1 For submission to **Biological Control**

2

3 Biological control of *Sirex noctilio* (Hymenoptera: Siricidae) in the northeastern United States
4 using an exotic parasitic nematode

5

6 David W. Williams^{1,*} and Ann E. Hajek²

7

8 ¹USDA, APHIS, CPHST Otis Lab, Buzzards Bay, MA U.S.A. 02542-1308

9 ²Department of Entomology, Cornell University, Ithaca, NY U.S.A. 14853-2601

10

11 * Corresponding author. *E-mail address*: david.w.williams@aphis.usda.gov (D.W. Williams),

12 Phone: 508-563-0943, Fax: 508-563-0903

13

ABSTRACT

The woodwasp *Sirex noctilio* is a devastating killer of pines in the southern hemisphere. Over the 20th Century, it has invaded exotic pine plantations in New Zealand, Australia, South America, and South Africa successively. In response to this woodwasp threat, in the 1960-70s CSIRO in Australia launched a major campaign of exploration for biological control agents in Europe, the Middle East, and North Africa, the presumed indigenous range of *S. noctilio*. The most promising natural enemy discovered was a nematode, *Deladenus siricidicola*, which lives inside a pine tree as a mycophagous form but becomes parasitic when near host larvae and sterilizes adult woodwasp females. Australian scientists developed a biological control management system based on the nematode, including how to rear and release it in the field. The CSIRO program has been a model for *Sirex* management in the pine-growing regions of the southern hemisphere since the 1960s. Thus, when *S. noctilio* was collected in North America in 2004, it seemed natural to try using the Australian model as a simple technology transfer. Unfortunately the transfer has not proved to be so simple. In the following, we present the results of six years of experimental controlled release studies, in which no nematodes were actually released into the field. One of the first discoveries was that another strain, a “non-sterilizing” strain, of *D. siricidicola* was already present in the areas invaded by *S. noctilio*. Our annual studies compared infection rates between trees inoculated with the Australian “Kamona strain” and controls without nematodes. In most cases, the treated trees had the higher rates of nematode parasitism, but the controls were not always zero, suggesting that the non-sterilizing strain was well-established in our study plots. Many of the Kamona-treated trees did not contain Kamonas, suggesting that the non-sterilizing strain may have out-competed the Kamonas. Even with Kamona treatments, there was relatively little sterilization, indeed much less than expected. It

seems likely that the *S. noctilio* strain invading North America is physiologically different from the Australian nematode and not susceptible to Kamona sterilization. Another interesting result was observed between host tree species. In a three-year study, nematode parasitism rates were consistently higher in *P. sylvestris* than in *P. resinosa* and significantly higher when the three years of data were pooled. We conclude with the observation that the Kamona strain is apparently not compatible with the *S. noctilio* invading North America and suggest that more exploration is needed in its area of origin for different and more effective nematode genotypes.

Key words: *Deladenus siricidicola*; *Sirex noctilio*; *Pinus*; nematode; Kamona strain; classical biological control

1. Introduction

The European woodwasp, *Sirex noctilio* L., is a killer of *Pinus* species in the southern hemisphere. It was first discovered outside its native range of Europe, North Africa, and the Middle East in New Zealand about 1900 (Rawlings, 1948). Because pine is exotic in the southern hemisphere, where it is principally grown in plantations, *S. noctilio* found a perfect environment in which to prosper, with abundant monocultures of its host and without a guild of natural enemies. This woodwasp was reported next in Tasmania in 1952 and shortly thereafter (1961) on the Australian mainland, where it continues to invade the eastern half of the continent (Neumann et al., 1987; Carnegie et al., 2005; Collet and Elms, 2009; Nahrung et al., 2015). By the 1980s, *S. noctilio* was discovered in South America, with the distribution now including Uruguay, Argentina, Brazil, and Chile (Iede et al., 2000). By 1994, *S. noctilio* was found in South Africa (Tribe, 1995). Woodwasp outbreaks have wrought destruction as the species traveled throughout the southern hemisphere (Hurley et al., 2007). *Sirex noctilio* was moved most recently to the northern hemisphere, where in 2004 and 2005 it was found invading the northeastern United States and Canada, respectively (Hoebeke et al., 2005; de Groot et al., 2006). Interestingly, this species is not considered a pest in Europe (Wermelinger and Thomsen, 2012).

In response to the obvious damage potential posed by *S. noctilio*, the Australian Congress of Scientific and Industrial Research Organization (CSIRO), along with the Commonwealth Institute of Biological Control, mounted a worldwide campaign in the 1960s and 1970s to explore for its natural enemies (Taylor, 1967). Ultimately ten species of parasitic Hymenoptera were imported, of which five became established, with variable impacts on the host (Taylor, 1976). However, the most generally successful natural enemy proved to be a nematode, *Deladenus siricidicola* Bedding, which was chosen from many candidate species primarily

because of its specificity to siricids, its ability to cause high infection rates (>95%), and its relatively minor effects on host growth and adult size (Bedding and Iede, 2005). The strain ultimately used for the CSIRO biological control program was collected originally in Sopron, Hungary, and a more recent reisolate now in use is named “Kamona”.

The nematode had the very convenient characteristic of being able to develop into two very different morphs: a fungus-feeding form or a parasitic form (Bedding, 1972). If an *S. noctilio* host was not near the oviposition site, the juvenile nematodes developed into the fungus-feeding form, eating the symbiotic white rot fungus, *Amylostereum areolatum* (Chaillat ex Fries) Boidin, carried by female *S. noctilio* and injected into the tree during oviposition. This fungus is necessary for the nutrition of larval progeny of *S. noctilio* (Madden and Coutts, 1979; Thompson et al., 2014). When host larvae were scarce, the nematode could produce multiple generations per year feeding on the fungus, a feature that proved to be very useful for its mass rearing (Bedding and Iede, 2005; Bedding, 2009). On the other hand, when a host larva was nearby, juvenile nematode development took a different pathway, leading to the production of an adult female of the parasitic form, which bored into the host larva using a stylet (Bedding, 1972). At host pupation, the female nematode grew rapidly and produced many juvenile offspring that entered the developing host eggs. Host development through the adult stage was not affected by the parasitism, and when the woodwasp female finally emerged, she flew and oviposited the infected eggs normally. The result was to reduce the woodwasp population while simultaneously dispersing the juvenile nematodes into new trees (Bedding, 2009).

