

# Symbionts mediate oviposition behaviour in invasive and native woodwasps

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- Abstract**
- 1 Globalization leads to the introduction of invasive species that are often accompanied by associated microorganisms, and this can lead to homogenization of both introduced hosts and microbes with the native biota. One such example is the invasive Eurasian woodwasp *Sirex noctilio*, which inoculates pines with an obligate nutritional mutualist, the white rot fungus *Amylostereum areolatum*.
  - 2 Although *S. noctilio* has been previously introduced outside of its native range, its arrival in North America was the first time that it was introduced to communities hosting native *Sirex* species and *Amylostereum* strains.
  - 3 We conducted experiments aiming to investigate acceptance versus avoidance of native and non-native *Amylostereum* strains and species during ovipositor drilling by females of *S. noctilio* and a native congener, *Sirex nigricornis*.
  - 4 *Sirex noctilio* preferred wood without prior fungal emplacement, whereas *S. nigricornis* preferred wood inoculated with one of the strains of *Amylostereum* that putatively invaded with *S. noctilio*.
  - 5 Drilling and presumed oviposition by both woodwasp species were highly aggregated.
  - 6 Based on the responses of these two *Sirex* species to the fungal strains and species included in the present study, the invasive *S. noctilio* would continue its present symbiont associations, whereas the native *S. nigricornis* would partly use the strain of fungal symbiont putatively introduced with *S. noctilio*.

**Keywords** *Amylostereum*, invasive symbiont, *Sirex* woodwasp, symbiont specificity, white rot fungus.

It is well established that increases in globalization provide invasion pathways through which invasive species such as insects are introduced (Hulme, 2009; Olden *et al.*, 2011; Lockwood *et al.*, 2013). However, the invasives that are reported and studied are generally larger organisms and the communities of microbes that are introduced along with their hosts are less well known. Mutualistic symbionts, either arriving with an introduced non-native species or acquired after introduction, can facilitate the colonization, population increase and spread of an invasive species (Lockwood *et al.*, 2013; Traveset & Richardson, 2014; Lu *et al.*, 2016). However, the presence or absence of mutualists accompanying invasive species can also disrupt invasions or can alter the impact of invasive species on ecological networks. Non-native or native mutualists combined with invasive hosts, as well as non-native mutualists combined with native hosts, have

impacted forest ecosystems around the world (Wingfield *et al.*, 2016).

Obligate symbionts must be present for the successful establishment and population increase of a non-native invader. The transmission of obligate symbionts from one generation to the next is often assured by vertical transmission, although obligate symbionts can also be transmitted horizontally (Frago *et al.*, 2012). Horizontal transmission in such instances has been supported not only by phylogenetic studies, but also by documented instances of host switching among host/symbiont associations that were previously considered to be highly specific (Caldera *et al.*, 2009; Hajek *et al.*, 2013; Kostovcik *et al.*, 2015). Symbiont switching could allow novel hosts to expand their niche breadth. However, mechanisms explaining how and when switching occurs remain to be clarified in many systems.

Our understanding of associations between insects and microorganisms acting as mutualists, pathogens or commensals is constantly increasing. Wood-boring *Sirex* woodwasps require

their nutritional fungal symbiotic mutualists, white rot fungi in the genus *Amylostereum*, for larval development within stressed trees. *Sirex noctilio*, an aggressive Eurasian species that is capable of killing healthy pines (*Pinus* spp.) under some conditions, has been introduced to areas with plantations of exotic pines in the Southern Hemisphere (Australasia, South America and South Africa) (Talbot, 1977; Spradbery & Kirk, 1978; Madden, 1988). In 2004, *S. noctilio* was first reported from northeastern North America where other species of *Sirex*, as well as pines (*Pinus*) and *Amylostereum*, are native (Hajek & Morris, 2014; Slippers *et al.*, 2015). More recently, *S. noctilio* has also been reported as being an invasive in northeastern China (Shi *et al.*, 2015). North American species of *Sirex* are not considered pests and do not attack healthy trees. In eastern North America, *Sirex nigricornis* specializes on pines and now co-occurs in trees with *S. noctilio* (Long *et al.*, 2009; Ryan *et al.*, 2012a; Hajek *et al.*, 2013). Further complicating these invasion dynamics is the fact that different genotypes of *S. noctilio* (Boissin *et al.*, 2012; Bittner *et al.*, 2017) and strains of its symbiont *Amylostereum areolatum* (Slippers *et al.*, 2002; Nielsen *et al.*, 2009; Bergeron *et al.*, 2011; Castrillo *et al.*, 2015), usually of unknown origins, have been introduced around the world, including North America.

Eclosing *Sirex* females acquire symbionts from *Amylostereum* lining their pupation chambers. Fungal cells are packed into mycangia at the base of the ovipositor and females then inject the fungus into new trees when ovipositing, which involves drilling through the bark and into the xylem with ovipositors ranging from 8–20 mm in length (Ryan & Hurley, 2012). Females also carry phytotoxic venom and insert this venom when drilling. The fungus alone has little effect on trees but the venom plus fungus acting together can cause tree death. The obligate ectosymbiotic fungi are assumed to be principally transmitted vertically (van der Nest *et al.*, 2012), although some horizontal transmission must occur (Hajek *et al.*, 2013; Wooding *et al.*, 2013). We hypothesize that horizontal acquisition of these ectosymbionts would be strongly impacted by the locations of oviposition by adults. In agreement, after mating, *S. noctilio* females are attracted to their symbiotic fungus, *A. areolatum*, over the congeneric fungus *Amylostereum chailletii* (Sarvary *et al.*, 2016). Ovipositors of *S. noctilio* are electrophysiologically active and have been shown to respond to chemicals associated with host trees, although the mutualistic fungus has not been tested (Hayes *et al.*, 2015). *Sirex noctilio* avoid drilling into wood inoculated with *Leptographium wingfieldii*, a species of ophiostomatoid fungus vectored by bark beetles (Ryan *et al.*, 2012b). This fungus can outcompete the *S. noctilio* symbiont, *A. areolatum* (Ryan *et al.*, 2011), and so it is appropriate for adult females to avoid ovipositing in areas where this bark beetle-vectored fungus already occurs.

