

ABSTRACT

Title of Document: Community Ecology and *Sirex noctilio*:
Interactions with Microbial Symbionts and
Native Insects

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Sirex noctilio is an invasive woodwasp with a global distribution that feeds on the sapwood of pine trees. Wood-feeding in the basal Hymenoptera (sawflies) arose out of sequential adaptations to feeding on nutrient poor and digestively refractive internal plant organs (xylem). Symbiotic association with White-rot fungi are thought to aid overcoming nutritional and digestive barriers, including exceedingly low nitrogen (N) and refractory lignocellulosic polymers. In this dissertation I evaluate wood-feeding relative to nutrition, symbiosis and biotic resistance to invasion of exotic North American habitats in *Sirex noctilio* [Hymenoptera: Siricidae]. I evaluated nutrient relations within fungal mutualism using: 1) functional morphological analysis of insect feeding, 2) sterol molecules to determine diet sources and 3) metagenomic and isotopic analyses for discovery of novel microbial associates and their associated nutrient pathways. Nutritional constraints of wood feeding are potentially compounded by the presence of diverse fungal and insect

communities as they divide the tree resource. I examined the role biotic resistance to *Sirex* and its fungal mutualist, *Amylostereum*, in North America using field and laboratory experiments. Morphological evidence supported a role for *Amylostereum* in external digestion of wood. Observational evidence confirmed *Sirex* larvae did not ingest wood biomass but preferentially extracted liquid substances via specialized structures of mandibles. Sterol analysis indicated plant compounds as the primary constituent of the diet, while metagenomic analysis of bacteria and their metabolic pathways showed a bacterial microbiome adapted to short chain plant polymers, starch and sugar metabolism. Stable isotopes suggested an additional symbiotic association with nitrogen fixing bacteria enriched the nitrogen deficient food substrate. These studies point toward herbivory with microbial supplementation of nutrients as a tri-partite relationship, pending conclusive identification of the bacterial symbiont for *Sirex*. Specific constraints of wood feeding by the *Sirex* symbiotic complex were antagonized by intraguild predation and fungal competition in North America. Competition interfered with *Amylostereum*, while intraguild predation accounted for an additional 15% mortality of larval populations. This research describes the evolutionary role of microbial symbionts in wood-feeding in the Hymenoptera and the internal and external constraints to foraging this ubiquitous, yet nutrient poor food resource.

COMMUNITY ECOLOGY AND *SIREX NOCTILIO*: INTERACTIONS WITH
MICROBIAL SYMBIONTS AND NATIVE INSECTS

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Dedication

To Abby, for a thousand smiles

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Chapter 1: Introduction and Project Summary

The European woodwasp, *Sirex noctilio* Fabricus (Hymenoptera: Siricidae) [hereafter; *Sirex*], is a polyphagous species that is an important pest of pine trees. In its native range of Europe, North Africa and Asia, *Sirex* is a secondary pest of *P. sylvestris*, *P. nigris*, and *P. pinaster* (Talbot 1977, Spradbery and Kirk 1978), but in the Southern Hemisphere, where it was accidentally introduced, it is highly invasive, causing up to 80% mortality in *Pinus* plantations (Corley et al. 2007, Dye et al. 2008, Collett and Elms 2009). The species of pine attacked in the non-native ranges native to North America are primarily *P. radiata* and *P. taeda*. In all, at least 23 species are susceptible to *Sirex*, including *P. resinosa*, *P. ponderosae*, *P. strobus*, *P. contorta* and *P. banksiana*. The susceptibility of North American pines to *Sirex* is of great concern to land managers (Ciesla 2003), a concern heightened by the discovery of *Sirex* in North America in 2004 (Hoebeke 2005, Wilson et al. 2009).

Sirex develops in the xylem of mature pine trees as larvae. Adult female *Sirex* typically select stressed trees for oviposition, upon which they drill into the sapwood and deposit a phytotoxin, a fungal mutualist and an egg. A single female may lay up to 450 eggs in an attacked tree depending on female *Sirex* body size and host tree vigor (Madden 1981). The Siricidae, like all Hymenoptera, are haplodiploid, with unmated females producing only male progeny. Sex ratios in *Sirex* are male biased ranging from 2:1 up to 12:1 (male:female) in certain populations. *Sirex* kills trees through combined action of phytotoxin and fungal mutualist. Phytotoxin causes a rapid, but temporary (~ 2 weeks) disruption of translocation within the attacked tree (Coutts 1969a, Fong and Crowden 1973). During initial pathogenesis caused by the

phytotoxin, the fungal mutualist of *Sirex*, *Amylostereum areolatum* (hereafter *Amylostereum*), establishes in the sapwood (Cou tts 1969b). The combined effects of multiple ovipositions, phytotoxic mucous and the phytopathogenic *Amylostereum* overwhelm tree defenses and gradually kill attacked trees (Cou tts 1969a).

The aggressive attack of living trees, wood-feeding and obligate association with white-rot fungi are characteristics of the Siricidae that differentiate this family from other sawflies (Smith 1988). *Sirex* is unique from other siricids in forming high density populations causing extensive tree mortality in invasive populations. Siricids feed within the xylem of living trees, but little is known about how they extract sufficient nutrients for growth from wood. It is presumed that fungal mutualists fill the gap between tree nutrients and larval demands, but formal tests have not been undertaken. In this dissertation, I present three chapters on the nutritional implications of xylophagy for *Sirex* (Chapters 2-4). *Sirex* preferentially feeds on living pines, but causes their death in the process of colonization and feeding. Numerous North American insects colonize dead pines. I looked at insect interactions (i.e. predation, parasitism and competition) in a North American pine ecosystem relative to *Sirex* colonization of living trees for evidence of biotic resistance to invasion in the diverse insect communities of a natural pine ecosystem (Chapter 5).

Insect adaptations to xylophagy include morphological, symbiotic and behavioral changes unique to feeding on woody tissue. Plant defenses aside, wood is notoriously nutrient poor and is constructed of digestively refractory lignocellulosic bonds (Mattson 1980, Walczyńska 2007, Geib et al. 2008). I present morphological and behavioral adaptations to wood-feeding in *Sirex* that sets the stage for how

nutrients are gained by *Sirex* (Chapter 2). Observational data revealed *Sirex* specifically fed in the transition zone between *Amylostereum* infected and *Amylostereum* free tissue. Larvae preferentially extracted liquid fractions from the xylem by pressing wood chunks between specialized plates of the mandibles. In this manner, liquid fractions were ingested while bulk xylem was dropped to the floor of the feeding chamber and eventually passed to the back of the chamber without ingestion. Larvae contained enlarged salivary glands and a reduced gut. Salivary excretions putatively play an important role in digestion of fungi (Talbot 1977), but evidence generated in assays of diet components using sterol molecules suggested otherwise (Chapter 3).

Due to the low nutrient content of wood it is presumed that fungal biomass is key ingredient in nutrition for *Sirex* (Talbot 1977). I investigated this assumption using the unique chemical structures of sterols synthesized in plants and fungi as a tracer of their presence in the consumer (Chapter 3). This showed that although fungal sterols were present in low concentrations, plant sterols were the primary diet constituents and sources of *Sirex* sterol nutrition. I concluded that fungal hyphae actively grow in the wood and produced lignocellulosic enzymes, but that fungal hyphae from *Amylostereum* did not constitute a major part of the larval diet for *Sirex*. This finding has major ecological implications as *Amylostereum* is essential to *Sirex* development, but by supplementing nutrition indirectly as an ‘external rumen’, not as a source of bulk biomass. The separation of nutrition from direct consumption of *Amylostereum* rekindled the question of how *Sirex* derived sufficient nutrients from the scarce nutrients in pine wood.

Many insects feeding on marginal resources harbor nutritional and digestive symbionts (Baumann et al. 1995, Aanen 2002, Pinto-Tomás et al. 2009). Associations with symbiotic bacteria can alter the physiological constraints on their host, but are not limited to species pairs, and may occur broadly as communities of microorganisms working in concert to the benefit of a host plant or animal (Ferrari and Vavre 2011). The bacterial community associated with larval and adult *Sirex* was sequenced using metagenomic techniques to describe community membership and community metabolic potential (Chapter 4). Additionally, targeted experiments were carried out to determine how *Sirex* copes with nitrogen (N) deficiency in wood, using stable isotope and ^{15}N assimilation studies. The bacterial pathways in *Sirex* agreed with both the morphological (Chapter 2) and sterol molecule (Chapter 3) analyses in that *Sirex*, which suggested very little in the way of digestive capability on long chain plant polymers, such as cellulose and hemicellulose. Isotopic analyses for N isotope natural abundance indicated N-fixation may play a central role in N budget for developing larvae. Pine wood was composed of 0.04% N, more than 100 times less %N than larvae (5.1% N). ^{15}N assimilation from $^{15}\text{N}_2$ labeled gas in larval *Sirex* relative to controls could only be derived from N-fixation. N-fixation is energy intensive and only carried out by anaerobic bacteria. Results of these studies coupled with morphological evidence in Chapter 2 suggest larvae may compensate for N deficiency through selective foraging on high energy bonds to fuel internal N-fixation by bacterial symbionts, but further investigation using feeding trials are warranted for confirmation of this hypothesis.

Sirex occupies a niche within the forest environment where living tree resources are colonized by eggs and the fungal mutualist, *Amylostereum*. The plethora of powerful lignocellulosic enzymes associated with White-rot fungi, such as *Amylostereum*, may be valuable tools in the acquisition of resources for larval *Sirex* (Leonowicz et al. 2001). Through fungal association *Sirex* gains nutritional advantages through an extended phenotype, but *Sirex* also gains complications as interactions that affect one member of a symbiotic complex consequently affect all members (Ferrari and Vavre 2011). In Chapter 5 I examine the role of interactions with pine dwelling insects and biotic resistance to the *Sirex* symbiotic mutualism in North America. Field surveys, experimental manipulations of *Sirex* attack on healthy trees and benchtop fungal competition trials indicated North American insects and fungi interact antagonistically with *Sirex* populations and their fungal mutualist in North America. Native cerambycid beetles were strongly attracted to trees experimentally exposed to *Sirex* oviposition, and within the xylem, actively preyed upon *Sirex* larvae. I provide the first evidence for intraguild predation of siricids by cerambycid larvae and estimate that it accounted for 15% *Sirex* larval mortality. *Sirex* larvae were negatively associated with scolytine bark beetles in surveys, and benchtop experiments demonstrated interference competition between the fungal symbionts associated with *Sirex* and bark beetles. The obligate role of *Amylostereum* in larval nutrition outlined in Chapters 2-4 coupled with evidence for antagonism from ubiquitous bark beetle fungal symbionts suggest *Sirex* may be negatively impacted by the presence of forest insects and fungi. Interactions include, but are not limited to bark beetles and their symbiotic fungal symbionts. Symbiosis may be

broadly defined as long-term associations between organisms, where mutual benefits are not a prerequisite for association, but are common. The association between *Sirex* and *Amylostereum* is a symbiotic association, but may be further described as an obligate mutualism in that both partners benefit from the association. These definitions of symbiosis are held throughout, with symbiosis being used to describe associations where mutual benefit are not fully understood. The diversity imbued by native forests, their insect communities and microbial associations may be a key difference between previous introductions and the current introduction to North America. It remains to be seen whether *Sirex* invasion dynamics will reach outbreak levels as it did in previous introductions.

Research presented in this dissertation spans the conceptual fields of community ecology and organismal biology. This research suggests it took multiple associations with microorganisms to overcome the nutritional deficiencies of feeding on pine xylem, but also that it may take a community of antagonists to limit *Sirex* populations in introduced ranges. A combination of genomic and isotope analyses elucidated the mechanism of wood feeding in *Sirex* and provide the first evidence of N-fixation in sawflies and describe the mechanisms behind evolution to wood feeding that set the stage for adaptations for symbiont theft and ultimately the evolution of parasitism in the Hymenoptera. The discovery of N- fixation in *Sirex* is likely the first evidence of a widespread phenomenon within internal feeding sawflies. Further research is needed to address the ubiquity of this association in basal Hymenoptera. Evolutionary adaptations to wood-feeding are linked to community interactions with other wood-boring insects as colonization by insects and associated microorganisms

fundamentally change the food resource and ultimately the fitness of heterospecific wood colonizers. I document the diverse interactions of North American insects and the invasive woodwasp *Sirex*, but highlight the potential for these interactions to act more broadly in the global distribution of *Sirex* and likely other wood-boring insects.

Chapter 2: Behavioral and morphological adaptations reveal insights into xylophagy and obligate symbiosis in the European woodwasp, *Sirex noctilio*

2.1 Abstract

Symbiotic associations with microorganisms and the diversity of functional capabilities they provide have facilitated the proliferation of insects in a wide variety of inhospitable environments. Woodwasps in the family Siricidae are broadly associated with mutualistic basidiomycete fungi integral in host colonization and nutrition, but mechanistic details of these interactions are poorly understood. In this study, I present new evidence from observations on morphological, behavioral and symbiotic adaptations within the siricid sawfly, *Sirex noctilio*, for xylophagy in pine trees. I examined the role of symbiosis in foraging for *Sirex* larvae by analyzing larval foraging location in xylem and the specialized morphological adaptations of mandibles and internal anatomy for nutrient extraction. I found larvae concentrated feeding near the border of fungal symbiont growth in the pine xylem, but that this behavior changed abruptly around the 8th larval instar. Separation from *Amylostereum* in 8th instar larvae correlates with the cessation of feeding and the initiation of pupation. Foraging larvae did not ingest bulk xylem tissue. Instead larvae processed xylem using mandibles specially modified for pressing liquid fractions from the xylem. When occluded, xylem sheared free by the chisel-like teeth of the apical margin of the left mandible is pressed between specialized structures on the occlusal

surface of the right and left mandibles releasing soluble fractions. Fluid extracts presumably drain toward the oral cavity via a sulcus on the occlusal surface of the left mandible. Fibrous lignocellulose was expelled directly from the oral cavity without ingestion and passed along the underside of the larvae via a coordinated peristaltic undulation of the abdomen to the rear of the feeding chamber. Larval guts lacked xylem and elaborate structures typical of cellulose fermentation. The observed suite of morphological and behavioral adaptations to xylophagy and symbiosis suggest *Sirex* relies heavily on external processing of xylem by enzymes derived from the fungal mutualist. This system is unique in that in contrast to most xylophagous insects, *Sirex* ingests a primarily liquid diet. Xylophagous insects are important players in ecosystem functioning. The diverse ways in which these insects process this extremely difficult food source are useful for understanding insect ecology, but also for development of biofuel technology and conservation of forest ecosystems.

2.2 Introduction

The xylem tissue of woody plants is one of the most abundant long lived biological materials on Earth and is an important storage reservoir in the global carbon cycle (Ryan et al. 2010). Cellulose digestion is a primary constraint on insect herbivore fitness and is biologically intractable for most insect herbivores without assistance from microbial associates (Martin 1991, Douglas 2009, Watanabe and Tokuda 2010). The lignocellulose complexes in the xylem of woody tissue presents a particular problem as lignin is generally only digestible to specific microorganisms (Breznak 1982, Klepzig et al. 2009, Watanabe and Tokuda 2010). Pine xylem is

composed of recalcitrant lignin and cellulose polymers and has a notoriously low nutrient content (Mattson 1980). Insects and fungi utilize xylem with the help of evolutionary adaptations specialized for coping with the polymeric and nutritional constraints of wood feeding (Watanabe and Tokuda 2010, Chiappini and Aldini 2012). In insects, adaptations to wood-feeding include a suite of behavioral, morphological, and associational adaptations (Warnecke et al. 2007, Chiappini and Aldini 2012). That the majority of wood decomposition is carried out by microorganisms and that the majority of insects feeding in wood are saprophagous speaks to the difficult nature of this resource for insect herbivores (Rayner and Boddy 1988, Jonsell et al. 1999, Boddy 2001, Waldrop and Firestone 2004), but insect saprophagy varies across degrees of decay for specialized insect groups (Haack and Mattson 1993, Jonsell et al. 2005, Saint-Germain et al. 2007). The order by which insects colonize wood may have much to do with specializations for particular food resources, as wood changes throughout the decay process.

Xylophagous microorganisms alter the structural and nutritional character of woody debris as they utilize the wood and degrade recalcitrant lignocellulosic polymeric bonds (Rayner and Boddy 1988, Boddy 2001). Insects benefit from associations with microorganisms by consuming microbes (e.g., fungi) either preferentially (mycetophagy hypothesis) or secondarily while consuming decaying organic matter (aka 'peanut butter on a cracker' hypothesis) (Anderson and Sedell 1979). Alternatively, insect herbivores feeding on chemically refractory resources may take advantage of the exogenous enzymes produced by microbes for their own digestion either externally (e.g. external rumen hypothesis) (Swift et al. 1979) or

internally (exogenous enzyme hypothesis) (Martin 1991). In derived associations, insects coevolve with specific microbial species, each contributing some essential factor to the relationship (e.g. nutritional mutualisms). In leaf-cutter ants, termites, and ambrosia beetles, mutualistic fungi digest plant tissue provided by the insect partner and incorporate it into fungal mycelia or specialized structures (e.g. gongylidia), which are then consumed by the insect (aka ‘fungus farming’) (Mueller and Gerardo 2002). Wood digestion in insects may also occur within specialized structures (fermentation chambers) that facilitate wood digestion by endosymbiotic bacterial associates (e.g. termite hindgut, (Warnecke et al. 2007). The precise nature of associations between most insects and their microbial partners are cryptic, but may be indirectly elucidated through evaluation of adaptive morphological and behavioral traits distinctive to particular life-history traits.

Fungal decomposition of wood alters the structural integrity and energy profile of wood through conversion of easily assimilable compounds to fungal biomass (Waldrop and Firestone 2004). Decrease in polysaccharide and starch content decreases overall nutrients, but degradation of recalcitrant bonds may facilitate bulk digestion of cellulose by insect herbivores. Wood decay is a slow process involving many transitions in substrate quality (Worrall et al. 1997), saprophytic insects colonize according to states of decay (early – late) that optimize their fitness (Jonsell et al. 1999, Saint-Germain et al. 2007). Upon tree death, the xylem tissue of pines (Pinaceae: *Pinus*) is rapidly colonized by endophytic and saprotrophic fungi which in turn attract insects adapted to feeding on fungal and fungal degraded wood tissue (Jonsell et al. 1999). Some insects, such as sawflies, are

more aggressive than others and primarily colonize living trees. Sawflies vector wood-rot fungal mutualists to trees which are injected along with eggs at oviposition. Fungal mutualists have putative roles in host colonization and cellulose digestion for sawflies (Talbot 1977). Characteristics of diet and foraging preferences of insects manifest in the biology of the insects and the morphology of feeding organs (e.g. mandibles, gut, and salivary glands) (Schmidt et al. 2000, Hochuli 2001). In this study, I show that morphological, behavioral and symbiotic associations of xylophagous insects can be used by ecologists as a means of assessing resource use and xylotrophic ecology of wood boring insects.

The organs under the greatest selection relative to forage resources are the mandibles. Mandibles interact directly with resources and mandible morphology provides important clues to the manner in which insects extract nutrients (Acorn and Ball 1991, Deligne 1999, Chiappini and Aldini 2012). In wood-feeding coleoptera, ‘wedge’ and ‘chisel’ shaped mandibles correlate with feeding upon deteriorating and sound wood, respectively (Chiappini and Aldini 2012). Differing modes of feeding rely on differential modifications of tree tissue. Cellulose digestion requires physical and chemical components to break apart indigestible lignocellulosic bonds from digestible cellulose and hemicelluloses. Morphological features typical to this mode of feeding include the raised mola on the occlusal surface of mandibles in cerambycid beetles for grinding woody material and the presence of expanded invaginations of the gut for housing microbial symbionts for fermentative digestion (Stehr 1987, Delalibera et al. 2005, Grünwald et al. 2010). In contrast, wood-feeding Lyctidae do not consume cellulosic material and presumably practice extra-oral digestion, which

again is reflected in mandible and gut morphology (Parkin 1940, Crowson 1981, Chiappini and Aldini 2012). Differential feeding modes offer alternative constraints. Fungal digestion of wood material reduces the structural integrity of lignocellulose and sometimes concentrates limiting nutrients in fungal hyphae (Boswell et al. 2002), but also reduces the concentration of easily assimilable sugars (Henn et al. 2002). The various degrees of saprophagy have functional and nutritional constraints, which in turn have important implications for forest ecology.

As a group, sawflies exhibit a diversity of behavioral, anatomical and symbiotic adaptations for xylophagy (Haack and Mattson 1993). Siricid woodwasps feed endogenously on the xylem of pine trees and are associated with phytopathogenic basidiomycetes that are involved in tree colonization and nutrition (Cartwright 1938). The low nutritive value of pine suggests fungal hyphae may be an integral part of larval diet (Talbot 1977), but sterol analyses fail to support this supposition (Thompson et al. 2013). Given the multiple ways in which fungal symbionts may be integral to xylophagy, I investigated the behavioral and morphological attributes of the invasive European woodwasp, *Sirex noctilio* [hereafter: *Sirex*], for foraging on nutritionally refractory pine xylem.

Sirex is an aggressive woodwasp of Eurasian origin that vectors a phytopathogenic fungal mutualist, *Amylostereum areolatum* [hereafter: *Amylostereum*]. *Sirex* is an early colonizer of pine xylem and readily kills weakened trees but does not colonize trees previously colonized by xylotrophic insects and fungi (Spradbery and Kirk 1978, Ryan et al. 2011). *Amylostereum* assists in colonization and is a putative source of nutrition for developing larval *Sirex* (Talbot

1977). In this study, I examined the symbiotic association between *Sirex* and *Amylostereum* in the xylem environment to investigate the nature of foraging and fungal enzymes in *Sirex* biology. I examined the functional morphology of mandibles and internal anatomy and their use in extricating nutrients from xylem tissue. I found that the unique constraints of xylophagy in this woodwasp are coupled with adaptations that include external digestion of tree tissue by the fungal mutualist. Unique morphological adaptations of the feeding structures of larvae for extraction of soluble nutrients from externally digested material form the basis for this symbiosis, as internal anatomy lacks the specialized morphology for digestion of cellulose material. The traits described here may apply broadly to woodwasps and has implications for the use of exogenous enzymes ('external rumen' hypothesis), the evolution of fungal mutualism in sawflies, and community assembly in dead and dying trees.

2.3 Methods

2.3.1 Sample Collection

Natural infestations of *Sirex* and *Amylostereum* were collected from red pines (*Pinus resinosa*) from May – August in 2010 and 2011. Trees showing signs *Sirex* oviposition on their trunk (n=30) were felled and trunks were sectioned to ~60 cm lengths (bolts). Representative subsamples (5-6 bolts/tree) were taken from each tree for extraction of larvae. Sampled pines ranged in diameter from 13-24 cm at breast height (DBH, ~ 1.3 m). Larvae were extracted from pine xylem by tracking larval galleries and sequentially splitting bolts (< 1 cm width). Bolts were randomly selected

from all possible bolts. All samples were collected in Tioga County, Pennsylvania, USA, [41°44'54" N; 77° 18'04" W].

2.3.2 Foraging Spatial Dispersion

I examined the location/pattern of larval foraging chambers relative to the fungal mutualist, *Amylostereum*, and its lignocellulosic enzymes using the methods described in (Thompson et al. 2013) for staining Laccase, a characteristic enzyme of white-rot fungi. Briefly, 1.5mL of the enzyme stain ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (1.175 mg/mL dH₂O) was drenched over the cross-section of xylem containing the foraging cavity of each larva. ABTS is colorless until exposed to oxidase enzymes, such as the multi-copper/phenol oxidase family of enzymes associated with white-rot fungi (e.g. Laccase) (Niku-Paavola et al. 1990). Laccase oxidized the ABTS solution turning it blue denoting the presence of *Amylostereum* in pine xylem. Laccase is primarily associated with basidiomycete white-rot fungi (Baldrian 2004), but not all fungi have been tested for enzyme activities. The efficacy of the ABTS at differentiating *Amylostereum* from heterospecific fungi was evaluated using *Ophiostoma ips*. *Ophiostoma ips* is a fungal symbiont of *Ips pini* and is the most common fungus encountered in pine trees colonized by *Sirex*. Laccase closely associates with fungal hyphae (Leonowicz et al. 2001). As such, foraging patterns of larval *Sirex* relative to fungal mutualist *Amylostereum* were evaluated by measuring the nearest distance (mm) from the anterior/head end of the feeding cavity of larvae to the border between *Amylostereum* and *Amylostereum*-free xylem tissue (n = 113) in naturally infested pines, as measured by the laccase enzyme assay. The position of larvae relative to

Amylostereum was taken as a proxy for foraging tendencies relative to new and old growth of *Amylostereum* in natural infestations.

Larval Siricidae bore characteristic linear feeding galleries in xylem, most common being a horse-shoe shaped gallery leading toward the center of the tree then back out to the periphery (Rafes 1960, Madden 1981). Larval ontogeny may effect foraging behavior (Hochuli 2001). I evaluated size/instar effects on foraging relative to *Amylostereum* by measuring the diameter of the larval feeding cavity prior to staining with ABTS. Madden 1981 showed larvae go through 8-12 larval instars depending on conditions within the tree and that larval tunnel diameter reasonably approximates larval instar for trees of a similar condition. Larval spatial relationship to *Amylostereum* during foraging was plotted to visualize the relationship between fungal mutualist and larvae in trees and was statistically analyzed by constructing linear models for size and spatial data. Approximate larval instar was plotted on top of larval foraging data for evaluation of ontogenetic effects on foraging behavior.