In brief, this nematode was used by CSIRO to implement a biological control system in Australia as follows (Haugen et al., 1990; NSCC, 2002; Bedding and Iede, 2005). Nematodes were mass reared during the summer. In the meantime, plantations were surveyed for woodwasp

hot spots. In the late spring to early summer, trap tree plots of ten pines were created near the hot spots. The trees were injected with an herbicide to stress them so as to render them attractive to *S. noctilio* females (which were active in the late summer) and eventually to kill them. During the fall to mid-winter, the trap trees, which were by then populated with woodwasp larvae and fungus, were felled and inoculated with nematodes, which developed and invaded host eggs during the remainder of their annual life cycle.

At the outset of the USDA's *S. noctilio* biological control program in 2005, development seemed to be simply a matter of technology transfer from CSIRO: purchase nematodes from Ecogrow, the licensed producer in Australia, culture and mass rear *D. siricidicola* according to the preexisting protocols (Bedding and Iede, 2005), purchase the hammers needed to punch holes for the inoculations, and inoculate the trap trees according to established methods on an annual calendar of operations (Williams et al., 2012). Following these steps, we could expect to obtain *D. siricidicola* infection rates of 90% or more in the *S. noctilio* population (Bedding and Akhurst, 1974). Unfortunately, this proved to be wishful thinking. Many factors, both abiotic and biotic, differed between Australia and eastern North America, including climate, *Pinus* species, species and strains of the symbiotic fungus, *A. areolatum*, and presence of several indigenous *Sirex* species (Williams et al., 2012). Changes of any or all of these factors had the potential to alter the dynamics of the tree-fungus-wasp-nematode ecosystem. In addition, a non-sterilizing strain of *D. siricidicola* was already present in *S. noctilio* populations in North America (Yu et al., 2009). In the following, we investigate differences in infection rates and egg sterilization in the U.S. region under invasion by *S. noctilio* and consider causes for low levels of efficacy, including pine species and the presence of other nematode strains and species.

2. Materials and methods

2.1 Sites

We used pine stands in central New York and the northern tier counties of Pennsylvania over the course of the six-year study (Fig. 1). Ideally we would have preferred to use the same sites for the duration of the study. However, this was not possible, especially not in New York State. Stands there were generally small and privately owned. Thus, we were not permitted to fell too many trees and risk overexploiting a stand. This was also the case with some stands in Pennsylvania. The only site we observed over most of the study years was Hills Creek State Park, PA.

Initially, the sites were centered near Fulton, NY (star on Fig. 1), where *S. noctilio* was first detected, but over time, experimental sites were generally moved southward, tracking the spread of the woodwasp population and our pest survey efforts.

Each year, we established three to five sites in largely unmanaged plantations of European Scots pine, *Pinus sylvestris* L., and North American red pine, *P. resinosa* Sol. ex Aiton, sampling 50-100 trees each year (Table 1).

2.2 Nematode and fungus cultures

We purchased nematode cultures from the company in Australia that mass produces *D. siricidicola* (Ecogrow Australia, Bondi Junction, New South Wales). These nematodes were of the Kamona strain, cultured in the laboratory on a fast-growing isolate of the fungus *A. areolatum*, on potato dextrose agar. We maintained nematode cultures on several North American isolates of *A. areolatum*, but primarily used two fungal isolates for mass-rearing. Both were isolated from the mycangia of *S. noctilio* females collected in the field in central New York and northern Pennsylvania. Following methods outlined in Bedding and Iede (2005), we mass-

reared the fungus and nematode in culture flasks on an autoclaved mixture of brown rice and wheat berries.

In 2008, a strain of *D. siricidicola* was discovered to be already present in many sites in Canada and the U.S. and presumably had been introduced with *S. noctilio* (Shields, 2009; Williams et al., 2009; Yu et al., 2009). Following Zondag (1975), we call it the “non-sterilizing strain” because it does not enter the woodwasp eggs.

2.3 Releases

Initially we planned timing for releases of *D. siricidicola* by extrapolation from the Australian experience, which indicated a time frame for releases from November to December. However, considering the large climatic differences between Australia and North America, the former having mild winters and the latter, very cold winters, we decided to move the date for releases to a warmer part of the season in the northeastern U.S. and release the nematodes in the fall. The release dates were as follows: 2 October 2007, 25 September 2008, 23 September 2009, 23 August and 26 September 2010, 27 August 2011, and 8 September 2012. We generally used naturally struck trees (i.e., as opposed to herbicided trap trees) for the releases as these provided more reliable infestations. We selected trees for inoculation primarily by the presence of resin beads (Rawlings, 1948). If trees met that criterion, we then examined their crowns. We chose trees with red or brown needles, but also accepted suppressed trees with green needles. We rejected trees without needles or with *Sirex* emergence holes. We then felled the trees, inoculated them with nematodes, and sealed the cut bole ends with a green wood end sealer (Waxlor®, Willamette Valley Company, Eugene, Oregon).