In the present study, we investigated the interactions between the native *S. nigricornis* and the invasive and now sympatric *S. noctilio*, as well as strains and species of obligate fungal mutualists. Specifically, we examined the ovipositor drilling behaviour of females from both woodwasp species in pine bolts inoculated with different strains and species of *Amylostereum* carried by either of these two woodwasp hosts. The objective of our study was to relate drilling behaviour by the two *Sirex* species with respect to their typical symbiotic fungal strain versus alternate strains and species of *Amylostereum* that occur

in North America. We expected the invasive *S. noctilio* to preferentially oviposit in wood inoculated with the putatively invasive strain of *A. areolatum* IGS-BD (AaBD) and the native *S. nigricornis* to preferentially oviposit into wood inoculated with native *A. chailletii* (Ach) or the native *A. areolatum* IGS-BE (AaBE) strain. Therefore, we hypothesized that each species would preferentially drill in association with known fungal strains/species rather than wood either not colonized by *Amylostereum* or colonized by *Amylostereum* species/strains not from the area of origin of that *Sirex*.

## Materials and methods

### *Sirex* collection and mating

*Sirex noctilio* were obtained by cutting down infested mature red (*Pinus resinosa*) and Scots (*Pinus sylvestris*) pines with signs of infestation (i.e. resin beads) in spring 2011 and 2012, from numerous locations in central New York and Pennsylvania. Logs were cut into 70-cm long bolts and ends were waxed to prevent contamination by other wood-rot fungi and to conserve moisture. Bolts were placed in fibre barrels (height 77.5 cm, diameter 51 cm) with window screening lids and kept in an unheated building from the harvest date until October. *Sirex noctilio* and *S. nigricornis* that had emerged from bolts were collected daily from barrels and, after emergence, were maintained individually at 4 °C in lidded clear plastic cups (volume 29 mL). Northeastern *Sirex nigricornis* emerging from wood that we had collected were not sufficiently abundant in 2011 and 2012 (Hajek *et al.*, 2017a) and so females for the present study were caught in flight intercept panel traps in Grant Parish, Louisiana, in 2011, and maintained at 4 °C until use.

For *S. noctilio*, recently emerged females were placed with a male until mating occurred and then were returned to 4 °C and were typically used for experiments 1–5 days later. A subsample of the trap-caught *S. nigricornis* females from Louisiana was placed with males, although they would not mate. Because newly emerged female *S. noctilio* fly to the tops of trees to find male leks and mate prior to dispersing (Madden, 1988), we hypothesized that these *S. nigricornis* females were caught in traps when dispersing and that they had already mated prior to dispersal. *Sirex* are haplodiploid and can successfully lay male eggs regardless of whether or not they have mated. There is evidence that *S. noctilio* lay more eggs when mated but still lay eggs abundantly when unmated (Madden, 1974; potentially mated = 156 eggs versus unmated = 140 eggs). Such a study has not been conducted with *S. nigricornis*, although we found that the trap-caught *S. nigricornis* drilled with their ovipositors during the present study but not as frequently as *S. noctilio*.

### Fungal inoculation of experimental bolts

The three strains of two *Amylostereum* species used in the present study are three of the four IGS strains that were isolated from mycangia of species of *Sirex* collected in North America from 2007–2012 (Nielsen *et al.*, 2009; Hajek *et al.*, 2013); only one of the two IGS strains commonly isolated from *S. noctilio* was used in the present study. Fungi were isolated using methods described previously by Thomsen and Harding (2011) (Table 1).

**Table 1** Fungal strains used in the experiments

Sirex host	Fungal species and IGS group	SAC number <sup>a</sup>	Location, County/State	Date collected
<i>Sirex noctilio</i>	<i>Amylostereum areolatum</i> BD (AaBD; introduced with <i>S. noctilio</i> )	101	Granby, Fulton, New York	27 December 2007
<i>Sirex nitidus</i>	<i>Amylostereum areolatum</i> BE (AaBE; native to North America)	81	Winterport, Waldo, Maine	10 September 2007
<i>Sirex nigricornis</i>	<i>Amylostereum chailletii</i> (Ach; native to North America)	91	New Haven, Oswego, New York	17 September 2007

<sup>a</sup>SAC, *Sirex*/*Amylostereum* Culture Collection, Hajek Lab, Cornell University.