2.3.3 Foraging *Sirex* & Internal Anatomy

Observations of larval feeding mechanics were made opportunistically on larval galleries that were only partially revealed and where larvae appeared undisturbed by splitting of the bolt (n = 5). Splitting of bolts is highly invasive, but when possible, larvae were observed foraging *in situ* for a period of ~15 min. Special attention was paid to excavation mechanics and the fate of chewed wood within the oral cavity. After observation, larvae were fully excavated and placed in 70% ethanol for dissection. Samples of frass packed in galleries were inspected for indications of manipulations in the mandibles or gut. The term frass is commonly to refer to insect

feces, but has multiple meanings in the literature (Weiss 2006). In the general context of *Sirex*, frass refers to the wood particles that are tightly packed into the trailing tunnel (gallery) left by foraging larva. Larval guts were dissected to examine ingested material in larvae and were supplemented by additional larvae extracted at the same time as larvae observed foraging. In total twenty larvae were dissected and gut contents evaluated. Internal organs were removed using fine tipped forceps and documented photographically using a Leica MZ APO stereo microscope (Leica Microsystems, Buffalo Grove, IL, USA). Microscopic anatomical features were slide mounted and analyzed using an Olympus BX51 compound microscope (Olympus America Inc. Center Valley, PA, USA). Images of internal anatomical features were photo documented and analyzed using Nikon Digital Sight DS Fi1 digital camera and Nikon NIS-Elements BR imaging software (Nikon, Melville, NY, USA). Images were illustrated using Adobe Illustrator CS6 (Adobe System Inc, San Jose). Specific attention was paid to functional morphology of feeding structures (e.g. mandible, foregut, midgut, hindgut and salivary glands). Internal and external morphological adaptations were photographically documented and described relative to putative functional morphology.

2.3.4 Statistical Analysis

Linear models were fit to the foraging data describing larval size/instar relative to spatial relationship with *Amylostereum* in the xylem. Larval foraging distance to the border of *Amylostereum* and the size of the larvae at the time of collection were response and predictor variables, respectively. Linear, exponential, and piecewise regression models were fit to the foraging data and evaluated for

explanatory power using AIC criteria in the package ‘AICc modavg’ (Mazerolle 2012). Plots of the data revealed a possible threshold effect in late instar larvae. A piecewise regression was performed for evaluation of threshold presence and location using maximum likelihood for locating possible breakpoints in larval behavior, as described in the package ‘Segmented’ (Muggeo 2008). Linear models for the separate segments in the threshold model were fit using the `lm` command in the base package and supplied as a single model to for model selection (R Development Core Team 2009).

2.4 Results

2.4.1 Foraging Spatial Dispersion

ABTS stains of laccase were specific to *Amylostereum* and did not stain the conspecific *Ophiostoma ips* in wood (Figure 1). Eighty-five percent of all larvae examined, were found within 10mm from the edge of *Amylostereum* and *Amylostereum*-free xylem in trees. Laccase assay revealed larvae prior to the 8th instar were located 2.4 ± 1.0 mm (mean \pm SE) inside the fungal hyphae/enzymes in xylem of red pines. However, larvae exhibited a non-random pattern within the xylem with respect to *Amylostereum* (Figure 2). Model selection results for linear models describing the relationship between *Sirex* and *Amylostereum* indicated a threshold effect in larval foraging behavior (Table 1). The observed pattern in larval foraging was punctuated by a distinct shift in foraging pattern at larger larval tunnel diameters (i.e. later instar larvae, size/instar relationship as described in (Madden 1981). The maximum likelihood estimate of the threshold breakpoint suggests a change in larval behavior at a larval tunnel diameter 4.7 ± 0.2 mm (mean \pm SE)(~ 8-9th larval instar).

At which point, larvae were found at increasingly greater distances from laccase stained *Amylostereum*. The threshold model was only marginally better than the exponential model overall with both models predicting separation of larvae and *Amylostereum* at approximately the same threshold. The threshold model outperformed the exponential model predominantly in early instar larvae. Decreased enzyme production was not documented in older fungal mycelia of laboratory cultures on sterilized red pine xylem.

2.4.2 Foraging *Sirex* & Internal Anatomy

Sirex larvae possess asymmetric mandibles aligned approximately opposed to each other, but having anterior margins aligned perpendicular to each along the vertical plane (Figure 3a). The right mandible possesses large teeth along the anterior margin that angle toward the back of the oral cavity and approximately fit into a sulcus on the occlusal surface of the left mandible. The left mandible forms a cavity between the anterior toothed margin and a ventral lobe that forms a flat shelf that extends toward and over the right mandible when occluded. The ventral surface of the overlapping left mandible forms a complimentary structure to a raised ridge on the dorsal plane of the right mandible, such that when mandibles are occluded xylem is presumably pressed releasing fluids which drain via the sulcus to the oral cavity (Figure 3b). The toothed chisel-like apex along the frontal margin of the left mandible excavates xylem in thin slices (Figure 3d (inset)). Chewed xylem (frass) sampled from the trailing gallery appears relatively un-manipulated.

During feeding it was observed that masticated xylem tissue was dropped from the mandibles without ingestion and passed to the rear of the growing tunnel via

peristaltic undulation of the abdomen (Figure 3c). At the posterior of the larvae, processed xylem mixed with faeces (combined = frass), and was tightly packed into the trailing gallery (Figure 3d). Dissections of larval guts did not reveal the presence of bulk xylem in the internal gut compartments, except an occasional fragment (< .01mm) found in the area of the proventriculus, but never exceeding 5 particles/larvae and frequently with none present at all.

Observations of the internal anatomy of *Sirex* indicate a limited capacity for ingestion of solid cellulosic material. The alimentary canal of *Sirex* is thin and threadlike from the oral cavity to the anus, and lacks distinct fermentation chambers or other gut elaborations typical of xylophagous insects (Figure 4a). Malpighian tubules branch from the midgut-hindgut border and extend the length of the body in both directions. The fat body of larval *Sirex* filled the majority of the body cavity and enveloped reticulate salivary glands (Figure 4b). Interspersed among the cells of the fat body are putative bacteriocytes (Figure 4b (inset)). The ducts within the salivary gland open into larger ducts that culminate at the salivary orifice (aka, sericos) located between the labial palps (Figure 5).

2.5 Discussion

Xylophagy is constrained by recalcitrant lignocellulose, nutrient deficiency, and chemical defenses of xylem tissues in host plants. Wood-boring insects cope with these barriers to xylophagy through a combination of morphological, behavioral and symbiotic adaptations (Klepzig et al. 2009, Chiappini and Aldini 2012). This study finds that the European woodwasp, *Sirex noctilio*, has a host of adaptations for xylophagy that are highly influenced by interactions with its white-rot fungal

mutualist, *Amylostereum areolatum*. In this study, we found support from morphological and foraging patterns for the hypothesis that *Amylostereum* acts as an external rumen for foraging sawfly larvae.

In *Sirex*, the hypothesis for external digestion is supported by: 1) foraging in areas with fungal enzymes; 2) limited gut lumen volume and lack of specialized fermentation chambers; 3) specialization of mandibles for squeezing fluids from the xylem; 4) little to no wood tissue in the gut; and 5) limited manipulation of frass in feeding galleries. This evidence combined suggests *Sirex* ingests only liberated organic compounds and monomers of fungal depolymerization reactions in the xylem (e.g. glucose, mannose, galactose, acetic acid, xylose, etc.; see review by (Kirk and Cullen 1998). These findings suggest outsourcing of digestive capability onto the fungal mutualist is supported by multiple evolutionary adaptations within the mandibles and gut for processing externally digested xylem and has implications for the evolution and ecology of xylophagy in this basal group of the Hymenoptera. The Siricidae are variously associated with *Amylostereum* as internal feeders on xylem tissue (Cartwright 1938). The association between *Sirex* and *Amylostereum* is possibly exemplary of the Siricidae in general and has implications for the origins and evolution of symbiosis in this important xylophagous family.

Association with microorganisms may indirectly benefit xylophagous insects through degradation of plant defensive and lignocellulose compounds or may directly benefit insect consumers through concentration or manufacture of growth limiting nutrients (Dowd 1992, Klepzig et al. 2009). *Sirex* larvae fed in close proximity to *Amylostereum* in pine xylem, although a characteristic pattern for separation from

Amylostereum was observed in larger larvae corresponding to later instars (> 8th instar). *Amylostereum* excretes lignocellulosic enzymes into the xylem as it externally digests xylem tissue for its own consumption (Kirk and Cullen 1998, Leonowicz et al. 1999). Larval *Sirex* fed near the edge of fungal growth in wood, where new fungal growth and enzyme production is predicted to be greatest (Blanchette 1991). Organic nutrients may also be concentrated this area as fungi translocate limiting nutrients to areas of active growth (Levi and Cowling 1969, Boswell et al. 2002). Paradoxically, sound wood is predicted to have higher proportions of easily digestible polysaccharides since fungi exhibit preferential digestion and assimilation of across a compound digestibility gradient with easiest assimilable compounds (e.g. starch and sugar) preceding more recalcitrant polymers (e.g. cellulose and hemicellulose) (Kirk and Cullen 1998). Evidence from sterol metabolism in *Sirex* shows primarily plant derived sterols in *Sirex* larvae and adults (Thompson et al. 2013), similar to profiles of ectophagous sawflies (Feldlaufer and Schiff 1996) and are suggestive of a herbivorous rather than mycetophagous diet. The observed preference for larval foraging in the zone bordering fungal enzyme and enzyme-free tissue may be adaptive for foraging on plant polysaccharides with the assistance of fungal enzymes, though the role of fungal input is currently unresolved. It is highly suggestive that fungal input is involved in feeding given the observed separation from *Amylostereum* in later larval instars of this study. Separation is suggestive of a cessation of feeding during the ontogenic shift toward pupation and the reproductive stage of the life-cycle. This shift away from the fungal symbiont may have practical implications as parasitic nematodes used in biological control in invasive populations use the fungal

symbiont as a food source (Bedding 1972), though specific research is need to address this component.

The gut of larval *Sirex* lacked secondary specializations, such as fermentation chambers, like those found in other xylo-/saprophagous insects (Warnecke et al. 2007). Limited volume of the gut of *Sirex* is suggestive of short retention time of ingested material and makes fermentative process in *Sirex* unlikely (Penry and Jumars 1987). Morphological evidence and feeding behavior are suggestive of a diet that consists primarily of starch and polysaccharides similar to that proposed for xylophagous Lyctidae and Bostrichidae (Chiappini and Aldini 2012). However, association with the mutualist *Amylostereum* by *Sirex* may provide a key difference in digestive capabilities between simple starch digestion and more complex digestion of hemicellulose, cellulose and lignin.

Modifications of mandibles to accommodate specific food resources are common in insects (Acorn and Ball 1991, Chiappini and Aldini 2012). The mandibles of *Sirex* have specialized features designed for pressing xylem tissue presumably to extract fluid fractions. Little to no lignocellulose was observed in the guts of larvae. After pressing between mandibles xylem tissue was discarded into the feeding gallery beneath the head capsule. Presumably the liquid and soluble organic fractions or the xylem are ingested by the larva. Frass was then pushed along the underside of the larvae to the back of the feeding chamber. Observations of frass showed largely intact pieces of xylem, some still bearing the tooth marks of the left mandible. Internal processing of xylem leaves characteristic signs on frass, of which the frass of *Sirex* did not have (Solomon 1977). The extraction of easily assimilable products from

exogenous fungal digestion is potentially adaptive in *Sirex*. Lignocellulose digestion is a slow process, but my feeding only high energy monosaccharide components *Sirex* may greatly enhance the ratio of effort invested to energy returned and may represent a novel strategy within the sawflies for xylophagy. This information does however suggest that *Sirex*, feeds within trees with minimal fungal decay, in which easily assimilable compounds are abundant, a strategy that fits descriptions of *Sirex* life-history/colonization traits (Spradbery and Kirk 1978).

In addition to the obvious traits of the mandibles and the intestinal tract, *Sirex* larvae possess large salivary glands. Excretions from these glands purportedly rapidly destroy fungal hyphae and it was previously believed that fungal hyphae were the primary source of nutrition for larvae (Talbot 1977), but recent analysis using sterol molecules detected only low biomass of *Amylostereum* in infested wood (Thompson et al. 2013). Conclusive evidence for fungal constituents in *Sirex* nutrition remains unknown. It is evident from this study that salivary glands are active in the feeding galleries, where saliva is spread on the surrounding walls of the tunnel giving a ‘water soaked’ appearance to the larval feeding chamber. Presumably the extracts ingested from material squeezed between the mandibles has been partially digested by *Amylostereum*, though excretions from salivary glands cannot be ruled out and have been shown to be active on fungal hyphae (Morgan 1968).

The digestive association between *Sirex* and *Amylostereum* has three important implications for the biology of *Sirex*. First, the characteristic region in the wood at which *Sirex* feeds is targeted to the zone where areas of active fungal growth meet fungal free zones in the xylem. As infestations within trees progress this zone

decreases due to the finite space within a given tree. If the pattern predicted in this study represents tracking of an optimal food resource for developing larvae, it holds that larval growth and development may be predicted by tree size and infestation intensity (e.g. (Madden 1981, Hurley et al. 2008). Second, external digestion places *Sirex* in a unique position whereby intra- and interspecific competitive interactions with *Amylostereum* may directly impact *Sirex* fitness through effects on nutrient assimilation. The level of infestation and co-colonizing insects and saprophytic fungi may have significant impact on resources quantity and quality for foraging larvae. Lastly, *Sirex* is obligately associated with *Amylostereum* and cannot exist where *Amylostereum* does not grow (Talbot 1977). From a practical point of view, enhancing resistance to *Amylostereum* will significantly impact the fitness of *Sirex* in invasive areas. Likewise, encouraging antagonisms to *Amylostereum*, such as fungal competitors, may produce a similar beneficial effect.

Differences in feeding modes among xylophagous insects reflect the various adaptations of species to food resources. Early colonizing insects have the opportunity to take advantage of easily assimilable plant compounds before they are metabolized by saprophytic fungi, but are only able to do so if they are able to overcome plant defenses. Late colonizing insects are not limited by chemical defenses, but must cope with the resilient compounds that linger through fungal decomposition, but may also benefit from consumption of fungal hyphae. The colonization patterns of xylophagous insects reflect these tendencies (Aukema et al. 2004, Jonsell et al. 2005). Neither early colonizing nor late colonizing insects are capable of metabolizing xylem without microbial associates. In the case of *Sirex* and possibly other Siricidae,

association with wood-rot fungi has led to evolutionary adaptations that inextricably link insect and fungus through mutual benefits. In this study we document a suite of adaptations in *Sirex* that suggest early colonization and fungal association are linked through external digestion of plant compounds in this insect-fungal mutualism which are suggestive of greater associations and behavioral tendencies of the Siricidae in general.

2.6 Figures & Tables

2.6.1 Tables

Table 1. AICc model selection of linear models predictive of larval foraging patterns in red pine relative to larval size and distance outside or within the fungal mutualist *Amylostereum*.

AICc model selection						
Model	K	AICc	Δ AICc	AICc Wt.	Cumulative Wt.	LL
Piecewise	4	757.31	0.00	0.85	0.85	-374.47
Exponential	4	760.77	3.46	0.15	1.00	-376.20
Linear	3	789.96	32.66	0.00	1.00	-391.87
Null	2	807.99	50.68	0.00	1.00	-401.94

2.6.2 Figures

Figure 1. Fungal cultures of *Ophiostoma ips* (a = before; b = after) and *Amylostereum* (c = before; d = after) at one week growth before and after addition of phenol oxidase stain, ABTS (2,2' - azino - bis (3 - ethylbenzothiazoline 6 - sulphonic acid) (conc. 1.175mg / mL); extent of hyphal growth on surface of wood (dotted line).

Figure 2. The position of larval feeding cavities in pine xylem relative to the fungal mutualist, *Amylostereum*, and tunnel diameter (mm). Xylem containing *Amylostereum* (grey) and without *Amylostereum* (white); larval feeding cavity (○); line of best fit from piecewise regression model (solid black line); maximum likelihood estimate for behavioral 'threshold' (vertical ---); larval instar (solid bar x-axis, size / instar described in Madden, 1981).

Figure 3. Larval *Sirex* feeding mechanics: a) asymmetric right (RM) and left (LM) mandibles; b) dorsal view of mandible occlusion; c) a peristaltic undulation of the abdomen moves pulverized xylem from beneath the head capsule to the posterior of the tunnel (1-3); d) chewed xylem accumulated in the feeding tunnel as tightly packed frass, which has sawdust like appearance approximately the width of the chiseled apical margin of the left mandible (inset).

Figure 4. The dissected alimentary canal of larval *Sirex*, with fat body removed (a); Fat body surrounding salivary glands with bacteriocytes dispersed throughout (b; inset); foregut (Fgt), midgut (Mdg), hindgut (Hdg), malpighian tubules (Mpt), fat body (FB), bacteriocyte (Oe)

Figure 5. *Sirex noctilio* head; (Ant) antennae, eye spot (Eye), clypeus (C), labrum (Lm), ventral view of left mandible (Md), Maxillary palp (Mx/Plp), Sericos/ Salivary orifice (Slo), Labial palp (Lbplp), Labium (Lb), Foreleg (Fl), Midleg (Ml); left mandible (inset).

