morphotypes	15	13	14

Cynipidae), seems to avoid high fungal endophyte space on the leaf. It is likely, however, that all galling insects encounter fungal endophytes in their galls during feeding. Even if galling sites are chosen that are comparatively free of fungal endophytes, galling itself may promote fungal growth (Butin 1992; Faeth & Hammon 1997). In this study, we found that the colonization frequency of fungal endophytes in gall tissue was extremely high for all aphid species, ranging from 95.7-98.6% (Table 3). This implies that the insects are likely encountering many fungal endophytes, though we do not know what effects the fungal endophytes of poplars may have on *Pemphigus*. Comparisons to other studies of related species may be useful as guides, but the tripartite relationship between an insect, a fungus and a vascular plant is complex and can depend on the particular species involved (Shorthouse & Rohfritsch 1992; Wilson 1995; Raman 2012; Raman et al. 2012).

The profiles of the fungal endophyte communities differ between the galls of the three aphid species. The leaf-gallers *P. populicaulis* and *P. obesinymphae* both have a significantly higher IR than the petiole-galler, *P. populitransversus*, which is consistent with the lower IR of petiole tissue itself. Fungal endophytes may be ecologically less dense or abundant in both the petiole tissue and petiole galls. Although IR is much higher in the galls of the leaf-galling aphid species, we found that they do not share the most similar fungal endophyte communities. *P. obesinymphae* galls contain a larger number of distinct fungal morphotypes (Table 4), while the fungal endophyte profile of *P. populicaulis* galls is more similar to that of the petiole galls of *P. populitransversus.* Possibly, the aphids themselves may be infecting the gall with different fungal endophytes. Aphids are notorious vectors of plant viruses (Nault 1997; Andret-Link & Fuchs 2005), but it is not known if fungal pathogens are also transmitted by aphids.

The fungal endophyte composition of the galls of *P. obesinymphae* is distinct from the other two aphid species, even though both *P. obesinymphae* and *P. populicaulis* share similar sites for gall induction at the base of the leaf lamina. We suspect that seasonal differences in the life histories of these aphids may also contribute to these differences. Gall-forming insects often have complex life histories that are closely matched to the

seasonal schedules of their host plants. Some aphid species, like *P. populicaulis* and *P. populitransversus*, alternate between woody and herbaceous host plants. In these, aphids return to their woody hosts in the autumn, where a sexually-produced egg is deposited and persists over the winter months until the following spring. *P. obesinymphae*, by contrast, overwinters as adults, and the sexual generation is therefore delayed until the spring. Thus, while *P. obesinymphae* and *P. populicaulis* are both leaf-gallers, they are forming galls on seasonally and developmentally distinct foliar tissue. Seasonal variation in fungal endophyte communities is well-described (Pehl & Butin 1994; Faeth & Hammon 1997; Wei et al. 2007). It is possible that the distinct *P. obesinymphae* profile is due to either seasonal differences (fungal endophytes of poplars differ from spring to summer), or that the summer flush leaves themselves actively recruit distinct fungal endophytes because of intrinsic differences from spring flush leaves.

The similarity coefficients suggest, however, that the distinctiveness of fungal communities inhabiting galls of different aphid species is not solely explained by seasonal or developmental traits of the poplar leaves they attack. The leaves associated with the galls of the three species are equally distinct in terms of similarity coefficients, regardless of the season in which they flush. The IR from *P. populitransversus* galls is significantly lower than either of the other two species, even that of the seasonally synchronous *P. populicaulis.* All three species exhibit fungal endophyte profiles that are more distinct from their associated plant tissue (*P. obesinymphae* and *P. populicaulis* vs. leaves; *P. populitransversus* vs. petioles) than different tissue types are to each other (e.g., between leaves and petioles). Thus, galls hold a different fungal endophyte community compared to the surrounding plant tissue.

Fungal endophyte communities in plant tissue

Because of the economic importance of poplars, there has been some study of the endophytic community of their foliar tissues (Bailey et al. 2004; Santamaria & Diez 2005; Doty et al. 2009; Albrectsen et al. 2010; Martin-Garcia et al. 2011). We isolated many OTUs with affiliation to fungal species previously described to occur in poplars,

such as *Alternaria, Aureobasidium pullulans* (Dothideomycetes: Dothideales), *Cladosporium, Cladosporium cladosporioides,* and *Epicoccum nigrum* (Dothideomycetes). Most of the common fungal OTUs were shared across plant tissues, indicating the cosmopolitan nature of many fungal endophytes (Table 7).

However, we found differences in CF, IR and number of distinct fungal endophytes in petiole tissue compared to that of the leaf or gall (Table 5). Previous work has found similar disparities in the fungal endophyte CFs of leaves and petioles (Mishra et al. 2012), but the pattern appears to be specific to the plant species (Suryanarayanan & Vijaykrishna 2001; Kumar & Hyde 2004). Variation in the fungal endophyte composition of plant tissues is common (Rodriguez et al. 2009).

In conclusion, our study constitutes the first comparative description of the natural communities of fungal endophytes in poplar galls. In galls of each of the aphid species, which are closely-related and share a common host plant and many ecological and life history characteristics, pair-wise comparisons between leaf, petiole and gall tissue indicated that galls were distinct. Gall-forming insects typically exhibit highly specialized, tissue-specific preferences for gall formation. Our results suggest that insect galls provide distinct opportunities for colonization or proliferation of non-overlapping sets of fungal endophytes on plants (Table 7). It has been suggested that insect-induced plant modifications, like galls, can affect biodiversity at higher trophic levels by adding habitat complexity and facilitating opportunities for finer niche partitioning. For example, Waltz and Whitham (1997) showed that the presence of the galls of another *Pemphigus* species, *P. betae*, corresponded to an increase in arthropod diversity. Similar results have been described in leafrollers and sawflies (Martinsen et al. 2000; Bailey & Whitham 2003). Our results suggest that the effects of galls on diversity extend not only to higher trophic levels, but downward to the fungal endophyte communities as well. The degree to which galls represent more than small-scale features amidst a large set of factors governing tree fungal endophyte ecology, and rather act as persistent drivers of fungal community structure and co-evolutionary change, would benefit from further study.

Acknowledgements

I would like to thank the co-authors of the study, Natalie Christian and Patrick Abbot, for their help with the experiments and writing of the manuscript. This work was supported by a National Science Foundation grant #IOS-1147033 and a Vanderbilt University Central Discovery Grant awarded to P.A. We are grateful to members of the Rokas lab, specifically John Gibbons and Jason Slot, for their help with the fungal cultures and identification. We would also like to acknowledge Hayden Hill for his help upkeeping the fungal cultures and Cassidy Cobbs for her feedback on the experimental design and data analysis. Lastly, we cannot forget to thank the reviewers of this paper, who provided us with helpful feedback and suggestions.

CHAPTER V

THE ROLE OF CONFLICT, COMPETITION AND KINSHIP IN THE EVOLUTION OF SOCIAL BEHAVIOR IN GALL-FORMING APHIDS

Abstract

Cooperation is inherently vulnerable to exploitation by cheaters because of the availability of a shared common good. Conflict occurs when cheaters enter a cooperative group, because the cheaters can bring about a 'tragedy of the commons' by exploiting the shared resources of the group. Aphids present a rare opportunity to study the effect of cheaters on groups due to multiple unique life history traits. Most social aphids live and clonally reproduce within hollow galls, where specialized first-instars forego reproduction to maintain and defend the gall against predators. The aphids are "fortress defenders," which means that they live within their food; therefore, their common good is the gall itself, as well as the cooperative defensive behaviors. The shared resources of the aphids can be exploited when neighboring aphids enter the gall. These cheater aphids exploit the cooperation of the natal aphids while refraining from participation in altruistic behaviors. Previous research in the lab has shown that the presence of cheaters negatively affects social aphids, but could not untangle if this cost was due to having any unrelated aphids within galls (even if all cheaters derive from a single clone), or if costs are a positive function of the number of clonal genotypes competing within groups. We first characterized how relatedness among cheaters affect the fitness of the individuals in the gall. Next, we characterized the consequences of cheaters for weakly and nonsocial species. We found that the relatedness of cheaters has no obvious fitness effects on the natal aphids, and, contrary to our expectations, the presence of cheaters has the strongest negative effect on nonsocial species, compared to the social or weakly social species. The results suggest that there is a more complex relationship between competition and relatedness than previously realized.

Introduction

On one level, the group with the highest fitness will be composed solely of altruistic individuals, because all individuals are cooperating and contributing to common resources. However, if a cheater enters the group either through mutation or migration, the cheater will be the most fit individual because the cheater gains the benefit of the common resource without paying a cost. This situation presents an "altruism paradox" (West et al., 2002); how does altruism arise if it is more beneficial to be the cheater than the altruist? There have been many proposed solutions to this problem, including policing, reciprocity, sanctions and punishments (Axelrod & Hamilton 1981; Frank 2003; Trivers 1971; West et al. 2007). However, the exact fitness cost of unrelated cheaters has been difficult to measure due to the natural history of many social species. Multiple matings are common in social Hymenopteran species making it difficult to know the relatedness among individuals *a priori* (Boomsma & Ratnieks 1996; Simmons 2005; Yasui 1998). Also, social groups usually do not allow unrelated individuals to enter the nest making it impossible to introduce cheaters (Holway et al. 2002; Howard & Blomquist 2005). Finally, the group size of some social insect groups make it difficult to measure the exact fitness effect of the cheaters (Bourke 1999).

