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Microbial Symbionts Shape the Sterol Profile of the Xylem-Feeding Woodwasp, *Sirex noctilio*

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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 by using GC-MS to quantify concentrations of free and bound sterol pools from multiple life-stages of *Sirex*, 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 μg/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*. Our 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.

Keywords *Sirex noctilio* · *Amylostereum* · Sterol metabolism · External rumen hypothesis · Invasive symbiosis · Mycetophagy

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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 ephemeral within terrestrial environments (Wheeler and Blackwell, 1984). Termites, leaf-cutter ants, and ambrosia beetles have evolved mutualisms with fungi that diminish the ephemeral nature of fungal resources and select 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 coopted independently 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, we 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, 1969; Talbot, 1977).

The symbiotic complex of *Sirex* and *Amylostereum* aggressively kills living trees through mass attack and shared biological weapons (Coutts, 1969). 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 the lignocellulosic compounds in the xylem tissue (Coutts, 1969). *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 larvae of a close relative of *Sirex* (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; but see Scully et al., 2012). Pine xylem also is 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 excrete substances that rapidly destroy the hyphae of *Amylostereum* in wood, thus further supporting the mycetophagy hypothesis. Alternatively, larvae may obtain nutrients indirectly from plant material via extracellular digestive enzymes derived from *Amylostereum* ("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, we 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 and Nes, 2003), and predatory and parasitic insects may obtain cholesterol directly 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 and Nes, 2003).

Plants, animals, and fungi synthesize and/or metabolize distinctly different sterol molecules for homeostasis (Fig. 1; Behmer and Nes, 2003). Plants contain little cholesterol (Hartmann, 1998), instead containing predominantly sitosterol and campesterol (Hartmann, 1998). In order to utilize sterols from plants, phytophagous insects metabolize phytosterols (typically sitosterol and campesterol) to cholesterol

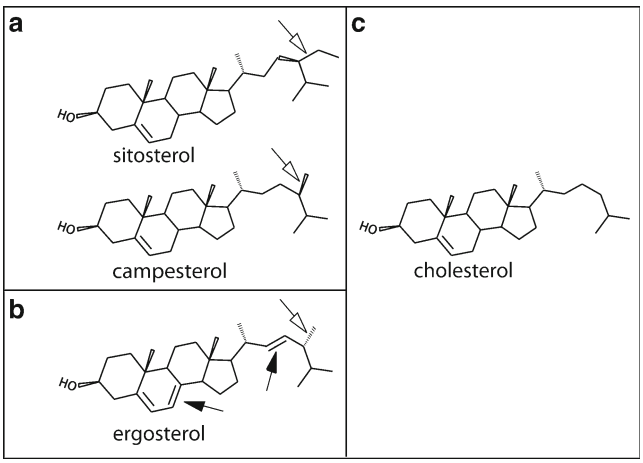

Fig. 1 Key dietary sterols available to, and recovered from *Sirex*. Phytosterols (**a**) and fungal sterols (**b**) are converted to cholesterol (**c**) via dealkylation of side-groups (*hollow arrows*) at C24 and, in the case of ergosterol, removal of double bonds (*filled arrows*) at C7 & C22

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 (Pasanen et al., 1999), 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 (Fig. 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, we investigated the sources of dietary sterols for *Sirex* feeding in red pines in North America. We use the distinct structural profiles of fungal and plant sterols to determine the origin of sterol products recovered from insects and to infer the relative dietary importance of plant and symbiont resources for *Sirex* larval development. This work tested 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 adult gender relative to alternative plant derived sterols using GC-MS to quantify sterol molecules. Sterol analysis is useful for not only determining nutrient sources for consumers, but also the metabolic intermediates that are formed in hormone and microbial metabolism of sterols. This study tested the direct (mycetophagy) -vs- indirect ('external rumen') hypotheses for the basis of symbiosis between *Amylostereum* and *Sirex* by using sterol molecules unique to each feeding method.

Methods and Materials

Study System Native to European and North African pine species, *Sirex noctilio* has been introduced to numerous continents outside its native range, including Australia, South Africa, South America, and most recently, North America (Carnegie et al., 2006). 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 (incl. *P. strobus*, P. *rigida*, *P. virginiana*, *P. loblolly*) (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).