2.4 Inoculation

Preparation of the nematodes for a release followed a precise procedure and recipe. harvesting of nematodes from culture flasks is detailed in Bedding and Iede (2005). Briefly, we washed the mass-reared nematodes from flasks with tap water, counted them, and placed them in breathable baggies in aliquots of 1×10^6 *D. siricidicola* per 10 ml for transport to the field. At a release site, we mixed the contents of one baggie with 5 g of polyacrylamide gel and 500 ml of water, whisked the mixture until the water was absorbed, and decanted it into plastic squeeze bottles. In the 2007, 2010, and 2012 releases, we used the nematode dosage recommended by CSIRO, that is, 1×10^6 per batch of inoculum, to treat 10-20 trees (Bedding and Iede, 2005). To prepare the trees, we felled them and punched 50 inoculation holes at 15 cm spacing along the lower tree trunk using a punch hammer (Bedding, 2009). For the occasional shorter tree, we had fewer than 50 holes, with the highest number determined by the height at which the stem diameter was ≈ 5 cm. We then injected approximately 0.45 ml of gel inoculum into each hole using the squeeze bottle. Unlike the nematode preparation, the nematode application method in the field is not so precise. The punch holes may vary in depth and the gel suspension may not completely fill the holes. In practice, we seemed to be using less inoculum per tree than reported in the literature, with some left over after a site was complete. Accordingly, we compensated for this apparently lower density of nematodes by increasing the dosages by two-fold in 2009 and 2011 and by three-fold in 2008. Rather than risk applying too few nematodes, we chose to add some in the release preparation.

Trees remained on the ground exposed to ambient conditions during the winter. Because we were unaware of the presence and importance of the non-sterilizing strain until 2009, we assumed in 2007 and 2008 that all nematode infections resulted from the Kamona inoculations and did not include uninoculated trees as controls. Starting in 2009, we created controls by

183 felling a third or half of the sample trees at each site and injecting them with pure gel (minus
184 nematodes).

185 2.5 Sample collection

186 Each year, we collected bolt samples from inoculated trees during late winter to early
187 spring (March – May). We cut three 68-cm bolts from each tree from within the inoculation
188 zone: one at the top (≈ 7.5 m above the base) and another at the bottom (≈ 0.5 m) and a third at
189 approximately the center (≈ 4.0 m). Remaining woody material, including the tree crown, was
190 chipped to 2.5 cm dimension so as to kill *S. noctilio* larvae and *D. siricidicola*, and thus, prevent
191 any escape of nematodes into the environment. We transported the bolts to the quarantine
192 laboratory, where we sealed the cut ends to slow desiccation and placed them in screened barrels
193 (48 cm diameter $\times$ 72 cm height) for adult emergence. During the next three months, we
194 collected the emerging insects and held them at -20°C before siricid identification and
195 dissection. All siricids, as well as parasitic Hymenoptera and Cerambycidae, were dissected to
196 assess possible infection by nematodes.

197 2.6 Analysis

198 The population size of *S. noctilio* was expressed as numbers of insects per m^3 of wood
199 (i.e., to account for size differences among trees). We did not record diameters and lengths of
200 each of the three bolts per tree during the first three years of the project. Instead we used
201 diameter breast height (dbh) as an aggregate estimate of sample diameter. Thus, sample volume
202 was estimated as simply,

$$\text{volume} = \pi \cdot (\text{dbh}/2)^2 \cdot \text{length}$$

where length = 2.04 m, the total length of the three bolts. From 2010 onward, we recorded lengths and diameters for all bolts and estimated the sample volumes with (presumably) greater accuracy as,

$$\text{volume} = \pi \cdot [(\text{diameter}_1/2)^2 \cdot \text{length}_1 + (\text{diameter}_2/2)^2 \cdot \text{length}_2 + (\text{diameter}_3/2)^2 \cdot \text{length}_3]$$

where the subscripts indicate the dimensions of the lower, middle, and upper bolts measured.

We explored these relative volume estimates by analyzing a data set with both dbh and individual bolt diameters (2011). We found a consistent difference between the two estimates for an individual tree, with dbh always yielding a higher estimate. Thus, we could transform the data using a simple correction factor. However, we have chosen not to do so, instead using what we believe be our best estimates for each year.

Statistical analyses were carried out in SAS 9.3 (SAS Institute, Inc., 2012; Schlotzhauer, 2009). Descriptive statistics were computed using PROC MEANS and PROC UNIVARIATE. Group comparisons were made with PROC TTEST or PROC NPAR1WAY, with the choice depending mainly on sample sizes and assessment of normality. The nonparametric analysis used a Wilcoxon Rank Sum test (Schlotzhauer, 2009) and included year/site comparisons with at least 7 trees

2.7. *Sirex* diagnosis and nematode collection

All emerging wasps were dissected to assess infection and sterilization. Specimens of both sexes were dissected with a minuten pin at a magnification of 45×. The presence or absence of nematodes within the hemocoel was first noted. Then the ovaries and eggs were examined in females and the testes in males. In assessing sterilization, we considered only the presence or absence of nematodes in eggs; we did not attempt to quantify numbers of eggs containing

nematodes for each female although recent research suggests that those numbers can be quite variable (Keeler, 2012; Nahrung et al., 2015). We generally examined at least ten individual eggs for each female. Because of difficulty in determining whether nematodes were inside an egg or merely between the outer chorion and inner ovariole wall, a compound microscope at 400× was sometimes needed for examinations.

To examine parasitism by the two strains of *D. siricidicola*, we used 417 male and 143 female *S. noctilio* from 2008-2012 for DNA analysis; these woodwasps were a subset of the total emergences given in Table 1 for these years. Woodwasps for DNA analysis were preserved in 95% ethanol in 10 mL scintillation vials and stored at -20° C.

2.8. Molecular identification of nematode strains

2.8.1. DNA extraction

Samples of nematodes were transferred to 180 ml tissue lysis buffer (ATL buffer, Qiagen, Valencia, CA) and stored at 4° C until DNA isolation and extraction were performed. Prior to extraction, tubes containing nematodes were maintained in a water bath at 56° C overnight for tissue lysis. DNA was then extracted using a QIAamp DNA Micro Kit (Qiagen) according to the protocol provided by the manufacturer. DNA was eluted with 30 mL sterile, distilled, molecular grade water (ddH₂O) and stored at -20° C in 1.5 mL QIAamp MiniElute columns until further use.