Because of some unique features of their life history, aphids present a rare opportunity to study the fitness effect of cheaters on groups. First, social aphids occur in confined tumor-like plant growths called galls. The gall provides protection and nourishment for its inhabitants (Stone & Schonrogge 2003). Within these galls, the stem mother or queen reproduces clonally. Since all the inhabitants of a gall are descended from a single stem mother, they are genetically identical to one another. This results in high relatedness among individuals. Second, most of the offspring stay in their natal gall, altruistically contributing to its upkeep or protection. These offspring act as soldiers and will aggressively defend the gall from intruders and other disturbances (Pike et al., 2008). Additionally, these individuals are responsible for expelling waste, cadavers, and performing other such maintenance duties (Benton & Foster 1992). Finally, it has been shown that some social aphid species have a "drifting period" at the beginning of the

galling season, in which some individuals will leave their gall for neighboring galls (Abbot et al. 2001; Grogan et al. 2010). The natal aphids do not seem to recognize these neighboring aphids as a threat and allow them into the gall. When these neighboring aphids arrive, they do not participate in altruistic behaviors, but instead exploit the cooperation of the natal social aphids while refraining from dangerous and costly defensive behaviors. The cheaters actively ingest phloem, produce waste in the form of honeydew, and take advantage of the free protection of the soldier aphids, leading to an acceleration of their own development (Abbot et al. 2001). The limited resources within a gall leave aphids highly vulnerable to cheaters or free riders. Cheaters pose two main issues to social groups: they fail to contribute to social activities, such as defense and housekeeping, and make the group too large, overburdening their limited resources (Aviles et al. 2002).

Previous work has shown that, as kin selection theory predicts, cheaters bring about a 'tragedy of the commons' within galls (Grogan et al. 2010; Rankin et al. 2007), incurring competitive clonal interactions within galls likely mediated by resource exploitation. However, this result was correlational, leaving open a critical set of questions only manipulative experiments can answer. An important one is: what are the consequences of conflict for the social benefits of cooperation? An array of arthropod predators and microbial parasites attack *Pemphigus* galls. If cheaters do not defend galls or perform the hygienic duties that soldiers commonly perform (such as removing cadavers and waste that potentially foul galls; worse, cheaters may even vector microbial pathogens; Stern & Foster 1996), then conflict should lead to higher rates of morbidity due to parasitic infection or predation. Moreover, previous work could not disentangle whether costs of conflict are simply a function of having any unrelated aphids within galls (even if all cheaters derive from a single clone), or if costs are a positive function of the number of clonal genotypes competing within groups (i.e, whether relatedness among cheaters is important). Our first experiment aims to untangle the effect of unrelated aphids on social goods in *Pemphigus obesinymphae*.

Next, we explored if conflict requires cooperation to be manifested. A general theoretical prediction is that social complexity and within-group conflict essentially co-evolve (Frank 1998). Social aphids offer a rare opportunity to test this theory because, unlike traditional eusocial species, sociality has evolved multiple times and there is a range of sociality, so we can compare the cost of conflict across species with varying degrees of social complexity (Lawson et al. 2014). Past research in the lab has shown a correlation between the presence of unrelated cheater aphids have a negative fitness effect in social aphid, *P. obesinymphae* (Grogan et al., 2010), but what effect do cheater aphids have on less social aphid groups? Since there are fewer resources to exploit in weakly and nonsocial populations, will the presence of cheaters have a negative fitness effect? In our second study, we focus on three closely related *Pemphigus* species: *Pemphigus obesinymphae, Pemphigus populitransversus,* and *Pemphigus populicaulis. Pemphigus obesinymphae* is considered highly social, because individuals fiercely defend the gall from predators and actively remove waste from the gall. *Pemphigus populitransversus* is considered weakly social, because individuals also fiercely defend the gall, but do not remove waste from the gall. *Pemphigus populicaulis* is traditionally considered nonsocial because individuals only weakly defend the nest, but do actively remove waste (Lawson et al. 2014).

Methods

Effect of relatedness of cheaters- field experiment

To determine if the cost of cheaters is merely due the presence of cheaters or if relatedness among cheaters can effect the fitness, we measured fitness correlates of galls after introducing aphids with varying degrees of relatedness. Fifty galls were flagged at two sites: Centre Pointe (July 9, 2013; N 35.9929, W 86.5985) and Enon Springs (July 10, 2013; N 35.9793, W -86.5332). Tanglefoot, a sticky substance that serves as a barrier to climbing insects, was applied to the petiole of each gall. The galls were divided into five groups and labeled with a group letter A-E; Group A served as the control. The treatment groups B-E had a total of 50 aphids from other clones introduced. Aphids from one

colony were introduced to group B, aphids from two colonies were introduced to group C, aphids from three colonies were introduced to group D, and aphids from four colonies were introduced to group E. For the control group A, 50 aphids were removed from the labeled gall and then re-introduced. The colonies introduced to the galls at Centre Pointe were collected from Ashland City Highway on July 8, 2013 to ensure there was no genetic overlap. Similarly, the colonies introduced to the galls at Enon Springs were collected on July 9, 2013 from Centre Pointe. After the non-related clones were introduced, the galls were monitored weekly for survivorship. Any dead galls were collected, missing galls were recorded, and Tanglefoot was re-applied. In September, organza bags were applied to each gall to collect winged alates leaving the galls. After one week the bags and galls were collected and taken to the lab for processing.

Effect of relatedness of cheaters- fitness measurements

The collected galls were stored in a freezer at -20°C until tallied. Galls from all control and treatment groups were analyzed together. The contents of each organza bag were recorded, including the number of winged alates and whether any predators were present. The gall was emptied and the contents were recorded, including the number of winged alates, the distribution of the instars (1^{st}, 2^{nd}/3^{rd}, and 4^{th}), the presence of predators, and the overall condition of the gall. The size of the gall was also measured and recorded. Once all of the galls were processed, those that contained winged alates were further examined to determine the number of embryos present in the winged alates. Because aphids have telescopic generations, the fitness of an individual can easily be determined by counting the number of embryos contained within its abdomen. Five winged alates were selected for embryo counting from each gall. The abdomen of each winged alate was removed and the number of embryos was recorded with the remaining head and thorax stored for later analysis.

Comparing the effects of conflict across groups with varying social complexities- field experiment

To explored if conflict requires cooperation to be manifested, we compared the effect of cheaters across three aphids species with varying degrees of social behaviors. On May 2, 2013, we flagged and numbered 60 *P. populicaulis* galls (N 36.09120, W 086.92250). On May 19, 2013, we flagged and numbered an additional sixty *P. populicaulis* galls on a second tree (N 36.09120, W 086.92250). Galls labeled with even numbers were left open to cheaters, and galls labeled with odd numbers had Tanglefoot® applied to them, thus inhibiting cheaters from entering. Organza bags were placed over each of the *P. populicaulis* galls on June 15, 2013, and the bagged galls were collected on June 24, 2013. Similarly, sixty *P. populitransversus* galls were flagged (N 36.21464, W 0.86.98225) on June 3, 2013; these galls were bagged on July 8, 2013 and collected. Sixty *P. obesinymphae* galls were flagged and labeled (N 36.21464, W 0.86.98225) on June 28, 2013, bagged September 10, 2013, and collected on September 17, 2013. Like *P. populicaulis,* both *P. populitransversus* and *P. obesinymphae* were numbered: evens were left open to cheaters and odds were closed to cheaters with the application of Tanglefoot®. The galls of all species were checked bi-monthly, when dead galls were collected and Tanglefoot® was reapplied. After collection, all galls were stored in a freezer at -20˚C.

Comparing the effects of conflict across groups with varying social complexities - fitness measurements

In the lab, galls were cut in half with a razor blade, and the winged alates, non-winged instars, and predators in each gall were tallied, as were the winged alates and other species present in the organza bags. All gall and bag contents were stored in 1.5 mL tubes at -20˚C for later analysis. To determine fecundity, we dissected and counted the embryos of the winged alates. Aphids have telescopic generations and their offspring begin developing within their abdomens when they are born. Therefore, the fecundity of an individual can be measured by counting the embryos in its abdomen.

Data Analysis

We measured multiple fitness correlates including the age distribution within the gall, number of winged alates and fecundity. To determine the fitness effect due to the presence or relatedness of cheaters, we compared the sum of the number of winged alates in the gall and in the bag, and the average number of embryos in the abdomen of each winged alate between groups. The total number of aphids and number of winged alates were analyzed as nonparametric equivalents of two-way ANOVAs. Whole model tests were followed by pairwise contrasts (Sokal et al., 1995). We analyzed the number of alive and dead galls using a contingency analysis. All statistical analyses were performed in JMP v. 7.01 (SAS, Cary, NC, USA). All reported *P*-values are two-tailed.

Results

Effect of relatedness of cheaters

We performed field-based manipulative experiments to isolate the effect of unrelated cheaters on the overall fitness of natal aphids in the social aphid *P. obesinymphae*. We found no significant difference in any of the fitness correlates measured. There was not a significant difference in the total number of aphids in the gall between control groups (all from a single stem mother) or any of the treatment groups (varying from 2-5 different clones) (Fig. 1a; ANOVA, F = 0.6303, df = 65, $P = 0.6428$). Also, there was not a significant difference in the number of winged alates produced per gall (Fig. 1b: Wilcoxon test, $\chi^2 = 1.4173$, df = 4, $P = 0.8412$). Finally, we found no significant difference between the percentage of dead galls collected for each treatment group (Fig. 2: Contingency test, $\chi^2 = 0.914$, df = 4, $P = 0.9225$).