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 diam 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 instars 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 instars (Behmer and Grebenok, 1998). The material commonly called 'frass' in this, and possibly other pine sawflies, is not true frass (see definition in 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 no greater than 1.5 cm behind foraging larvae. Adult male and female *Sirex* were collected at random from the original *Pinus* bolts upon emergence (N=18 and 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 5 mm increment borer (Haglof ®) to a depth of 3.5 cm (bark removed).

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 then were 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 2 wk 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 were 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 first were annotated and then identified by best-hit comparison using blastn search in the nucleotide database in Genbank (Altschul et al., 1990), with a (≥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 1 mm^3 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 then placed in the dark to grow under sterile conditions for 2 wk. 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 cm^3 cubes of xylem either containing or devoid of fungal hyphae. *Ophiostoma* sterols have been analyzed successfully 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 U. Wisconsin-Madison. The remaining six *Amylostereum* strains were isolated from pure cultures isolated from dissected lab reared female *Sirex*, as described above.

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 dH_2O), 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). We first verified the presence of the common lignolytic enzyme laccase and its activity on ABTS using laboratory strains of *Amylostereum areolatum* obtained from Dr. Aaron Adams of the University of Wisconsin. Subsequent field collected fungal strains described in the methods section were tested for laccase activity using methods similar to Niku-Paavola et al., 1990 at 1/8th concentration (1.375 mg/ml). Using this technique we 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.

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 Missisauga Ontario,CA), with subsequent extraction in 30 ml of 95 % ethanol. These samples were shaken and incubated in the dark at room temperature for 24 h, and the ethanol was evaporated to dryness under nitrogen. The residue was resuspended in 70 % methanol:water, to which 10 ug of cholestane were 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 resuspended 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 h to replace the fatty acid moiety present at C3 with a free hydroxyl group. The fraction containing the glycosylated sterol was resuspended in 8 ml of 100 % methanol, containing 10 % HCl, and incubated in a shaking

water bath (225 rpm) at 55 °C for 2.5 h 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,USA).

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 diam and 0.25 μm film thickness. The running conditions were: inlet 260 °C, transfer line 280 °C, column 80 °C (1 min), ramp at10°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) (detailed methods. 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 (μg/g).

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 (μg/g) using GLM and one-way ANOVA with multiple comparisons using Tukey's HSD. The concentration of sterol in *Pinus* (μg/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). We 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.

Results

Sirex Sterols Sterols were identified in three forms (free, acylated, and glycosylated). However, we found 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*, we observed three strong trends. First, cholesterol was the dominant form in *Sirex* tissues and the only sterol quantifiable from eggs (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 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 (Fig. 2a). In contrast, *post hoc* comparisons of acylated sterols showed males differed from females (P=0.005, df=30), female *Sirex* were different from eggs (P=0.002, df=30), and larvae marginally different from eggs (P=0.06, df=30, Fig. 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 found only in adult female *Sirex* (Fig. 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 (Fig. 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