*Amylostereum areolatum* IGS BE (AaBE) was isolated from *Sirex nitidus* from Maine and this strain is assumed to be native, having been isolated from this native *Sirex* species in an area without invasive *Sirex* (Nielsen *et al.*, 2009; Hajek *et al.*, 2013). AaBE was the most common strain of *A. areolatum* isolated from *S. nigricornis* from New York and the only strain isolated from *S. nigricornis* from Louisiana (Hajek *et al.*, 2013). The *A. chailletii* (Ach) strain used had been isolated from *S. nigricornis* from New York State and is considered native because *S. noctilio* has never or rarely been reported as being associated with this fungal species, which was known from North America before the invasion of *S. noctilio* (Gilbertson, 1984; Nielsen *et al.*, 2009; Hajek *et al.*, 2013; Wooding *et al.*, 2013). *Amylostereum areolatum* IGS BD (AaBD), isolated from *S. noctilio* in New York State, is assumed to have been introduced with *S. noctilio* to North America (Slippers *et al.*, 2002; Hajek *et al.*, 2013). Fungal isolates were stored at  $-81^{\circ}\text{C}$  and then thawed for the experiments. Fungal cultures were grown on 50% potato dextrose agar (PDA) at  $23^{\circ}\text{C}$  in darkness for at least 2–3 weeks. Circular plugs of each fungal strain were cut from mature cultures with a 10-mm diameter cork borer to use for inoculating bolts.

We generally based the experimental methods on those reported by Fukuda & Hijii, 1997, who compared the oviposition behaviour of two Japanese siricids. We conducted experiments to evaluate the ovipositor drilling behaviour of *S. noctilio* or *S. nigricornis* in response to pine bolts inoculated with either of the three fungal isolates versus controls. *Pinus sylvestris* (2011) and *P. resinosa* (2012) used for experimental bolts were harvested from Arnot Teaching and Research Forest, Cornell University, Van Etten, New York. Mature, healthy trees with no previous signs of *Sirex* oviposition were felled. For assays with *S. noctilio*, trees were felled in mid-summer and cut into 70-cm long bolts approximately 2 weeks before an experiment began. After cutting, bolts were allowed to dry in an enclosed space for 1 week prior to fungal inoculation. Adult *S. nigricornis* were available much later, in November. For trials with *S. nigricornis*, pines were felled in mid-summer, ends were waxed and trees were stored in a growth chamber at  $15^{\circ}\text{C}$  for 3 months. When *S. nigricornis* were available, wood was moved to ambient temperature and waxed ends were removed. Wood was then cut into bolts and, 1 week later, fungus was inoculated.

For all experimental bolts, 5–7 days prior to a study, bolts (mean  $\pm$  SE diameter:  $19.1 \pm 0.4$  cm for *S. noctilio* and  $21.6 \pm 0.5$  cm for *S. nigricornis*) were randomly assigned to one of four treatments: inoculation with plugs of fungus (AaBE, Ach or AaBD), or uninoculated PDA, with one treatment per bolt. A 10-mm diameter cork borer was used to remove 16 ‘plugs’ of bark and outer cambium, approximately 10 mm deep. Fungal

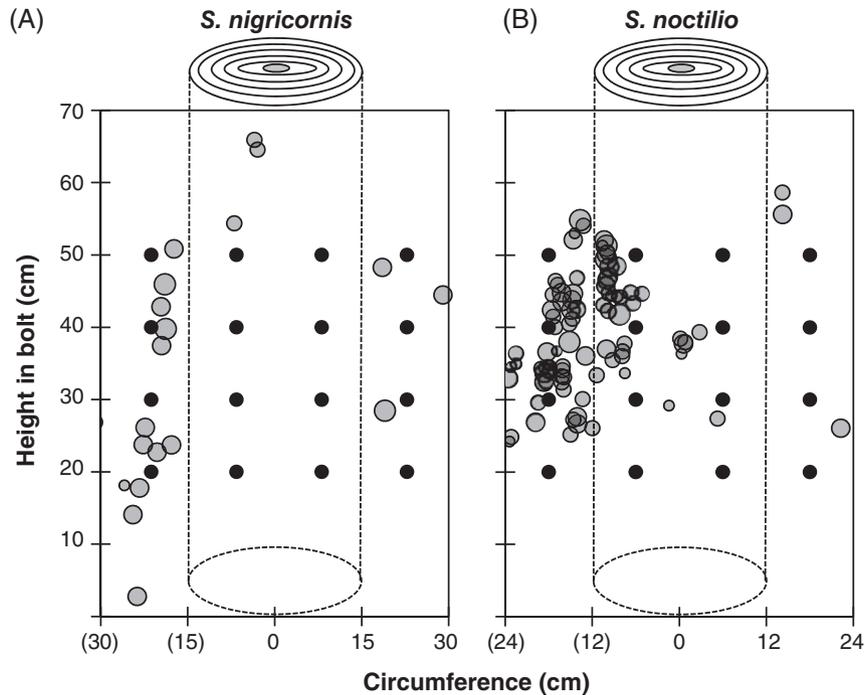
plugs with consistently mature growth were cut from culture plates and placed into holes, with mycelium facing inward. For control treatments, plugs of uninoculated PDA were inserted using the same methods. To conserve moisture and avoid contamination, each fungal plug was then covered with the bark portion (approximately 3 mm thick) of the original wood plug and taped in place. Four columns of four fungal plugs each were spaced at each  $90^{\circ}$  around the bolt circumference, with plugs approximately 10 cm apart vertically (Fig. 1). Plugs at the end of each column were 20 cm from the ends of the bolts, aiming to decrease potential effects of the cut ends.

Parts of each wood plug not placed over the fungal plug were weighed and dried in an oven at  $80^{\circ}\text{C}$  for 48 h. The wet and dry weights of the wood plugs were used to calculate percentage moisture [% moisture = (wet weight – dry weight)/wet weight  $\times$  100] and all 16 values for each bolt were averaged. All bolts used for studies had moisture levels of 46.2–56.7%. Preliminary analyses indicated no significant differences in oviposition behaviour according to bolt diameter or moisture (data not shown).