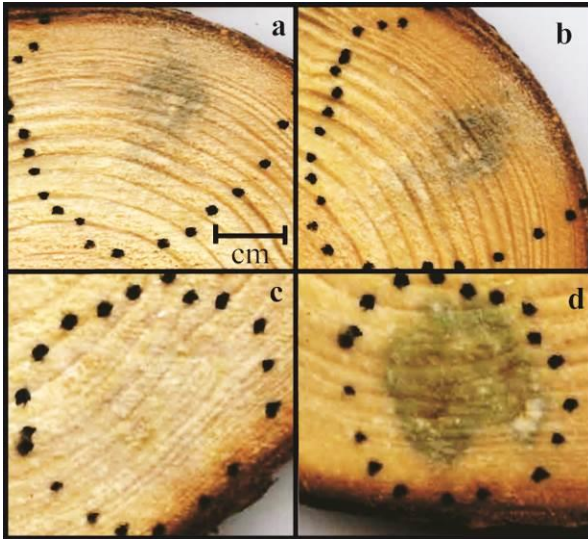


Figure 1

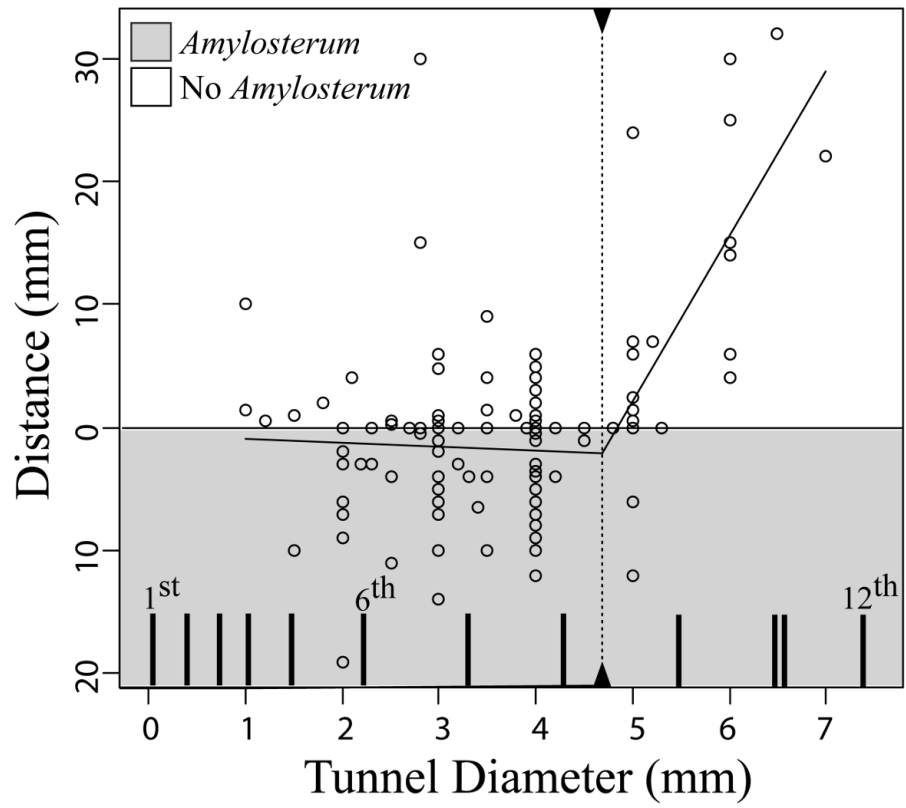


Figure 2

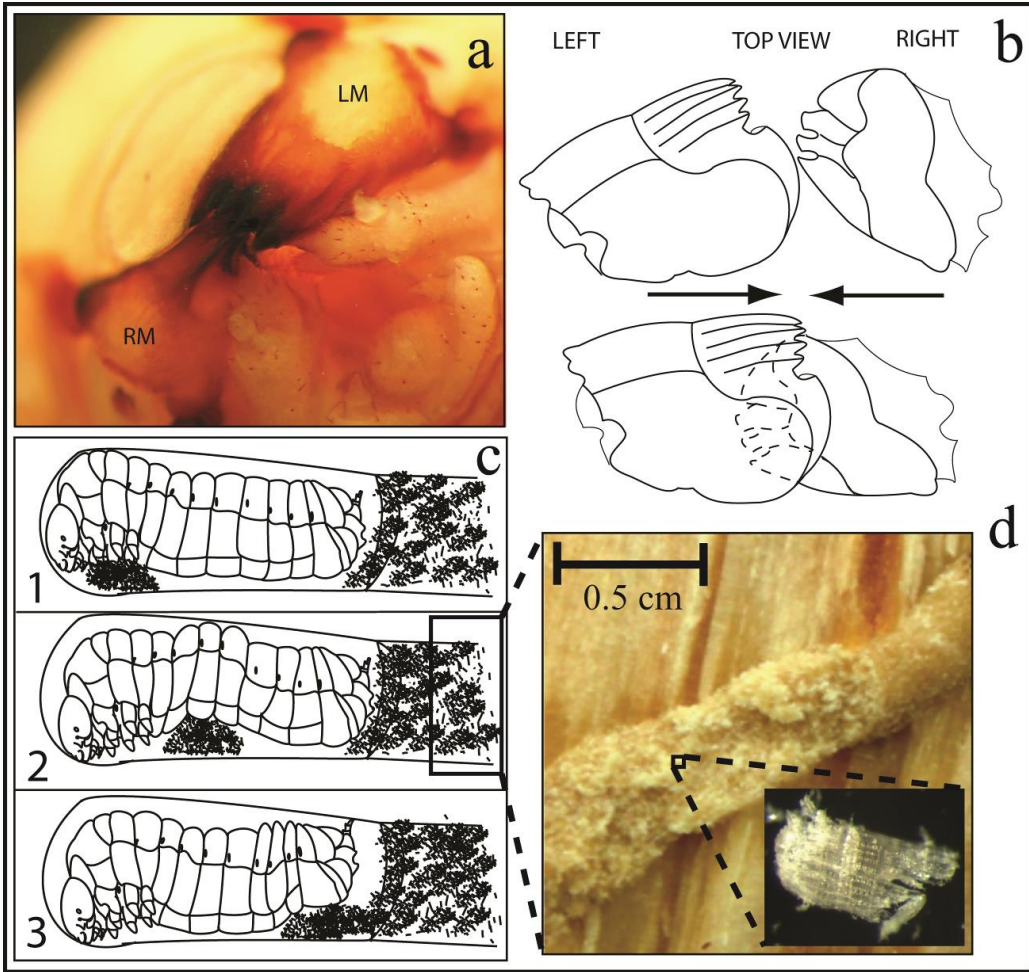


Figure 3

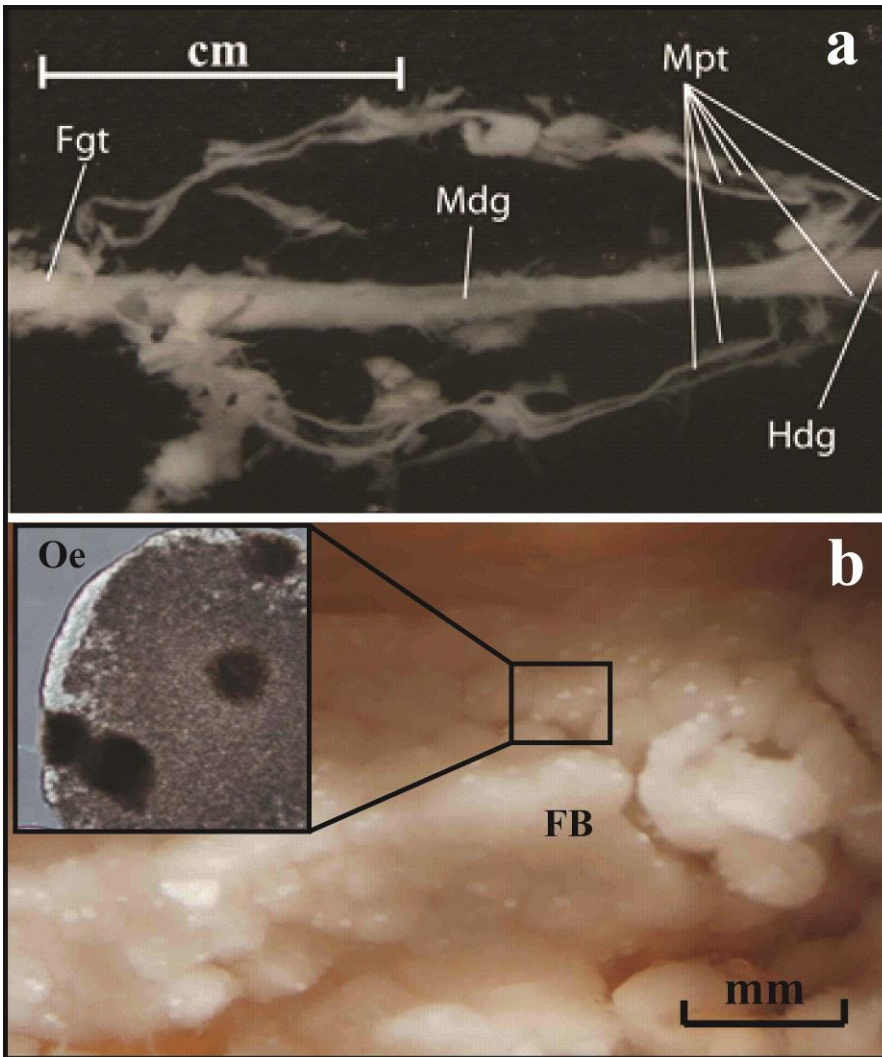


Figure 4

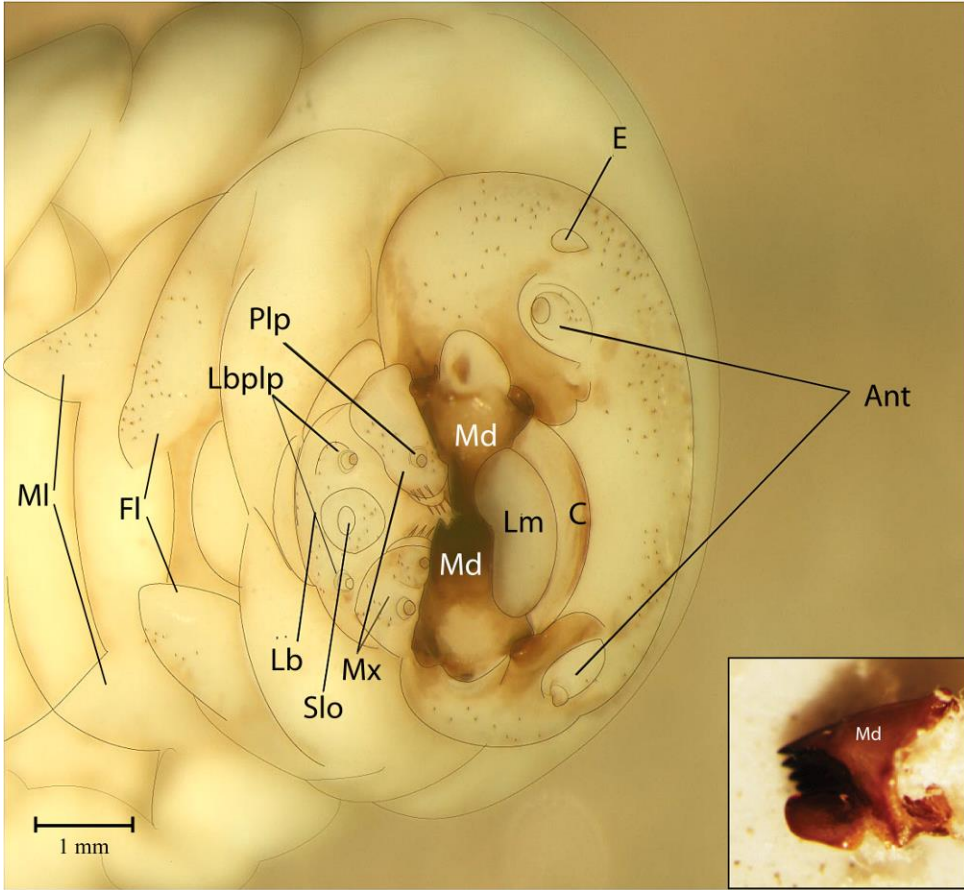


Figure 5

Chapter 3: Microbial symbionts shape the sterol profile of the xylem profile of the xylem-feeding woodwasp, *Sirex noctilio*

3.1 Abstract

The symbiotic fungus *Amylostereum areolatum* is essential for growth and development of larvae of the invasive woodwasp, *Sirex noctilio*. In the nutrient poor xylem of pine trees, upon which *Sirex* feeds, it is unknown whether *Amylostereum* facilitates survival directly through consumption (mycetophagy) and/or indirectly through digestion of recalcitrant plant polymers (external rumen hypothesis). We tested these alternative hypotheses for *Amylostereum* involvement in *Sirex* foraging using the innate dependency of all insects on dietary sources of sterol and the unique sterols indicative of fungi and plants. We tested alternative hypotheses using GC-MS to quantify concentrations of free and bound sterol pools from multiple life-stages of *Sirex*, and its food sources, and waste products in red pine (*Pinus resinosa*). Cholesterol was the primary sterol found in all life-stages of *Sirex*. However, cholesterol was not found in significant quantities in either plant or fungal resources. Ergosterol was the most prevalent sterol in *Amylostereum* but was not detectable in either wood or insect tissue ($<0.001\mu\text{g/g}$). Phytosterols were ubiquitous in both pine xylem and *Sirex*. Therefore, dealkylation of phytosterols (sitosterol and campesterol) is the most likely pathway to meet dietary demand for cholesterol in *Sirex*. Ergosterol concentrations from fungal-infested wood demonstrated low fungal biomass, which suggests mycetophagy is not the primary source of sterol or bulk nutrition for *Sirex*. These findings suggest there is a potentially greater importance for fungal enzymes,

including the external digestion of recalcitrant plant polymers (e.g. lignin and cellulose), shaping this insect-fungal symbiosis.

3.2 Introduction

Associations between insects and fungi are ubiquitous in nature and range from non-specific polyphagy to obligate mutualism (Jonsell and Nordlander 2004). Foraging on fungi comes with substantial metabolic considerations, as fungi are prolific producers of enzymatic and toxic metabolites (Martin 1979) and, depending on the species, may be extremely ephemeral within terrestrial environments (Wheeler and Blackwell 1984). Termites, leaf-cutter ants, and ambrosia beetles evolved mutualisms with fungi that diminish the ephemeral nature of fungal resources and selected for more edible features within a stable symbiotic association, better known as ‘fungal agriculture’ (Mueller and Gerardo 2002). In these symbiotic associations fungi serve as direct sources of nutrition and/or as exogenous sources of digestive or otherwise beneficial enzymes (Currie et al. 2003, Suen et al. 2011).

Microbial metabolic pathways have been independently coopted by insects numerous times throughout evolutionary history (McCutcheon et al. 2009). Nutritional mutualisms between insects and bacteria are especially widespread on low quality resources, supplying essential amino acids (Moran et al. 2005), altering resource phenology (Kaiser et al. 2010), and protecting resources from spoilage (Kaltenpoth et al. 2005). Equally well documented, symbioses between insects and fungi are environmentally ubiquitous and taxonomically diverse ranging from leaf galling flies (Kobune et al. 2011), ambrosia beetles (Batra 1966), termites (Aanen

2002), and sawflies (Cartwright 1938) to multiple lineages of ants (Schultz and Brady 2008). Opportunities for associations between insects and microorganisms are ubiquitous, but the diversity of mechanisms for maintenance of many of these associations remains poorly understood. In this study, I explored hypotheses for the nutritional basis for the symbiosis between the European woodwasp, *Sirex noctilio* [Hymenoptera: Siricidae; hereafter *Sirex*], feeding on the nutrient poor xylem tissue of red pines [*Pinus resinosa*; hereafter *Pinus*] and its obligate fungal symbiont, *Amylostereum areolatum*, [phylum Basidiomycota; hereafter *Amylostereum*] (Coutts 1969b, Talbot 1977).

The symbiotic complex of *Sirex* and *Amylostereum* aggressively kills living trees through mass attack and shared biological weapons (Coutts 1969b). For its part, *Sirex* conditions host trees for fungal symbiont establishment by introducing a potent phytotoxin during oviposition that rapidly, but only temporarily, stops translocation (Madden 1977). In the tree's weakened condition packets of *Amylostereum* introduced alongside eggs kill the host tree through blockage and cavitation of xylem channels, while simultaneously metabolizing lignocellulosic compounds in the xylem tissue (Coutts 1969b). *Amylostereum*, like most white-rot fungi, produces a multitude of extracellular lignocellulosic enzymes that digest the wood surrounding the fungal hyphae (Leonowicz et al. 2001). In addition to demonstrable phytopathogenic functions, *Amylostereum* has been implicated as an essential source of dietary nutrition (Madden 1981) and as an indirect source of wood-degrading enzymes for siricid larvae, as was suggested for the close relative of *Sirex*, *Sirex cyaneus* (Kukor and Martin 1983). However, the relative importance of these dietary pathways – i.e.,

plant vs. fungal nutrition – has not been quantified for the *Sirex-Amylostereum* symbiosis or Siricidae in general.

Sirex larvae are presumed to feed on fungal mycelia because pine xylem is highly indigestible and has exceedingly low nutritive value on its own (Mattson 1980). Pine xylem is composed predominantly of recalcitrant cellulose and lignin polymers (Nairn et al. 2008) and is indigestible without the aid of microbial symbionts (Farrell et al. 2001, Scully et al. 2012). Pine xylem is also largely deficient in nutrients, including amino acids and sterols (Geib et al. 2008), which are essential for insect herbivore growth and development. *Sirex* without *Amylostereum* invariably dies (Madden 1981). It is therefore postulated that the fungus serves as a direct source of nutrients for larvae via grazing fungal mycelia (mycetophagy) in wood (Francke-Grosman 1939). *Sirex* contains well developed salivary glands (Maxwell 1955) that secrete substances that rapidly destroy the hyphae of *Amylostereum* in wood. Activity of secretions on fungal hyphae has been used as evidence for fungal feeding (mycetophagy) (Morgan 1968). Alternatively, larvae may obtain nutrients indirectly from plant material via extracellular digestive enzymes produced by *Amylostereum* during wood digestion (“the external rumen hypothesis,” Swift et al. 1979; see also Martin 1987). Foraging characteristics of *Sirex* and *Amylostereum* in wood suggest a central role for *Amylostereum* in larval nutrition, but the relative input from both plant and fungal sources remains untested. Here, I used the distinct biochemical signatures of plant- and fungal-derived sterol compounds to assess the importance of these pathways for *Sirex* nutrition.

Sterol molecules and their metabolic intermediates are highly informative in determining the sources of dietary sources in insect symbioses (Bentz and Six 2006, Kobune et al. 2011). Despite their integral role in cell membrane fluidity, permeability, and signaling, insects are incapable of producing sterol molecules *de novo* and must acquire sterols from external sources (Clayton 1964, Behmer and Nes 2003). Cholesterol is the sterol recovered from most insects (Behmer & Nes 2003) and predatory and parasitic insects may directly obtain cholesterol from food items, but phytophagous and mycetophagous insects must either synthesize cholesterol by modifying the chemical structure of ingested sterols or, less commonly, use alternate sterols to maintain homeostasis (Behmer & Nes 2003).

Plants, animals, and fungi synthesize and/or metabolize distinctly different sterol molecules for homeostasis (Figure 1; Behmer & Nes 2003). Plants contain little cholesterol (Hartmann 1998), instead they contain predominantly sitosterol and campesterol (Hartmann 1998). To utilize sterols from plants, phytophagous insects metabolize phytosterols (typically sitosterol and campesterol) to cholesterol via dealkylation of the side chain through multiple enzymatic steps (Ikekawa et al. 1993, Schiff and Feldlaufer 1996, Ciufo et al. 2011). Fungi predominantly produce ergosterol, which is characterized by a double bond at the 5, 7, and 22-positions, and an alkyl group on the side-chain at the 24-position (Figure 1b). Ergosterol utilization by insects is not common, perhaps due to an inability in most insects at reducing a double bond at the 7-position (Behmer and Nes 2003). However, insects with fungal symbionts, such as in leaf-cutter ants, anobiid beetles and ambrosia beetles, commonly utilize ergosterol as a metabolite (Maurer et al. 1992, Nasir and Noda

2003). In each of these cases, the use of ergosterol is indicative in the sterol profile by the presence of 7-dehydrocholesterol or similar ergosterol derivatives. The requirement for cholesterol in insects is particularly interesting for phytophagous and mycetophagous insects, as it is not commonly encountered in nature, but holds some structural specificity in insect homeostasis.

In this study I investigate the sources of dietary sterols for *Sirex* feeding in red pines in North America. I use the distinct structural profiles of fungal and plant sterols to determine the origin of sterol products recovered from insects and infer the relative dietary importance of plant and symbiont resources for *Sirex* larval development. This work tests the role of the fungal symbiont, *Amylostereum*, in woodwasp nutrition by quantifying the amount of fungal biomass available in pine xylem and the amount assimilated into insect tissues at multiple life-history stages (egg-adult) and gender relative to alternative plant derived sterols using GC-MS to quantify sterol molecules. Sterol analysis is useful not only determining nutrient sources for consumers, but also for determining the metabolic intermediates that are formed in hormone and microbial metabolism of sterols. This study tests the direct (mycetophagy) -vs- indirect ('external rumen') hypotheses for the basis of symbiosis between *Amylostereum* and *Sirex* using sterol molecules unique to each feeding method.

3.3 Methods

3.3.1 Study System

Sirex is native to European and North African ecosystems, where it feeds predominantly on Scots pine (*P. sylvestris*). *Sirex noctilio* has been introduced to

numerous continents outside its native range, including Australia, South Africa, South America, and most recently, North America. In North America, red pine (*Pinus resinosa*) is the most commonly utilized native host (Hajek et al. 2009). Red pine is found throughout the current introduced range of *Sirex* and overlaps the ranges of several other susceptible and economically important species including *P. strobus*, *P. rigida*, *P. virginiana*, *P. taeda* (Carnegie et al. 2006). For this study, samples of *Sirex* and *Pinus* host material were collected from the southern boundary of the known distribution of *Sirex* in North America (41°44'54.98" N; 77° 18'04.94" W Tioga County, Pennsylvania).

3.3.2 Sample Collection

Sirex eggs, larvae, and adults were sampled from 15 naturally infested pines in May of 2010. Trees were felled and the trunk of the tree was sub-sectioned into bolts ~60 cm in length for larval extraction and adult rearing. Bolts ranged from 15 to 32 cm in diameter at mid-line. Mid-late larval instars (~4th - 11th) were extracted from 10 randomly chosen bolts from among the 91 total available from all trees. Larvae were collected by splitting pine bolts along the grain to reveal larvae in feeding galleries. Larvae (n = 18) were collected, along with ~1.0 g of frass material (n = 8). Early instar larvae were not sampled due to difficulty in locating and collecting sufficient material. In addition, maternal inputs of cholesterol in eggs may confound observations in early instar larvae (Behmer and Grebenok 1998). The material commonly called 'frass' in this, and possibly other pine sawflies, is not true frass (Weiss 2006), but instead is a masticated, processed wood material that passes predominantly externally along the underside of the insect body and then blends with

a small volume of excrement at the posterior of the abdomen near the anus. For simplicity, 'frass' is used here to describe the chewed pulped wood mixed with excrement that accumulates in the galleries behind foraging larvae. Frass was collected from immediately behind foraging larvae up to a distance of 1.5 cm away from the feeding chamber. Adult male and female *Sirex* were collected at random from the original *Pinus* bolts upon emergence (n = 18 & n = 15 respectively). Ovaries were dissected and eggs were removed from a total of 18 females for analysis of egg sterol, but sample size constraints necessitated pooling of eggs from groups of females (6 females/sample; mean dry wt. 2.33 mg; n = 3). Additional samples of food source (pine xylem/sapwood) was removed from healthy trees (n = 6) and *Sirex* attacked trees (n = 7) at breast height (~1.3 m) using a 5mm increment borer (Haglof[®]) to a depth of 3.5 cm (bark removed).

3.3.3 Fungal Isolation

Following a procedure similar to Thomsen and Harding (2011), pure cultures of the fungal symbiont, *Amylostereum*, were obtained from recently emerged adult females (n = 6) via dissection of the mycangial organs located at the base of the ovipositor. Briefly, mycangia from individual surface sterilized female *Sirex* were dissected using sterile forceps, placed in sterile PBS (1X Phosphate Buffered Saline) and mechanically disrupted using sterile forceps. Samples were then vortexed for 3 min. and spread plated in a 1:10 dilution onto Yeast Malt (YM) agar (Difco[®]). Fungal colonies were picked from initial isolation plates and transferred onto new YM plates for isolation of pure cultures and biomass production. *Amylostereum* isolates from the seven original female extractions were grown for two weeks before

being scraped from the surface of growth media using sterilized stainless steel micro spatulas (Fisher Scientific) and placed in 70% ethanol at -20°C until sterol analysis. Agar 'control' samples were subjected to identical treatment minus fungal growth and submitted for comparison as a negative control to sterol profiles of fungal cultures on agar. Nuclear DNA of fungal isolates was extracted using the enhanced yield protocol of the Mo Bio PowerSoil[®] DNA Isolation Kit (Mo Bio Laboratories). Fungal isolates of *Amylostereum* were identified by amplification and sequencing of the rDNA internal transcribed spacer region using the ITS1-4 primer pair according to the protocol of (Gardes and Bruns 1993). Sequences were first annotated and then identified by best-hit comparison using blastn search in the nucleotide database in Genbank (Altschul et al. 1990), with a ($\geq 90\%$) sequence similarity cutoff at the nucleotide level to its closest database hit. The seven sequences extracted from seven female *Sirex* were deposited in Genbank[®] under accession numbers JX035728-JX035739.

Analysis of fungal biomass in pine wood was obtained by transferring a 1mm³ plug of hyphae from the edge of active pure cultures of *Amylostereum* and placing it directly onto the exposed sapwood of a 1 cm thick slice of autoclave sterilized *Pinus* and placed in the dark to grow under sterile conditions for 2 weeks. Sterol profiles were obtained for wood with fungal cultures of *Amylostereum* (n = 7), *Ophiostoma ips* (n = 6) [hereafter: *Ophiostoma*], and *Pinus* without fungal inoculation (n = 7) by cutting out 1 cm³ cubes of xylem either containing or devoid of fungal hyphae. *Ophiostoma* sterols have been successfully analyzed from wood (Bentz and Six 2006) and acted as a positive control for extraction and analysis of fungal sterols. Samples

were stored in 70% ethanol at -20°C until GC-MS analysis. Fungal free samples of *Pinus* served as a control baseline for the plant sterol profile. *Ophiostoma* and one *Amylostereum* strain used in this study were obtained from Dr. Aaron Adams of the University of Wisconsin-Madison. The remaining six *Amylostereum* strains were isolated from pure cultures isolated from dissected lab reared female *Sirex*, as described above.

3.3.4 Tracking Fungal Growth

White rot fungi of the phylum Basidiomycota are among the most prolific decomposers of woody plant material and are associated with numerous lignocellulosic enzymes (Leonowicz et al. 1999, 2001). *Amylostereum* is a white rot fungus that digests lignin with the help of the excreted lignolytic enzyme laccase (Bordeaux 2008). In vitro, the activity of laccase can be measured using the compound 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) in solution (1.376 mg/ml dH₂O), which laccase metabolizes to form a blue/green product. The enzymes produced by white rot fungi are required for the breakdown of wood compounds, such as lignin and cellulose, for metabolic purposes but also for passage of hyphae through the wood matrix (Blanchette 1991).

Fungal enzymes have been used to track fungal growth in woody tissues (Srinivasan et al. 1995). I first verified the presence of the common lignolytic enzyme laccase and its activity on ABTS using laboratory strains of *Amylostereum areolatum* obtained from Dr. Adams. Subsequent field collected fungal strains described in the methods were tested for laccase activity as described in Niku-Paavola et al. 1990 at 1/8th concentration (1.375mg/mL). Using this technique I tracked the growth of

Amylostereum prior to sterol analysis. ABTS solution was made fresh for each sample and samples were taken only where both visual confirmation of fungal hyphae and ABTS blue product were observed. Stains of natural infestations showed similar activity in the presence of ABTS to lab cultured strains.

3.3.5 Sterol Analysis

Insect samples were surface washed to remove external contaminants, dried, and ground prior to analysis. Insect samples and fungal cultures were disrupted for sterol analysis with 30 ml of 95% ethanol and 8, #5 glass beads (Sigma, St. Louis, MO, USA) in a modified pneumatic paint shaker, while structurally resilient plant material was ground to a fine powder using an 8 inch half round file coupled with repeated grinding bouts on a commercial grain mill (KitchenAid Mississauga Ontario), with subsequent extraction in 30 ml of 95% ethanol. These samples were shaken and incubated in the dark at room temperature for 24 hours, and the ethanol was evaporated to dryness under nitrogen. The residue was re-suspended in 70% methanol:water, to which 10 µg of cholestane was added as an internal standard and the free sterol was extracted with water-equilibrated hexane. The hexane fraction was subdivided into two equal fractions for plant and fungal samples to facilitate the examination of both free and acylated sterol, while the methanol:water fraction was used for the examination of glycosylated sterol. Separate individuals were used with animal tissues for examination of free and acylated fractions. Subsequently, all sample fractions were evaporated to dryness. The sample fraction containing the free sterol was re-suspended in a minimal volume of hexane and conjugated and analyzed by GC-MS (see below). The sample fraction containing the acylated sterol was

resuspended in 8 ml of 70% methanol:water containing 5% KOH and incubated in a shaking water bath (225 rpm) at 55°C for 2.5 hours to replace the fatty acid moiety present at C3 with a free hydroxyl group. The fraction containing the glycosylated sterol was resuspended in 8ml of 100% methanol, containing 10% HCl, and incubated in a shaking water bath (225 rpm) at 55°C for 2.5 hours to replace the carbohydrate moiety present at C3 with a free hydroxyl group. Subsequently, the free sterols were extracted from the chemically treated samples with water -equilibrated hexane and the hexane layer was washed to neutrality with hexane - equilibrated water. The sterols contained in the 3 fractions were converted to their respective tmsi derivatives by overnight incubation with a 2:1 excess volume v/v of BSTFA+TMCS, 99:1 (Sylon BFT) (Supelco Inc. Bellefonte, PA).

All conjugated sterol was processed by GC-MS using an Agilent 6850N GC coupled with a 5973 mass selective detector (Agilent Technologies, Inc., Santa Clara, CA, USA). The GC-MS was equipped with a fused capillary EC-5 column (30 m) (Alltech, Nicholasville, KY, USA) with a 0.25 mm internal diameter and 0.25 μm film thickness. The running conditions were: inlet 260°C, transfer line 280°C, column 80°C (1 min), ramp at 10°C min^{-1} to 300°C, 300°C (20 min), with helium (1.5 ml min^{-1}) as carrier gas. The Agilent 5973 mass selective detector maintained an ion source at 250°C and quadrupole at 180°C. Steroids were identified and quantified by GC-MS using Selected Ion Monitoring (SIM) protocols for each steroid identified (Rahier and Benveniste 1989). Authentic steroid standards were purchased commercially (Sigma and Steraloids).

Sirex larval, adult (male and female), healthy wood, frass and fungal cultures were quantified for ‘free’ sterols (unbound 3’ hydroxyl group), and two conjugated (bound) classes: ‘acylated’ (bound 3’ hydroxyl group, typically lipid bound) and ‘glycosylated’ (bound 3’ hydroxyl group, typically carbohydrate bound). Sterol quantities were analyzed by life-stage and sex for animal samples as percent sterol composition (%). Wood and fungal food resource samples were weighed prior to analysis for determination of sterol concentration ($\mu\text{g/g}$).

3.3.6 Statistical Analysis

Differences in sterol composition were analyzed on an individual sterol basis across *Sirex* life-stages and adult *Sirex* gender using generalized linear models (GLM) for percentage data with multiple comparisons of means under standard assumptions (R Development Core Team 2009). Relative proportion of sterols specific to life stages and gender in adults were evaluated using one-way ANOVA (F-tests), followed up with Tukey HSD test for comparisons between groups with significant ANOVAs (R Development Core Team 2009). In particular, eggs represented the basal sterol profile required for development, while those collected for mid-late stage larvae represent sterols associated with foraging and development. Comparison of gender specific sterols in adults represents sterols unique to reproductive ecology of mature *Sirex*. Free and acylated sterol fractions were analyzed in separate models as these fractions represent specific features of their function in cell metabolism and support. Data on proportions of sterol in *Sirex* life-stages exhibited skew characteristic of bounded data and were arcsin square-root transformed prior to analysis. In cases where sterol derivatives were rare, binomial distributions were used

in the GLM along with logit transformation. Multiple comparisons tested for differences among life stages and sexes, except where limited by the absence of extracted sterol classes, which occurred for all molecules except cholesterol. Experimentwise error for multiple pairwise comparisons was controlled using Tukey's HSD test for multiple comparisons (R Development Core Team 2009).