2.8.2. PCR and molecular analysis of *D. siricidicola*

The internally transcribed mtCO1 sequence was amplified for all nematode isolates. The primers used for this region were the CO1-F1 primer (5'CCTACTATGATTGGTGGTTTTGGTAATTG'3) and the CO1-R1 primer (5'CAGGCAGTAAAATAAGCACGAGAATCT A'3) (Ye et al., 2007). Procedures described by Morris et al. (2013) were used for amplification. Amplification was carried out in 0.2 mL Thermowell tubes (Corning Inc., Corning, NY) in a total reaction volume of 25 µL, with PCR mixtures containing: 1 x PCR buffer with 25 mM MgCl₂ (Qiagen); 200 µM each of dATP, dCTP, dGTP, and dTTP (Qiagen); 0.5 µM of each primer; 70-100 ng extracted nematode DNA; and 2.5 U Taq PCR enzyme (Qiagen). Amplification was performed in a thermal cycler (PTC-100 Peltier, MJ Research, Boston, MA) with the following parameters specific for the mtCO1 region: Denaturation at 94° C for 5 minutes, 35 cycles of 50 s DNA denaturation at 94° C, 45 s annealing at 63 ° C, 1 minute 30 second extension at 72° C, followed by a final extension at 72° for 10 minutes. The samples were then purified using a QIAQuick PCR Purification Kit (Qiagen) according to manufacturer's instructions and eluted in Elution Buffer (Buffer EB). DNA concentrations were quantified by UV spectrophotometry using a Thermo Scientific NanoDrop™ 1000. Purified and quantified PCR products were subjected to 60V electrophoresis on a 1.5% (w/v) ethidium bromide-stained agarose gel for 1 hour and 40 minutes. Gels were then visualized under UV illumination and analyzed for the presence of CO1 bands.

2.8.3. Restriction analysis

Strain identification of nematodes was assessed qualitatively using a combination of restriction enzyme digests. The first method was based on the use of Aci1 (New England

Biolabs, R0551L, CutSmartTM Buffer) to cut the mtCO1 sequence, thus using the polymorphism described by Yu et al. (2009) to differentiate between Kamona and the non-sterilizing strain of *D. siricidicola*. This method is described in detail in Morris et al. (2013). The second test employed the enzyme BsrG1 (New England Biolabs, R0575S, NEBuffer 2.1) to differentiate between Kamona and *D. proximus* Bedding, a native nematode species that infects the native siricid *S. nigricornis*, which occurs in the same trees as *S. noctilio* (Bedding, 1974). All digests were incubated overnight at 37 °C and then subjected to 60V electrophoresis on a 1.5% (w/v) ethidium bromide-stained agarose gel and visualized under UV illumination after 1 hour and 40 minutes.

3. Results

The numbers of siricids (primarily *S. noctilio*) that emerged in our sample barrels and were dissected ranged widely, from < 10 to >700 per site per year (Table 1). Despite our best efforts to identify trees attacked by *S. noctilio* and the large numbers of trees we sampled (464), the percentages of uninfested trees averaged $36.4 \pm 19.7\%$ and ranged up to 83.3% (Table 1). The percentages of trees containing nematode-infected *S. noctilio* averaged just $28.1 \pm 20.5\%$.

During the course of the study, *S. noctilio* densities declined steadily overall, with over 1,000 woodwasps per m³ in 2007 through as low as 40.0 per m³ in more recent years (Table 1). Despite the wide range in woodwasp density among sites, the rates of infection (average percentages of total infected *S. noctilio* per infested tree per site in trees treated with *D. siricidicola*) were relatively consistent. During 2007 and 2008, the years for which we had no control trees, the average infection rates across sites were 21.8 ± 28.0 and $22.7 \pm 26.4\%$, respectively (Table 2). Nematode strain was determined in 2008. Almost half of the nematodes

at the Hills Creek site (48.6%) were Kamonas. The percentages of Kamonas decreased to 0.0 at sites closer to the putative original release in Fulton, NY.

In the later years, 2009-2012, as expected, infection rates in the uninoculated control trees were consistently lower than or about equal to the nematode-treated trees (Table 3). However, the nematode infection rate was significantly higher ($p = .017$) for treated trees (median = 27.0) than for control trees (median = 0.0) only at Hills Creek in 2011. Surprisingly, the DNA analyses revealed that, in spite of the higher infection rates in trees inoculated with Kamonas, most of those infections were not caused by Kamonas (Table 3). With the exception of the population at the Ole Bull State Park, PA site in 2010, which had 100% infection by the Kamona strain, the average infection rates in *S. noctilio* due to that strain ranged from just 0.0 to 33.3% (Table 3).

We caught a possible glimpse of the southward leading edge of the *S. noctilio* infestation in the period 2009-2011, from observations of the spatial distributions of nematode strains. The populations at Hills Creek State Park, PA, exhibited a decrease from 16.7 to 0.0 to 0.0% infection by Kamonas from 2009 to 2010, and then 2011, respectively (Table 3). Presumably, the Kamona strain released in that site was overwhelmed by the non-sterilizing strain brought in by the advancing *S. noctilio*. By contrast, the Ole Bull site, which is about 60 km to the southwest of Hills Creek (Fig. 1D), had only Kamonas in 2010, suggesting that we recovered the nematode strain that we had released and that the non-sterilizing strain had not yet arrived. To the southeast of Hills Creek, the Hypocrite Trail site (Fig. 1F) harbored nematodes that were 33.3% Kamona in 2012.