#### Ovipositor drilling trials

Numbers of *Sirex* available for experiments varied by year and species. In 2011, 24 *P. sylvestris* bolts (six inoculated with each of the three fungal treatments or controls) were arranged randomly, in a  $3.3 \times 3.3$  m mesh tent in a greenhouse and 24 female *S. noctilio* were released into the tent for 48 h. This was repeated, with new *S. noctilio* and new bolts (48 *S. noctilio* females in total). Four replicates of 12 *S. nigricornis* (48 *S. nigricornis* females total) were conducted in the same way. In 2012, this experimental design was used again with 16 *P. resinosa* bolts for each of two replicates with 26 or 35 females of *S. noctilio* in the test with the bolts for 30 h.

After *Sirex* were removed from tents, the bark was peeled from each bolt using fine chisels and drill holes were located and counted using binocular magnifiers ( $\times 3.5$ ; Optivisor, Donegan Optical Co., Lenexa, Kansas). When *S. noctilio* females drill into wood, they can create one solitary drill, which generally does not contain an egg but rather contains only fungus and venom (Madden, 1974); this is thought to pre-condition the wood before an egg is laid. Alternatively, when a female inserts her ovipositor into the wood, she can make several deeper drills at differing angles, after only one insertion of the ovipositor through the bark. In these cases, there are often two to five drills in a cluster, and eggs plus venom are generally deposited in the earlier drills (one egg/drill). The last drill of clusters with multiple



**Figure 1** Example of the distribution of oviposition by *Sirex nigricornis* (A) and *Sirex noctilio* (B) on two different bolts used in tent studies. Black dots indicate the placement of fungal treatments or controls around the bolt. The grey dots indicate the location of drill clusters around the bolt, and their size is proportional to the number of drills per drill cluster.

drills only includes fungal cells and venom. *Sirex nigricornis* is assumed to follow a similar pattern (F. Stephen, personal communication). Thus, in experimental bolts, when one drill hole was isolated from others or several drill holes were present in a small group (approximately 1–2 mm apart), these were each defined as a drill cluster. Therefore, clusters with only one drill represent the insertion of fungus plus venom, whereas clusters with two or more drills indicate injection of venom and fungus and oviposition. The number of drill holes in each cluster (from one to five) was recorded and the location of each drill cluster on each bolt was spatially referenced by measuring its distance and azimuth from the nearest fungal plug, which was also spatially referenced (Fig. 1).

#### Statistical analysis

We assessed the main effects of treatment and species, as well as their interaction, on the number of clusters per bolt, which was considered as a counting process and analyzed using Poisson regression. We combined data for 2011 (both species) and 2012 (*S. noctilio* only) given that the experiments were conducted very similarly in both years. The significance of main and interaction effects was assessed using  $Z$ , which refers to the ratio of the parameter coefficient to its standard error, as a test statistic in Poisson regression. We tested the null hypothesis that each parameter coefficient was 0 and constructed contrasts in post-hoc analyses (Agresti, 1996).

We also examined the main effects of treatment and species, as well as their interaction, on the number of drills per cluster. The number of drills per cluster ranged from one to five, although,

biologically, the drills per cluster can be considered as a binary response. Thus, using this binary response variable, we tested the significance of main and interacting effects using nominal logistic regression. The significance of effects was based upon the likelihood ratio and odds ratios were constructed when appropriate (Agresti, 1996).

We investigated main effects of treatment and species, as well as their interaction, on the minimum distance between drill clusters and control or fungal plugs. We did this aiming to determine whether these two woodwasp species were avoiding fungi that they did not prefer by drilling further from those fungal inoculations or were drilling closer to inoculations of fungi that they preferred. The minimum distance (cm) was transformed using  $\log_{10}$  to normalize the distribution. We used an analysis of variance to test significance and post-hoc tests were conducted on the least squares means using Tukey's honestly significant difference test. This and previous analyses were conducted in R (R Development Core Team, 2015).

We examined the degree of spatial aggregation in the location of drill clusters to determine whether *Sirex* females showed a spatial preference with respect to ovipositional behaviour. The location of drill clusters in each bolt was considered as a spatial point process and spatial aggregation was estimated through the  $L$ -function using the spatstat package (Baddeley *et al.*, 2015) in R (R Development Core Team, 2015). The surface of each bolt was considered a cylindrical coordinate system and each drill cluster was assigned unique spatial coordinates based upon its distance and direction from the nearest plugs. A two-dimensional representation of the distribution of oviposition on two bolts is shown in Fig. 1. We first estimated the  $L$ -function separately for

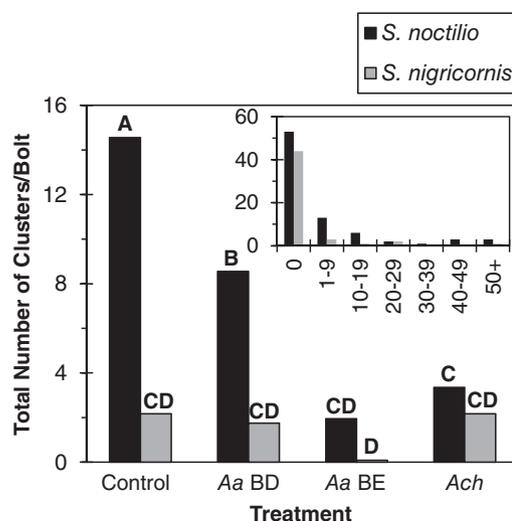
each year and species (*S. noctilio* in 2011 and 2012; *S. nigricornis* in 2011) and tested the significance of empirical estimates of the *L*-function against 200 Monte Carlo simulations of complete spatial randomness. We then examined spatial aggregation in location of drill clusters within a species but for each treatment separately to determine whether there was a tendency for *S. nigricornis* or *S. noctilio* females to aggregate their drill clusters qualitatively differently, depending on bolt treatment.