In contrast to samples from animal tissue, differences in plant and fungal sterols collected from natural and lab settings were measured for concentration of sterols not relative proportions. Skew in concentration of sterols in plants and fungi were corrected prior to statistical tests with log transformation. Samples from artificial media (YM agar) with and without *Amylostereum* were analyzed for sterol concentration ($\mu\text{g/g}$) using GLM and one-way ANOVA with multiple comparisons using Tukey's HSD. The concentration of sterol in *Pinus* ($\mu\text{g/g}$) was analyzed for sterol concentration in *Pinus* xylem tissue with and without fungal cultures using one-way ANOVA. Ergosterol can be used as a surrogate for measuring fungal biomass in wood (Pasanen et al. 1999). I evaluated fungal biomass in wood and the change in phytosterol concentration due to fungal enzymes using natural field collected samples (described above) and benchtop fungal growth chambers. Fungal and phytosterol concentrations were evaluated independently for healthy *Pinus*, *Ophiostoma* and *Amylostereum* inoculated *Pinus* using one-way ANOVA.

The effect of fungal and *Sirex* feeding modifications on naturally attacked *Pinus* xylem tissue were analyzed using GLM for the phytosterols campesterol and sitosterol in a one-way ANOVA with three factor levels: healthy *Pinus*, *Sirex* attacked *Pinus*, and *Sirex* frass.

3.4 Results

3.4.1 *Sirex* Sterols

Sterols were identified in three forms (free, acylated and glycosylated). However, I found very little evidence of sterols bound to sugar (glycosylated), thus we evaluated only unbound (free) and fatty acid bound (acylated) sterols. The manner in which conjugated and free sterols are processed and the unpredictable nature of the chemical extractions therein do not permit statistical comparison between sterol pools. However, qualitative comparisons across free and acylated sterol pools are made where appropriate.

With respect to the sterol profiles of *Sirex*, I observed three strong trends. First, cholesterol was the dominant form in *Sirex* tissues and the only sterol quantifiable from eggs (Figure 2). Second, phytosterols (campesterol, sitosterol and stigmasterol) and fungal sterols (ergosterol) were recovered in the larvae and/or adults, but only in the conjugated form (Figure 2). Third, a diverse range of atypical insect sterols/steroids (cholestanol, cholestan-3-one, cholest-4-en-3-one, cholest-5,7,-dien-ol and cholest-3,5-dien-ol) were recovered from larvae and adults (Figure 2). With respect to the total sterol profile, larval and female profiles consisted primarily of cholesterol and cholestanol, while males had mainly cholesterol and cholestan-3-one. The sterol profile of adult female *Sirex* was the most diverse of all sterol profiles, containing metabolic intermediates, phytosterols and ergosterol in the acylated analysis (Figure 2).

Significant changes in percent sterol profiles of *Sirex* life-stages are representative of relative changes in sterol proportions between sterol pools and are

not necessarily indicative of changes in total sterol quantity. However, changes in proportions may be indicative of greater behavioral or metabolic states in certain life-stages. In *Sirex*, like most insects, cholesterol is the dominant sterol, but sterol proportions differed significantly between life stages in both free ($F^{(3,19)} = 3.9$, $P = 0.02$) and acylated sterol pools ($F^{(3,30)} = 6.9$, $P = 0.001$). Post hoc comparisons of free cholesterol using Tukey's HSD test showed only adult males ($P = 0.03$, $df = 19$) differed significantly from the egg stage (Figure 2a). In contrast, post hoc comparisons of acylated sterols showed males differed significantly from females ($P = 0.005$, $df = 30$), female *Sirex* were significantly different from eggs ($P = 0.002$, $df = 30$) and larvae marginally different from eggs ($P = 0.06$, $df = 30$, Figure 2b) in cholesterol concentration. The sterol profiles of larvae and adult female *Sirex* were nearly identical in proportions of free sterols (sterols with free 3' hydroxyl groups), differing in only the level of cholest-5,7-dien-ol, which was only found in adult female *Sirex* (Figure 2a). Adult males differed markedly from other life-stages with undetectable levels of cholestanol and cholest-4-en-3-one, and in having an elevated proportion of the steroid precursor cholestan-3-one in the free analysis ($F^{(2,17)} = 3.7$, $P = 0.04$), with proportions of free cholestan-3-one highest in males (Figure 2a), but reversed and greater for females compared to others in the acylated analysis ($F^{(2,28)} = 18.9$, $P = <0.001$). Adult male *Sirex* differed from all others in having the steroid precursor cholest-3,5-dien-ol in the free sterol fraction (Figure 2a). The sterol profile of *Sirex* eggs was nearly entirely composed of cholesterol, though trace amounts of phytosterols could be found within the background at concentrations of <0.001 ug/gram. Plant sterols were not detected in the analysis of free sterols of any

Sirex life-stage, but were detected in acylated analysis where differences between life-stage differed significantly ($F^{(2,28)} = 6.9$, $P = 0.004$; Figure 2b). In post hoc comparisons sitosterol had a higher proportion, particularly in adult males over females and larvae (male-larvae: $P = 0.004$, $df = 28$; male-female: $P = 0.049$, $df = 28$).

3.4.2 Sterol in Food Resources

Growth of *Amylostereum* fungal cultures in the lab on YM agar and sterilized pine xylem, were visually confirmed prior to analysis of sterol profiles (colonies measured ~4 cm in diameter at 2 weeks growth). Cholesterol was detected in analysis of YM agar media and was subsequently removed from analyses of sterols from fungi growing on YM agar. The dominant sterol in *Amylostereum* cultures grown on YM agar is ergosterol (acylated, 28.7 ± 5.4 $\mu\text{g/g}$; free, 18.9 ± 5.1 $\mu\text{g/g}$ (mean \pm SEM)). No other sterols were detected above the 0.001 $\mu\text{g/g}$ threshold.

As expected phytosterol concentrations were low in the free sterol fraction of *Pinus*, suggesting the majority were locked in cell membranes. Base saponification released acylated sterols and significantly increased detection, thus assays for plant tissue were analyzed only for the acylated sterol pool as it likely represented a more complete profile of sterols in *Pinus*. *Pinus*, with and without fungal cultures, differed only in the presence or absence of ergosterol associated with fungal hyphae (Figure 3). Campesterol levels appeared to decrease in *Ophiostoma* inoculated xylem tissue, but differences were not statistically significant. *Amylostereum* cultures on sterilized wood contained only trace amounts (< 0.005 $\mu\text{g/g}$) of ergosterol, while *Ophiostoma* cultures showed a clear ergosterol signal (1.2 ± 0.34 $\mu\text{g/g}$) (Figure 3 (arrow)).

Acylated sterol profiles from healthy and *Sirex* infested environmental samples of *Pinus* in nature, again indicated sitosterol and campesterol were the dominant sterols found in pine xylem tissue (18.48 ± 2.3 and 2.7 ± 0.4 $\mu\text{g/g}$ respectively; Figure 4). Concentrations of these compounds did not change in *Pinus* attacked by *Sirex* relative to non-attacked trees. *Sirex* frass, however, was depleted in campesterol compared to unmolested *Pinus* and *Sirex* attacked *Pinus* xylem ($F^{(2,15)} = 5.9$, $P = 0.013$). Post hoc comparisons clearly indicate the effect of *Sirex* manipulation of wood; Tukey HSD: healthy *Pinus* – frass ($P = 0.03$, $df = 18$) and *Sirex* attacked *Pinus* – frass ($P = 0.04$, $df = 18$), but did not differ in sitosterol (Figure 4). Frass further differed from other wood samples by containing measurable concentrations of both ergosterol (0.96 ± 0.64 $\mu\text{g/g}$) and cholesterol (0.72 ± 0.35 $\mu\text{g/g}$) in addition to typical phytosterols of *Pinus* (Figure 4).

Staining for the presence of *Amylostereum* in cultures using the ABTS indicator for the lignocellulosic enzyme, laccase, depicted the presence of *Amylostereum* in both artificial cultures (Figure 5a) and in natural infestations (Figure 5b). Laccase, like most enzymes, deteriorates over time, thus measures of activity on ABTS depict active fungal cultures in wood. Samples taken within enzyme stain guaranteed accurate sampling of fungal infested tissue.

3.5 Discussion

3.5.1 Sterol Ecology of *Sirex*

Symbioses between insects and fungi have played an integral role in the ecology and evolution of insects. Among insect-fungal symbioses the cultivation of

fungal symbionts as food has received much attention (Mueller and Gerardo 2002). Contrary to this popular ‘fungus farming’ model of symbiosis documented in ants, termites, and ambrosia beetles where mycetophagy is the dominant mode, analysis of sterol molecules associated with *Sirex* show little support for fungal feeding in this sawfly-fungus mutualism. *Sirex* propagates *Amylostereum* with high fidelity and needs *Amylostereum* to successfully colonize host pine trees, but data on ergosterol concentration in wood indicated *Amylostereum* persisted in low abundance in the xylem, which could preclude a role in bulk nutrition, but possibly not key metabolites. Ergosterol concentrations are nearly undetectable in both wood infested with *Amylostereum* and larvae of *Sirex*. These data suggest either rapid metabolism of the limited fungal biomass in wood or exclusion of this resource from the diet of *Sirex*. Alternatively, the arsenal of lignocellulosic enzymes associated with *Amylostereum* may play an important role in nutrient acquisition (“external rumen hypothesis,” Swift et al. 1979, and "ruminant hypothesis," Nobre and Aanen 2012).

Insects cannot synthesize sterol molecules *de novo*. As such they must rely on external sources to meet dietary sterol demands. Fungal consumption is readily apparent in mycetophagous insects via ergosterol derivatives and end metabolism sterols (e.g., $\Delta^{5,7}$ -sterols) (Nasir and Noda 2003). In this study, I found no evidence for significant utilization or metabolism of ergosterol compounds as ergosterol was found at extremely low levels in both food resources and foraging larvae (<0.0001 $\mu\text{g/g}$) and ergosterol derivatives were not detected in insect tissues. Sterol analysis indicated low (<0.005 $\mu\text{g/g}$) but persistent fungal biomass in both natural and lab cultures of wood containing *Amylostereum*. Limited fungal biomass may explain a

large part of vanishingly low concentrations of ergosterol in the sterol profile of consumers, but is also indicative of a larger role for plant tissue in the diet of *Sirex*.

The sterol profile of *Sirex* was similar to that of many phytophagous insects. Sitosterol and campesterol are the most prevalent sterols in *Pinus* and were likewise the only phytosterols found in larval and adult *Sirex* (with the exception of ergosterol associated with mycangia of adult females, see below). Interestingly, phytosterols were only recovered in the acylated fraction indicating *Sirex* binds at least some phytosterols to lipid, usually fatty acids. Little is currently known about the functional significance of sterol conjugation in insects, but it may be a mechanism used by insects to “flag” sterols for sequestration, metabolism or excretion (Behmer and Nes 2003). *Sirex* seems capable of converting phytosterols to cholesterol given that sitosterol and campesterol are common in *Pinus* wood, but occur at low quantities in larvae. Metabolism of phytosterols to cholesterol is documented in phytophagous insects and has been reported in other sawflies (Schiff and Feldlaufer 1996), but not for wood feeding sawflies and not across life-stages. Interestingly, the concentration of campesterol, but not sitosterol, was reduced in the frass compared to *Pinus* wood. However, there was a relatively similar proportion of campesterol and sitosterol in larvae, despite a higher proportion of sitosterol relative to campesterol in undigested wood. Together, these findings suggest that in *Sirex* campesterol may be more readily absorbed than sitosterol, but that campesterol might not be metabolized to cholesterol as readily as is sitosterol. Many aspects of insect sterol physiology and biochemistry are poorly understood, so further research is needed to more broadly understand sterol absorption and metabolism in insects, especially in *Sirex*.

3.5.2 *Amylostereum* in *Pinus*

The sparse growth and low biomass for *Amylostereum* indicated by sterol analysis in wood tissue was checked against extraction efficiency of another common wood-rot fungus, *Ophiostoma*, grown under identical conditions. Extraction was not of concern as sterols from *Ophiostoma* grown on wood showed a consistent signal. *Ophiostoma* biomass in wood and on artificial media was relatively equivalent to that of *Amylostereum* by visual inspection, but *Amylostereum* had significantly lower ergosterol concentrations. I confirmed ergosterol was the major sterol associated with *Amylostereum* on artificial media where biomass was presumably higher and confirmed *Amylostereum* presence in wood cultures and in natural infestations using enzyme assays of wood analyzed by GCMS. It is also of note that phytosterol concentrations in both natural and bench-top experiments did not differ significantly between *Sirex* infested and uninfested tissues, suggesting oxidative modifications of sterols was not prevalent in wood inoculated with *Amylostereum* despite documentation of this in other white-rot fungi (Gutiérrez et al. 2002). As a result esterified sterols found in *Sirex* are not likely derived from fungal metabolism in wood.

3.5.3 Bacteria Modified Sterols

Interestingly a number of esterified sterol derivatives (e.g. cholestanol, cholestan-3-one, cholest-4-en-3-one) were found in *Sirex*. These derivatives are very unusual in insects, unless the insects eat food containing them (Jing et al. 2013). They are also unusual in plants, but have been reported in tobacco plants expressing the chloroplast-targeted 3-hydroxysteroid oxidase gene (pMON33814) from an

Actinomyces spp. bacteria (Heyer et al. 2004). A genomic analysis on fauna associated with *Sirex* indicated a rich bacterial flora, including *Actinomyces* spp. (Adams et al. 2011). The presence of the unusual sterols in *Sirex* may be a result of microbial modification of sterols in the gut lumen (Heyer et al. 2004), although background for this type of association needs further investigation.

3.5.4 Insect Sterol Pathways

Modified sterols found in adult and larval, but not egg, tissues may represent the sequestration of harmful sterols and/or hormonal signaling pathways (Eliyahu et al. 2008). Consistent with hormonal hypotheses, adult *Sirex* showed higher accumulations of sterol esters, including two sex specific sterols; cholest 3,5 dien-ol (male) and cholest 5,7 dien-ol (female). Sex-specific sterols could be related to recently discovered contact pheromones in both adult female *Sirex* cuticle (Böröczky et al. 2009) and volatile lek signaling pheromone isolated from adult male *Sirex* (Cooperband et al. 2012). The role of esterified sterols in *Sirex* physiology requires more investigation, but their potential in defining symbiotic associations and pheromone signaling are potentially rewarding as *Sirex* is an invasive pest with global distribution.

Dietary sterols have been used to investigate diet characteristics in numerous mycetophagous symbiotic relationships (e.g. bark beetles (Bentz and Six (2006), but the absence of a definitive fungal sterol signal in adult and mid-late instar larval *Sirex* put into question mycetophagy in this primitive hymenopteran. This study does not preclude the potentially important role of fungal mycelia in early instar larvae and host colonization. First and second instar larvae are believed to forage directly on

fungal hyphae in their oviposition tunnel before entering sapwood (Madden 1981), but conversion from ergosterol to cholesterol requires significant metabolic investment and is not common in insects (Behmer and Nes 2003). First instar larvae putatively feed solely on mycelia of *Amylostereum* (Madden 1981). Mycetophagy in early instars may represent a transient state whereby *Sirex* initially benefits from fungal inputs, but ultimately shifts to the more abundant, but less digestible food source with increasing processing capacity at larger body size (Singer 2001). In regard to sterols in early instars, maternally supplied cholesterol in the eggs may carry through confounding assumptions from early instars (Behmer et al. 1999).

The obligate symbiosis between *Sirex* and *Amylostereum* was not apparent from the sterol profile of *Sirex*, but is strikingly apparent in the consistent ergosterol signal detected from the mycangia of adult females. Adult females transfer *Amylostereum* to future generations via secretion from mycangia. The mycangia of adult female *Sirex* purportedly foster *Amylostereum* proliferation in mycangia with nutrient rich secretions (Cartwright 1938). If higher nutrient levels lead to higher fungal biomass in mycangia a similar mechanism may explain the detectable concentrations of ergosterol in frass. Elevated levels of nitrogen found in a related study (Chapter 4) and the presence of cholesterol in frass are indicative of insect inputs to frass that may increase the substrate nutrient quality for *Amylostereum*. Enhanced nutrients could be responsible for the enhanced growth of *Amylostereum* in frass. Higher densities of *Amylostereum* in frass does not directly translate to *Sirex* nutrition, but may increase contact between larvae and *Amylostereum*, which may in turn facilitate symbiont acquisition in adult females prior to exiting host trees.

The consumption of biologically available resources and their assimilation into growth and reproduction pathways is central to all subsequent behavioral, biological, and evolutionary processes. Integral to this phenomenon is the discrepancy between that which is consumed and the dietary needs of the consumer. Where dietary demands are not met, whether through nutrient deficiency or inhibitory chemical and physical barriers, symbiotic associations have an opportunity to amend shortcomings (Douglas 2009). The symbiotic association between *Sirex* and its fungal symbiont, *Amylostereum*, is a prime example of symbiosis in recalcitrant low nutrient conditions. Here I find little support from sterol analysis for a direct dietary role of the fungal symbiont, but multiple lines of evidence for the ‘external ruminant hypothesis’ (Nobre and Aanen 2012). Low *Amylostereum* biomass but ubiquitous enzyme production from *Amylostereum* supports the hypothesis that the symbiont is more important for transfer of digestive potential than for bulk nutrition in *Sirex noctilio* and potentially other Siricids, though transfer of specific metabolites should not be ruled out.

3.6 Figures & Tables

3.6.1 Figures

Figure 1. Key dietary sterols available to and recovered from *Sirex*. Phytosterols (a) and fungal sterols (b) are converted to cholesterol (c) via dealkylation of side-groups (hollow arrows) at C24 and, in the case of ergosterol, removal of double bonds (filled arrows) at C7 & C22.

Figure 2. The total proportion (mean \pm SEM) of sterols extracted from the free sterol pool (a) of various life-stages of *Sirex* qualitatively differs from the conjugated sterol pool (b) in some esterified sterols, but most strikingly in phytosterols (gray shading) in *Sirex*. Asterisks denote significance between life-stages (Tukey's HSD, $p < 0.05$). Trace levels of sterols are omitted.

Figure 3. Sterol concentration (mean \pm SEM) from base saponified samples of fungal inoculated pine xylem (*Pinus* + *Amylostereum* / *Ophiostoma*) and un-inoculated control xylem (*Pinus*). Ergosterol was present in trace quantities ($< 0.005 \mu\text{g/g}$) from *Pinus* + *Amylostereum* and in greater quantities in *Ophiostoma* (filled arrow). Sterol concentrations were not statistically different (Tukey HSD, $p > 0.05$).

Figure 4. Sterol concentration (mean \pm SEM) from samples of pine xylem with *Amylostereum* (*Pinus* w/*Sirex*), without *Amylostereum* (*Pinus* healthy) and *Sirex* frass. Asterisks denote significance between life-stages (Tukey's HSD, $p < 0.05$).