We collected the longest time series for a single site during 2008-2012 in a *P. resinosa* plantation at Hills Creek State Park, PA (Table 1). As with the overall results, the trend in mean density of *S. noctilio* started out high in 2008 ($937.9 \pm 716.2\%$ *S. noctilio* per m^3) and generally decreased from 2009 to 2012: 379.2, 431.3, 212.1, and 297.8%, respectively. However, the *S. noctilio* infection rates for treated trees were more variable at Hills Creek, ranging from approximately 0.0 in 2009, 2010, and 2012 (medians, Table 3) to 27.0% in 2011 (median, Table 3), possibly associated with the smaller sample sizes in later years. In addition, the control trees at Hills Creek had nearly zero nematodes in the period from 2009 through 2012. The molecular analysis of subsamples of reared female *S. noctilio* revealed that, in the best case, at Hypocrite Trail in 2012 (excluding the year 2010 at Hills Creek, which had just one sample), only 33.3% of all of the treated trees contained the Kamona strain, with 66.7% of the remaining trees being the non-sterilizing strain.

Sterile *S. noctilio* eggs containing juvenile nematodes were relatively rare (Table 4); we found just 44 sterile females in six years of sampling. Thus, we were unable to analyze their occurrence statistically and present only counts. The 228 *S. noctilio* carrying nematode-infected eggs collected altogether after Kamona treatments had an overall sterilization rate of just 18%. As expected, none of the 20 nematode-infected *S. noctilio* females emerging from control trees were sterile.

We collected data on *S. noctilio* infection rates in both *P. sylvestris* and *P. resinosa* only during 2007–2009. During that time, woodwasp density exhibited a steady downward trend in *P. sylvestris*, whereas it had a peak in 2008 in *P. resinosa* (Fig. 2A). In all three years, mean infection rates in trees treated with Kamonas were consistently numerically higher in *P. sylvestris* than in *P. resinosa*, but not significantly so when testing years separately ($p > 0.05$)

(Fig. 2B). When we pooled data across the years, we found the means to be significantly different (t -test, $p = .018$) with $24.7 \pm 29.0\%$ infection in *P. sylvestris* and $14.5 \pm 21.6\%$ in *P. resinosa*.

4. Discussion

Studies of typical biological control systems often involve two species: for example, a parasitoid and host, a pathogen and host, a predator and prey, or an herbivore and plant (Hajek, 2004). The system centered on *S. noctilio* is considerably more complex, with five interconnected components: a nematode with two very different phenotypes (i.e., so as to make it virtually two separate species), a host/herbivore (i.e., *S. noctilio*), a plant pathogen/*Sirex* symbiont/nematode food (i.e., *A. areolatum*), and a plant (i.e., *Pinus* spp.) (Bedding and Iede, 2005). Changes in any of the components, as well as other biotic and abiotic factors, may alter the dynamics of the system (Slippers et al., 2012). In Australia, trees are grown in monoculture with uniform management. In the CSIRO biological control program for *S. noctilio*, the same fungus and nematode strains are used from year to year, the infection rate of the nematode is carefully monitored and maintained, and the nematode is applied according to uniform protocols (Haugen et al., 1990). Unfortunately, the Australian system has not always worked well in other parts of the southern hemisphere (Hurley et al., 2007; Hurley et al., 2008).

However, the situation is especially complex and unpredictable in North America because of its diverse native pine forests, multiple strains and species of the symbiotic fungus, non-sterilizing *D. siricidicola* strain, and occurrence of several native *Sirex* species as well as the invasive *S. noctilio*. Moreover, based on microsatellite analysis of limited samples (Boissin et al., 2012), scenario testing suggests that North America might have undergone two separate

invasions by *S. noctilio*. One strain is potentially similar to that found in South America (which originated in Europe), whereas the other is European. Having at least two genotypes of the woodwasp adds to the complexity of the biological control system. For example, Bedding (1972) reported finding a Belgian nematode attacking a Belgian strain of *S. noctilio* but not sterilizing it, whereas the same nematode sterilized an Australian *S. noctilio* strain. Thus different strains of *S. noctilio* can be susceptible or resistant to sterilization by the same strain of *D. siricidicola*.

Two shorter studies were undertaken in other laboratories during the time of this study, and they provide some interesting comparisons and contrasts with ours because both evaluated the non-sterilizing strain of *D. siricidicola*. The first study (Kroll et al., 2013) was conducted in 2011 in the same general areas in New York and Pennsylvania as this study and with the same two *Pinus* species. The authors reported that 44.0% of their trees contained *S. noctilio* infected with nematodes and that infection rates averaged 27.9% across all sites. By comparison, the mean infection rate was $28.1 \pm 20.5\%$ across trees in our study and $17.6 \pm 13.6\%$ across all sites and years (Table 1). The second study (Ryan et al., 2012) was conducted in southern and central Ontario, Canada, at sites farther north than New York. The authors reported that 38% of female woodwasps had nematodes in the hemocoel (but never in the eggs). By contrast, just $23.6 \pm 13.2\%$ of females in our study contained nematodes during the same two years. It must be stressed that all of our trees at this time were inoculated with the Kamona strain, but we recovered Kamonas at only two of the five release sites (Table 2, 2008).

The feature of the relationship between *S. noctilio* and *D. siricidicola* most relevant to biological control is female sterilization. In a seminal paper on the biology of *D. siricidicola*, Bedding (1972) noted that successful sterilization is dependent on a critical synchrony between the release of juvenile nematodes from the mother into the host hemocoel and the development

of the host eggs. If juveniles are released too early, the eggs may not be sufficiently mature to assure normal nematode growth, and if too late, the juveniles may not be able to penetrate the fully developed chorion and enter the egg. This synchrony (or lack thereof) seems to vary between strains and species of the nematode and the woodwasp. A spectrum of host effects can result, ranging from a few shrunken, malformed eggs without nematodes to a complement of fully formed egg shells neatly packed with juveniles. Abiotic factors, especially temperature, also undoubtedly affect the physiological processes involved in sterilization. A recent paper (Yousuf et al., 2014) investigated the effects of four constant temperatures in the range 24° to 28° C on *S. noctilio* development and *D. siricidicola* parasitism. The authors observed that a relatively small increase in temperature (+4° C) could lower *S. noctilio* egg numbers, induce abnormal egg formation, and decrease larval development and adult size. In addition, higher temperatures reduced the rates of infection and sterilization by *D. siricidicola*.