## Results

With regard to the number of drill clusters per bolt, we observed significant effects of species ( $Z=9.04$ ,  $P<0.001$ ), treatment ( $Z=8.40$ ,  $P<0.001$ ) and a species-by-treatment interaction ( $Z=4.58$ ,  $P<0.001$ ). Significant differences among treatments and between species are noted in Fig. 2. The pattern of fungal choice by *S. noctilio* differed from *S. nigricornis*. In particular, choice tests showed that *S. noctilio* most frequently drilled into control bolts, with intermediate drilling into bolts with the invasive *AaBD* and the least drilling into bolts with the native *AaBE* and *Ach* (Fig. 2). Interestingly, there were some drills in bolts inoculated with each type of fungus. By contrast, *S. nigricornis* created many fewer drills than *S. noctilio* and displayed an approximately equal number of drills in each treatment, except there were almost no drills in bolts inoculated with the native *AaBE*.

We also observed a significant effect of species ( $G^2=6.6$ ,  $P=0.01$ ), treatment ( $G^2=29.9$ ,  $P<0.01$ ) and a species-by-treatment interaction ( $G^2=33.8$ ,  $P<0.01$ ) on the binary response that reflects the number of drills per cluster (1 drill = fungus plus venom versus 2+ drills = venom and 1 egg/drill until the last drill of fungus plus venom) (Fig. 3). Merging data across treatments, *S. noctilio* females were overall 4.0 times (95% confidence interval = 2.5–6.6) more likely to inject venom and oviposit ( $\geq 2$  drills/cluster) than were *S. nigricornis* females. Within *S. noctilio*, although there were differences in the numbers of clusters by treatment, with a strong preference for control bolts or bolts treated with its putatively invasive symbiont, *AaBD* (Figs 2 and 3B), there was no effect of treatment with regard to the proportion of clusters with 1 or 2+ drills ( $G^2=2.6$ ,  $P=0.46$ ) (Fig. 3B). By contrast, there was a significant effect of treatment for *S. nigricornis* ( $G^2=36.6$ ,  $P<0.01$ ); *S. nigricornis* females were 52.7 times (95% confidence interval = 6.4–431.8) more likely to both inject venom and presumably oviposit in bolts treated with *AaBD* than the other treatments combined (Fig. 3A).

Mean distances between drill clusters and control or fungal plugs, by treatment and species, are presented in Table 2. We observed a significant effect of treatment ( $F=5.50$ ;  $df=3$ , 617;  $P<0.01$ ), an insignificant effect of species ( $F=0.35$ ;  $df=1$ , 617;  $P=0.55$ ) and a significant treatment-by-species interaction effect ( $F=2.77$ ;  $df=3$ , 617;  $P=0.04$ ) in the distance of drill clusters from fungal or control plugs (Table 2). *Sirex nigricornis* drilled furthest from control plugs, and closest to *AaBD*, the fungal strain assumed to have been introduced to North America with *S. noctilio*. This is consistent with our findings that *S. nigricornis* also made more 2+ drill clusters in this treatment compared with others. In *S. noctilio*, females drilled closest to *AaBD* and *AaBE* and furthest from *Ach*.

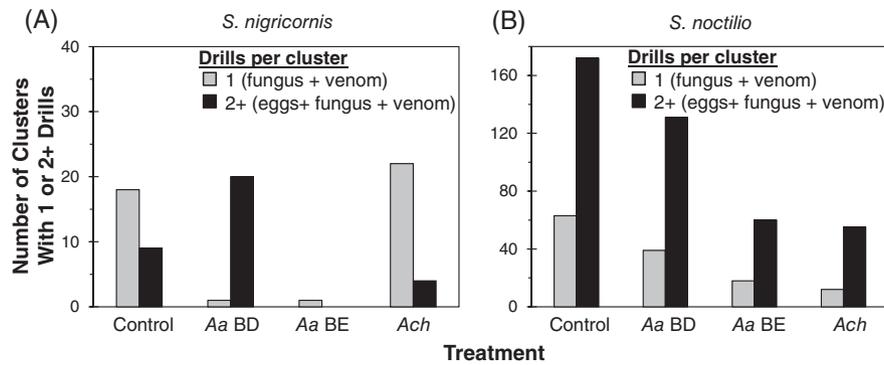


**Figure 2** The number of drill clusters per bolt for *Sirex noctilio* and *Sirex nigricornis* by treatment (*AaBD* = *A. areolatum* introduced with *S. noctilio*; *AaBE* = *Amylostereum areolatum* native to North America; and *Ach* = *Amylostereum chailletii*). Different uppercase letters represent significant differences among treatments and species ( $P<0.05$ ). Inset: showing, by *Sirex* species, the number of bolts (*y*-axis) having 0 clusters, 1–9 clusters, ..., and 50 or more clusters (*x*-axis), and also illustrating the preponderance of 0 data.