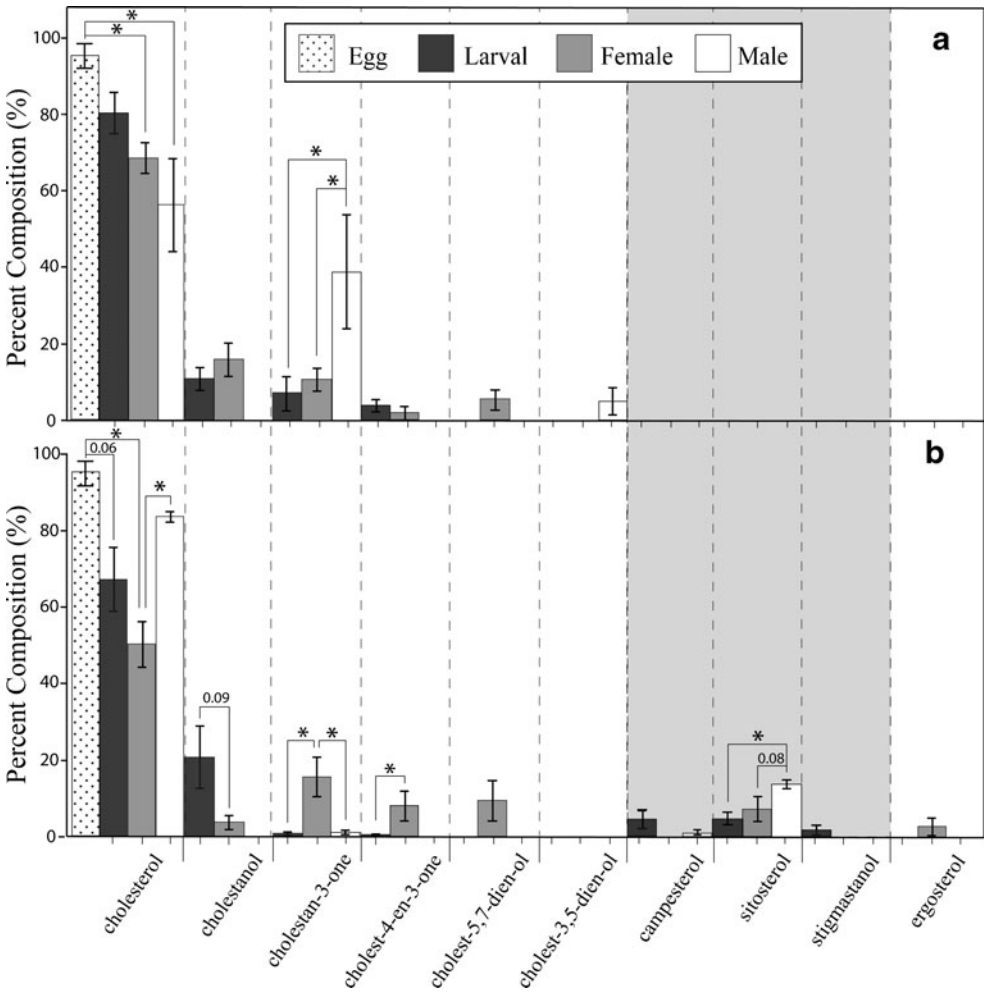

Fig. 2 The total proportion [mean±SEM] of sterols extracted from the free sterol pool (**a**) of various life-stages of *Sirex* qualitatively differs from the conjugated sterol pool (**b**) in some esterified sterols, but most strikingly in phytosterols (*gray shading*) in *Sirex*. *Asterisks* denote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

in the free sterol fraction (Fig. 2a). The sterol profile of *Sirex* eggs was nearly entirely composed of cholesterol, although 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; Fig. 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).

Sterol in Food Resources Growth of *Amylostereum* fungal cultures in the lab on YM agar and sterilized pine xylem, were confirmed visually prior to analysis of sterol profiles (colonies measured ~4 cm diam at 2 wk growth. Cholesterol was detected in analysis of YM agar media, and was subsequently removed later analyses of fungal growth on YM agar. The dominant sterol in *Amylostereum* cultures grown on YM agar is ergosterol (acylated, 28.7±5.4 μg/g; free, 18.9±5.1 μg/g (mean±SEM)). No other sterols were detected above the 0.001 μg/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 (Fig. 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 μg/g) of ergosterol, while *Ophiostoma* cultures showed a clear ergosterol signal (1.2±0.34 μg/g) (Fig. 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 μg/g respectively; Fig. 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 (Fig. 4). Frass further differed from other wood samples by containing measurable concentrations of both ergosterol (0.96±0.64 μg/g) and cholesterol (0.72±0.35 μg/g) in addition to typical phytosterols of *Pinus* (Fig. 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 (Fig. 5a) and in natural infestations (Fig. 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.

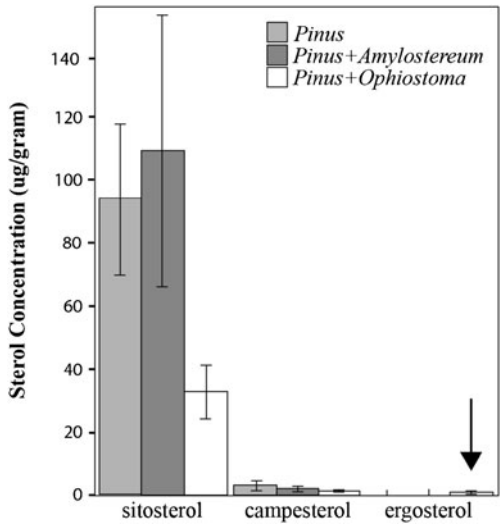

Fig. 3 Sterol concentration [mean±SEM] from base saponified samples of fungal inoculated pine xylem (*Pinus+Amylostereum/Ophiostoma*) and un-inoculated control xylem (*Pinus*). Ergosterol was present in trace quantities (<0.005 μg/g) from *Pinus+Amylostereum* and in greater quantities in *Ophiostoma* (*filled arrow*). Sterol concentrations were not statistically different (Tukey HSD, P>0.05)