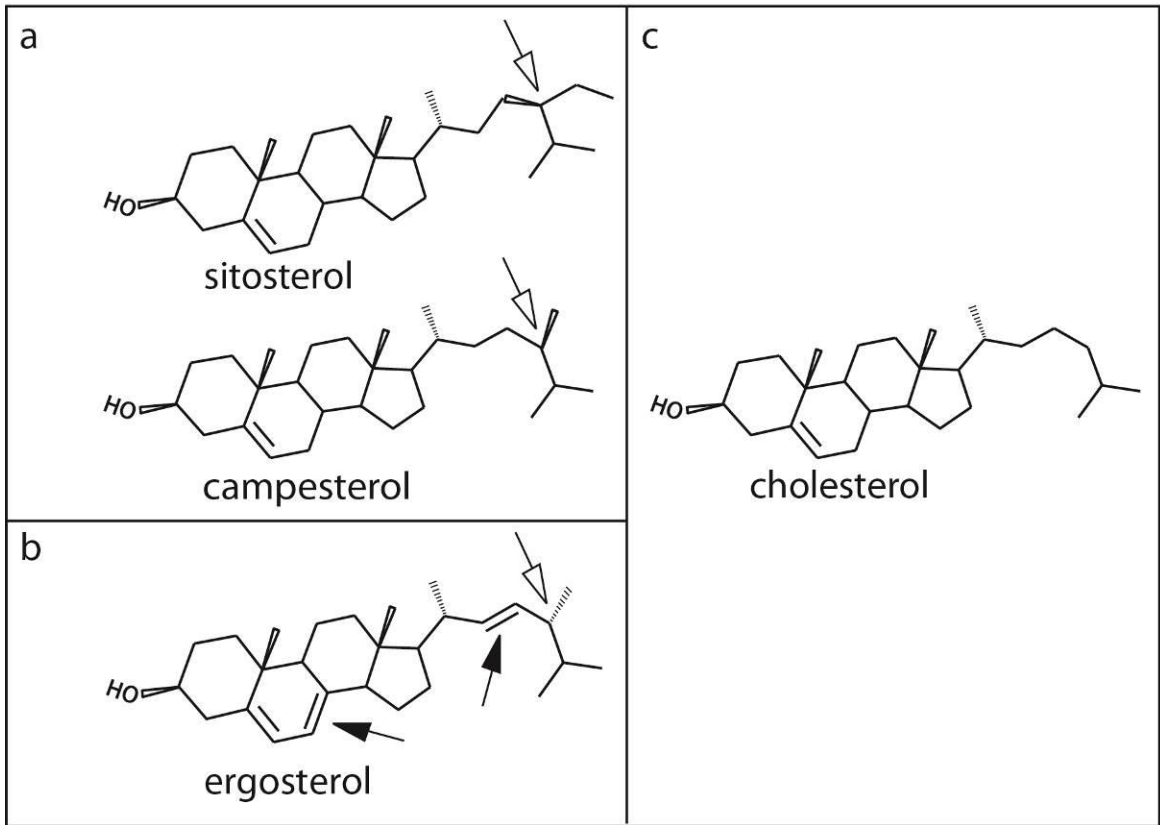


Figure 1

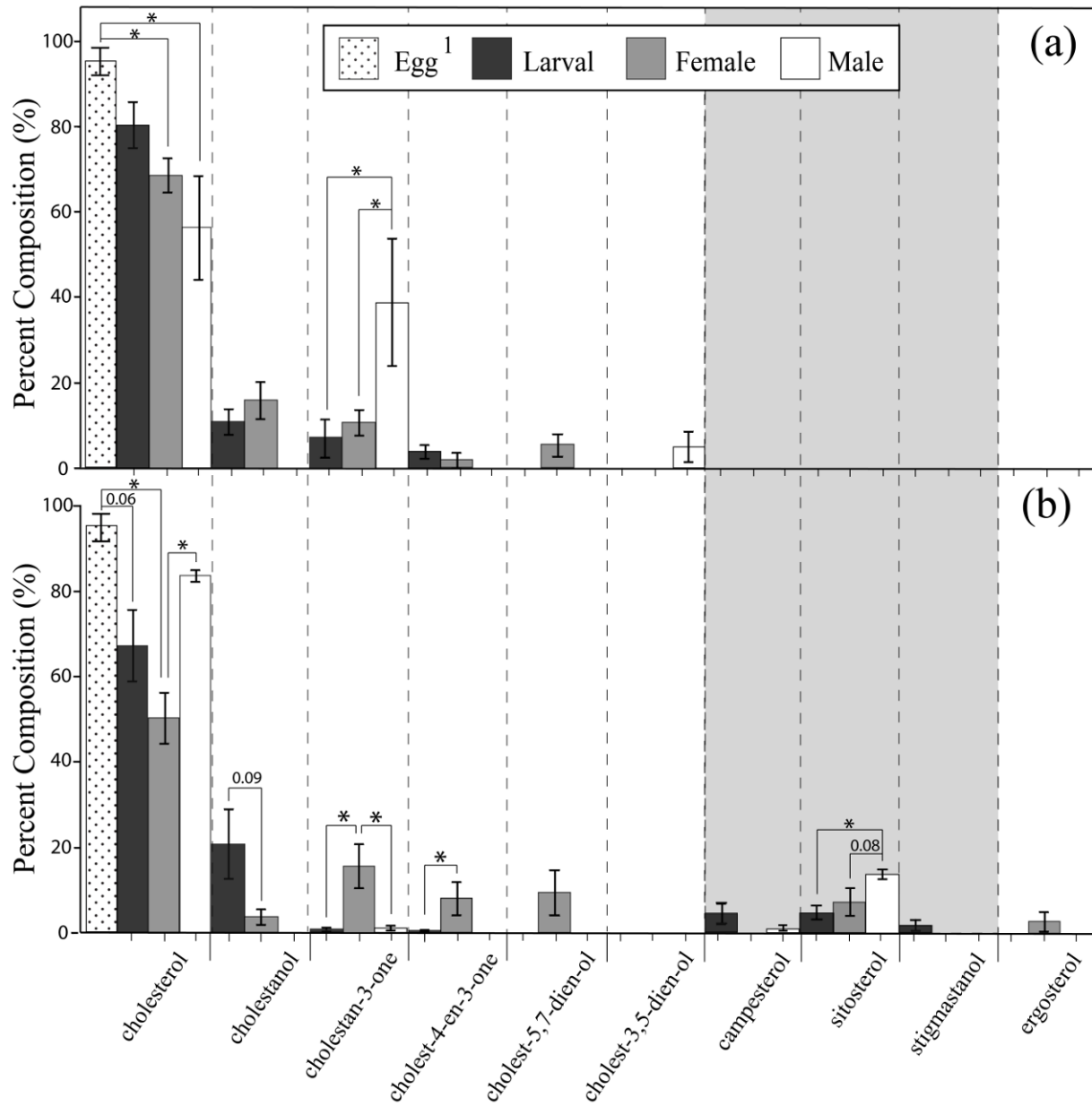


Figure 2

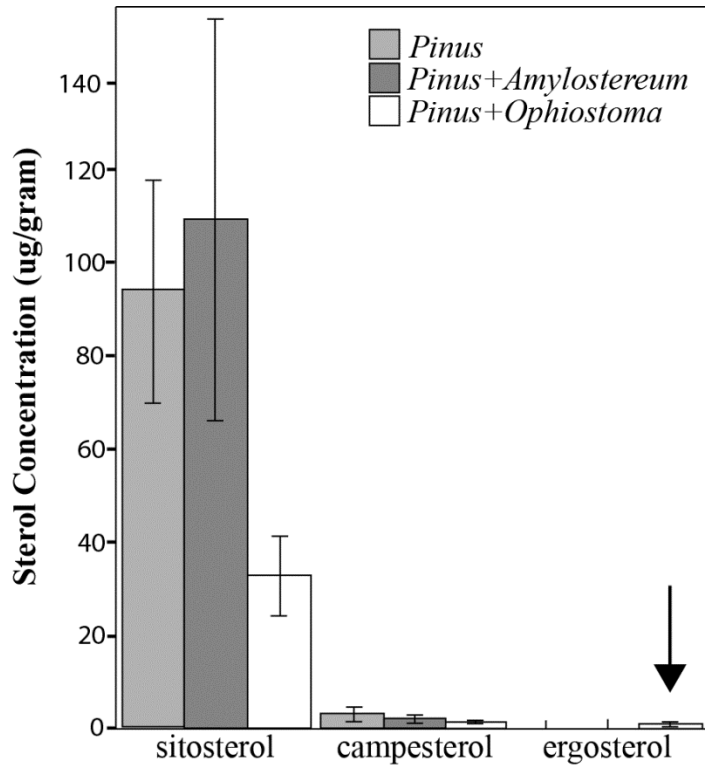


Figure 3

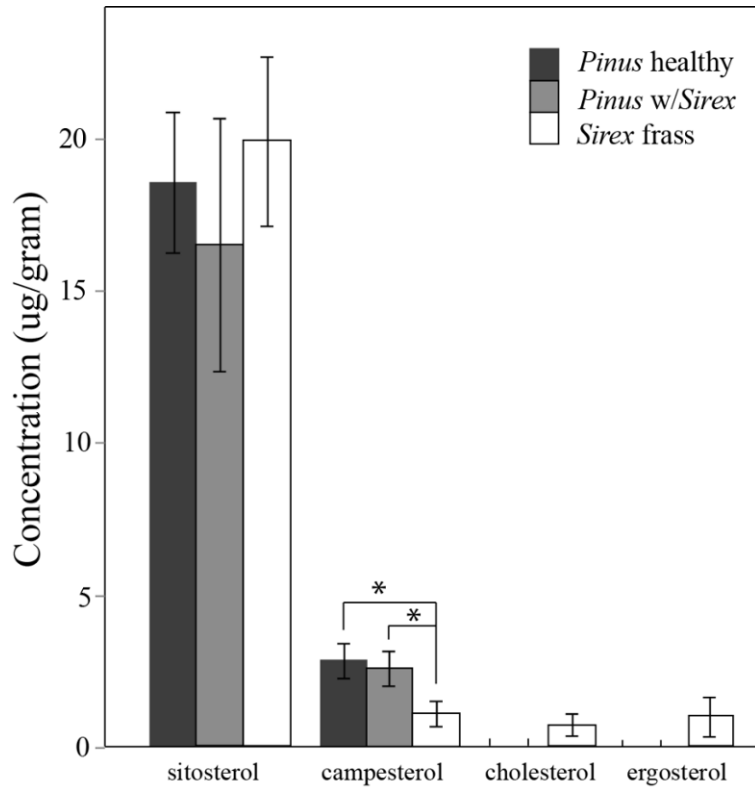


Figure 4

Chapter 4: Nitrogen fixation, external digestion and the microbiome of the wood-feeding sawfly, *Sirex noctilio*.

4.1 Abstract

Nitrogen (N) is limiting in terrestrial ecosystems, but at 0.03-0.1% N wood is among the lowest N containing biological materials. Wood-feeding in insects is compounded by the ubiquity of lignin polymers. Woodwasps (Hymenoptera: Siricidae) are wood-feeding sawflies that feed on the xylem of pine trees, with the assistance of a white-rot fungal mutualists. Nutritional adaptations to wood-feeding in woodwasps are poorly understood. Using metagenomic and stable isotope analysis I explored the role of bacterial associates in wood-feeding for the woodwasp, *Sirex noctilio*. Bacterial associates of *Sirex* were adapted to metabolism of simple carbohydrates, not fermentation of complex lignocellulose typical of other wood-feeding insects. Isotopic analysis showed bacterial nitrogen fixation supplies the majority of N in the diet of larvae. N-fixation has never been described in sawflies, but is likely widespread given the N deficiency of wood food sources. N-fixation in *Sirex* is supported by external digestion of wood by a wood-rot fungal mutualist (*Amylostereum areolatum*) that liberates simple carbohydrates for digestion by internal bacterial associates. This microbial assembly line enables woodwasp development on the unlikely food source of pine wood. The relationship described here is likely ubiquitous in xylem-feeding sawflies and represents a major evolutionary adaptation for feeding on nutrient poor plant tissues.

4.2 Introduction

Wood is among the most nutrient poor and enzymatically refractory biological materials on Earth. In terrestrial ecosystems, wood is abundant, but the use of wood as a food resource is largely restricted to fungal decomposers due to digestive and nutrient barriers. Nitrogen (N) is a limiting nutrient in most ecosystems (Vitousek and Howarth 1991, Menge et al. 2012), but is of particular concern for phytophagous insects in terrestrial ecosystems due to the high C:N ratio of terrestrial plants (Elser et al. 2000). Wood is extremely imbalanced even among plant tissues and is among the most N deficient biological materials at ~ 0.03-0.1% N compared to ~1.0% N in other plant tissues (Mattson 1980, Meerts 2002). In contrast, insect herbivores typically contain ~6.0 % N. This equates to a ~100 fold difference between wood-feeding insects and their food source. Compare this to a ~6 fold difference typical of leaf-feeding herbivores (Mattson 1980, Menge et al. 2012). In addition to N imbalance, wood contains the highest levels of lignin in plant tissues (~26-30%) along with high levels of cellulose and hemicellulose (Campbell and Sederoff 1996). Lignin is enzymatically refractory and digestible only to select wood-rot fungi (Leonowicz et al. 1999, Sánchez 2008). In wood feeding insects, lignin degradation is thought to occur only with assistance from microbial associations (Geib et al. 2008). Nutritional challenges of feeding on wood are overcome through compensatory feeding, efficient nutrient recycling, or symbiotic associations (Potrikus and Breznak 1981, Tayasu et al. 1994, 2002, Saint-Germain et al. 2007, Grünwald et al. 2010), but the severity of N deficiency in wood and the difficulty of processing and digesting wood limits

strategies for compensatory feeding and extreme N-deficits may prevent effective recycling.

Most wood-decay occurs as a result of microbial metabolism (Blanchette 1991). Insects are generally thought incapable of digestion of wood (Martin et al. 1991, Geib et al. 2008), but wood-feeding occurs in three terrestrial insect orders (Schulmeister 2003, Inward et al. 2007, Hunt et al. 2007), with most occurring in the presence of wood-rot fungi (Kukor et al. 1988). The siricid woodwasps of the basal Hymenoptera (sawflies) are among twenty three insect families that actively feed on wood (Smith 1988, Lieutier 2004). Wood-feeding occurs in thirteen Coleoptera, two Blattodea, three Lepidoptera and three Hymenoptera families in North America (McMinn et al. 1993). Of these families only Cerambycidae, Curculionidae, Buprestidae and Siricidae actively colonize the woody stems of living trees. Other wood-feeders in the sawflies arose through sequential steps from foraging externally to gradually more concealed plant parts including steps with leaf-rolling and stem boring (Smith 1988). Although internal feeding may provide increased protection from natural enemies (Stone and Schönrogge 2003), shifts toward feeding on internal plant organs, such as wood, pose nutritional barriers and internal tissues are generally lower in nitrogen (N) and higher in lignin and cellulose than external tissues (Mattson 1980). The adaptations for wood feeding are thought to be conferred by mutualistic associations in the siricid woodwasps (Talbot 1977), but recent evidence suggests fungal mutualists are only part of the picture (Thompson et al. 2013). The evolution of wood feeding in basal Hymenoptera is of particular importance as it is the adaptation to wood feeding that established the ecological context for the evolution of

parasitism and the radiation of Hymenoptera into one of the largest and most ecologically important insect orders.

Wood-feeding in the Hymenoptera is restricted to the families Anaxyelidae, Siricidae, and Xiphydriidae of the super family Siricoidea (collectively woodwasps) (Smith 1988, Schulmeister 2003). The Siricidae is the most speciose of the three families and is characterized by an ability to colonize living trees with the help of a basidiomycete fungal mutualist (Cartwright 1938). Mutualistic fungi are thought to provide the majority of nutrients to siricid larvae developing in pine wood (Talbot 1977), similar to fungal grazing in ‘fungus farming’ leaf-cutter ants, ambrosia beetles and some termites (Mueller and Gerardo 2002, Mueller et al. 2005), but fungal inputs are not detectable in larval tissues nor in wood surrounding foraging larvae (Thompson et al. 2013). Concentration of N in hyphae of wood-rot fungi has been documented, but it is unknown if N can be concentrated in quantities sufficient to overcome the deficit in wood tissue on which siricid larvae feed. In bark beetles and leaf-cutter ants, fungal nutrition alone is insufficient to remediate N deficiencies for insect hosts, and mutualistic N-fixing bacteria supply the balance (Peklo and Satava 1949, Pinto-Tomás et al. 2009).

Among wood-feeding insects, symbiotic N-fixation has been documented in termites (Tayasu et al. 1994), bark beetles (Bridges 1981), stag beetle larvae (Kuranouchi et al. 2006) and larvae of several longhorn beetle species (Grünwald et al. 2010). The prevalence of nitrogen fixation demonstrated to date in wood-feeding insects, as compared to foliage-feeding insects, suggests N-fixation is a critical adaptation enabling utilization of this low quality food resource. However, N-fixation

is energy intensive, requiring 12-24 ATP per N₂ molecule reduced to inorganic N compounds (Stam et al. 1987). As such, N-fixation is rapidly switched off where biological N is available (Kessler et al. 2001). An ability to fix atmospheric N has never been demonstrated in the basal Hymenoptera despite wood-feeding in three sawfly families (Siricidae, Anaxyelidae, Xyphidriidae). In this paper I examine nitrogen dynamics and symbiont relations in the siricid woodwasp, *Sirex noctilio* [hereafter: *Sirex*] for evidence of bacterial fixation of atmospheric (N²) gas supporting larval development in the nitrogen poor tissue of pine xylem.

In this study I document metagenomic, isotopic and experimental evidence of N-fixation in a wood-feeding sawfly. The gut of *Sirex* is atypical of fermentative digestion (Chapter 2). I assayed the bacterial community of *Sirex* and its carbohydrate metabolic pathways using metagenomic evidence. Bacterial associates were typical of internal associates of insects and animals, but were not as diverse as those commonly associated with wood digesting insects (Warnecke et al. 2007, Grünwald et al. 2010, Burnum et al. 2011). Furthermore, analysis of metabolic pathways suggests bacterial metabolism is targeted primarily at digestion of starch and sugar. Bacterial carbohydrate metabolic pathways indicate little capacity for fermentative breakdown of cellulose in wood biomass. N-limitation is typically met with efficient processing for extraction of this limiting nutrient. *Sirex* frass was enriched with N compared to food sources. *Sirex* and frass were tested for isotopic fractionation using stable isotope analysis. Stable isotopes indicated *Sirex* was diluted in ¹⁵N rather than enriched. Isotopic dilution occurs through inputs of ¹⁴N from atmospheric sources. Fixation of Atmospheric N was confirmed using gaseous ¹⁵N isotopic assimilation

experiments on *Sirex* larvae. Heavy isotope ^{15}N was elevated in ^{15}N treatments relative to controls providing evidence that bacterial N-fixation plays a role in nitrogen metabolism for larvae living on the N-depleted wood of pine trees. Absence of fermentation chambers negates efficient processing of wood for limiting nutrients.

I used isotopic assays of foragers and food sources to show that growth limiting N came from bacterial N-fixation and could not possible be derived in sufficient quantities from food sources. N-fixation was experimentally tested using ^{15}N isotope assimilation in gas tight chambers. The results of this study demonstrate the first example of N-fixation in wood-feeding sawflies adding to the growing body of literature showing multi-partite associations between insect-fungal mutualisms and their emergent importance in the evolutionary history of wood-feeding insects.

4.3 Methods

4.3.1 Sample Collection

Sirex larvae were collected from natural infestations on Red pines (*Pinus resinosa*) in Tioga County, PA (41°44' N; 77°18' W, May 2011). Sections of the trunks of 15 trees (15-32 cm diameter), showing resin beads from *Sirex* oviposition in the previous year, were felled, sectioned and transported to a quarantine facility in the Dept. of Entomology, University of Maryland, College Park, MD, 20742 (Permit # P526P-10-02796). In total, ninety bolts of *Sirex* infested red pine were collected. Larvae were isolated for experimentation by splitting bolts with the grain to reveal larvae in feeding cavities. An additional sample was collected from four trees two

years prior in the same area for metagenomic analysis of bacterial assemblages associated with larvae.

4.3.2 Microbiome Extraction

The bacterial microbiome was identified from a pooled sample of larval *Sirex* (n = 6) collected from natural infestations of red pines (n =4), as described above. Larvae were surface sterilized in 70% ethanol and washed prior to maceration and extraction of bacterial community DNA. Bacterial DNA was selectively isolated from the much larger pool of eukaryotic DNA using differential centrifugation as described in (Suen et al. 2010). Briefly, larvae were macerated in 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄) containing 0.1% Tween and then centrifuged for 5 minutes at 40×g to separate out bacterial cells. Bacterial cells formed a layer at the bottom of this mixture. The top layers were removed and washed three additional times to remove all remaining bacteria. The final solution containing bacterial cells was then centrifuged for 30 minutes at 2800×g, re-suspended in 1X PBS containing 0.1% Tween and filtered through a 100 um filter. Total DNA from this resulting sample was then extracted using a Qiagen DNeasy Plant Maxi Kit (Qiagen Sciences, Germantown, MD, USA). Extracted bacterial DNA from *Sirex* larvae was used to create a shotgun library which was then sequenced using a single pyrosequencing plate on a Roche 454 FLX GS Titanium sequencer (Margulies et al. 2005). Raw sequence reads generated for this microbiome will be deposited in NCBI's Short Read Archive.

4.3.3 Microbiome Community

The community bacterial metagenome of *Sirex* was binned into phylogenetic groups using marker genes for taxonomic classification in the program MetaPhyler (MetaPhylerV1.25.tar.gz; 16). Briefly, 31 marker genes provide phylogenetic reference for NCBI BLAST based identification of taxonomic groups based on taxonomic rank, reference gene, and sequence length, learned from the reference marker genes. The reference database includes marker genes from all complete genomes, several draft genomes and the NCBI nr protein database. The query metagenomic sequences were mapped to the reference marker genes using BLASTX. MetaPhyler classifies each sequence individually based on its best reference hit. The stringent classification strategy employed by MetaPhyler avoids assigning an organism to a lower-level taxonomic group if the evidence does not support this assignment, allowing identification of novel organisms or taxa.

4.3.4 Microbiome Carbohydrate Metabolism

The predicted proteome from the bacterial portion of the fungus garden community metagenome was annotated using the carbohydrate active enzyme (CAZy) database (Cantarel et al. 2009) following the approach of (Suen et al. 2010). In short, a local database of all proteins corresponding to each CAZy family from the CAZy online database (<http://www.cazy.org/>) was constructed, and this was used to align the predicted proteome of the bacterial portion of the larval community metagenome using BLASTP (e-value of 1e-05). This proteome was then annotated against the protein family (Pfam) (Punta et al. 2012) database (<ftp://ftp.ncbi.nih.gov/pub/mmdb/cdd/>) using RPS-BLAST (Marchler-Bauer et al.

2007) (e-value: 1e-05). A CAZy to Pfam correlation list was then compiled based on the secondary annotations provided through the CAZy online database. Finally, only those proteins that had significant BLAST hits to a protein from our local CAZy database and its corresponding Pfam were retained and designated carbohydrate-associated enzymes.

4.3.5 Nitrogen in *Amylostereum*

I tested N translocation in *Amylostereum* fungal cultures (n = 6) growing on 1 cm x 12 cm diameter slices of sterilized red pine xylem. Fungal cultures were isolated from adult female *Sirex* collected and identified in a previous study (Thompson et al. 2013). *Amylostereum* was inoculated onto the sterilized pine xylem and allowed to grow for two weeks after which blocks of wood ~0.25 cm³ were cut from the center, border and outside fungal growth for varying aged samples of wood decay. Samples were then dried in a convection drying oven (Lab-Line) at 45°C for two weeks before being ground to a powder with a mortar and pestle using dry ice. Total percent N from wood and fungal samples was analyzed on subsamples of *Amylostereum* on wood as described below. N concentrations in differing fungal growth zones were analyzed using generalized linear models for total percent N with a Poisson error distribution.

4.3.6 Nitrogen Concentrations in Larvae and Diet

Larvae and frass from natural infestation of red pine in Tioga County, PA were collected and dried as described above. Cores of xylem were collected from a healthy red pine in the vicinity of *Sirex* collection sites using a 5mm increment borer (Haglof Inc., Sweden) drilled to a depth of 5 cm in the sapwood. Samples were held

on ice during transport, dried and ground as previously described and frozen at -20°C until isotope analysis on a Carlo Erba 2500 Elemental Analyzer at the University of Maryland's Central Appalachians Stable Isotope Facility (Frostburg, MD). Wood, frass and larval tissue were ground as describe above and packaged into tin capsules prior to isotope analysis. All samples were submitted in a single submission and included control samples of insect (small hive beetle, *Aethina tumida*) and plant (*Metrosideros polymorpha* foliage) as sample controls in addition to carbon and nitrogen laboratory controls used at CASIF.

Means and standard deviations of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were calculated for larvae (n = 6), frass (n = 6), *Amylostereum* (n = 5) and healthy wood (n = 5). Isotopic fractionation in consumers causes an enrichment of ^{15}N . N-fixation causes the opposite effect due to incorporation of ^{14}N (dilution effect), justifying a one-way test for isotope discrimination. Concentrations of $\delta^{15}\text{N}$ between consumer (*Sirex* larvae) and food source (*Amylostereum*- infested and healthy red pine xylem) were compared using a one-tailed t-test between food sources. Wood did not differ statistically in $\delta^{15}\text{N}$ concentration between fungal infested and fungal-free samples and was subsequently combined for comparison with larvae. Isotope groups were checked for homogeneity of variance prior to analysis of variance and outliers were evaluated using Cook's distance criteria (Cook 1977), checked against internal standards for isotope concentrations and removed prior to statistical analysis if found aberrant.

4.3.7 Nitrogen Budget

The nitrogen budget of *Sirex* relative to food resource was calculated using the relative nitrogen-use efficiency (NUEa) versus the total amount of nitrogen available

to larvae in excavated galleries (n = 16). Adult *Sirex*, frass and healthy xylem were collected at the time of *Sirex* emergence and analyzed for percent C and N on a Carlo Erba elemental analyzer (Dept. of Earth Sciences, Dartmouth College, NH). Larval galleries corresponding to the collected adults were sequentially sectioned from point of oviposition to adult emergence holes for volume of wood in gallery (C_{wood}). Nitrogen assimilation (AN) was calculated using the difference in nitrogen content between frass and healthy wood given by:

$$AN = \% N_{wood} \cdot (C_{wood} \cdot NUE_a) \quad \text{Eq. 1}$$

where:

$$NUE_a = \frac{\% N_{wood} - \% N_{frass}}{\% N_{wood}} \quad \text{Eq. 2}$$

4.3.8 ¹⁵N Assimilation/Nitrogen Fixation

Endogenous N-fixation was experimentally tested on larvae using the ¹⁵N assimilation assay. Freshly extracted larvae from natural infestations of red pine were confined in gas-tight chambers with atmospheres enriched with ¹⁵N. *Sirex* larvae were placed in 12 mL gas-tight exetainer vials (Labco, UK) with a sheet of sterilized filter paper (Whatman) moistened with sterile deionized water, where ¹⁵N and ¹⁴N gas were introduced into larval chambers of treatment and control groups (respectively). N₂ gas was introduced to a volume approximating the natural N₂ concentration of air (~80%). The remaining chamber headspace was filled with O₂ (~20%).

In total, thirty *Sirex* larvae were removed from galleries in red pine and randomly placed into vials of three treatment groups. Only larvae measuring ~1.5 cm in length were included to control for any effects of larval instar or size (Madden 1981). Treatment groups consisted of living larvae exposed to ^{15}N , living larvae exposed to $^{14}\text{N}_2$, and autoclave sterilized larvae (121°C & 15atm for 15min) exposed to $^{15}\text{N}_2$. Autoclave sterilization killed larvae and their bacterial associates. Each treatment received ten larvae. Assimilation vials were then placed in the dark at 28°C for the duration of the experiment (65hrs) to minimize stress and disruption of fixation (Prestwich and Bentley 1981, Pinto-Tomás et al. 2009). At the termination of the experiment, condition of the larvae was evaluated by response to poking with a dissection teasing needle. Dead, pupated and otherwise unresponsive larvae were removed from the experiment prior to analysis of isotope concentration. Six complete reps of each treatment remained for isotope analysis. N-fixation rate was calculated using ^{15}N incorporation into larval tissue by analyzing the ratio of ^{15}N to ^{14}N ($\delta^{15}\text{N}$) in larval tissues. Ratios were measured in parts per thousand (‰) as is typical for isotope studies. Samples of larvae were dried, ground and quantified for isotope concentrations on a Carlo Erba Elemental Analyzer (as described above).

Insect and headspace samples were analyzed at the University of California-Davis, Stable Isotope facility using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) (solids) and a Thermo- Finnigan GasBench + PreCon trace gas concentration system interfaced to a Thermo-Scientific Delta V Plus isotope-ratio mass spectrometer (Bremen, Germany). The fraction of nitrogen derived from

nitrogen fixation (FNA) in the larvae was calculated according to the equation (Robertson et al. 1999):

$$FNA = \frac{AE_L}{AE_A} \quad \text{Eq. 3}$$

The percent ^{15}N excess in larvae (AE_L) was divided by the percent excess ^{15}N in headspace of respective treatments (AE_A) (Myrold, D.D. et al. 1999). Differences in the ratio of ^{15}N to ^{14}N ($\delta^{15}\text{N}$) measured as concentrations in part per mil were evaluated using Tukey HSD test for multiple mean comparisons.

4.3.9 N-fixation Budget Estimation

The estimated amount of N_2 fixation in *Sirex* can be calculated using the nitrogen derived from the atmosphere ($\%N_{dfa}$), through N fixation as described in Tayasu et. al., 1994, by the following equation:

$$\%N_{dfa} = \frac{\delta^{15}N_{food} + \Delta_{dig}}{[(\delta^{15}N_{food} + \Delta_{dig}) - \Delta_{fix}]} \cdot 100\% \quad \text{Eq. 4}$$

where: Δ_{dig} is $[(\delta^{15}\text{N}_{Sirex} - \delta^{15}\text{N}_{food})]$ represents the isotopic discrimination during the digestion of wood material by *Amylostereum* and Δ_{fix} is the isotopic discrimination from N_2 fixation. Since *Sirex* feeds in both fungal infested and non-infested wood tissue (Chapter 2), I used a mean $\delta^{15}\text{N}$ value calculated from $\delta^{15}\text{N}$ of fungal infested and uninfested wood (3.14 ‰). The $\delta^{15}\text{N}$ value of *Sirex* larvae in this study was -1.34

‰. For the Δ_{fix} I used the estimated values for a range of N-fixation ($-2 < \Delta_{\text{fix}} < -0.02$) as described in (Tayasu et al. 1994).

4.4 Results

4.4.1 Microbiome Community

The bacterial microbiome of larval *Sirex* was relatively species poor. Metagenomic analysis revealed only 12 genera of bacteria (Table 1). Intracellular α -proteobacteria (*Ehrlichia* and *Rickettsia*) were the most abundant bacteria found in larvae, followed by *Burkholderia*, *Cyanothece* and *Staphylococcus*. The top five genera found in this study accounted for ~72% of all bacterial DNA sequences extracted from larval *Sirex*. A majority of bacterial genera found in this study were facultative or obligate anaerobes suggesting they were primarily collected from internal environments (Table 1, ‘Sources’).