For each species and year, we observed significant spatial aggregation (e.g. significantly different from complete spatial randomness) in the location of drill clusters when pooling over treatments at spatial scales  $>0.6$  cm (Fig. 4). When considering each species and their degree of aggregation by treatment, the behaviour of the *L*-function was qualitatively similar across treatments, including control bolts, for *S. nigricornis* (Fig. 5A). For *S. noctilio*, *L*-function estimates were similar among the fungal-treated bolts, which were qualitatively different from control bolts, even though we still detected significant spatial aggregation in control bolts relative to complete spatial randomness (Fig. 5B).

## Discussion

The invasive and native *Sirex* species both discriminated among pine bolts inoculated with different fungal treatments, although they made very different choices. *Sirex noctilio* consistently drilled most into control bolts rather than into bolts inoculated with *Amylostereum*. However, the proportion of drill clusters that were pre-conditioning (one drill/cluster) versus presumably egg-laying ( $\geq 2$  drills/cluster) did not differ across treatments. Less drilling by *S. noctilio* into bolts inoculated with *A. areolatum* could have been an active choice because we know that *S. noctilio* females can detect *A. areolatum* volatiles (Fernández Ajó *et al.*, 2015; Sarvary *et al.*, 2016). Drilling by *S. noctilio* in bolts inoculated with *AaBD*, which is assumed to have been introduced to North America with *S. noctilio*, was intermediate. Avoidance of the other two fungal treatments, both of which could have been novel to *S. noctilio*, is easy to envision because the presence of these other fungi could have limited successful colonization by the fungus being inoculated at oviposition. We



**Figure 3** Number of drill clusters with either one drill (fungus plus venom only) or two or more (presumably adding oviposition) for (A) *Sirex nigricornis* and (B) *Sirex noctilio*, and by treatment (AaBD = *Amylostereum areolatum* putatively introduced with *S. noctilio*; AaBE = *Amylostereum areolatum* native to North America; and Ach = *Amylostereum chailletii*). Although *S. noctilio* was significantly more likely than *S. nigricornis* to oviposit, the relative proportion of clusters with 1 versus 2+ drills did not differ among treatments in *S. noctilio*. By contrast, the proportion of oviposition (2+ drills) by *S. nigricornis* was significantly higher than single drills in bolts treated with AaBD.

**Table 2** Mean  $\pm$  SE minimum distance (cm) between drill clusters and treatment plugs in *Sirex nigricornis* and *Sirex noctilio*

<i>Sirex</i> species	Treatment	Number of clusters	Mean $\pm$ SE distance <sup>a</sup>
<i>Sirex nigricornis</i>	Control	27	9.45 $\pm$ 0.77 A
	<i>Amylostereum areolatum</i> BD (AaBD)	21	6.50 $\pm$ 0.99 B
	<i>Amylostereum areolatum</i> BE (AaBE)	1	8.50 $\pm$ NA <sup>b</sup>
	<i>Amylostereum chailletii</i> (Ach)	26	8.25 $\pm$ 0.91 AB
<i>Sirex noctilio</i>	Control	235	7.55 $\pm$ 0.22 AB
	<i>Amylostereum areolatum</i> BD (AaBD)	170	7.01 $\pm$ 0.27 B
	<i>Amylostereum areolatum</i> BE (AaBE)	78	6.92 $\pm$ 0.40 B
	<i>Amylostereum chailletii</i> (Ach)	67	8.70 $\pm$ 0.42 A

<sup>a</sup>Different uppercase letters denote significant differences. Analyses were conducted on the transformed data, whereas raw data (means  $\pm$  SE) are reported within Table 2.

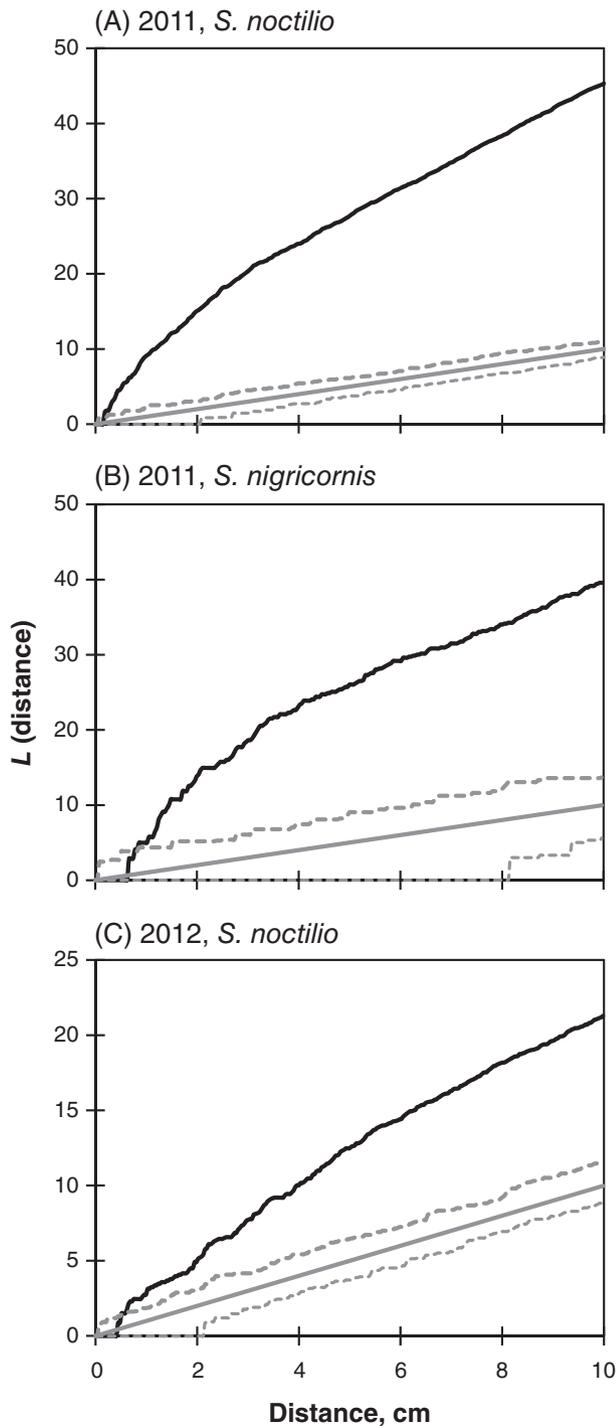
<sup>b</sup>As a result of only one cluster, this treatment was excluded from analyses; the mean distance represents the measurement for this single cluster.