Comparing the effects of conflict across groups with varying social complexities

We then compared the effect of the presence of cheaters in three *Pemphigus* species with differing degrees of sociality based on the presence of soldiers (social, weakly social, and non-social). We found a significant difference between the total number of aphids in *P. populicaulis* galls that were open to cheaters versus closed to cheaters, but not a

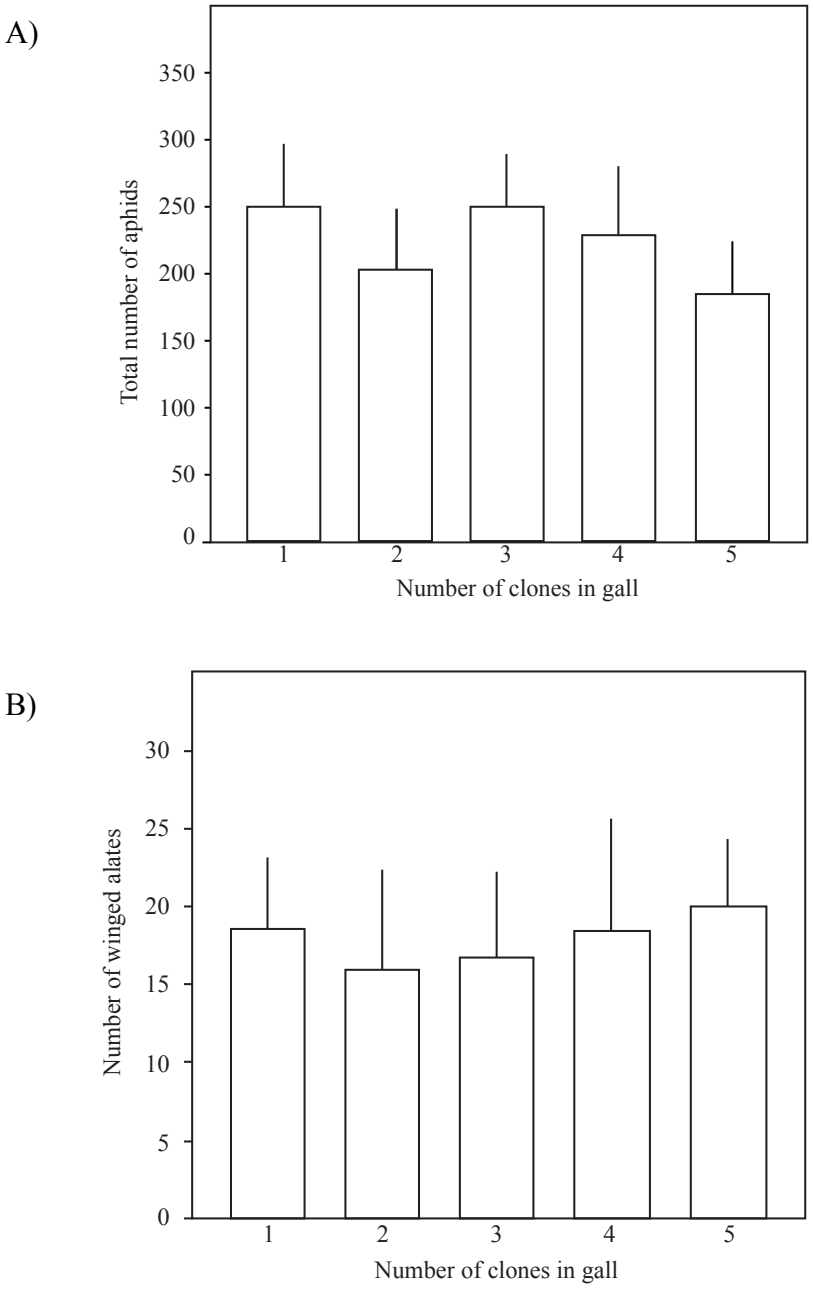

Fig. 1. (A) Average (± SE) number of aphids or (B) winged alates in galls with varying degrees of clonal diversity.

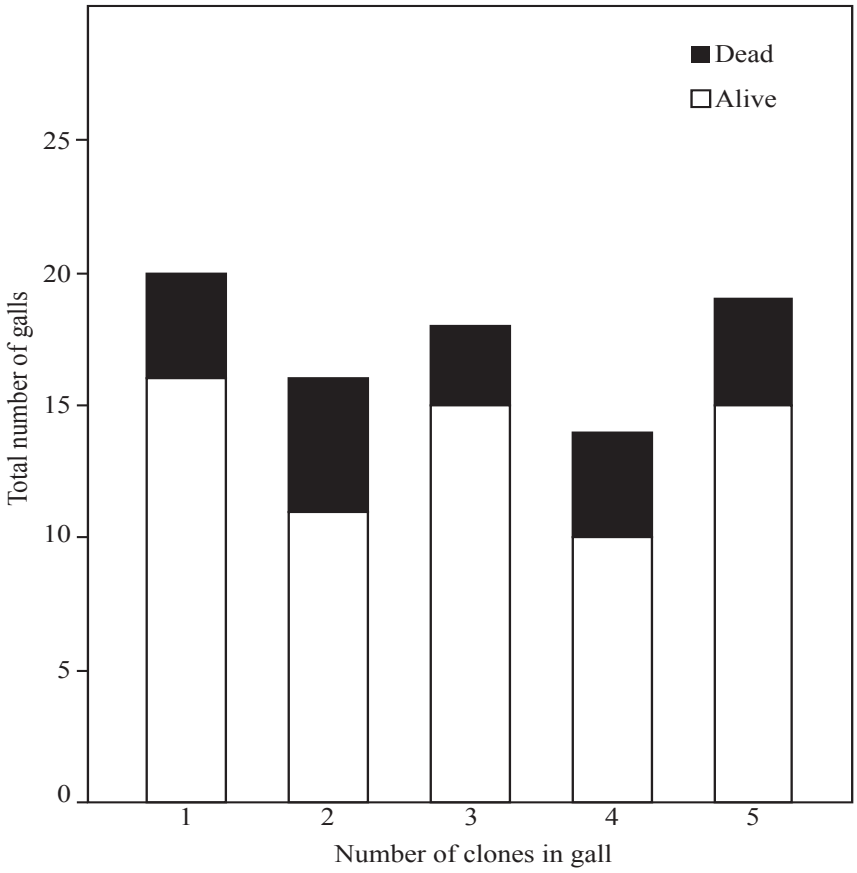

Fig. 2. Survivorship of galls with varying clonal diversity. Black bars indicate the number of dead galls and white bars indicate the number of alive galls.

significant difference in total number of aphids in *P. populitransversus* (Fig. 3; Wilcoxon test, df = 1, $P = 0.0131$) or *P. obesinymphae* galls (Wilcoxon test, df= 1, $P = 0.0679$; ANOVA, df = 1, $P = 0.1388$). The difference in total number of aphids in *P. populicaulis* galls was due to significant differences in the distribution of later instars between open and closed galls. Late instars, 2^{nd} - 3^{rd} (Table 1; Wilcoxon test, df = 1, $P = 0.0347$) and 4^{th} (Table 1; Wilcoxon test, df = 1, $P = 0.0142$), were significantly different between galls open and galls closed to cheaters, but 1^{st} instars were not significantly different (Table 1; Wilcoxon test, df = 1, $P = 0.9506$). The distributions of *P. populitransversus* and *P. obesinymphae* aphids were also not significantly different between galls that were open and closed to cheaters (Table 1). We found a similar pattern in the total number of winged alates between galls that were open and closed to cheaters across aphid species. There was a significant increase in total numbers of winged alates in *P. populicaulis* galls closed to cheaters compared to galls open to cheaters (Fig. 4; Wilcoxon test, df= 1, $P = 0.0025$), but no significant differences between galls that were open to cheaters and galls that were closed to cheaters in *P. populitransversus* (Wilcoxon test, df = 1, $P = 0.9399$) or *P. obesinymphae* (Wilcoxon test, df = 1, $P = 0.8779$).

The difference in total number of aphids and winged alates between galls that were open to cheaters and galls that were closed to cheaters was not due to a difference in the number of predators. There was not a significant difference in number of predators between galls that were open to cheaters and galls that were closed to cheaters for any species (Fig. 5; *P. populicaulis* open versus closed, Wilcoxon, $\chi^2 = 3.1633$, df = 1, $P = 0.0753$; *P. populitransversus* open versus closed, Wilcoxon, $\chi^2 = 0.0590$, df = 1, $P = 0.8081$; *P. obesinymphae* open versus closed, Wilcoxon, $\chi^2 = 0.3011$, df = 1, $P = 0.5832$). However, *P. obesinymphae* had significantly more predators than *P. populicaulis* and *P. populi-transversus* (Fig. 5; Overall Wilcoxon, $\chi^2 = 12.4108$, df = 2, $P = 0.002$; *P. populicaulis* versus *P. obesinymphae*, $P = 0.0008$; *P. obesinymphae* versus *P. populitransversus*, $P = 0.0239$; *P. populicaulis* versus *P. populi-transversus,* $P = 0.8193$).