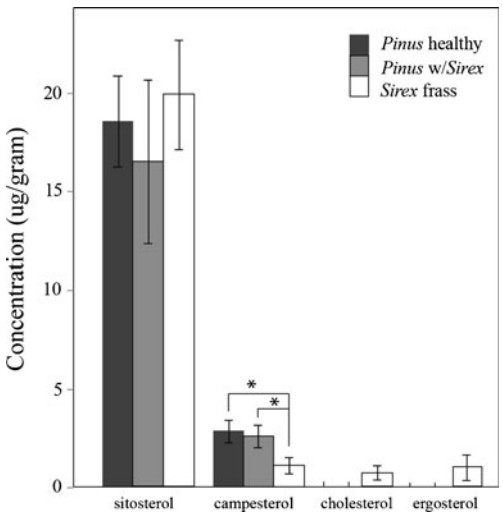

Fig. 4 Sterol concentration [mean±SEM] from samples of pine xylem with *Amylostereum* (*Pinus* w/*Sirex*), without *Amylostereum* (*Pinus* healthy) and *Sirex* frass. *Asterisks* denote significance between life-stages (Tukey's HSD, P<0.05)

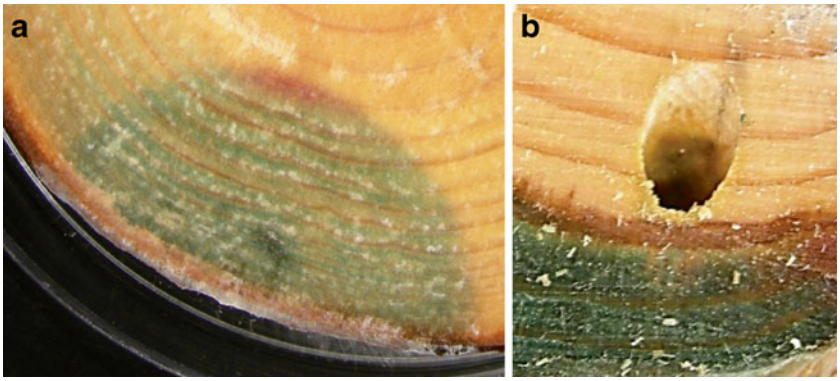

Fig. 5 *Amylostereum* cultures in wood stain blue-green in the presence of ABTS (1.375 mg/ml) due to extracellular lignolytic enzymes. Stains of cultures grown on sterilized wood (**a**) were identical to *Amylostereum* stained in natural infestations next to *Sirex* larval tunnels (**b**). The change ("due to extracellular lignolytic enzymes") modifies the text to better indicate the nature of the interaction, but only minimally adds to the text length

Discussion

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 a food resource for host insects has received much attention (see review by: 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 a 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, we 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 μg/g) and ergosterol derivatives were not detected in insect tissues. Sterol analysis indicated low (<0.005 μg/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 (Ikekawa et al., 1993). Sitosterol and campesterol are the prevalent sterols in *Pinus*, and were 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 recovered only in the acylated fraction, indicating *Sirex* binds at least some phytosterols to lipid, usually fatty acids. We currently know little 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 *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*.

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. We 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.

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 unusual in insects, unless the insects have been eating foods containing them (Jing et al., 2012). They also are 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; and unpublished metagenome by B. Thompson). The presence of the unusual sterols in *Sirex* may be a result of microbial modification of sterols in the gut lumen (e.g., Heyer et al., 2004), although background for this type of association needs further investigation.

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 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 instars and host colonization. First and second instars 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 instars 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 (e.g., 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 fugal biomass in mycangia, a similar mechanism may explain the detectable concentrations of ergosterol in frass. Elevated levels of nitrogen found in a related study (B. Thompson, unpublished data) 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 translate directly to *Sirex* nutrition, but may increase contact between larvae and *Amylostereum*, which in turn may facilitate symbiont acquisition in adult females prior to exiting host trees (Talbot, 1977).

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, we found 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, although transfer of specific metabolites cannot be ruled out.

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