know that if fungi vectored by bark beetles (Ryan *et al.*, 2011; Yousuf *et al.*, 2014) or other strains or species of *Amylostereum* (Nielsen *et al.*, 2009; Hajek *et al.*, 2013; Castrillo *et al.*, 2015) are already present, these can compete with and exclude *A. areolatum* strains. We also observed that *S. noctilio* drilled furthest from Ach, which is a species that it never or rarely carries (Nielsen *et al.*, 2009; Hajek *et al.*, 2013; Wooding *et al.*, 2013). Therefore, the choice of control bolts by *S. noctilio* could be a successful strategy to improve fitness because other fungi would not be present to compete with the fungus being inoculated.

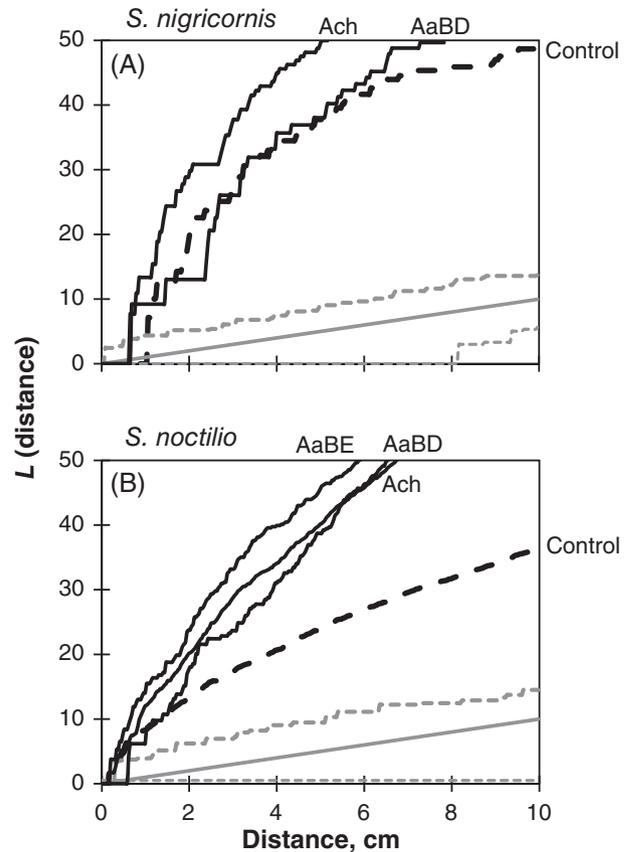
Consistent with its being aggressive, *S. noctilio* made more total drill clusters than *S. nigricornis* and more clusters presumably with eggs ( $\geq 2$  drills/cluster). This trend of abundant drilling by *S. noctilio* has also been reported during comparisons with less aggressive European woodwasps (Spradbery, 1973). The low numbers of drills by *S. nigricornis* compared with *S. noctilio* could also be a result of other causes; unavoidably, we did not know whether *S. nigricornis* females had mated or not and being unmated might have influenced their reproductive behaviour to some extent, as has been reported for *S. noctilio* (Madden, 1974).

Recent studies are now reporting symbiont switching in different systems (Kiers *et al.*, 2010; Garnas *et al.*, 2016) and the present study confirms this possibility for *S. nigricornis*. *Sirex nigricornis* created the most drills into bolts inoculated with AaBD (Fig. 3A), a fungal strain putatively introduced to North America with *S. noctilio* (Nielsen *et al.*, 2009; Hajek *et al.*, 2013).

Almost all of these drills were clusters of  $\geq 2$ , suggesting that eggs were laid. Preference for this fungal strain by *S. nigricornis* in the present study is consistent with the fact that larvae of this native *Sirex* are known to develop in the same trees as *S. noctilio* (Long *et al.*, 2009; Ryan *et al.*, 2012a; Hajek *et al.*, 2013) and horizontal transmission of *Amylostereum* between *Sirex* species appears to occur within trees (Hajek *et al.*, 2013; Wooding *et al.*, 2013). *Sirex* species were previously considered specific to single *Amylostereum* species (Talbot, 1977; Gilbertson, 1984), although studies of *Sirex* and *Urocercus* have demonstrated flexibility in the *Amylostereum* species being carried (Wooding *et al.*, 2013; Fitza *et al.*, 2016; Hajek *et al.*, 2017b), in particular by two *Sirex* native to North America, including *S. nigricornis* (Hajek *et al.*, 2013; Olatinwo *et al.*, 2013). Although relatively little is understood about the biology and behaviour of *S. nigricornis*, flexibility in symbiont use could be advantageous now that the competitive *S. noctilio* is present. Native *Sirex* species usually develop in stressed trees, comprising an ephemeral resource, whereas *S. noctilio* is considered to be better able to successfully attack healthier trees than *S. nigricornis*. *Sirex nigricornis* adults generally emerge and fly later in the year than *S. noctilio* (Ryan *et al.*, 2012a; Haavik *et al.*, 2013; Hartshorn *et al.*, 2016; Hajek *et al.*, 2017a) and so *S. noctilio* emerges and finds sites for oviposition before *S. nigricornis*. *Sirex nigricornis* drilled and presumably oviposited close to locations where AaBD is present in wood which suggests that this species might be willing to take