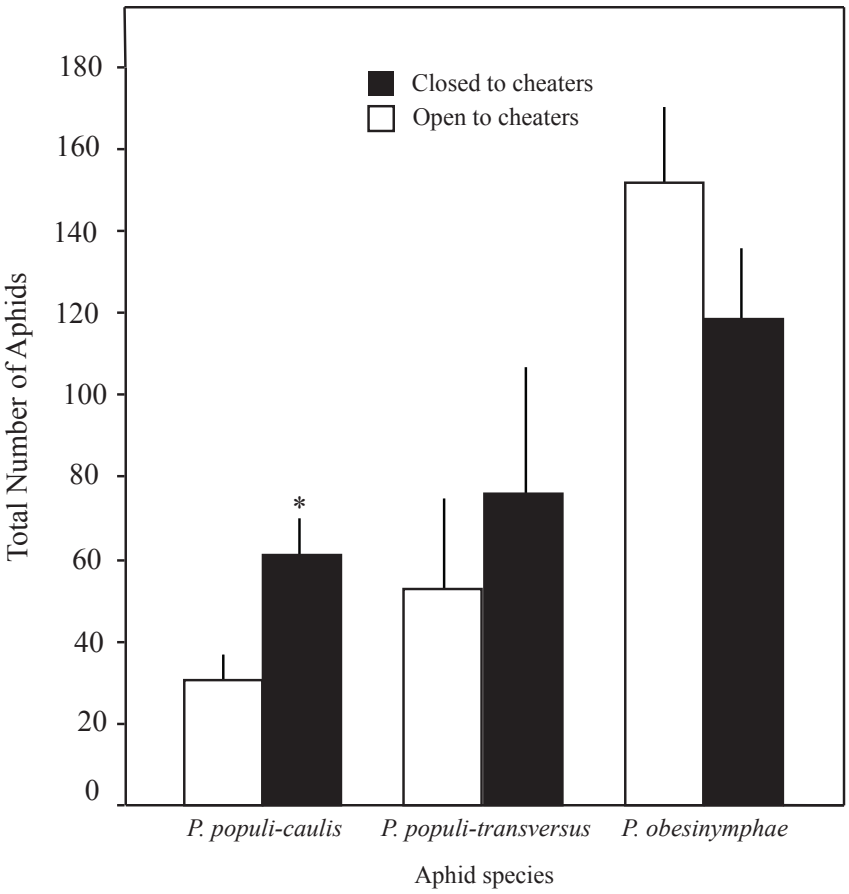

Fig. 3. Average (±SE) number of aphids in galls of three *Pemphigus* aphid species either unmanipulated, and cheaters can freely enter the gall (white bars) or closed to cheaters with a sticky barrier (black bars). Asterisks indicate a significant difference ($P < 0.05$).

Table 1. Mean number (±SE) of either 1st, 2nd/3rd, or 4th instar in the galls of three *Pemphigus* aphid species.

	Total number of 1st instars		Total number of 2nd/3rd instars		Total number of 4th instars	
	Open to cheaters	Closed to cheaters	Open to cheaters	Closed to cheaters	Open to cheaters	Closed to cheaters
P. populicaulis	14.21 ± 2.96	17.66 ± 3.55	3.31 ± 1.38	5.68 ± 1.46*	3.196 ± 1.39	6.489 ± 1.63*
P. populitransversus	36.10 ± 14.81	46.69 ± 20.54	3.75 ± 2.75	4.67 ± 2.88	2.38 ± 1.38	2.56 ± 2.10
P. obesinymphae	56.69 ± 11.92	51.04 ± 11.42	35.24 ± 6.17	35.24 ± 5.67	25.96 ± 6.63	14 ± 3.3

Each gall was either unmanipulated to allow cheaters to freely enter the gall (open) or treated with a Tanglefoot barrier to keep cheaters from enter the natal gall (closed). Asterisks indicate a significant difference ($P < 0.05$).

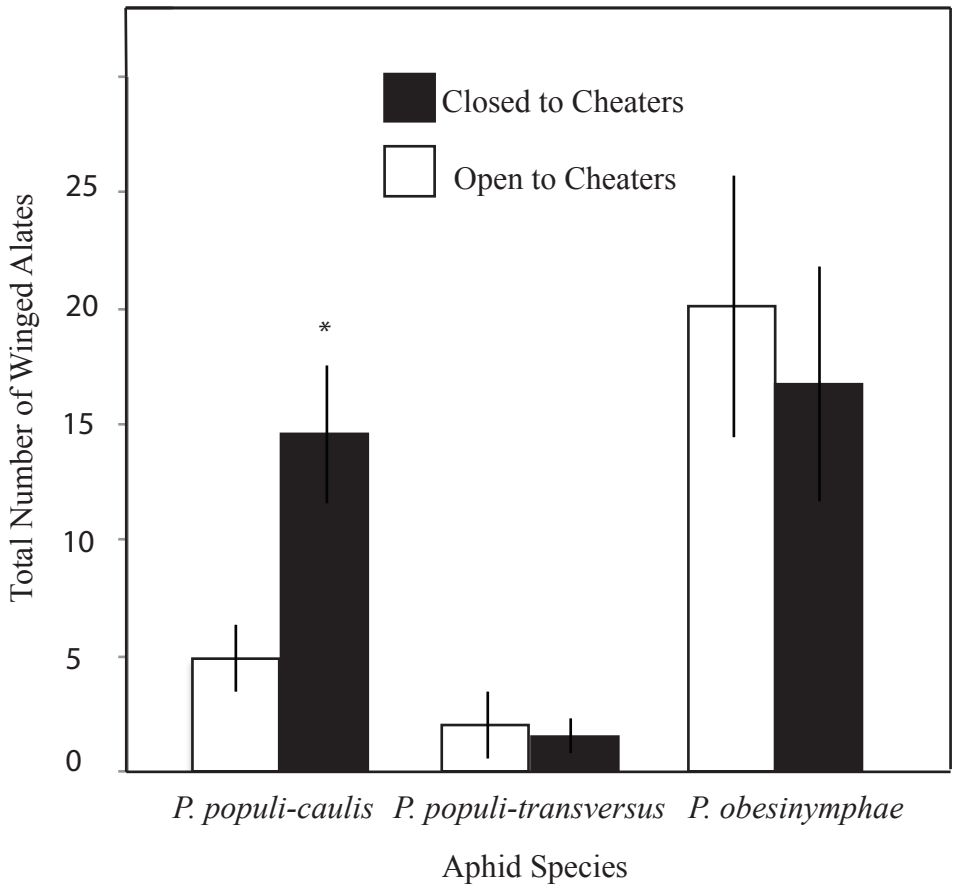

Fig. 4. Average (±SE) number of winged alates in galls of three *Pemphigus* aphid species either unmanipulated, and cheaters can freely enter the gall (white bars) or closed to cheaters with a sticky barrier (black bars). Asterisks indicate a significant difference ($P < 0.05$).

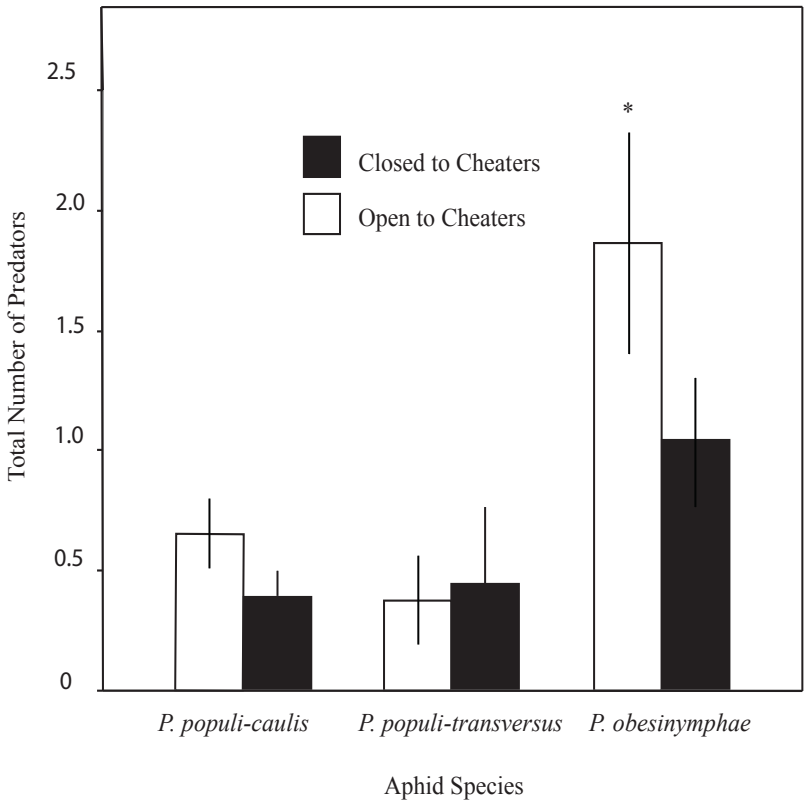

Fig. 5. Average of the total number of predators counted in galls of three *Pemphigus* aphid species either unmanipulated, and cheaters can freely enter the gall (white bars) or closed to cheaters with a sticky barrier (black bars). Asterisks indicate a significant difference ($P < 0.05$).

Another measure of fitness is the condition of the whole gall. *P. obesinymphae* galls had significantly more dead than alive galls when left open to cheaters (Fig. 6**;** Contingency test, df = 1, χ^2 =9.54, *P* = 0.0085). Neither *P. populicaulis* (Fig. 6; Contingency test, df = 1, χ^2= 1.824, *P* = 0.1768) or *P. populitransversus* (Fig. 6; Contingency test, df= 1, χ^2= 0.346, *P* = 0.5564) galls had a significant difference in the number of dead galls when closed versus open to cheaters.