In an evolutionary context, non-sterilization is not necessarily a bad thing for the woodwasp population. In fact the occurrence and persistence of *D. siricidicola* in other parts of the world suggest that it may be a useful adaptation (Bedding, 1972; Bedding, 1974; Nuttall, 1980). Indeed, by the time we started our work, the non-sterilizing *D. siricidicola* strain had become well established in central New York (Zylstra and Mastro, 2012) and southern Ontario. At woodwasp oviposition, juvenile non-sterilizing nematodes slide alongside the ovipositor with the egg, and thus, have a live host available for parasitism as soon as the first instar ecloses and begins to feed (Bedding, 1972; Slippers et al., 2012). This can be especially advantageous for the nematode at low woodwasp densities, when single *S. noctilio* females attack a tree. Because non-sterilization does not require the exacting physiological and abiotic conditions (i.e., that sterilization does) and may provide a selective value, non-sterilization or partial sterilization may

be the prevalent state in nature (Keeler, 2012). By contrast, a sterilizing strain such as Kamona, having killed its host (egg), must depend upon a woodwasp female with living eggs to oviposit into the same tree. For this reason, Bedding (1972) refers to *S. noctilio* as being more “social” and therefore better adapted for complete sterilization. Thus, the sterilizing strain seems to disperse more slowly and is more virulent.

From the standpoint of forest managers, sterilization of *S. noctilio* is highly desirable. Using trap trees and a virulent sterilizing nematode strain is an augmentative biological control strategy that is aimed at establishment and recycling in the environment for some unknown time. From an operational standpoint, this is relatively easy in a place like Australia. Hot spots are identified in a plantation through aerial survey. Trap trees are then created and inoculated with nematodes, and the female *S. noctilio* from the infestation emerge and are attracted to oviposit in the nearby trap trees. By contrast the situation in North America is quite different, with diverse forests in diverse landscapes in a range from natural to highly managed (Dodds and de Groot, 2012). Such a scenario over vast regions cannot be treated augmentatively. (However, note that the Australians have attempted to use such an approach to “over flood” the “defective strain” of nematode that was inadvertently released in the State of Victoria (Bedding and Iede, 2005).) The only really feasible approach in North America is inoculation along the leading edge of the woodwasp invasion with a goal of classical biological control, which means permanent establishment. This possibility is unfortunately confounded by the presence of the non-sterilizing strain and its ability to hybridize with the Kamona strain (D. Williams, unpublished results). At this point, we do not know what the consequences of strain hybridization may be for the infection of *S. noctilio* and this may be the reason for the low rates of sterilization that we have observed (Table 4).

Our controlled releases of the Kamona strain produced wide-ranging rates of infection, from near 0.0 to 43.7% (Tables 2, 3). Ultimately we were not granted permission to leave the nematodes in the field and follow the development of their “outbreak” in *S. noctilio* populations. Therefore, we could not observe any possible buildup over several seasons, which is what is expected in Australia. However, the results of Australian researchers give us some hope. Bedding and Akhurst (1974) reported an infection rate of 31% in a small plantation plot in 1970. This was followed by rates of 47% in 1971 and 92% in 1972. Clearly, the *D. siricidicola* Kamona strain alone is capable of rapid population growth.

Although comparisons of nematode infection rates inside treatment and control trees were generally not different from a statistical standpoint, they exhibited the same trends; the infection in the Kamona treatment was generally numerically higher than the non-sterilizing infections in the controls (Table 3). Before we learned about the presence of the non-sterilizing strain, our hypothesis was that the nematode treatments should yield some positive infection rate and any nematodes discovered in dissections should be identified as Kamonas. Conversely, the controls should have an infection rate of zero. But plainly, the non-sterilizing strain was well established by the time we started our study (Zylstra and Mastro, 2012). The rather low infection rate for the controls (which received only gel) along with the low contribution of Kamonas in the treated trees suggests that the treated trees were a better environment for the non-sterilizing strain than the control trees.

As an interesting aside, one tree each at Bell Hill and Ole Bull in 2010 contained infected females of *Sirex nigricornis* F. (seven altogether). In all cases, their eggs were sterile, suggesting that this native woodwasp is susceptible to both nematode strains. Unfortunately these

nematodes were not included in DNA analyses, but due to sterilization we assume that they were Kamona.

A recent review hypothesized that pine species could influence *D. siricidicola* (Slippers et al. 2012) and it is now clear, based on several studies, that levels of nematode infection in *S. noctilio* can be associated with pine species. We report that over 3 years, more *S. noctilio* emerging from *P. sylvestris* than *P. resinosa* were infected with *D. siricidicola* (Fig. 2). Investigating these same pine species, in 2008 in Ontario, Canada, Ryan et al. (2012) found significantly higher nematode infection levels in *S. noctilio* emerging from 34 *P. sylvestris* trees compared with 4 *P. resinosa*. Ryan et al. (2012) also reported that in 2007, similar *D. siricidicola* infection levels were found in *Pinus banksiana* Lambert and *P. sylvestris*. In Australia, Nahrung et al. (2015) found more *D. siricidicola* infection in *S. noctilio* emerging from *Pinus radiata* D. Don compared with *Pinus taeda* L., and reported that higher levels of blue stain fungi in *P. taeda* could have influenced this.

We speculate that the relatively higher rates of nematode infection reflect the European origins of *S. noctilio* and *P. sylvestris*. Some aspect(s) of their long coevolution has set this pattern, whereas *S. noctilio* has only very recently encountered *P. resinosa* and not had time to adapt to it. Possible explanations include differences in the trees' tracheid structures and numbers, which may slow nematode dispersal through *P. resinosa* wood (Irbe et al., 2013; Havimo et al., 2009), or differences in chemical composition between tree species, or different growth rates on different isolates of the symbiotic fungus (Morris et al., 2012). Regardless, it seems that the species of pine can influence the levels of *S. noctilio* infection by *D. siricidicola* and this deserves more studies including more species of pines.