**Figure 4** *L*-function estimates (solid black line) of the spatial location of drill clusters for *Sirex noctilio* in 2011 (A) and 2012 (C), as well as for *Sirex nigricornis* in 2011 (B). The grey solid line represents the theoretical expectation under complete spatial randomness (CSR), whereas the dashed grey lines are the upper and lower expected values under CSR based upon 200 simulations. In all cases, there is evidence of significant spatial clustering of drill locations at distances > 0.8 cm.



**Figure 5** *L*-function estimates of the spatial location of drill clusters for (A) *Sirex nigricornis* (2011) and (B) *Sirex noctilio* (2011–2012) by treatment. The grey solid line represents the theoretical expectation under complete spatial randomness (CSR), whereas the dashed grey lines are the upper and lower expected values under CSR based upon 200 simulations. The dashed black lines represent control bolts, whereas the solid black lines are labeled by bolt treatment (AaBD = *Amylostereum areolatum*, introduced with *S. noctilio*; AaBE = *Amylostereum areolatum*, native to North America; and Ach = *Amylostereum chailletii*). In all bolt treatments, including the controls, there was evidence of significant spatial clustering, although the pattern was different in *S. noctilio* fungal treatments relative to the controls. AaBE treatments for *S. nigricornis* are not shown because there was only one drill cluster.

advantage of fungal symbionts already established by *S. noctilio*. Because *S. nigricornis* attacks trees after *S. noctilio*, however, the amount of the tree acceptable to and available for *S. nigricornis* will be, in part, determined by acceptable areas of tree that are unoccupied after oviposition by *S. noctilio*.

We found that drills by both species were aggregated. In Tasmania, Madden (1974) reported a tendency for uniform distributions of *S. noctilio* drills in *Pinus radiata*, although, in more resistant trees, drilling was aggregated. The advantages of drill aggregation could be that larvae would potentially be taking advantage of areas where *Amylostereum* and venom had been introduced at more than one drill cluster, compensating for situations where no or little fungus or venom were inserted, or so that eggs are laid where *Amylostereum* is already well established. In agreement with our findings, in the Southern Hemisphere, it was very common for *S. noctilio* to make more

single drills to pre-condition wood than clusters with numerous drills, which are assumed to indicate oviposition (Coutts & Dolezal, 1969; Madden, 1974).

Whether the introduction of *S. noctilio* and the *A. areolatum* strains putatively introduced with it will affect population densities of *S. nigricornis* cannot be conclusively determined because no data are available regarding *S. nigricornis* densities prior to the invasion by *S. noctilio*. Native North American siricids are not considered pests and are relatively poorly studied with regard to their life history and ecology. Thus, the consequences of invasion by the congeneric *S. noctilio* to *S. nigricornis* populations remain unknown. We do know that relatively few *S. nigricornis* emerge from trees co-infested by *S. noctilio* (A. E. Hajek, unpublished data; Ryan *et al.*, 2012a; Hajek *et al.*, 2013, 2017a). However, changes in the fitness of *S. nigricornis* when switching to what is assumed as being the invasive symbiont requires further study. Indeed, no experimental studies have been conducted for either of these *Sirex* species to define fitness relative to association with different fungal strains or species. Conversely, our results do not suggest that *S. noctilio* will preferentially switch to adopt the native *A. areolatum* (AaBE) or *A. chailletii*.

It is generally assumed that *S. nigricornis* prefers to oviposit when trees are very stressed, more so than *S. noctilio* (Hajek *et al.*, 2017a). The reason behind less total drilling by *S. nigricornis* compared with *S. noctilio* could have been that the age of wood being offered was not optimal. The possibility also remains that *S. nigricornis* females could have drilled less than *S. noctilio* overall because *S. nigricornis* females used in studies were collected from traps were older, whereas *S. noctilio* females were collected directly after emergence from wood.

Three strains of *A. areolatum* are assumed to have been introduced to North America with *S. noctilio* (Bergeron *et al.*, 2011; Castrillo *et al.*, 2015), adding to at least one native strain of *A. areolatum* and native *A. chailletii* (Nielsen *et al.*, 2009; Olatinwo *et al.*, 2013). Our experimental studies confirm that *Sirex*/*Amylostereum* mutualism is not as specific as previously assumed and this community of strains and species of mutualists will be used by both these invasive and native woodwasps to some extent. The divergent biologies of these two woodwasps may mean that they will use different resources within the same regions (i.e. predominantly attacking trees with differing levels of vigour), perhaps with differential assistance by the mutualists acquired either vertically or horizontally. Regardless, it remains highly likely that biotic homogenization will continue as more species of *Sirex* or *S. noctilio* strains from new locations carrying different strains of *A. areolatum* (Slippers *et al.*, 2002) are introduced to new regions. Increased biotic homogenization will add yet another aspect of uncertainty to the invasiveness of *S. noctilio*, related to the use of *A. areolatum* by native *Sirex* and the associated challenges posed by the potential use of biological control with a nematode that switches between parasitism of *S. noctilio* and feeding on *A. areolatum*.

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