Discussion

Effect of relatedness of cheaters

It has long been accepted that high relatedness plays an important role in the development of social behavior (Hamilton 1964; Aoki & Moran 1994; Aviles 2002; Abbot 2009; Bourke 2011). However, because of the natural history of many social organisms, quantifying the effect of varying relatedness *a priori* has proven difficult. Studies of social bacteria have found altruistic behaviors, such as quorum sensing and multicellular fruiting bodies used for dispersal, increase as relatedness increases (Diggle et al. 2007; Vos & Velicer 2009), but these are the really the only examples of manipulative studies of relatedness in natural populations. Social aphids offer a unique opportunity to explore the effect of relatedness in social insects. First, aphids are clonal, thus relatedness is easily calculated. Second, there is little evidence of kin recognition in aphids, which allows us to introduce unrelated aphids into the gall and measure the fitness effect of the introduced aphids (Miller 1998; Shibao 1999). Past research found a negative correlation between the diversity of clones in a gall and multiple fitness measures, but the study was unable to determine if decreased relatedness led to decreased fitness or if the decrease in fitness led to increased clonal diversity (Grogan et al. 2010). For example, under stressful environmental conditions, cottonwoods, the primary host plant of *Pemphigus* aphids, begin to senesce and drop leaves (Killingbeck 1996). Aphids within the gall would

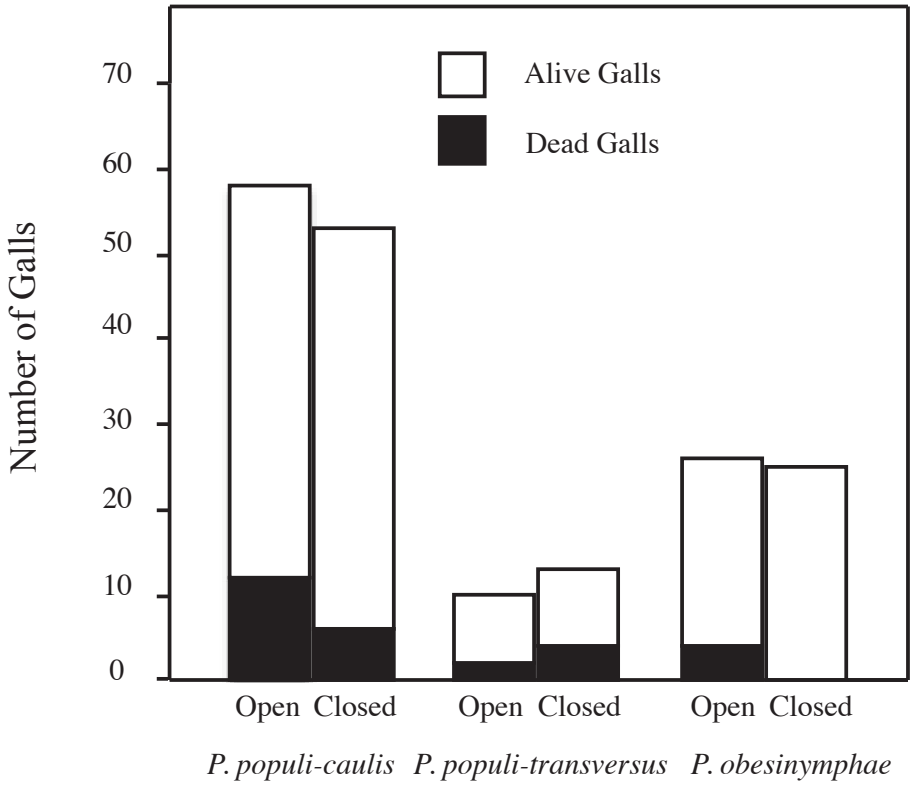

Fig. 6. The total number of alive and dead galls for three *Pemphigus* aphid species. Open galls allowed for cheaters and natal aphids to freely enter and leave the gall, while a Tanglefoot barrier prevented movement in galls closed to cheaters (closed).

receive less nutrients, becoming less fit and begin to leave the gall for neighboring galls. The decreased fitness of the aphids would lead to increased clonal diversity. To tease apart these possibilities, we experimental manipulated the clonal diversity within galls.

We found no significant fitness effect of clonal diversity on overall gall fitness (Fig. 1). These results disagree with previous findings in the lab (Grogan et al. 2010). We believe that this disagreement is due to the fact that we did not distinguish between the fitness of natal and introduced aphids. Using restriction enzymes, we are currently working to genotype the winged alates and stem mother from each group to determine which aphids are natal versus introduced. By identifying which gall the aphids originated from we can (1) insure that the introduced aphids remained in the gall for the duration of the experiment and (2) separate the fitness of the natal from the introduced aphids. We predict that by comparing the fitness of the natal aphids exclusively in control and treatment groups, we will find a negative fitness effect of unrelated aphids, similar to previous findings in the lab (Grogan et al. 2010). The question remains if the presence of unrelated aphids alone leads to a decrease in fitness in natal aphids or if this fitness effect can be compounded by increased clonal diversity. By comparing the natal aphid fitness across treatments, ranging from a clonal diversity of two to five, we can begin to answer this question. Because of the *a priori* nature of the manipulation, this experiment is the first of its kind in social insects and will help us understand the cost of conflict in social groups.

Comparing the effects of conflict across groups with varying social complexities

Competition within social groups can be costly to the collective group fitness (Hardin, 1968; Rankin et al., 2007). Past research in the lab has shown that competition in social aphids has a negative fitness effect on the natal aphids (Grogan *et al.,* 2010), but the consequences of social exploitation in weakly and nonsocial aphids are unknown. We

compared fitness correlates in groups with and without cheaters across social, weakly social, and nonsocial *Pemphigus* species to determine the effect of the presence of cheaters in altruistic groups. Since there are fewer resources to exploit, we predicted that the presence of cheater aphids will have less of an effect on weakly and nonsocial aphid groups. Similarly, we hypothesized that the presence of cheaters will be more detrimental to social aphid groups, since there is a common good to exploit.

Interestingly, we found that in *P. populicaulis*, the least social species, there was a significant decrease in the number of both total aphids and winged alates when cheaters were present (Fig. 3 and 4). Since *P. populicaulis* has less cooperative behaviors than the other species tested, soldiers are less aggressive, we expected the presence of cheaters to have little or no effect on natal aphid fitness. Our results imply that there is a cost of cheaters in the a species with less cooperative behaviors. Past research in the lab has shown that *P. populicaulis* displays some altruistic housekeeping behaviors and weak defense (Lawson et al. 2104). These behaviors could create a common good that cheaters exploit. These results support past research in the lab that *P. populicaulis* is not actually a nonsocial, but a weakly social species.

We found no significant fitness effects of the presence of cheaters in *P. populitransversus*, the weakly social species, or *P. obesinymphae*, the highly social species (Fig. 3 and 4). It is important to note that the sample size for *P. populitransversus* was lower than other two species (N= 9 galls closed to cheaters; N= 8 galls open to cheaters). Because the results were trending towards a negative fitness effect of cheaters, we predict that if we increase the sample size of *P. populitransversus,* we would find a slight negative fitness effect of cheaters on the natal aphids. Because *P. populitransversus* has moderately aggressive soldiers and less housekeeping behaviors compared to the other species, we predict that the fitness effect of the presence of cheaters would be less than that of the species with very aggressive soldiers and increased housekeeping behaviors (Lawson et al. 2014)

Since *P. obesinymphae* aphids are social and have a common good to exploit, we expected the presence of cheaters to have a negative fitness effect on natal aphids. Instead, we saw no significant difference in the total number of aphids or total number of winged alates in the presence of cheaters (Fig. 3 and 4). This contrasts past research in the lab, possibly because the experiment only looked at the fitness of the gall over one week at the end of the season. Similarly to the first experiment on the clonal diversity of the cheaters, we plan to use genotyping to tease apart the fitness of the natal aphids from the introduced aphids. We predict that the fitness of the introduced aphids will be higher than the natal aphids and when this is accounted for the fitness of the natal aphids will be higher in galls closed to cheaters than open to cheaters.

Conclusions

Hamilton developed an equation to explain how natural selection could favor cooperation if $rb > c$, where c is the cost to the altruist, b is the fitness to the beneficiary, and r is their genetic relatedness. This rule illustrates the fine balance between benefit and cost, cooperation and conflict and how increased relatedness can lower the fitness cost of altruistic behaviors (Hamilton 1963). Studies on the evolution of social behavior often focus on measuring the benefits of social living, the b term of Hamilton's rule, because the cost can be difficult to measure. In this study, we aimed to tease apart the complex relationship between cost and relatedness. One of the main cost of social behavior is unrelated individuals exploiting the shared resource of the group. By manipulating relatedness *a priori*, we were able to measure the fitness cost of decreased relatedness. This is one of the first studies of its kind in social insects. Next, we explored if conflict can occur when there is no shared good. Intriguingly, our preliminary results indicate that conflict can occur in species with less social behaviors, implying the group living alone, not highly social behaviors, is sufficient for conflict to arise.

Acknowledgements

I would like to thank the co-authors of this study for their help. Catie Mitchel and Emily Neil helped with the data collection and the writing of the manuscript. Stephanie Chaing and Catie Mitchel did the genotyping work and Patrick Abbot helped with the development of the project and the writing of the manuscript.

CHAPTER VI

DISCUSSION AND FUTURE DIRECTIONS

Aphids offer many unique opportunities to explore the unifying principles that guide the evolution of altruistic behavior itself (Strassmann & Queller 2010). The pea aphid (*Acyrthosiphon pisum*) is the model organism for the study of insect-plant interactions and insect symbioses. Understanding sociality in aphids therefore offers the promise of conceptual linkages between social evolution and disparate fields of biology. There is some evidence, for example, that aphid 'queens' regulate soldier development by withholding nutritional symbionts, thereby consigning their daughters to a sterile dead-end (Fukatsu & Ishikawa 1992). Aphids also offer key experimental advantages for the study of social behavior. For example, aphid clonality permits genotype-specific, "common garden" manipulations - but in a social insect. And, the transitional, 'on-the-cusp' nature of aphid sociality allows for much-needed reconstruction of the decisive factors that govern the tipping points back and forth between altruism and alternative life history strategies (there is general agreement that such transitional species offer key experimental pay-offs (West et al. 2002; Nowak et al. 2010)).

The inspiration for many of these experiments can be found in the work of W.D. Hamilton, the 'father' of the modern study of sociality. In various places Hamilton dwelled on aphids: he wrote often in vivid terms of a view of aphid social evolution in which cheating, conflict, defense, and intraspecific competition are the central drivers (1963 & 1964). At the heart of his organismal interest in aphids, you can find the pillars of modern sociobiology, including many of the open questions that remain today. This research integrated behavior, social evolution and aphids in unprecedented and exciting ways. This thesis utilized aphids to address three emerging themes in social evolution.