The complete “*Sirex* microecosystem” is clearly very complex. It may be helpful to simplify it by looking at its individual paired variables. As one example, consider the set of relationships as a 2×2 matrix with host species (*S. noctilio* vs. the native *Sirex* spp.) as one variable and sterilizing vs. non-sterilizing as the other. Studies might include the effects of co-occurrence of the *D. siricidicola* strains in the same host. Or the possible differences in behavior of Kamona in the native *Sirex* spp. vs. *S. noctilio*. Our results suggest a need for more basic studies to understand the ecological relationships between the nematode species and strains and the *Sirex* spp., both native and European. Such studies have not been undertaken, and may be difficult to quantify, but they could be very useful for our understanding.

5. Conclusions

Our overall assessment is that the nematode biological control system developed by CSIRO hasn’t worked well “off the shelf” in the U.S. A possible solution is to undertake further exploration in Europe to find a strain of *D. siricidicola* better adapted to the strain of *S. noctilio* and environmental conditions in North America. On the other hand, we may not have given the system a chance to work. We were unable to gain permission for full field releases over time. Following Clausen’s (1951) rule, a maxim of biological control, “if imported natural enemies are destined to become completely successful in host population regulation they will do so either within three host generations or within three years.” (DeBach, 1974). The 3- year progression of nematode infection rates reported by Bedding and Akhurst (1974) and cited above would seem to confirm the rule. The infection rates in our study were typically low ($\approx 21\%$) but consistent across years, so they might increase appreciably after a few seasons. Of perhaps greater concern

was the generally low rate of sterilization by the Kamona strain. Clearly sterilization is the lynchpin of the Australian system and we need to understand its apparent failure in North America. Another linked problem is the presence of the non-sterilizing strain. We know from laboratory studies that this strain will hybridize with Kamona, but we do not know the ramifications of its doing so. Hybridization may result in the loss of desirable characteristics of the Kamonas. Alternatively, the non-sterilizing strain may simply out-compete Kamona. All this notwithstanding, we cannot remove this well-established strain. The Australian experience of over-flooding their “defective strain” was similar to what we face, but in a smaller and simpler system. It is hard to conceive of now eliminating such an entrenched organism (i.e., the non-sterilizing strain of *D. siricidicola*) from the diverse and spatially complex forests of North America.

Acknowledgments

Many people assisted in the laborious field work to collect and the tedious lab work to process samples needed for this project. In particular, we thank Carrie Crook, Kelley Zylstra, Mike Crawford, and Robin Tait of the USDA, APHIS Syracuse Laboratory and Brad Regester, Bill Laubscher, Benn Carlson, Scott Stitzer, Joe Gaughan, and Tim Marasco of the Pennsylvania Division of Forest Pest Management for all of their hard work. In addition, we thank E. Erin Morris, Abigail Finley, and Christopher Iriarte for their painstaking work on the molecular analysis. We thank Russ Bulluck of USDA, APHIS for his suggestions on the manuscript. We are also grateful to Vic Mastro, Director (retired) of the APHIS CPHST Otis Laboratory, and Donald Eggen, Chief of the PA Division of Forest Pest Management, for their support.

References

- Akhurst, R.J., 1975. Cross-breeding to facilitate the identification of *Deladenus* spp., nematode parasites of wood wasps. *Nematologica* 21, 267-272.
- Bedding, R.A., 1968. *Deladenus wilsoni* n. sp. and *D. siricidicola* n. sp. entomophagous-mycetophagous nematodes parasitic in siricid woodwasps. *Nematologica* 14, 515-525.
- Bedding, R. A., 1972. Biology of *Deladenus siricidicola* an entomophagous-mycetophagous nematode parasitic in siricid woodwasps. *Nematologica* 18, 482-493.
- Bedding, R. A., 1974. Five new species of *Deladenus* (Neotylenchidae), entomophagous-mycetophagous nematodes parasitic in siricid woodwasps. *Nematologica* 20, 204-225.
- Bedding, R. A. 1993. Biological control of *Sirex noctilio* using the nematode *Deladenus siricidicola*. In: Bedding, R. A., Akhurst, R.J., Kaya, H.K. (Eds.) *Nematodes and The Biological Control of Insect Pests*. CSIRO Publications, Australia, pp. 11-20.
- Bedding, R. A., 2009. Controlling the pine-killing woodwasp, *Sirex noctilio*, with nematodes. In: *Use of Microbes for Control and Eradication of Invasive Arthropods*, Hajek, A.E., Glare, T.R., O'Callaghan, M. (Eds.), Springer, Dordrecht, pp. 213-235.
- Bedding, R. A. , Akhurst, R.J., 1974. Use of the nematode *Deladenus siricidicola* in the biological control of *Sirex noctilio* in Australia. *J. Austr. Entomol. Soc.* 13, 129-135.
- Bedding, R. A., Akhurst R.J., 1978. Geographical distribution and host preferences of *Deladenus* species parasitic in siricid woodwasps and associated hymenopterous parasitoids. *Nematologica* 24, 286-294.