4.4.2 Microbiome Carbohydrate Metabolism

Analysis of functional carbohydrate metabolic pathways using gene annotations from the Carbohydrate Active Enzyme database (CAZy) revealed a majority of genes in the microbiome were involved in starch and sugar metabolism (~40%) (Table 2). Genes involved in binding and metabolism of cellulose, hemicellulose and xylan were the second most abundant (~30%), while the remainder of the genes were involved in binding and metabolism of chitin, peptidoglycan and phenolic compounds (Table 2). There was congruence between genes for the

construction of carbohydrate binding modules (CBM) and the carbohydrate enzymes (carbohydrate esterases (CE), glycoside hydrolases (GH), polysaccharide lyases (PL).

4.4.3 Nitrogen Budget

The nitrogen content of larval *Sirex* was $5.13 \pm 0.11\%$ N (mean \pm se). The xylem of healthy red pines examined in this study contained $0.04 \pm 0.01\%$ N. Pine xylem with *Amylostereum* growing on it was not significantly different from healthy pine in total percent nitrogen. Percent N did not differ between younger and older areas of fungal growth ($t = -1.1508$, $df = 3.7$, $p = 0.32$). Larval frass was significantly higher $0.07 \pm 0.02\%$ N compared to both healthy and fungal infested wood (respectively Tukey HSD, $p = 0.002$, $p = 0.004$; Figure 1). Given the higher concentration of N in the frass compared to dietary sources, the nitrogen use efficiency (NUE_a) for *Sirex* larvae was negative (-0.75%). Negative N use efficiency indicates a net contribution of N rather than a net extraction of N from food source.

4.4.4 Nitrogen Fixation/Trophic Position

Pine wood was not significantly different in $\delta^{15}\text{N}$ with or without the presence of *Amylostereum* and was grouped for statistical analysis (mean = 4.11 ± 1.2 ‰). *Sirex* larvae had a $\delta^{15}\text{N}$ of -1.12 ± 0.4 ‰, representing a dilution effect of -5.0 ‰ ^{15}N relative to food sources. The difference between *Sirex* and diet constituents was significant ($t = 4.2517$, $df = 9.851$, $p\text{-value} < 0.001$; Figure 2). Isotopic dilution of ^{15}N was also present in larval frass (-4.9 ‰). Absolute difference due to N inputs from N-fixation were equivalent to an isotopic shift of greater than one full trophic level in the opposite direction of typical trophic level shifts.

4.4.5 ^{15}N Assimilation

Heavy ^{15}N isotope was enriched in non-sterilized *Sirex* larvae relative to ^{14}N and autoclave sterilized controls ($F_{(2,21)} = 3.8, p = 0.038$). Over the 65 hr treatment larvae treated with ^{15}N gas showed increased $\delta^{15}\text{N}$ ratio ($0.0015 \pm 0.0006 \text{ ‰}$) over control treatments. Differences in ^{15}N were significant by post hoc multiple comparisons for autoclave sterilized larvae (Tukey HSD, $p = 0.05$; Fig. 4) and ^{14}N chambers (Tukey HSD, $p = 0.06$; Figure 3). $\delta^{15}\text{N}$ in treatment tissue (AE_p) differed by 0.00015% from the control ^{14}N treatment group. The volume of ^{15}N gas in the headspace (AE_A) differed by .067%. Therefore, the calculated $\delta^{15}\text{N}$ fixation rate during this experiment is 0.0002% of total larval N over the 65hr treatment period.

The estimated input of fixed N on the isotope ratios observed in larvae given the isotope ratios of larval *Sirex* and wood and fungal food resources and using the range of fixation ratios (-0.02 - -2.0), as described in Tayasu et al 1994, amounted to an estimated $\%N_{\text{dfa}}$ of 94 – 115% of larval N budget is derived from N-fixation. The estimated N from fixation was calculated using the average $\delta^{15}\text{N}$ for healthy and fungal infested pine tissue (3.59‰ $\delta^{15}\text{N}$), and a trophic discrimination factor (Δ_{dig}) of ~4.93‰. The significant negative signal in the ratio of ^{15}N to ^{14}N is characteristic of the isotopic fractionation typical to metabolic discrimination for the more reactive ^{14}N during nitrogen fixation by the enzyme nitrogenase.

4.5 Discussion

The xylem of woody plants contains 20-30% lignin, 40% cellulose, 30% hemicellulose and 0.04-0.1% nitrogen (Mattson 1980, Campbell and Sederoff 1996,

Meerts 2002). The combination of digestive and nutritional barriers prevents most herbivores from utilizing wood as a food resource, though many insects feed as saprophytes on dead wood in association with wood-rot fungi (Saint-Germain et al. 2007, Weslien et al. 2011). Despite its ubiquity in terrestrial ecosystems, terrestrial wood feeding has arisen in only three insect orders, Blattodea, Hymenoptera and Coleoptera (Schulmeister 2003, Inward et al. 2007, Hunt et al. 2007). Only 23 insect families contain members that colonize wood in North America (McMinn et al. 1993). Of those 23 wood-feeding families only four colonize living wood the rest are associated with colonizing rotting wood (Klepzig et al. 2009). Adaptations for wood-feeding and the ubiquity of wood in terrestrial environments are thought to be responsible for historical radiations of terrestrial insects (Schulmeister 2003, Inward et al. 2007, Hunt et al. 2007). In the Hymenoptera, adaptations for colonization and wood-feeding in the Siricidae (woodwasps) and the extreme nutritional deficiencies surrounding wood-feeding led to subsequent adaptations for cheating around nutritional constraints, such as parasitism of the fungal mutualists (e.g. Xiphydriidae) and eventually parasitism of conspecific wood-feeders (e.g. Orussidae) (Smith 1988, Schulmeister 2003).

Symbiotic N-fixation is energy intensive requiring 12-20 ATP per molecule of N fixed (Stam et al. 1987). In *Sirex*, high energy molecules are acquired by larvae through ingestion of plant molecules externally digested by fungal mutualists (Chapter 2 & 3). Unlike other wood feeding insects, the gut of *Sirex* is thin and lacks fermentative chambers (Chapter 2). Morphologically a thin alimentary tract increases passage rate, precluding time intensive processing such as fermentation of cellulose

(Snodgrass 1935). Metabolic genes within gut bacterial communities reflect their metabolic environment (Woyke et al. 2006, Turnbaugh et al. 2006, Warnecke et al. 2007). Analysis of the microbiome of *Sirex* supports a hypothesis for preferential ingestion of easily assimilable compounds. Although low in both abundance and diversity, bacterial associates of *Sirex* contained genes that reflected processing of predominantly starch, glucose and easily assimilable mono- and disaccharides.

Siricid sawflies engage in mutualisms with basidiomycete fungi that actively digest wood (Leonowicz et al. 1999). Larval *Sirex* fed within the active zone of fungal digestive enzymes (Chapter 2). Fungal enzymes contributed to digestion in *Sirex*, but not to the N budget. Nitrogen concentration was not different across old and young areas of fungal growth. Growth of *Amylostereum* is sparse in wood (Thompson et al. 2013). It is possible differences in concentration are not detectable until fungal colonies reach high densities, but this would not help *Sirex* larvae as they feed preferentially at the border of fungal growth in wood (Chapter 2).

Nitrogen concentrations in healthy pine wood were extremely poor (0.04% N), but larval *Sirex* contained N concentrations typical of herbivorous insects ~5.1% N. N assimilation by insects living on N poor food sources is predicted to be high, but in this study, I found larval frass had a higher concentration of N than the food products from which it was produced. Increased N in frass suggested *Sirex* obtained N from an additional source. The negative isotopic shift of frass and *Sirex* larvae suggested the additional source was from fixation of naturally ^{14}N biased atmospheric N. Analysis of larval N budget predicted the volume of wood consumed by larvae would provide no more than 10% of the total N needed by larvae.

Bacterial N fixation creates a characteristic isotopic shift (dilution) toward ^{14}N due to the isotopic fractionation due to the higher reactivity of ^{14}N in the rate determining step of metabolic reactions (Unkovich 2013). Incorporation of predominantly ^{14}N into host tissue caused a dilution effect in *Sirex* larvae that suggested atmospheric sources of N. *Sirex* was below its food source by a full trophic level indicating significant input of fixed N in its diet. Frass exhibited a dilution effect as well, suggesting extra N in frass was supplied by inputs from the larvae excreting excess waste products of metabolism, most likely uric acid. Tests for biological N-fixation using a ^{15}N isotope assimilation assay confirmed the presence of N-fixation in larvae. Larvae in the presence of excess $^{15}\text{N}_2$ exhibited elevated concentrations of ^{15}N in their tissues relative to negative controls.

Calculated fixation rates from $\delta^{15}\text{N}$ ratios in food and larval tissues suggested >90% of nitrogen in *Sirex* larvae was derived from bacterial fixation pathways. N-fixation can only be carried out by bacteria and only in the absence of oxygen. The location of N-fixation in *Sirex* is currently not known, but mycetocyte-like organs identified in larval morphological examination (Chapter 2) suggest N-fixation takes place in the salivary glands. Preliminary data using primers for the nitrogenase gene indicate the presence of a nitrogenase gene associated with mycetocytes in the salivary glands (Thompson unpublished). Fixation in salivary glands may be ideal given the readily available source of N_2 gas entering through the tracheal system and has been identified in fungus growing termites (Gomathi et al. 2005). Identification of bacteria and location within the body are the logical next step. Numerous members identified in the metagenome of *Sirex* are candidates for nitrogen fixation, including

Ralstonia, *Ehrlichia*, *Rickettsia* and *Burkholderia* (Harriott et al. 1995, Lilburn et al. 2001, Verma et al. 2004, Perlman et al. 2006, Kikuchi et al. 2011, Bowman 2011).

By colonizing living pines, siricid sawflies occupy wood that has largely been unmolested by saprophytic fungi and as such is expected to have a higher content of easily assimilable starch and sugar compounds, but also higher levels of defensive chemistries (Boddy 2001, van den Brink and de Vries 2011). Fungal mutualists liberate easily assimilable starch and sugars for siricids, but may also play a role in detoxification. Fungi were thought to play a major role in N concentration for siricid larvae, but this pathway was not evident from this study. Instead phylogenetic ecological evidence from the basal sister group to the siricids the Stem sawflies (Hymenoptera: Cephidae) suggest feeding in N poor habitats preceded associations required for the metabolism of wood, though further research to determine this point is needed (Gauld and Bolton 1988). It is likely that further examination of wood-feeding and stem boring sawflies will reveal N-fixation in related groups. The basal Hymenoptera (sawflies) exhibit a gradual trend from external foraging to enclosed and eventually internal feeding (Schulmeister 2003). In *Sirex*, as it has been increasingly found in herbivorous insects, multiple associations with microbial partners enrich the capabilities of their host and enable the colonization of inhospitable resources. Further investigation will likely show that what has been found here is generally ubiquitous within the Siricidae and across insects feeding on nutrient poor and enzymatically refractory resources.

4.6 Figures & Tables

4.6.1 Tables

Table 1. Bacterial microbiome of larval *Sirex* identified to Phylum and Genus level using MetaPhyler. Percentage of genomic reads of the bacterial metagenome coding to a particular Genus (Percentage) and the historical habitat (Habitat) and reference for known ecological roles of the genus (Source).

Phylum	Genus	Percentage	Habitat	Source
α - Proteobacteria	<i>Ehrlichia</i>	18.5%	Intercellular pathogen / symbiont	(Bowman 2011)
α - Proteobacteria	<i>Rickettsia</i>	16.9%	Intercellular pathogen / symbiont	(Perlman et al. 2006)
β - Proteobacteria	<i>Burkholderia</i>	13.8%	Ubiquitous / animal associated environments	(Coenye and Vandamme 2003)
Cyanobacteria	<i>Cyanothece</i>	12.8%	Marine environments / endosymbionts	(Mazard et al. 2004)
Fermicute	<i>Staphylococcus</i>	10.8%	Animal associated environments	(Grice and Segre 2011)
γ - Proteobacteria	<i>Salmonella</i>	6.9%	Animal associated environments	(Winfield and Groisman 2003)
β - Proteobacteria	<i>Ralstonia</i>	6.8%	Soil / insect / plant xylem pathogenic	(Remenant et al. 2011)
Archaeota	<i>Pyrococcus</i>	6.0%	Hydrothermal vents / bacteriocyte	(Heddi et al. 2005)
δ - Proteobacteria	<i>Desulfovibrio</i>	3.0%	Environmental / animal gut environment	(Sato et al. 2009)
β - Proteobacteria	<i>Dechloromonas</i>	2.1%	Ubiquitous environmental / animal associated	(Salinero et al. 2009)
Chloroflexi	<i>Dehalococcoides</i>	2.0%	Environmental / animal associated	(Mohr and Tebbe 2006)
γ - Proteobacteria	<i>Escherichia</i>	0.4%	Ubiquitous / animal associated	(Winfield and Groisman 2003)

Table 2: Carbohydrate active enzymes (CAZy) of the *Sirex* microbiome. Genes families (CAZy Family) are involved in carbohydrate binding (CBM) and carbohydrate metabolism (carbohydrate esterase (CE), glycoside hydrolase (GH), polysaccharide lyase (PL)); raw count of genes (# Genes), percent of all CAZymes (Percent CAZy)

CAZy Family	Substrate	# Genes	Percent CAZy
CBM (32,35,47,48, 51)	Starch and sugar	15	10.27%
CBM (5)	Chitin	1	0.68%
CBM (57, 6)	Plant polymers (cellulose, hemicellulose, xylan)	11	7.53%
CE (1)	Plant polymers (cellulose, hemicellulose, xylan)	3	2.05%
CE (10)	Phenolic compounds	17	11.64%
GH (0, 1, 3, 30, 43, 44)	Plant polymers (cellulose, hemicellulose, xylan)	31	21.23%
GH (2, 9,13, 20, 27, 31, 35, 37, 47, 84, 89, 99)	Starch and sugar	50	34.25%
GH (18)	Chitin	6	4.11%
GH (23, 73)	Peptidoglycan	12	8.22%
Totals		146	99.98%

4.6.2 Figures

Figure 1. The total percent nitrogen of food sources and frass in natural infestations of *Sirex* in red pines (*Pinus resinosa*). Letters represent groups that are significantly different by Tukey HSD test, $p \leq 0.05$.

Figure 2. $\delta^{15}\text{N}$ stable isotope values of larvae and food sources; * one-way test for trophic enrichment between consumer (*Sirex*) and diet resources (Pine & Pine + Fungal symbiont); $t = 4.2517$, $df = 9.851$, $p\text{-value} < 0.001$. Dietary sources (Pine + Fungal Symbiont & Pine) were not statistically different and were combined for comparison to $\delta^{15}\text{N}$ of *Sirex* larvae (*Sirex*).

Figure 3. Larvae exposed to ^{15}N labeled gas (N_2) (Challenged) had a higher $\delta^{15}\text{N}$ than either gas exposed sterilized (^{15}N Autoclaved) or ^{14}N treated (14N Control) larvae; letters represent statistically significant groups by Tukey HSD, $p \leq 0.05$.

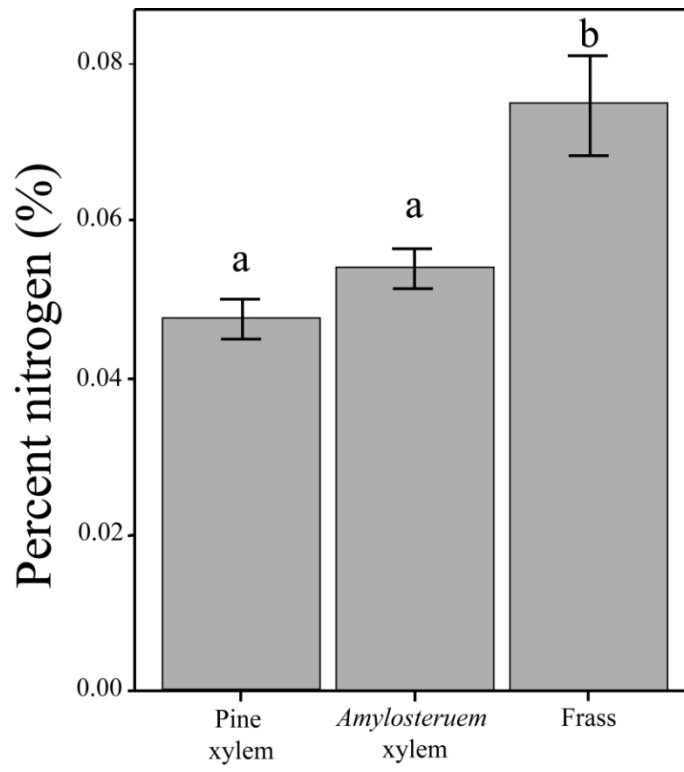


Figure 1

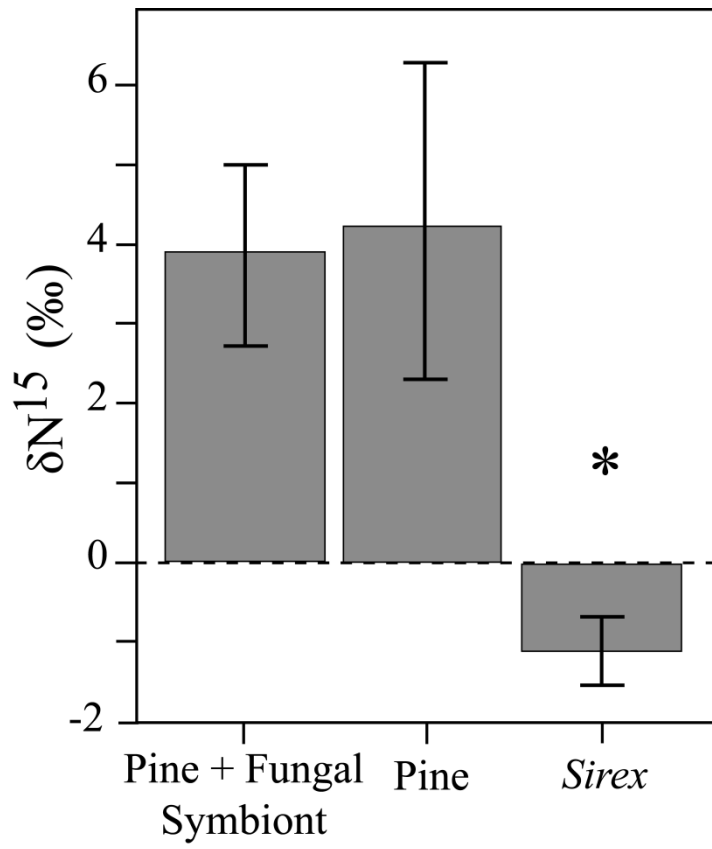


Figure 2

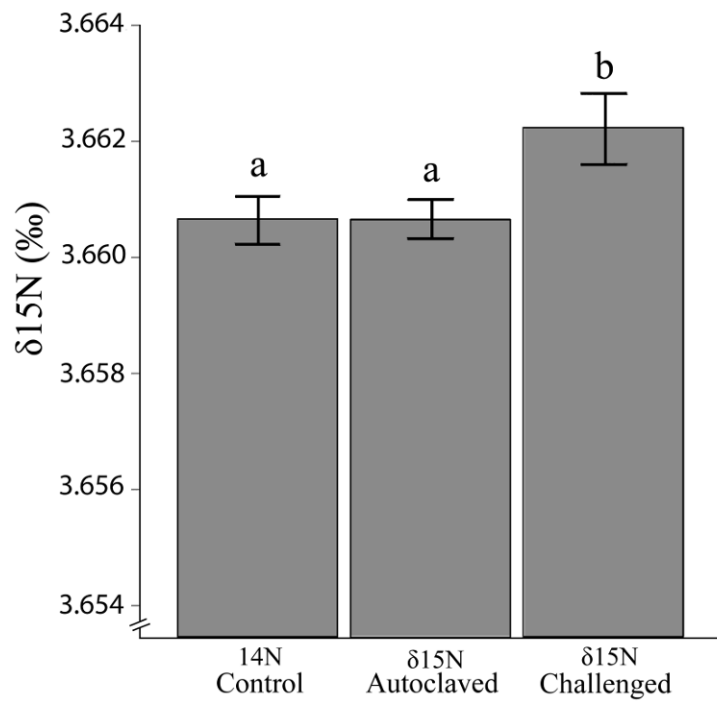


Figure 3

Chapter 5: Two is company, three's a crowd: biotic resistance to the European woodwasp (Siricidae: *Sirex noctilio*) and its fungal mutualist in North America

5.1 Abstract

The hypothesis of biotic resistance to invasion by non-native species is typically evaluated at the individual species level, but where symbiotic associations are concerned, fitness is dependent upon all partners. In this study, I combined survey data with field and benchtop experiments to evaluate biotic resistance to the invasion of the European woodwasp, *Sirex noctilio*, and its basidiomycete mutualist *Amylostereum areolatum* in North America. Intraguild predation on larval *Sirex* by presumed saprophytic larval *Xylotrechus* (Coleoptera: Cerambycidae) was ubiquitous in natural infestations. Multi-model inference showed intraguild predation was positively correlated with *Sirex* density, but negatively correlated with the abundance of *Ips* bark beetles (Coleoptera: Curculionidae: Scolytinae). Intraguild predation was negatively influenced by *Ips* through negative correlation between *Sirex* and *Ips* densities in pines. In paired experimental manipulations of *Sirex* attack of healthy red pines (*Pinus resinosa*) repeated over three consecutive years, I evaluated insect community attraction to *Sirex* colonization of red pine. I recovered 213 insect species from *Sirex* attacked pines. Canonical Correspondence Analysis (CCA) of species collections relative to the number of *Sirex* ovipositions and tree diameter revealed attraction of saproxylic species to *Sirex* attacked trees. Parasitic and predatory insects

showed variable attraction within guilds, with most species unresponsive to *Sirex* attack of trees. *Ips* was abundantly attracted to *Sirex* attacked trees in field manipulations. In benchtop trials with the fungal symbionts from *Sirex* and *Ips*, the fungal symbiont of *Ips* (*Ophiostoma*) outcompeted the symbiont of *Sirex* (*Amylostereum*) for space in wood in every instance except where *Amylostereum* was given one week of advanced growth. Although *Sirex* and *Ips* larvae feed in distinctly different microhabitats within trees (xylem and phloem, respectively), *Ophiostoma* extends into the microhabitat of *Sirex* and interferes *Amylostereum*. The dominance of *Ophiostoma* was dependent upon colonization timing which suggests mechanisms for how and when competition will negatively impact *Sirex* populations, such as that predicted by intraguild predation models. The patterns of interaction observed in this study suggest multiple pathways for biotic resistance to *Sirex* in North America. In previous introductions, pine plantations harbored low insect diversity precluding the saproxylic biotic resistance pathways documented in this study.

5.2 Introduction

Variable success of introduced species in recipient environments is a focal point of ecological research and theory (Lonsdale 1999, Paine 2006, Richardson and Pyšek 2008, Pyšek et al. 2010). Understanding the factors behind species range limits and expansion events requires knowledge of how both abiotic and biotic factors limit establishment, population growth and expansion in novel environments (Kellermann et al. 2009, Moeller et al. 2012). Abiotic variables are powerful determinants of species transport and colonization (Theoharides and Dukes 2007). If a species persists

through the gauntlet of environmental filters, antagonistic interactions within recipient communities have increasing influence on species distribution and abundance in the introduced range (Case et al. 2005). Theory suggests that predation, parasitism and competition within diverse communities may affect invasibility in a similar manner to the effects on range boundaries of species in native ranges (Shea and Chesson 2002, Holt and Barfield 2009). Species introduced to diverse environments face diverse interactions that may increase mortality and competition on already small and potentially genetically homogenous founding populations (Klüber and Eckert 2005, Dlugosch and Parker 2008, Ahern et al. 2009, Kinziger et al. 2011). Where introduced species are obligately associated with microbial mutualists, the totality of interactions on all members of the symbiotic complex may determine invasibility.

Microbial symbionts are often overlooked in invasive process, but have played a key role in invasiveness in plant and insect systems (Richardson et al. 2000, Rudgers et al. 2005). Each partner in a symbiosis may be differentially affected by novel biotic and abiotic factors in the recipient community, leading to differential adaptation of the host and its microbial mutualist and altered invasion dynamics (Yan et al. 2005, Hulcr and Dunn 2011, Lu et al. 2011). Novel environments can select for novel characteristics in one or both partners in introduced ranges (Callaway et al. 2004, Callaway and Ridenour 2004), such as increased pathogenicity of fungal symbionts (Yan et al. 2005). Alternatively, symbiotic associations may confer a disadvantage if biotic resistance to one member of the symbiotic complex inhibits the establishment of the whole complex.