First, if the canonical expression of altruistic behavior is self-sacrifice for the family group, then characterizing the adaptations for defense and care of the nest means identifying the very traits that have evolved under selection for altruist traits. In Chapter

II, I demonstrated that defining sociality in aphids depends critically on what trait is being measured. In addition, these data contribute to evidence suggesting that most galling aphid species, including those not traditionally described as nonsocial, may express some form of social behaviour. Most social insects outside of the Hymenoptera remain poorly studied, and even basic natural history information is often lacking, or largely anecdotal (Costa 2006). As studies of these 'other social insects' are undertaken, the lesson from aphids is that the nonsocial species tend to be particularly poorly studied, if at all. If the goal is to understand social transitions, studies of nonsocial species are as necessary to comparative studies as studies on social species. Second, aphids illustrate the degree to which the antecedents to advanced sociality may be identified in nonsocial species.

In Chapter III and IV, I further explored the two axes of sociality of aphids: defense and housekeeping. In Chapter III, I identified the mechanisms by which aphid soldiers overwhelm predators much larger than themselves. My results indicate that the soldiers overwhelm the tightly regulated phenoloxidase cascade leading to an increase in toxic quinones, eventually causing death. The next step would be to identify the compounds aphid soldiers use to induce this response. In Chapter IV, I explored how the role of increased housekeeping in *P. populicaulis, P. populitransversus* and *P. obesinymphae* have impacted the fungal endophyte community within the gall. These results were the first of their kind exploring the fungal endophyte community within the gall and surrounding plant tissue across multiple gall-inducing species.

Finally, the importance of role of kin selection in the evolution of sociality has recently become a hotly debated topic (Abbot et al. 2011; Boomsma et al 2011; Strassmann et al. 2011; Ferriere & Michod 2011; Herre & Wcislo 2011; Nowak et al. 2010). In chapter V, I began to explore the fine balance between relatedness and conflict in galling aphids. Preliminary results disagreed with previous findings in the lab. As described in discussion, we are using restriction enzymes to genotype winged alates from the treatment groups to tease apart the fitness of the natal aphids from the cheater aphids.

These results will help clarify (1) if the treatments worked and non-natal aphids were introduced and (2) allow us to compare the fitness of the natal aphids to the non-natal aphids, instead of comparing the fitness of the entire gall. I predict that the genotyping will reveal that the fitness of the cheaters is much higher than the natal aphids in both *P. populitransversus* and *P. obesinymphae*. These results would support previously reported results (Grogan et al. 2010).

Future studies

The three major themes I believe need to be further explored in the field of social behavior in aphids are defense, caste determination and sociogenomics. In Chapter III, I began to explore how social aphids mount a successful defense. Soldiers use their plant feeding mouthparts as needle-like weapons to wound invaders inducing a massive melanization response in victims (Ch. III, Fig. 1 and 2). Melanization is a common response to injury or infection in insects. Phenoloxidase is the terminal enzyme in the melanization cascade and hyperactivation of the cascade results in a toxic accumulation of quinones in insects (Shin et al. 2011). The next question in how are aphids soldiers able to induce this melanization response?

We have some preliminary evidence that, remarkably, aphids harvest secondary compounds from their host plants, which may induce massive melanization. Poplars, the primary host of most *Pemphigus* aphid species, have an array of defensive chemicals, including phenolic compounds, proteinase inhibitors, chitinases, and polyphenol oxidases to defend against plant-feeding insects, like aphids (Philippe & Ramirez 2009). We found that soldiers inject poplar-derived fatty acids in *Drosophila* larvae following attack (Fig. 1). The isolated compound contained tetradecanoic acid, octadecanoic acid and oleic acid.

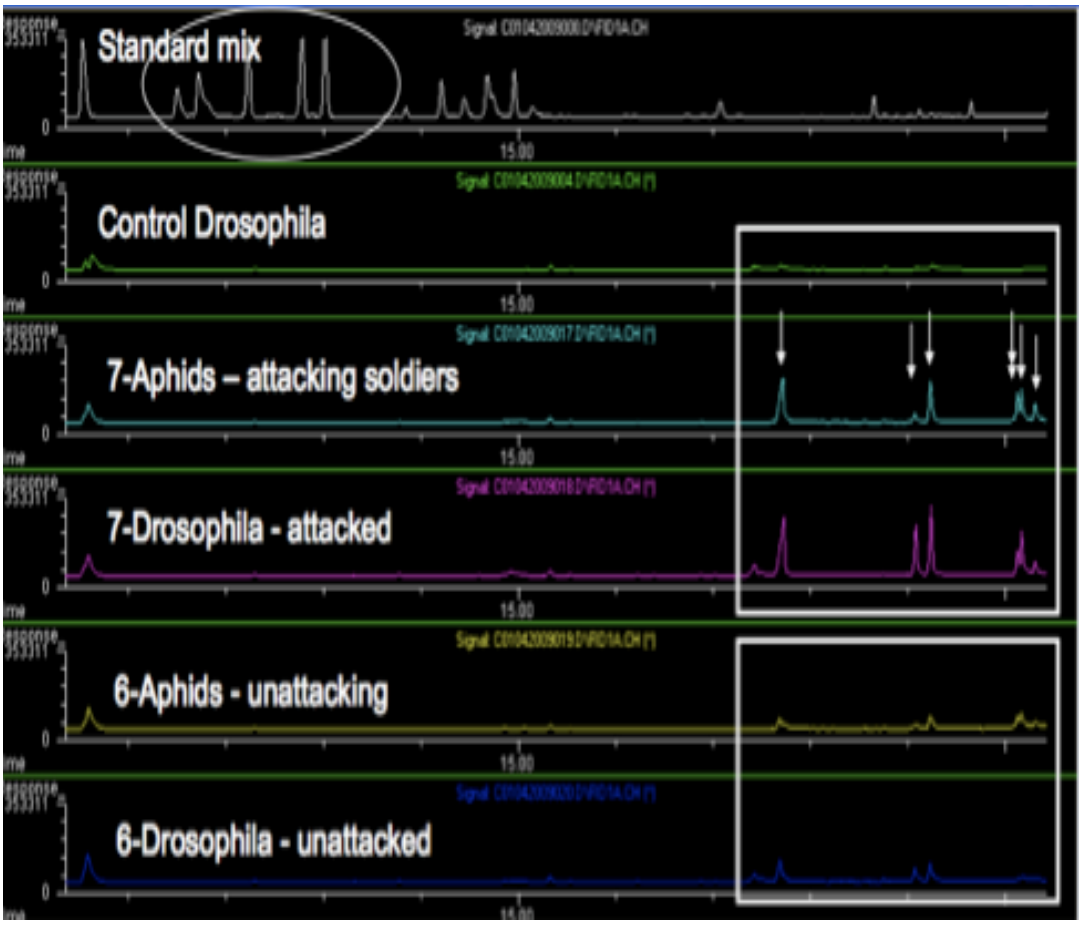

Fig. 1. Total ion chromatograms from GC, in which six samples are overlaid. Smaller molecular weight compounds are rightward, larger compounds leftward. The arrows point to compounds common in *Drosophila* larvae and aphid soldiers that attacked them.

While the mechanism is not yet clear, we were particularly interested in oleic acid because previous studies have shown experimental inoculation of oleic acid into 4th instar *Aedes aegypti* mosquitoes reduces survivorship relative to controls (Ramsewak et al. 2001). To investigate further, we injected *Drosophila* larvae with oleic acid or a PBS control, extracted hemolymph and quantified absorption patterns. Hemolymph of the larvae injected with oleic acid and the control were used in phenoloxidase assays that measured the transformation of L-DOPA to dopachrome as a function of optical density. We found a significant difference in absorption values between larvae injected with oleic acid and PBS controls (Fig. 2). This result suggests that the oleic acid indeed induces the melanization cascade, as we suspected. To put it another way, a key innovation in social evolution in aphids could be that they are co-opting phytochemistry for defense.

We are also exploring other possible candidates that aphids are using as effector molecules. Like many toxins and venoms, the aphid soldiers seem to induce a system wide failure in their victims (Fry et al. 2009). Of particular interest are proteases. Past research has shown that soldiers of one social aphid species, *Tuberaphis styraci*, produce an unusual cysteine protease that they secrete when they attack invaders with their mouthparts (Kutsukake et al. 2004). However, other social aphids apparently do not share this particular proteolytic enzyme (Kutsukake et al. 2008), nor have we found evidence of it in *Pemphigus.* Aphids therefore may employ diverse, lineage-specific biochemical strategies for defense against natural enemies. Another point of interest is that proteases have been shown to induce melanization, which often leads to death (Harrison & Bonning 2010). For example, the entomopathogenic fungi *Metarhizium anisopliae* protease, PR1A, digests cuticle proteins and is vital in the penetration of host cells. The expression of PR1A in the hemocoel of infected *Manduca sexta* leads to degradation of hemolymph proteins and increased melanization. It has been hypothesized that the protease triggers the melanization cascade by the cleavage and activation of prophenoloxidase, which contributes to the toxicity of PR1A (Harrison & Bonning 2010). Another cathepsin, ScathL from the flesh fly, *Sarcophaga peregrine*, when expressed in the virus AcMLF9 and introduced to multiple insect orders, including the tobacco

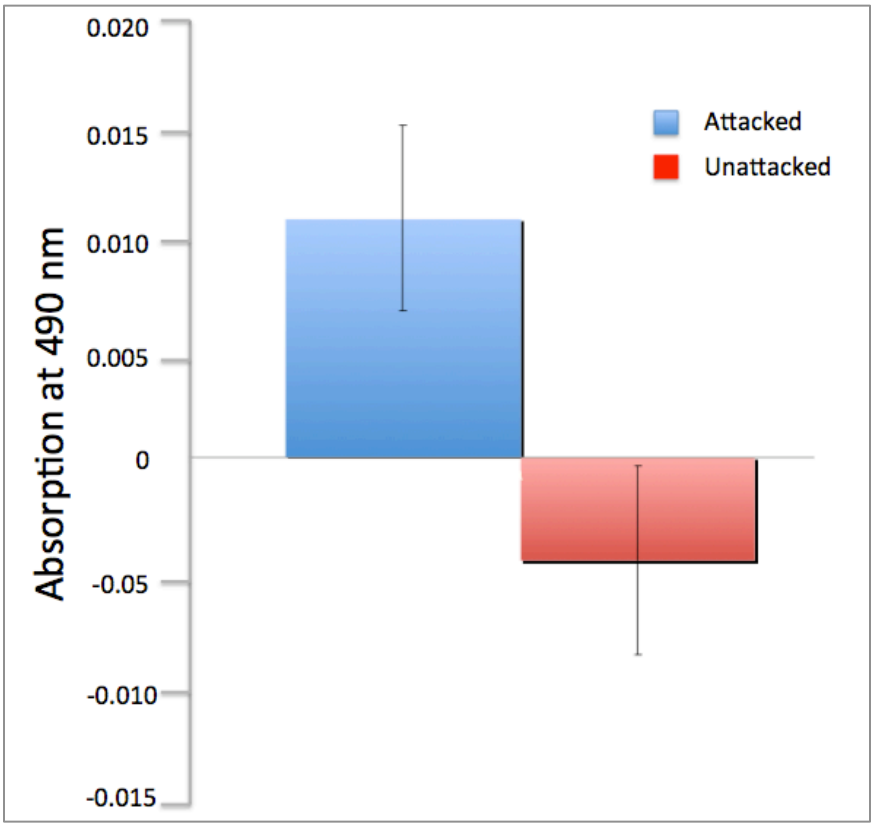

Fig. 2. There was a significant difference in absorption values between *Drosophila* injected with oleic acid and those injected with PBS. This suggests that the injection of the oleic acid triggered a melanization-like response in the *Drosophila* larvae.

budworm, *Heliothis virescens,* the tomato moth*, Lacanobia oleracera*, and the pea aphid, *Acyrthosiphon pisum*, causes significant melanization and death in all insects tested (Cerenius et al. 2008; Harrison & Bonning 2010).

A second major underexplored theme in the understanding the evolution of social behavior in aphids is caste determination. Caste determination, overlapping generations and cooperative brood care are the textbook definition of eusociality. This strict definition has lead to debate over the use of "eusociality" (Costa & Fitzgerald 2005; Crespi & Yanega 1995; Sherman et al. 1995). Historically, there has been debate about whether social aphids are truly eusocial, and some prefer the moniker "highly social". Because of the complexity of the aphid life cycle, including parthenogenesis and multiple host plants, very few aphid species have overlapping generations. Cooperative brood care does not apply to aphids, because unlike most highly social insects, aphids are hemimetabolous, not holometabolous, meaning aphid young are precocial, not altricial.

Division of labour and caste determination have been briefly described in some aphid species, but there has been no studies investing how castes are determined. In termites, bees and ants, the caste system is a result of phenotypic plasticity caused by a variety of environmental and genetic cues (Hamilton 1964; Crozier & Pamilo 1996; Evans & Wheeler 1999). Multiple different tasks have been described in social aphids, including defense, gall repair, gall upkeep, drifting and nest guarding (Abbot et al. 2001; Kutsukake et al. 2004; Kutsukake et al. 2009; Lawson et al. 2014). Although multiple tasks have been described, many questions remain involving task allocation: How do individual aphids decide which task to do? Can one aphid perform more than one task? Who decides which task needs to be done? Is task allocation controlled by physical interactions or hormonal regulation? Do individuals have morphological adaptations for tasks? To begin to explore these questions, we must first identify all the tasks that occur within the gall. Task allocation seems to be species specific. In *Pemphigus* aphid galls, there seem to be four main tasks: reproductive stem mother or queen, defenders (usually made up of the 1st instars), drifters (which leave the gall for neighboring galls), and

housekeepers (it unknown whether these individuals also serve as defenders). After identifying which individuals are responsible for each task, we could compare gene expression and hormone regulation across "castes," using similar methods to other eusocial taxa (Weil et. al 2007). By understanding how social aphids allocate tasks, we will have a better understanding of the social organization and communication in aphids.

A final theme of interest in the evolution of social behavior is the genetic basis of sociality or sociogenomics (Robinson et al. 2005; Toth et al. 2007). It has been argued that examining social behavior at the molecular level can help us "to understand how complex and highly derived patterns of social behavior have evolved from simpler ancestral behavior, and explain the evolutionary relationships of apparently similar behaviors across distantly related taxa," (Robinson et al. 2005). In aphids, the publication of the pea aphid genome has opened the door to numerous opportunities for the study of hemimetabolous insects, including the molecular basis of sociality (the pea aphid is not a social aphid). To examine the genetic basis of social behavior in aphids, we began a pilot project to compare transcriptomes of a social and nonsocial aphid species. We generated over 6.6 billion bp from *P. obesinymphae* 1st instar larvae (incidentally, the first glimpse at the transcriptome of a social aphid), and we have recently generated an equivalent number from of a nonsocial aphid (*P. populicaulis*). Currently, we are working to assemble and to annotate these data. This work would strengthened by additional runs on *P. obesinymphae* and *P. populicaulis* and by the addition of a second social species (*P. spyrothecae*) and second nonsocial species (*P. betae*). From these data, we will then target groups of candidate genes important in social behavior. Using reciprocal BLAST, we can match orthologs sequences across species, then compare expression values. Those highly over- or underexpressed between the social and nonsocial species can serve as genes of interest (Robinson et al. 2005).

APPENDIX A

SODIUM-SPECIFIC FORAGING BY LEAFCUTTER ANT WORKERS (*ATTA CEPHALOTES,* HYMENOPTERA: FORMICIDAE)

Abstract

1. Sodium is often a limiting nutrient for terrestrial animals, and may be especially sought by herbivores. Leafcutter ants are dominant herbivores in the Neotropics, and leafcutter foraging may be affected by nutritional demands of the colony and/or the demands of their symbiotic fungal mutualists. We hypothesized that leafcutter colonies are sodium limited, and that leafcutter ants will therefore forage specifically for sodium. 2. Previous studies demonstrated that leafcutter *Atta cephalotes* Linnaeus workers preferentially cut and remove paper baits treated with NaCl relative to water control baits. *Atta cephalotes* colonies in this study were presented with baits offering NaCl, $Na_2 SO_4$, and KCl to test whether leafcutters forage specifically for sodium. Sucrose and water were used as positive and negative controls, respectively. 3. *Atta* foragers removed significantly more of the baits treated with NaCl and $Na_2 SO_4$ than the KCl treatment, which did not differ from water. The NaCl and $Na_2 SO_4$ treatments were collected at similar rates. We conclude *A. cephalotes* forage specifically for sodium rather than for anions (chloride) or solutes in general. This study supports the hypothesis that leafcutter ants are limited by, and preferentially forage for, sodium.

Introduction

The demand for sodium, a key animal nutrient, is expected to vary geographically and among trophic levels. Aerosol deposition of salt declines exponentially with distance from oceanic sources (Stallard & Edmond, 1981). Sodium concentration in consumers' tissues can be 1000 times higher than producers, so herbivores are expected to be more sodium deprived than carnivores (National Research Council, 2005; Kaspari et al., 2008). Recent studies of salt foraging in ant communities support these predictions. More herbivorous ant species at inland sites showed the strongest sodium preferences

(Kaspari et al., 2008). Leafcutter ants (*Atta* and *Acromyrmex*) were poorly sampled by these studies that used vials as baits, but leafcutters are among the most herbivorous ants (Holldobler & Wilson, 2011). Leafcutter foraging preferences can be experimentally tested in the field by presenting paper baits at foraging trails. Leafcutters clear foraging trails to facilitate transport of leaf fragments to the nest (Kost et al., 2005). Leaf fragments are fed to symbiotic fungi. Fungus is the primary larval food source, whereas adult workers subsist mainly on liquid leaf exudates (Richard et al., 2005; Caldera et a*l*., 2009). Their diet suggests leafcutters should be among the most salt-seeking ant foragers (Kaspari et al., 2008; Russell et al., 2009). In previous studies, *Atta* foragers cut and removed fragments from papers treated with various chemical compounds (Costa et al., 2008; O'Donnell et al., 2010). In a previous experiment, sodium chloride-treated baits were preferred over water- treated baits by *Atta cephalotes* Linnaeus workers (O'Donnell et al., 2010). This study did not determine whether the leafcutters were acquiring sodium (Na) or chlorine (Cl), and the study did not rule out a general response to solutes. To test whether *A. cephalotes* workers forage specifically for sodium, we presented paper baits treated with aqueous solutions of sodium sulphate (Na_2SO_4), sodium chloride (NaCl), potassium chloride (KCl), and water-treated negative controls. We expected *Atta* foragers to harvest sodium chloride and sodium sulphate over potassium chloride and water.

Methods

Data were collected between 22 and 23 March 2012 at La Selva Biological station. Proximity to the Caribbean coast (approximately 50 km) means aerosol sodium deposition is relatively high at La Selva (Kaspari et al., 2008).