Microbial mutualisms in insects are involved variously in host colonization, nutrition, and resistance to natural enemies (Klepzig et al. 2009). External symbionts that derive benefit from insect association through transport to and/or colonization of plant resources but which are not protected within internal compartments (mycetomes/bacteriomes) of their host insect, such as filamentous fungi found in bark beetles, woodwasps, termites, and leaf-cutter ants, are subject to interactions with species within the local environment and the consequences therein (Cartwright 1938, Graham 1967, Klepzig et al. 2004). Interactions between mutualists and biotic antagonists (i.e. competitors) have consequence for colonization and range expansion of both partners (Bleiker and Six 2009, Moeller et al. 2012). Eusocial insects (e.g. leaf-cutter ants & termites) manage external antagonism through coordinated labor ‘fungus farming’ (Mueller and Gerardo 2002), but this technique is not available to solitary insects. Instead, solitary insects are mobile and colonize new substrates on a yearly basis providing new hosts for fungal mutualists (Spradbery and Kirk 1978, Bleiker and Six 2009), which avoids the need to combat antagonists.

Microbial mutualists can significantly improve resource quality for insect partners, but may also lead to increased interaction with antagonistic species (Martinez et al. 2006, Boone et al. 2008, Weslien et al. 2011). For tree killing insects, such as bark beetles and some woodwasps, colonization of trees creates opportunity for assemblages of insects and fungi specialized for feeding on undefended and otherwise decaying plant matter (saprophytes) (Jonsell and Nordlander 2004, Jonsell et al. 2005). Opportunistic saproxylic species may facilitate, compete with, or even kill heterospecific species through intraguild predation and competitive interactions

(Dodds et al. 2001, Boone et al. 2008, Fukami et al. 2010, Coyle and Gandhi 2012). These interactions may be especially important in nutrient poor environments where external symbionts contribute significantly to host insect nutritional demands. In wood-rot fungi, assembly history and competitive ability factor into insect and fungal assemblages (Saint-Germain et al. 2007, Fukami et al. 2010, Weslien et al. 2011).

I assessed the importance of community interactions for a globally invasive woodwasp and its fungal mutualist in red pine (*Pinus resinosa*) in their introduced North American range. North America is biologically diverse compared to previous introduction areas for the European woodwasp, *Sirex noctilio*, [hereafter, *Sirex*]. In previous introductions, *Sirex* outbreak populations were punctuated with extreme pine mortality (15 - 80%) and abnormally high population density (Wermelinger et al. 2008, Villacide and Corley 2012). Population dynamics of *Sirex* in North America have thus far followed a significantly different trajectory causing only 3 - 8% mortality and typically targeting only weak and/or stressed trees (Dodds et al. 2010) as is more typical of its native range (Spradbery and Kirk 1978, Wermelinger et al. 2008). Although variation in the invasiveness of *Sirex* in introduced ranges is likely affected by both abiotic and biotic factors, climatic conditions across the introduced range have been predictive of invasiveness (Carnegie et al. 2006).

The introduction of *Sirex* to North America differs from previous introductions to the Southern hemisphere in two major ways: 1) pines are not native to any of the previous introductions and occurred as monotypic pine plantations; and 2) habitats in the Southern hemisphere locations lacked many elements of a diverse pine-adapted insect community (e.g. saproxylic/herbivorous insects, parasitoids,

predators, etc.). In contrast, North America is a center of diversity for *Pinus* spp. and contains diverse assemblages of potential competitors and natural enemies similar to the native European range of *Sirex* (Wermelinger et al. 2008). The presence of diverse insect communities and associated microorganisms and the coincident observed decreased invasiveness of *Sirex* suggest native diversity may inhibit *Sirex* in North America, though formal description of mechanisms behind decreased invasiveness remains unclear.

In this study I assessed biotic resistance to invasion by the symbiotic insect-fungal mutualism between *Sirex* and the basidiomycete white-rot fungus *Amylostereum areolatum* [hereafter: *Amylostereum*], using a combination of observational and experimental approaches to quantify the roles of competition, predation and parasitism in introduced populations of *Sirex* in North America. Specifically I: 1) quantified patterns of association between native insects and *Sirex* in natural infestations of red pine; 2) tested community attraction to *Sirex* colonization of trees in a three year replicated field study; and 3) experimentally tested the mechanism of competition between *Amylostereum* and, *Ophiostoma*, a native fungal associate of bark beetles using benchtop experiments. These studies indicated substantial overlap in pine colonization between *Sirex* and North American insects and highlighted diverse pathways for biotic resistance in the species rich native pine forests of North America.

5.3 Methods

5.3.1 Study System

Sirex has a broad host range among North American pine species (incl. *Pinus resinosa*, *P. strobus*, *P. rigida*, *P. taeda*, *P. radiata*). Red pines (*P. resinosa*) are ubiquitous within the current range of *Sirex* in North America and are frequent hosts for *Sirex* (Hajek et al. 2009, Eager et al. 2011). *Sirex* kills living red pines through the action of a phytotoxic mucous and the phytopathogenic white-rot fungus, *Amylostereum* injected into the xylem of target trees along with eggs during oviposition. Larvae feed in close association with *Amylostereum* in pine xylem (Chapter 2 & 3). *Sirex* attacked trees rapidly wilted due to the effects of the phytotoxin, but are eventually killed by phytopathogenicity of *Amylostereum* (Talbot 1977, Kukor and Martin 1983). I studied the effects of *Sirex* attack on living red pines and the response of native insects in a mixed red pine hardwood ecosystem at the leading edge of the current range (as of 2009) of *Sirex* in North America (Tioga County, Pennsylvania, USA [41°44' N; 77° 18'04" W]). *Sirex* introduction to the study area preceded this study by approximately 1-2 years.

5.3.2 Community Survey

Native North American insect species coexisting with *Sirex* in red pines were assessed by felling *Sirex* killed red pines and capturing insects emerging from *Sirex* killed trees using emergence chambers (see below). Trees were felled (n = 12) from May- July of each year and sectioned to 50 cm lengths in years 2010 & 2011. Up to five bolts from each tree were held in emergence traps (~96 total bolts) constructed from 27 gal storage totes (Home Depot, HDX Model # 207585) and fit with

ventilation holes and a collection chamber consisting of a clear cup (SOLO[®]) with the bottom removed and the mouth with aluminum screen glued across it. Emerging insects were drawn into the collection chamber by placing a 75 watt lamp facing the emergence chambers. Insects were collected once a week from May – October and stored in 70% ethanol. The observed rearing period overlapped the natural emergence period of *Sirex* by three months. In 2011, all ‘emergence’ bolts were peeled and split to < 1 cm diameter at the end of the season to remove adult and larval insects. A subset of bolts was haphazardly chosen from all possible bolts and split prior to emergence of *Sirex* for dissection and evaluation of within tree interactions. All insects were identified to genus/species wherever possible.

Emergence phenology of saproxylic insects from red pines was constructed for the most abundant species of parasitoids (*Ibalia leucospoides* & *Rhyssa lineolata*) and saproxylic beetles, *Ips pini* (*Ips*), *Tetropium cinnamopterum* (*Tetropium*), Melandryidae, using emergence dates and abundance data from emergence chambers. A subset of 36 additional logs was held outdoors under ambient conditions in emergence chambers at the study location for comparison to chambers held inside. Emergence phenology did not differ appreciably between indoor and outdoor samples and was combined for phenology assessment. Insect emergence phenology from red pine bolts was constructed using the R package ‘Phenology’ (Girondot 2013). Cerambycidae (longhorned beetles) are saproxylic, feeding on woody plants either healthy or in various stages of decay. Intraguild predation is a recurrent theme among saproxylic insects and cerambycid larvae (Dodds et al. 2001, Ware and Stephen 2006, Schoeller et al. 2012). *Xylotrechus saggitatus* [hereafter: *Xylotrechus*], is a

longhorned beetle associated with dead pines of the east coast of North America (Arnett et al. 2002). *Xylotrechus* was frequently encountered as larvae in the trunk of dead *Sirex* attacked red pines. Larvae of *Sirex* and *Xylotrechus* leave characteristic tunnels (galleries) in wood. I followed galleries of *Xylotrechus* and *Sirex* through the xylem looking for signs of intraguild predation. Bolts were sequentially split to reveal the galleries of wood boring larvae. Signs of intraguild predation in galleries included direct observations or latent indicators, such as abrupt disappearance of larval *Sirex* at junctions of *Sirex* and *Xylotrechus* tunnels. Predation of *Sirex* was only concluded if mandibles and the anal spine of *Sirex*, which are not digested by *Xylotrechus*, could be recovered from a suspect site. Intraguild predation was often preceded by *Xylotrechus* larvae following the larval tunnels of *Sirex*. Counts of community members and intraguild predation were standardized by tree surface area for each bolt prior to statistical analyses.

Ips pini abundance in study bolts was calculated from counts of emergence holes. Bolt moisture was measured prior to log dissection, but correlated closely with bolt diameter and was not used for population models. *Xylotrechus* has a 2 -3 year life-cycle and was counted solely from larval populations (Arnett et al. 2002). Predation on *Sirex* by *Xylotrechus* larvae was modeled using generalized linear models with the log-link function and a Poisson distribution to minimize deviance in the residuals using the R program for statistical analysis (R Development Core Team 2009). Candidate models for predation were evaluated for differing combinations of plant and insect community factors and compared using AIC model selection in the ‘AICcmodavg’ package in R (Mazerolle 2012). Generalized linear models were fit for

predation by density of *Sirex* and *Sirex* density by density of bark beetles as these variables came out as significant in model selection. These variables were then plotted to investigate the nature of the relationship between these key variables.

5.3.3 Fungal Competition

Ips pini [hereafter: *Ips*] and its associated blue stain fungal symbiont *Ophiostoma ips* [hereafter: *Ophiostoma*] were commonly encountered in field surveys of *Sirex* infested trees. When *Ips* occurs in *Sirex* attacked pines, emergence of adult *Sirex* is typically poor (K. Dodds, unpublished). I examined competitive interactions between the symbionts of *Sirex* and *Ips* in controlled laboratory settings to examine functional mechanisms for this phenomenon. A pure culture of *Ophiostoma* was obtained from Dr. Aaron Adams (Univ. of Wisconsin). Pure cultures of *Amylostereum* were obtained from wild caught adult female *Sirex* as described in (Thompson et al. 2013). Collected females were killed via freezing at -20°C for 2 minutes immediately prior to dissection. Females (n = 6) were then surface sterilized in 70% ethanol and washed twice in sterile 1M phosphate buffered saline (pH 7.4) (Sigma). Mycangia were then extracted using ethanol sterilized stainless steel forceps as described in (Thomsen and Harding 2011). Mycangia were mechanically disrupted in PBS and mixed thoroughly prior to transfer to artificial media for fungal colony selection and purification. Liquid suspensions from each extraction were transferred to semi-solid potato dextrose agar (Sigma-Aldrich) as 20µL volumes and spread plated plate to separate colonies. Plates were incubated at room temperature for three days. Three colonies were then taken from each plate and serially transferred for using autoclave sterilized toothpicks picking up a small outer portion of each fungal

colony (Thompson et al. 2013). Nuclear DNA of fungal isolates was extracted using the enhanced yield protocol of the Mo Bio PowerSoil[®] DNA Isolation Kit (Mo Bio Laboratories). Fungal isolates of *Amylostereum* were identified by amplification and sequencing of the rDNA internal transcribed spacer region using the ITS1-4 primer pair according to the protocol of (Gardes and Bruns 1993). Sequences were first annotated by visual inspection of trace files and then identified by best-hit comparison using blastn search in the nucleotide database in Genbank (Altschul et al. 1990), with a ($\geq 90\%$) sequence similarity cutoff at the nucleotide level to the closest database match. The sequences from the six fungal samples extracted from female *Sirex* were deposited in Genbank[®] under accession numbers JX035728- JX035739.

Fungal cultures of *Amylostereum* were assayed for competitive ability against *Ophiostoma* on autoclave sterilized pine xylem and on artificial media (PDA). *Amylostereum* isolates were assessed for competitive ability via inoculation into sterile plastic petri plates containing either sterilized agar or wood (petri size 90 X 15mm (agar), 150 X 15mm (wood)) and either a control (sterile agar) or a challenger (agar plug with *Ophiostoma*). Sterilized pine xylem consisted 1cm thick slices from the trunk of a single ~14cm diameter healthy red pine that were autoclave sterilized (121°C x 15atm x 1hr). *Amylostereum* and *Ophiostoma* were inoculated to challenge arenas as ~1mm³ plug of agar containing fungal hyphae taken from the edge of active growing pure fungal cultures. Distance was used as a proxy for investigating differential competition at various fungal colony sizes. *Amylostereum* isolates were inoculated on the growth medium at a set distance apart (0, 1.5, 3.0cm) from either a challenger or a negative control. Cultures were then placed in the dark at room

temperature (20°C) and monitored daily for colony growth. Competition between fungi was assessed at the end of each trial as the relative area of fungal colonies on the surface of their substrate relative to negative controls. Relative colony area (mm²) was assessed for both *Amylostereum* and *Ophiostoma*. Colonies were grown for 11 days, at 0 and 1.5 cm separation, and 16 days with 3 cm separation to allow sufficient time for competitive interactions. Fungal colonies were not space/resource limited in any of the trials.

5.3.4 Community Attraction

Using a paired treatment design, I evaluated the attraction of insect species to *Sirex* attack/oviposition on red pines (*Pinus resinosa*). Healthy red pines positioned < 10m apart were paired into treatment groups of similar diameter (< 4cm difference). Treatment pairs were separated by a minimum of 25m. In 2009 (n = 20) and 2010 (n = 14) trees of each pair were randomly assigned to either *Sirex* attacked or no insect (control). *Sirex* attacked treatments were caged with five unmated adult females for a period of 72 hrs, over which time females oviposited into the trunk of treatment trees. Cages for *Sirex* attacked and control trees were affixed at ~1.3m above the ground and covered approximately 60cm of the tree trunk. Cages and insects were removed after 72hrs and the number of ovipositions tabulated. A strip (15 x 91.4cm) of steel 1.25cm gauge hardware cloth was then affixed vertically over the area of the tree receiving the greatest wounding in the *Sirex* attack treatment. Sticky traps were affixed in an identical orientation on the paired Control treatment tree. Hardware cloth strips were coated with Tangle-Trap paste (Contech Inc.) to trap visiting insects. All treatments were initiated on the same day and roughly corresponded to the peak

emergence season for *Sirex* in this area (Approximately August 1st – 15th). Traps in all years were visited bi-weekly from initiation to the first week of November. Traps were visually inspected and all Hymenoptera and Coleoptera were removed using forceps and placed in Citrasolv (Citra Solv LLC) for removal of Tangle-Trap. Insects were later transferred to 70% ethanol for long-term storage and identification. Insects were identified to genus or nearest species level identification for statistical analysis.

In 2011, treatment trees (n = 24) were divided equally among three treatments, the two original treatments and an additional mechanical damage treatment. Mechanical damage treatment was added to assess whether insect responses relative the specific damage caused by *Sirex* oviposition (i.e. physical+ phytotoxic + fungal damage) differed from simple tree wounding responses. Mechanical damage treatment mimicked physical damage caused by *Sirex* and consisted of puncturing the trunk of pines using a 16 gauge drill bit (~1.3mm) to a depth of 1 cm (approximate depth of *Sirex* ovipositions). Mechanical damage intensity was scaled to that of the orientation and number of ovipositions on the paired *Sirex* attacked treatment tree in the treatment group. ‘Mechanical Damage’ was performed 24hrs prior to removal of cages and the fixation of sticky-traps.

Analysis of catches from sticky-traps were restricted to the insect orders Coleoptera and Hymenoptera. These speciose groups contained the majority of trap catches in this study and constitute the majority of pine forest herbivores, predators, and parasitoids known to associate with the trunks of pine trees (Vanderwel et al. 2006, Saint-Germain et al. 2007, Coyle and Gandhi 2012). Shannon’s diversity (H)

and Pielou's evenness (J) were computed for the insect communities of each treatment group using the 'vegan' package in R (Oksanen et al. 2011).

Sticky-trap data was pooled by treatment group for each year and analyzed using ordination. Variation between years due to the use of live females and variable performance at study initiation precluded pooling treatments across all years.

Community attraction to treatment groups was assessed using canonical correspondence analysis (CCA) in the vegan package (Oksanen et al. 2011).

Ordinations were performed on species counts relative to the continuous variables, oviposition number and tree diameter for each treatment group. CCA was performed for full community data (all species) and for subgroups consisting of functional feeding groups (guilds). Insects were grouped into saprotrophic, predatory, parasitic and herbivorous feeding guilds based on known ecology of the species or closest relative, often at the genus or family level. Rare species present special problems for statistical analysis and are difficult to assess in terms of ecosystem processes (Barlow et al. 2010). Singleton and doubleton species were removed prior to ordination to improve assessment of the roles of more abundant species. Permutation tests were performed on the constraining variables (oviposition & tree diameter) using the ANOVA function and default settings for number of permutations (Oksanen et al. 2011). Permutation tests from ordinations were performed on full community and guild datasets within each year. Herbivore and predator guilds were poorly represented in insect collections and were not amenable to permutation analysis. Species feeding on the wood of pines that are typically associated with fungi were grouped within the guild 'Saprophytes'.

Univariate species responses were constructed for the most abundant insect species collected in traps. Univariate responses were statistically evaluated between treatment groups using ANOVA F-tests with a significance level of $p < 0.05$ (R Development Core Team 2009). Treatments were compared within collection year. The three species showing the greatest response to *Sirex* oviposition (*Xylotrechus saggitatus*, *Ips pini* and *Ibalia leucospoides*) were analyzed using generalized linear models with the Poisson error distribution. Homogeneity of variances were checked prior to statistical analysis as described previously.

5.4 Results

5.4.1 Community Survey

Red pines killed by *Sirex* contained numerous species of parasitic Hymenoptera and saproxylic Coleoptera. *Sirex*'s peak emergence from natural infestations was from 22nd July - 5th August 2011 (Figure 1a). The peak emergence of *Sirex* overlapped the emergence phenology of *Ibalia leucospoides* (Ibaliidae: Ibaliinae) & *Rhyssa lineolata* (Ichneumonidae: Rhyssinae), the most abundant parasitoids of *Sirex* in North America (Hajek et al. 2009). Parasitoid phenology tracked preferred larval hosts with larger *R. lineolata* emerging slightly before and during the emergence of *Sirex* when *Sirex* were at their largest. Emergence of the early larval instar specialist *I. leucospoides* [hereafter; *Ibalia*] was nearly identical to that of *Sirex* (Figure 1b).

Saproxylic species colonize dead or dying trees and exhibit 1-3 year life-cycles in dead wood. The native bark beetle, *Ips pini* [hereafter; *Ips*], is typically

bivoltine, but maintained a consistent emergence from May – September from red pine bolts of this study. The emergence phenology of *Ips* overlapped *Sirex* peak emergence in this study (Figure 1c). Emergence of the cerambycid beetle (*Tetropium cinnamopterum*) was synchronized with *Sirex* emergence during the study period, as was the emergence of False Darkling beetles (Melandryidae: Melandryinae) (Figure 1c). Study logs were dissected after emergence subsided for collection of un-emerged insects. Dissection revealed numerous cerambycid beetle larvae belonging to the genera *Graphisurus* and *Xylotrechus*, under bark and within xylem (respectively).

Starting populations of *Sirex* in trees of this study was estimated from emerged adults, parasitoids and killed larvae at approximately 616 individuals. Of this starting population only 359 *Sirex* emerged from the trees as adults (~58%). Natural enemies claimed ~42% of *Sirex* larvae in this survey. *Ibalia* parasitized ~19% of *Sirex* larvae and was the greatest source of mortality, followed by intraguild predation (~15%) and parasitism by *R. lineolata* (~6%). The intraguild predator, *Xylotrechus*, was the second most abundant insect collected from red pine xylem (n= 355 larvae). Intraguild predation decreased with increasing bolt diameter. As such predation rate was standardized across all bolt diameters by surface area. Standardized predation of *Sirex* by *Xylotrechus* larvae (Figure 2a) accounted for losses of $\sim 8.9 \pm 1\%$ of the *Sirex* population per m² tree surface (mean \pm SE) across all tree diameters. *Xylotrechus* have a growth span of >1 year as larvae in dead timber and were recorded entirely as larvae in the community survey. Estimates of intraguild predation rates described here are conservative given the difficulties of identifying latent evidence of predation.

Predation of *Sirex* increased with increasing density of *Sirex* in the tree stem (Figure 2b). Model selection indicated typical factors such as prey and predator density were important in the best models, while an interaction between *Ips* and *Sirex* was the key difference in the top two models (Table 1). *Sirex* density was negatively correlated with increasing tree diameter and *Ips pini* (density / m²) ($y = 404 - 44 x^2$ & $y = 2.07 - 0.01 x^2$ respectively; Figure 3).

5.4.2 Fungal Competition

Amylostereum and *Ophiostoma ips* colonies grew in a radial pattern from their points of inoculation, but halted upon contact with the competing fungus in all but the trials with no separation between inoculations of competitors (i.e. 0 cm trials, agar and sterilized xylem). Signs of competition were evident at the interface where colonies met and consisted of orange pigmentation of the hyphae of *Amylostereum* and the surrounding media and melanization of *Ophiostoma* hyphae in proximity to *Amylostereum*. Competitive interactions were not as pronounced in xylem cultures, but fungi were once again unable to over grow their competitor. *Amylostereum* was outcompeted on xylem at 0 cm (t-value = -4.2, *df* = 4, *p* = 0.01) and 1.5 cm (t-value = -3.6, *df* = 5, *p* = 0.02), but was not affected at 3.0 cm separation between colonies (Figure 4a). *Ophiostoma* growing on xylem was affected negatively by the presence of *Amylostereum* only at a distance of 1.5 cm (t-value = -3.5, *df* = 5.0, *p* = 0.02; Figure 4b).

Amylostereum was similarly inhibited by the presence of *Ophiostoma* on PDA despite faster growth rates. *Amylostereum* was quickly surrounded by *Ophiostoma* at 0 cm and 1.5 cm negatively affecting radial growth (t-value = -11.2, *df* = 6.5, *p* <.001

& 1.5 cm; t-value = -6.6, $df = 4.7$, $p = 0.001$, respectively; Figure 4c). *Ophiostoma* growth was negatively impacted by the presence of *Amylostereum* on agar at 1.5 cm (t-value = -4.5, $df = 4.7$, $p = 0.007$) and 3.0 cm (t-value = -7.3, $df = 5$, $p = 0.007$) (Figure 4d). The effect of differential substrates on growth was evident in the relative growth rates. Radial growth of *Amylostereum* and *Ophiostoma* was 1.0 and 4.0 (mm/day) respectively on nutrient rich PDA. In contrast, radial growth on nutrient poor xylem was 0.45 and 0.9 (mm/day) (*Amylostereum* and *Ophiostoma*, respectively).

5.4.3 Community Attraction

Treatment trees were established across a wide range of tree diameters (14-27 cm). Female *Sirex* oviposition on treatment trees varied from as few as four to as many as 160 ovipositions on an individual tree, with the number of ovipositions apparently dependent upon fitness of female *Sirex* and target trees at the start of the experiment (Madden 1974). On average larger trees received fewer ovipositions than smaller trees. Across years oviposition rate was highly variable. *Sirex* oviposition was the lowest in 2010. Insect attraction to *Sirex* attacked pines generally increased with increasing oviposition number. Attraction to *Sirex* attacked red pines was highest amongst saproxylic insects in 2009 and 2011 and was significantly different from un-attacked/ healthy pines (Table 2). Attraction of particular saproxylic species was consistent across all years, but as a community, was only significantly different from the control treatment in years 2009 and 2011. Insect communities visiting artificially/mechanically damaged pines of the additional treatment in 2011 were not different from those visiting *Sirex* attacked pines of the same year at guild and full

community levels ($F_{(2,13)} = 0.9, p = 0.75$; $F_{(2,13)} = 1.0, p = 0.5$ respectively) and were combined for comparison with healthy trees. The combined damage treatments indicated the characteristic attraction within the saproxylic community, but also a significant attraction within the parasitic community in 2011. In canonical correspondence analysis, oviposition number explained the majority of the variance in the insect community visitation of trees across all years and feeding guilds (Table 2). Herbivorous and predatory species were not collected in numbers high enough for permutation tests and were only included in full community ordinations. In all 213 insect species were collected on *Sirex* attacked red pines, though only a dozen responded in abundances of greater than 10 insects in a given year.

Species specific responses to the treatments were evident from ordination plots and were tested using generalized linear models for individual species response to treatments. Species responses were similar across years (for simplicity only 2011 is presented). The saproxylic cerambycid beetle, *Xylotrechus*, was significantly attracted to *Sirex* attacked (Poisson GLM $z = 3.0, df = 15, p = 0.002$) and mechanically damaged ($z = 4.5, df = 15, p < 0.001$) red pine, relative to healthy controls in *a priori* planned contrasts (Figure 5a). *Xylotrechus* was not specific to damage type and responded strongly to both mechanically damaged and *Sirex* attacked trees. Apparent increase in attraction to mechanically damaged trees over those attacked by *Sirex* was not statistically significant. Mechanically damaged trees tended to release more sap than *Sirex* damaged trees (Personal observation). *Ips pini* preferentially targeted stressed pines and was found in the highest densities on *Sirex* damaged trees ($z = 3.2, df = 15, p = 0.001$; Figure 5b). *Ips* was only marginally different on mechanically

damaged trees ($z = 1.8$, $df = 15$, $p = 0.06$). The response of the specialist parasitoid *Ibalia* was strongest to *Sirex* attacked trees ($z = 4.1$, $df = 15$, $p < 0.001$), but was not statistically different from mechanically damaged trees (Figure 5c). *Xylotrechus* caught in sticky traps on *Sirex* attack trees correlated with the number of ovipositions ($y = 0.68 + 1.02x$, $n = 25$, $p < 0.001$) (Figure 6). The response of *Ibalia* and *Ips* was highly variable and did not strongly correlate with *Sirex* oviposition.

5.5 Discussion

Damage caused by invading species is conditioned upon their ability to spread and reproduce in novel habitats (Melbourne and Hastings 2009, Rhainds et al. 2011). Adaptation to local conditions can increase invasibility (Hufbauer et al. 2012), but may be hindered by interactions with native species (Kennedy et al. 2002). Enemy release is a prominent mechanism used to explain emergence of invasiveness and increased mortality in introduced ranges, but does not always predict why some species become invasive while others do not (Colautti et al. 2004). In this study I document the occurrence of biotic resistance to invasion mediated through unexpected pathways from opportunistic saproxylic insects.

Dying trees go through a succession of saproxylic inhabitants, each focusing on particular resources that may be ephemeral through time (Grove 2002). I find that trees killed by the invasive woodwasp, *Sirex noctilio*, are colonized by a diverse assemblage of insects specialized on dead wood. Saproxylic insects were rapidly attracted to *Sirex* attacked trees. The response within the saproxylic guild was not specific to *Sirex* attack, but more generally to tree wounding. Species responses were

highly variable within guilds, with certain species in the parasitic guild responding strongly to *Sirex* attacked trees. The cerambycid beetle, *Xylotrechus saggitatus*, was the second most abundant saproxylic species found in the xylem of *Sirex* killed red pines, after *Sirex* and was found on *Sirex* attacked trees less than one week after trees were injured in field treatments. Cerambycid beetles, such as *Xylotrechus*, are commonly found in dead timber, but the rapidity of their response suggest they are early colonists that respond quickly and specifically to wounded trees (Erbilgin et al. 2003, Miller 2006). *Xylotrechus* attraction proceeded intraguild predation which by our conservative estimates accounted for ~15% mortality in *Sirex* populations. The predation rate by *Xylotrechus* on *Sirex* is a hidden factor in biotic resistance to invasion of North American forests, and one that should be taken into account when calculating sources of mortality, such as parasitism. Intraguild predation by cerambycid larvae is common in the literature, but had not been documented for sawflies, though small population densities in native sawflies make it difficult to assess (Dodds et al. 2001, Ware and Stephen 2006, Schoeller et al. 2012). Estimates of intraguild predation from this study are conservative, yet represent a level of mortality comparable to levels measured for parasitoids used in biocontrol of *Sirex* (e.g. *Ibalia* and *Rhyssa*)(Zylstra and Mastro 2012). Intraguild predation rate was heavily influenced by *Sirex* population density in infested bolts, which surprisingly was influenced by the presence of bark beetles.

Bark beetles are aggressive herbivores of pine phloem that utilize pheromones to initiate mass attacks that overwhelm pine defenses through massive fungal inoculation. An apparent competitive interaction between *Sirex* and *Ips* manifested in

a negative correlation between *Sirex* and bark beetle populations in red pines. Female *Sirex* actively make decisions about the quality host trees prior to oviposition of eggs and avoid trees with fungal growth (Ryan et al. 2011), but *Ips pini* was preferentially attracted to trees wounded by *Sirex* in this study. The competitive ability of fungal mutualist is important in regard to colonization timing. *Ips* and *Sirex* inhabit spatially discreet locations within the tree (phloem and xylem respectively), but the same is not true for their respective symbionts. Blue-stain fungi associated with bark beetles penetrate deep into sapwood. In benchtop trials, *Ophiostoma ips* outcompeted *Amylostereum* for xylem in all instances except where *Amylostereum* was able to establish well ahead of competitive interactions. It is unknown whether both insects are affected by the presence of symbiont competitors or only *Sirex*, but the reliance of *Sirex* on *Amylostereum* as an external source of easily digestible plant compounds puts *Sirex* in a vulnerable position in regard to fungal competition (Chapter 2 & 3). *Sirex* preferentially colonizes living trees in native and introduced ranges (Spradbery and Kirk 1978, Ryan et al. 2011, Villacide and Corley 2012). The colonization of living trees is suggestive of strategies for finding and establishing in new resources, rather than taking over already established resources, which may have something to do with the competitive abilities of *Amylostereum* and its central role in nutrition.

In laboratory trials *Amylostereum* (*Sirex* symbiont) was competitively inferior to *Ophiostoma* (*Ips* symbiont), but results were contingent upon colony size indicating assembly history may be important. *Ophiostoma* outcompeted *Amylostereum* when colonies met at a small sizes. At larger colony sizes *Amylostereum* was not negatively impacted by the presence of *Ophiostoma*,

suggesting colonization timing is important in structuring the outcome of competitive interactions. *Ophiostoma* had a faster growth rate than *Amylostereum*. However, *Amylostereum* appeared to have secondary metabolites capable of inhibiting the growth of *Ophiostoma* hyphae in culture. The colonization patterns of these fungal symbionts will largely be dependent upon phenology of insect hosts, which share identical preference of stressed pine trees and have overlapping emergence phenologies. *Sirex* emergence from trees peaked slightly later in the summer than *Ips* populations. The timing of colonization and the nature of the community colonizing trees has long term implications on populations of insects within the trees (Fukami et al. 2010, Weslien et al. 2011). In successful attack of trees by *Sirex*, colonized trees typically die slowly, possibly buying time for *Amylostereum* to develop. The tolerance of *Amylostereum* for pine defensive chemicals is currently unknown, but may be an important variable in the success of this symbiosis.

Sirex noctilio is native to pine ecosystems of Europe and Asia where it occurs in low abundance on various pine species, similar to its North American relatives (Spradbery and Kirk 1978, Wermelinger et al. 2008), but upon introduction to managed pine plantations of the Southern Hemisphere, *Sirex* reached high population densities and caused extensive tree mortality (Villacide and Corley 2012). In North America diverse mechanisms interact through multiple species and feeding guilds to antagonize *Sirex* populations. Diversity increases connectivity between trophic levels and may confer stability to ecosystems by reducing extreme dynamics within populations (McCann 2000). It is therefore important to study alternative mechanisms of biotic resistance in addition to traditional natural enemies (e.g. predators). *Sirex*

preferentially colonizes living trees as a food source, killing them in the process (Spradbery and Kirk 1978). Saproxylic species though commonly thought to infest and ingest dead wood, overlapped with *Sirex* in red pines and may prove to be one of the missing components in understanding the invasive ecology of this aggressive tree-killing herbivore. The foraging strategy of *Sirex* for attacking living trees and the weak competitive ability of *Amylostereum* suggest the success of *Sirex* and possibly other woodwasps may hinge on establishment of fungal symbionts in the absence of dominant fungal competitors and larval predators. The impact of fungal competition on *Sirex* populations remains to be investigated, but the results of this study suggest such alternative mechanisms of population regulation, such as competition with nutritional symbionts and intraguild predation may help explain the divergence in invasive ecology between North American and previous introductions.

This study highlights the potential for diverse native communities to buffer species invasions through shared resource cues and resource partitioning. The strong response of saprotrophic species makes intuitive sense, since these species are unable to access tree resources until tree defenses are undermined. With their rapid and direct response to aggressive tree attack, saprotrophic species open the possibility for competitive and even predatory interactions and may play a key role in moderating the destructive potential of tree killing insects. The implications for diverse interactions from opportunistic insects and fungi likely extend beyond this particular example and may be a broad phenomenon in stabilizing ecosystems.

5.6 Figures & Tables

5.6.1 Tables

Table 1. I modeled intraguild predation per m² on *Sirex noctilio* by *Xylotrechus saggitatus* as a function of environmental (tree diameter) and biotic (*Xylotrechus*, *Ips pini*, *Sirex*) factors. I ran all combinations of additive and two way interactive effects. Only the top four models are presented. K is the number of model parameters. QAICc is the model likelihood. Δ QAICc is the QAICc scaled so the lowest value is zero (for ease of interpretation). QAICc wt is the model weight. The 'Full Model' contains all factors and their interactions.

<i>Predation ~</i>	K	QAIC _c	Δ AICc	QAICc Wt
<i>Xylotrechus + Sirex + Ips + (Sirex * Ips)</i>	6	140.5	0.0	0.68
<i>Sirex + Ips + (Sirex * Ips)</i>	5	152.1	11.6	0.0
<i>Full Model</i>	17	156.9	16.4	0.0
<i>Xylotrechus + Sirex</i>	4	168.2	27.8	0.0

Table 2. The pine insect community visiting *Sirex* attacked ('*Sirex*') or healthy ('Healthy') red pines from 2009-11 by Canonical Correspondence Analysis. Insect communities were assessed in full (All guilds) and as subsets for feeding guild (Saproxyllic, Parasitic, Predatory, Herbivorous) for each year (2009-2011). Predatory and herbivorous insects were not caught in sufficient numbers for guild level CCA and permutation tests. Insect community responses to *Sirex* oviposition explained the greatest proportion of variance. Total constrained variance explained by *Sirex* oviposition and tree diameter (Constrained %). Species response vectors were analyzed using permutation tests for significance of oviposition treatment (Permutation p-value); (*) p-value ≤ 0.05 . Tree diameter was not a significant predictor of community patterns and was excluded for simplicity; (Δ) mechanical/artificial damage treatment not statistically different from *Sirex* treatment and was combined for year 2011 analyses.

Comparison	Year	Community /Guild	Species #	Constrained (%)	Permutation p-value
<i>Sirex attacked -vs- Healthy</i>	2009	All guilds	33	26	0.76
		<i>Saproxyllic</i> *	8	26	0.01*
		<i>Parasitic</i>	14	24	0.47
<i>Sirex attacked -vs- Healthy</i>	2010	All guilds	39	33	0.16
		<i>Saproxyllic</i>	7	26	0.17
		<i>Parasitic</i>	22	29	0.79
<i>Sirex attacked -vs- Healthy</i>	2011 ^{Δ}	All guilds*	57	12	0.01*
		<i>Saproxyllic</i> *	22	12	0.04*
		<i>Parasitic</i> *	20	11	0.03*

5.6.2 Figures

Figure 1. Emergence phenology (mean \pm 95%) for *Sirex noctilio* (filled circles) (a), parasitoids (b) and saproxylic beetles (c) emerging from *Pinus resinosa* (mean \pm 95%). Mean *Sirex* emergence (solid black line); *Sirex* emergence 95% confidence interval (gray shading and dashed lines).

Figure 2. *Xylotrechus* predation on *Sirex* larvae; typical predation event (a) and the occurrence of predation (counts/ m²) relative to *Sirex* density (counts/ m²) in xylem (b).

Figure 3. *Sirex* populations relative to a) red pine bolt diameter and b) *Ips pini* bark beetle density m².

Figure 4. Total colony area for *Amylostereum* and *Ophiostoma ips* grown on sterilized red pine xylem (a,b) and potato dextrose agar (c,d), both crossed with the competitor and alone (n = 6). Cultures were inoculated at increasing distances apart and grown for 11 days (0 & 1.5 cm) and 16 days (3 cm); * denotes t-test $p \leq 0.05$.

Figure 5. Attraction of *Xylotrechus saggitatus* (a), *Ips pini* (b), and *Ibalia leucospoides* (c) to ‘Healthy’ unwounded pine trees or trees wounded by either ‘*Sirex*’ or artificially simulated *Sirex* attack ‘Mechanical damage’ in study year 2011; All species F-test_(2,21) > 3.5, p -value ≤ 0.05 . (*) individual contrasts p -value ≤ 0.05 .

Figure 6. Number of *Xylotrechus* visiting red pines relative to the number of *Sirex* ovipositions (2009-2011). Oviposition numbers were significantly different between years ($F_{(1,21)} = 14.0, p = 0.001$) with exceptionally low ovipositions in year 2010 (red), and average in 2011 (green) and high numbers of ovipositions in 2009 (black).

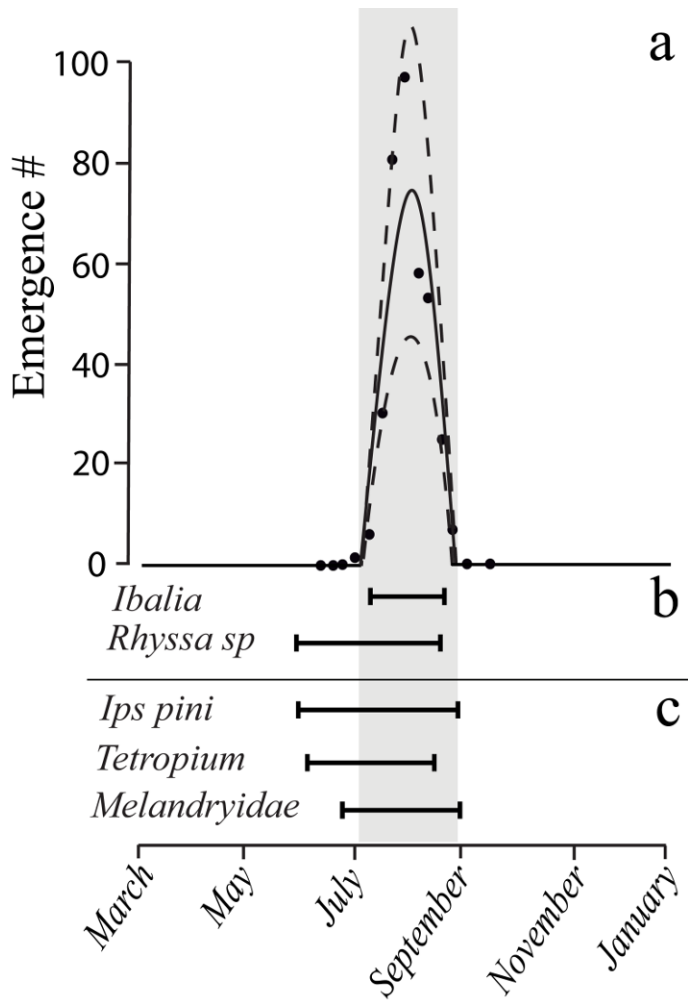


Figure 1

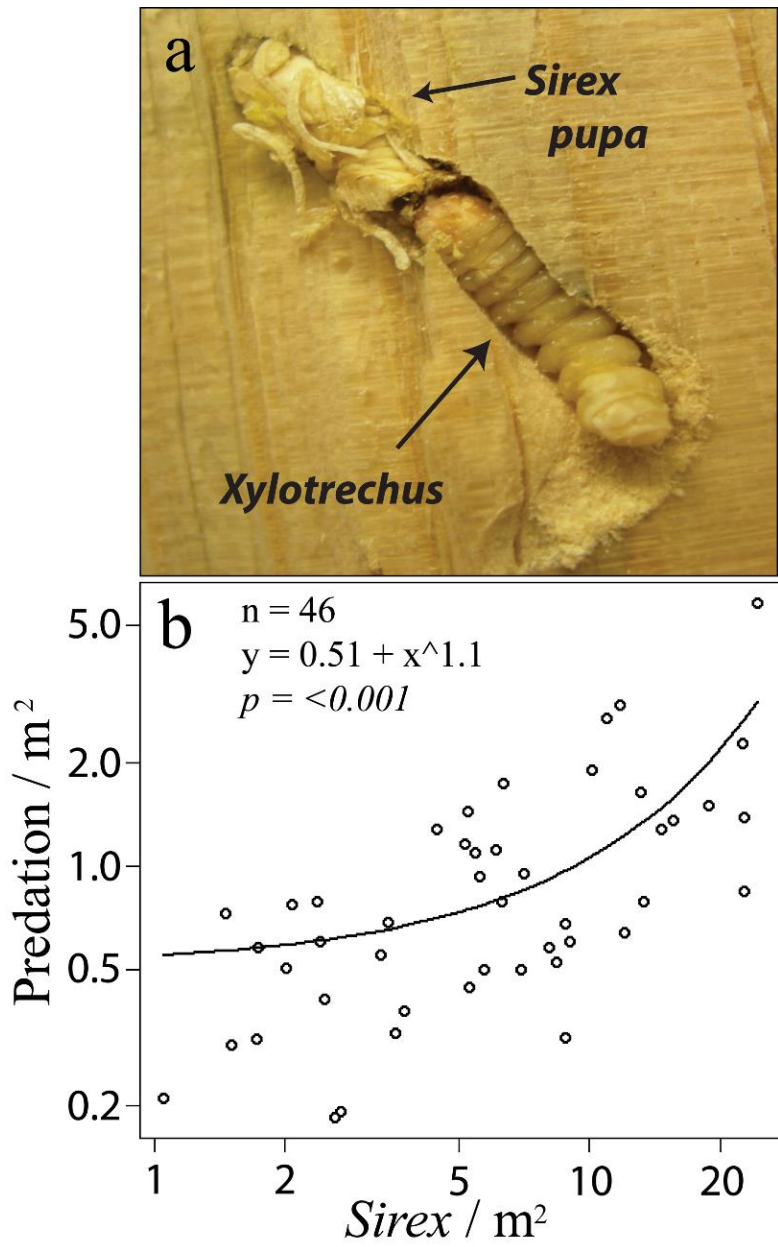


Figure 2

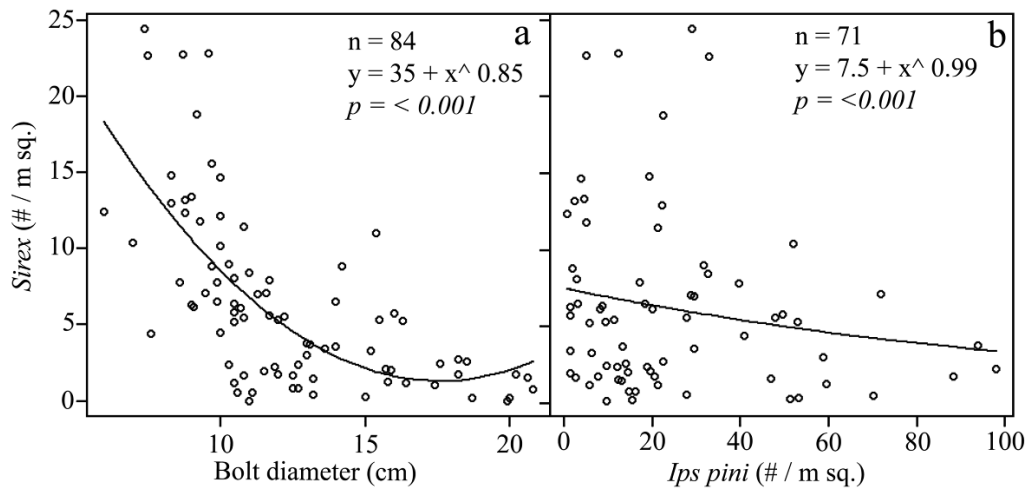


Figure 3

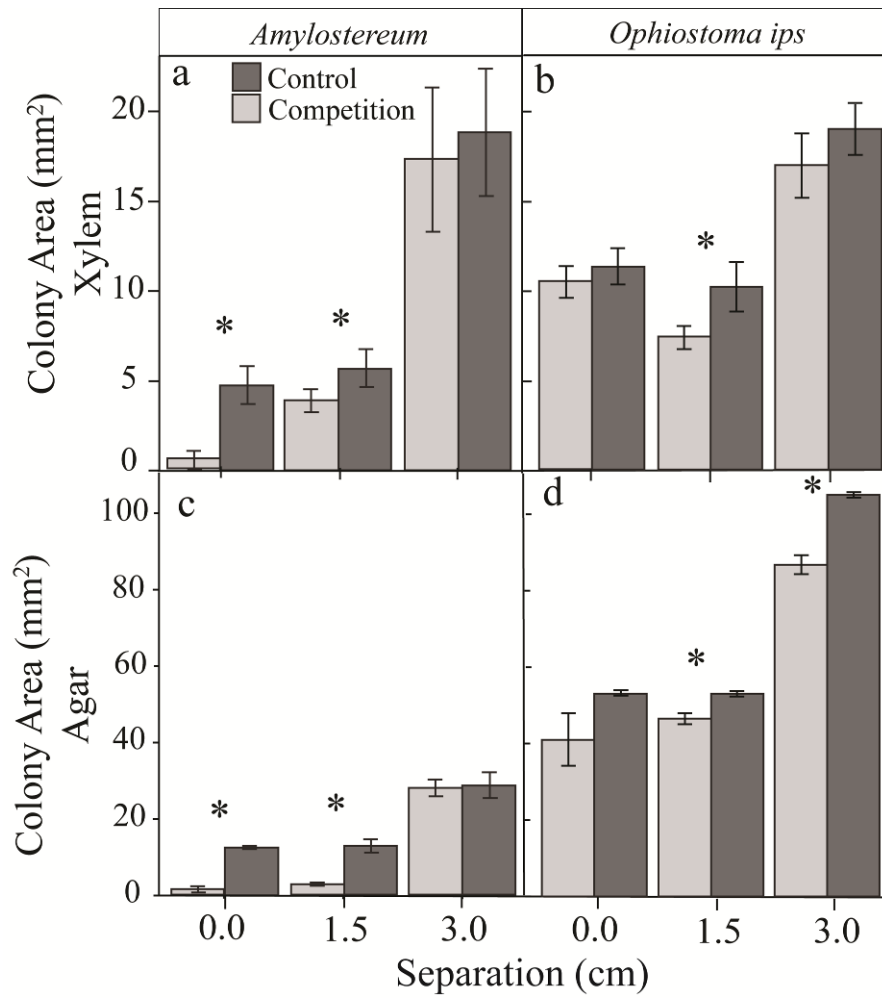


Figure 4

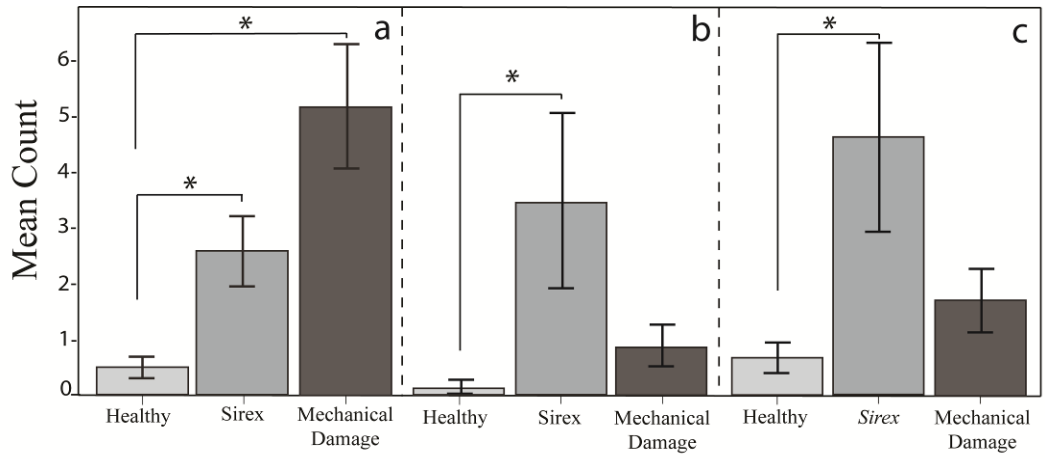


Figure 5

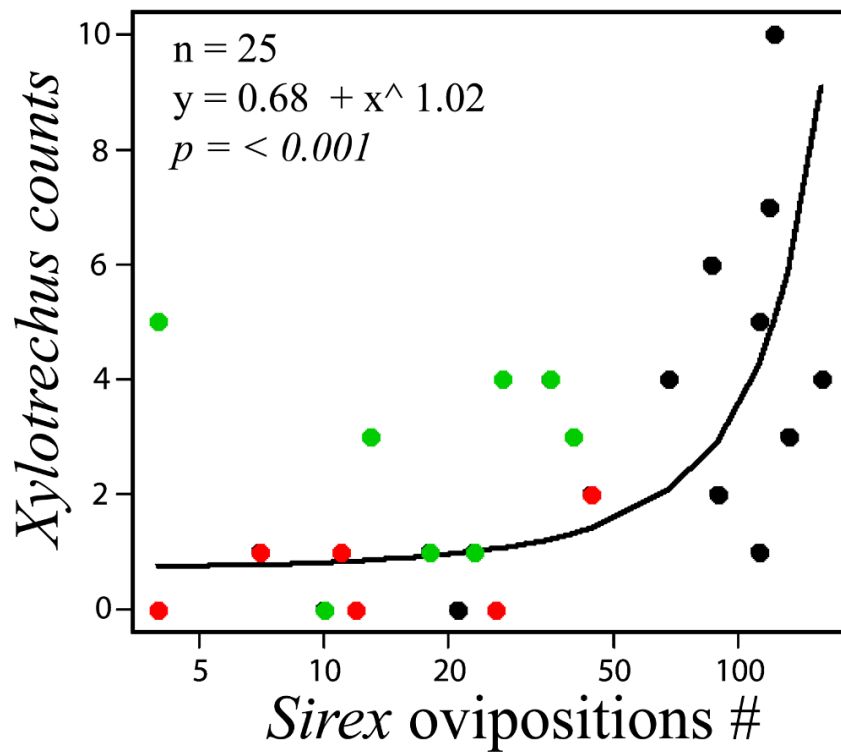


Figure 6

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