Five actively foraging *A. cephalotes* colonies were selected as subjects. All colonies had cleared foraging trunk trails at least 10 cm wide. Workers carrying leaf fragments were tracked to their nest mounds to confirm they came from different colonies. Subject colony nest mounds ranged from 100 to 600 m from each other. Each colony was used for one trial. Baits were single pieces of circular filter paper (Whatman no. 4 qualitative

papers, 125 mm diameter) soaked in aqueous solutions. Bait papers were soaked to saturation in approximately 250 ml of one of five solutions: 1 M sucrose (positive control), three 1 M salt solutions (treatments: NaCl, KCl, and $Na_2 SO_4$), and tap water (negative control). We then dried each paper for 2–4 h in a convection oven at 40 ºC. After drying an assistant labeled each paper in pencil with a letter code representing the treatment. Letter codes varied among trails and researchers were blind to the treatments. Bait papers were weighed to the nearest 0.01 g on an electronic balance for pre-trial weight. We used a Licor LI-3100C automated area meter to measure areas of bait papers. The areas of four pieces of filter paper were measured and the mean resulting area (121.08 cm^2) was used as the pre-trial area estimate for all baits.

For each trial, five baits, one per treatment, were placed along a single *A. cephalotes* foraging trunk trail. The first bait was placed 3 m from a nest entrance; baits were separated by a 3 m distance. The order of treatment placements was randomized. Baits were placed with the centre of the paper within 5 cm of the trail edge. We staked each bait paper to the ground with a metal surveyor flag. *Atta* foraging was strongest after dark, so baits were placed at approximately 22.00 hours local time and left overnight (O'Donnell et al., 2010).

Bait papers were collected the next morning at approximately 08.00 hour local time, after approximately 10 h had elapsed. The remaining bait papers were dried for 1–2h at 40ºC and allowed to cool to ambient temperature. Papers were reweighed on the same digital balance used before trials. We estimated the weight of bait paper removed by the ants as the difference between before and after weights. The area of the remaining paper was measured with the same area meter used before trials. We estimated the area of bait paper removed as the difference between before and after areas. We assumed all paper removed had been harvested by *Atta* workers because most bait papers showed cutting and fragment removal typical of *Atta* leaf harvesting, and *Atta* workers were observed cutting papers in all trials.

General linear models (GLM; SAS v. 9.2) (SAS Institute, Cary, NC, USA) were used to analyse the effect of colony identity and treatment on the log-transformed weight and area of paper removed by the ants. The data were log transformed to reduce inequality of variance among treatments. We did not measure forager traffic along the trails, and baits were not presented for identical time periods among colonies (times were constant within colonies). Uncontrolled colony differences were accounted for by including colony identity as a covariate first in the statistical model. The positive control sucrose treatment was used primarily to verify bait attraction and foraging activity. We excluded the sucrose data from the analyses because the relatively high removal of sucrose-treated paper could mask differences among the other treatments (O'Donnell et al., 2010).

Results and Discussion

The measures of weight removed and area removed were highly correlated within baits ($r = 0.96$, $n = 20$, $P < 0.0001$). In all colonies ants removed more of the sucrose positive control than any other bait (Fig. 1). There was no significant effect of colony on weight removed ($F_{4,12} = 1.88$, $P = 0.18$) or area removed ($F_{4,12} = 2.40$, $P = 0.11$). Treatments differed highly significantly in the weight of paper removed ($F_{3,12} = 10.05$, $P = 0.0014$) and area removed ($F_{3,12} = 5.68$, $P = 0.012$). *Post hoc* pair-wise means comparisons (Ryan–Einot–Gabriel–Welsch multiple range test, $\alpha = 0.05$) indicated both weight and area removal of NaCl and Na_2SO_4 treatments were not significantly different, and more paper was removed from each of these treatments than the KCl and water (negative control) treatments (Fig. 1). The KCl treatments did not significantly differ from the water treatments.

The foraging behaviour of leafcutters is of particular interest because these ants are dominant primary consumers in the Neotropics (Herz et al., 2007; Costa et al., 2008). We extend previous findings on *Atta* salt attraction (O'Donnell et al., 2010) and demonstrate that *Atta* forage specifically for sodium. Similar sodium-specific foraging has been documented for ant communities in general (Kaspari et al., 2009). Sodium sulphate was as attractive to *Atta* foragers as sodium chloride. In contrast, potassium chloride was not

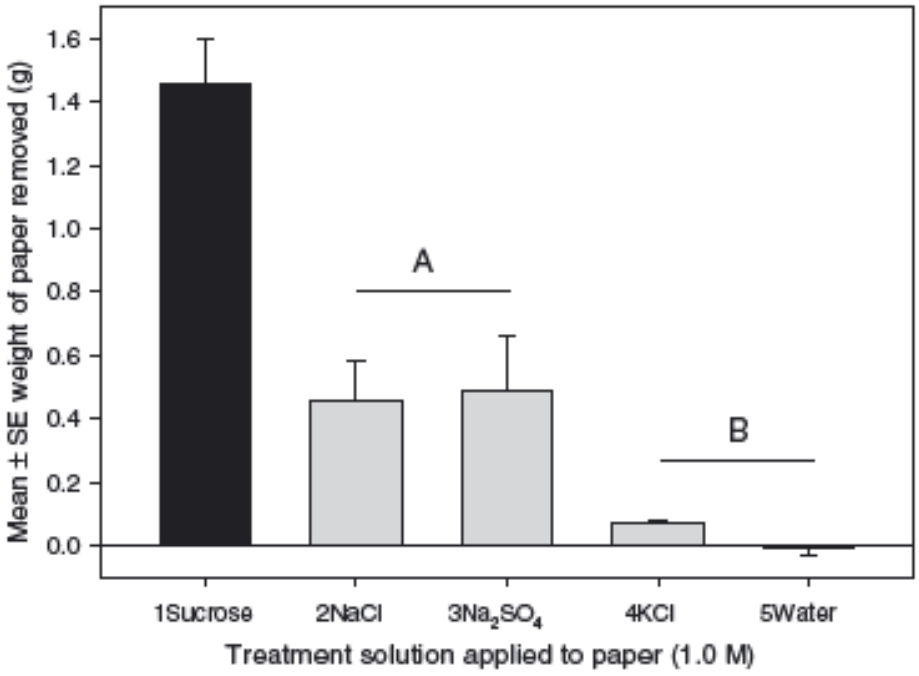

Fig. 1 Bar graph showing amounts of paper baits removed by *Atta cephalotes* foragers. Baits were treated with sucrose (black bar, positive control), water (open bar, negative control), and metallic salts (grey bars, treatments). Horizontal lines and letters above bars show the results of *post hoc* tests for pair-wise comparisons of means; treatments with the same letter were not significantly different.

significantly more attractive than water. *Atta* workers invest time and energy into cutting and carrying materials offering only sodium rewards, supporting the hypothesis that a sodium-limited diet drives specific foraging for this resource. Although potassium chloride bait removal did not differ significantly from the negative control (water), the slightly higher removal of KCl bait paper could indicate a weak attraction to potassium, or a weak general response to solutes or electrolytes.

The fact that foragers both cut and carry off fragments of bait papers suggest their responses were similar to those towards living leaf tissue. We do not know how the bait paper fragments were treated after removal. Discarded paper fragments were not seen near the nest entrances, suggesting the fragments were carried into the nests. It remains unknown whether the *Atta* foragers gather the sodium for their own consumption or for the benefit of their symbiotic fungus. Fungi may also forage for salt: tropical leaf litter fungi harvest sodium, and medium for growing *Atta* 's symbiotic *Leucocoprinae* fungi must include sodium for successful fungal growth (Cromack et al., 1977; Silva-Pinhati et al., 2005). Leafcutter workers can alter their long-term foraging preferences to fit the requirements of the fungal garden (North et al., 1999; Herz et al., 2008). In the short term, leafcutter foragers sometimes harvest plant matter for their own consumption (Seal & Tschinkel, 2007; Herz et al., 2008). Moreover, the fungal garden houses a complex community of other microbes, which could also play a role in the foraging decisions of the *Atta* workers (Caldera et al., 2009). Observations on captive *Atta* colonies may indicate how salt-treated baits are handled inside the nest (Dussutour et al., 2009). It may also be possible to track sodium flow in *Atta* colonies and their symbionts using sodium radioisotopes (Fassbender *et al*., 2010).

Sodium is a critical but often limiting nutrient to animals in tropical rain forests because it is rapidly leached away by rain water. Ant communities generally respond relatively weakly to sodium baits in coastal areas with high aerosol salt deposition (Kaspari et al., 2009; Dudley et al., 2012). Sodium was a highly attractive resource to *Atta* at our site approximately 50 km inland on a narrow oceanic isthmus. Our data

suggest the extreme herbivorous leafcutter ant diet promotes sodium-specific foraging even in areas of relatively high sodium availability.

APPENDIX B

LIST OF PUBLICATIONS

I. Chavarria Pizarro, L.*, H. McCreery*, **S.P. Lawson***, M. Winston*, and S. O'Donnell. (2012) Sodium-specific foraging by leafcutter ant workers (*Atta cephalotes*, Hymenoptera: Formicidae). *Ecological Entomology*, 37: 435-438. *authors contributed equally

II. **Lawson, S.P.,** N. Christian, and P. Abbot. (2014) Comparative analysis of the biodiversity of fungal endophytes in insect-induced galls and surrounding foliar tissue. *Fungal Diversity*, 66(1): 89-97.

III. **Lawson, S.P.,** C. Graham, A. Legan, and P. Abbot. (*Accepted*) Comparative phenotyping across a social transition in aphids. *Animal Behaviour.*

IV. **Lawson, S.P.,** L. Sigle, A. Legan, and P. Abbot. (*In preparation*) The basis of an effective defense in social aphids.

V. **Lawson, S.P.,** E. Neil, C. Mitchell, and P. Abbot. (*In preparation*) The role of conflict, competition and kinship in the evolution of social behavior in gall-forming aphids.

VI. Miller, D.G., **S.P. Lawson**, H. Estby, D. Rinker, and P. Abbot. (*In preparation*) Phylogeny and ecology of *Tamalia* aphids (Hemiptera: Aphididae).

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