- 538 Bedding, R. A., Iede, E.T., 2005. Application of *Beddingia siricidicola* for *Sirex* woodwasp
539 control. In: Grewal, P.S., Ehlers, R.-U., Shapiro-Ilan, D.I. (Eds). Nematodes as Biocontrol
540 Agents. CABI International, London, pp. 385-399.
- 541 Boissin, E., Hurley, B., Wingfield, M.J., Vasaitis, R., Stenlid, J., Davis, C., de Groot, P.,
542 Ahumada, R., Carnegie, A., Goldarazena, A., Klasmer, P., Wermelinger, B., Slippers, B.,
543 2013. Retracing the routes of introduction of invasive species: the case of the *Sirex noctilio*
544 woodwasp. Molecular Ecol. doi: 10.1111/mec.12065
- 545 Bordeaux, J.M., Dean, J.F.D., 2012. Susceptibility and response of pines to *Sirex noctilio*. In:
546 The *Sirex* woodwasp and Its Fungal Symbiont: Research and Management of a Worldwide
547 Invasive Pest. B. Slippers et al. (Eds)., Springer-Verlag, New York, pp. 31-50.
- 548 Carnegie, A.J., Bashford, R., 2012. *Sirex* woodwasp in Australia: current management strategies,
549 research and emerging issues. In: The *Sirex* woodwasp and Its Fungal Symbiont: Research
550 and Management of a Worldwide Invasive Pest. B. Slippers et al. (Eds), Springer-Verlag,
551 New York, pp. 175-202.
- 552 Clausen, C.P., 1951. The time factor in biological control. J. Econ. Entomol. 44, 1-9.
- 553 Collett, N.G., Elms, S., 2009. The control of *sirex* woodwasp using biological control agents in
554 Victoria, Australia. Agric. For. Entomol. 11, 283-294.
- 555 Corley, J.C., Villacide, J.M. 2012. Population dynamics of *Sirex noctilio*: influence of diapause,
556 spatial aggregation and flight potential on outbreaks and spread. In: The *Sirex* woodwasp
557 and Its Fungal Symbiont: Research and Management of a Worldwide Invasive Pest. B.
558 Slippers et al. (Eds)., Springer-Verlag, New York, pp. 51-64.

- 559 DeBach, P., 1974. Biological control by natural enemies. Cambridge Univ. Pr., Cambridge.
- 560 de Groot, P., Nystrom, K.L., Scarr, T.A., 2006. Discovery of *Sirex noctilio*
561 (Hymenoptera:Siricidae) in Ontario, Canada. Great Lakes Entomologist 39, 49-53.
- 562 Dodds, K.J., de Groot, P. 2012. *Sirex*, survey and management: challenges of having *Sirex*
563 *noctilio* in North America. In: The *Sirex* woodwasp and Its Fungal Symbiont: Research and
564 Management of a Worldwide Invasive Pest. B. Slippers et al. (Eds), Springer-Verlag, New
565 York, pp. 265-286.
- 566 Hajek, A., 2004. Natural enemies. An introduction to biological control. Cambridge University
567 Press, New York.
- 568 Haugen, D.A., Bedding, R.A., Underdown, M.G., Neumann, F.G., 1990. National strategy for
569 control of *Sirex noctilio* in Australia. Austral. For. Grower 13, No 2.
- 570 Havimo, M., Rikala, J., Sirvio, J., Sipi, M., 2009. Tracheid cross-sectional dimensions in Scots
571 pine (*Pinus sylvestris*) – distributions and comparison with Norway spruce (*Picea abies*).
572 Silva Fennica 43, 682-690.
- 573 Hoebeke, E.R., Haugen, D.A., Haack, R.A., 2005. *Sirex noctilio*: discovery of a palearctic siricid
574 woodwasp in New York. Newsl. Michigan Entomol. Soc. 50, 24-25.
- 575 Hurley, B.P., Slippers, B., Wingfield, M.J., 2007. A comparison of control results for the alien
576 invasive woodwasp, *Sirex noctilio*, in the Southern Hemisphere. Agric. For. Entomol. 9, 159-
577 171.
- 578 Hurley, B.P., Slippers, B., Croft, P.K., Hatting, H.J., van der Linde, M., Morris, A.R., Dyer, C.,
579 Wingfield, M.J., 2008. Factors influencing parasitism of *Sirex noctilio* by the nematode

- 580 *Deladenus siricidicola* in summer rainfall areas of South Africa. *Biological Control* 45, 450-
581 459.
- 582 Iede, E.T., Klasmer, P., Pentead, S. do R.C., 2000. *Sirex noctilio* in South America: distribution,
583 monitoring and control. In: XXI International Congress of Entomology. Foz do Iguacu, Pr.
584 Agosto 2000. Embrapa Soja Anais, Londrina-PR. p. 474.
- 585 Irbe, I., Sable, I., Treimanis, A., Jansons, A., Grinfelds, U., 2013. Variation in the tracheid
586 dimensions of Scots pine (*Pinus sylvestris* L.) and lodgepole pine (*Pinus contorta* Dougl. var.
587 latifolia Engelm) trees grown in Latvia. *Baltic Forestry* 29, 120-127.
- 588 Keeler, D.M., 2012. Flight period and species composition of *Sirex* (Hymenoptera: Siricidae and
589 associated *Deladenus* (Nematoda: Neotylenchidae) within Arkansas pine forests. Unpubl.
590 MS thesis, University of Arkansas.
- 591 Kroll, S.A., Hajek, A.E., Morris, E.E., Long, S.J., 2013. Parasitism of *Sirex noctilio* by non-
592 sterilizing *Deladenus siricidicola* in northeastern North America. *Biological Control* 67,
593 203-211.
- 594 Madden, J.L., 1988. *Sirex* in Australasia. In: Berryman A.A. (ed. Dynamics of forest insect
595 populations: patterns, causes, implications. Plenum Press, New York.
- 596 Madden, J.L., Coutts, M.P., 1979. The role of fungi in the biology and ecology of woodwasps
597 (Hymenoptera: Siricidae). In: Insect-fungus symbiosis: Nutrition, mutualism, and
598 commensalism. L.H. Batra (Ed.), John Wiley & Sons, New York.

- 599 Morris, E.E., Jimenez, A., Long, S.J., Williams, D.W., Hajek, A.E., 2012. Variability of growth
600 of *Deladenus siricidicola* on strains of the white rot fungus *Amylostereum areolatum*.
601 BioControl DOI 10.1007/s10526-012-9447-1
- 602 Morris, E.E., Kepler, R.M., Long, S.J., Williams, D.W., Hajek, A.E., 2013. Phylogenetic
603 analysis of *Deladenus* nematodes parasitizing northeastern North American *Sirex* species. J.
604 Invertebr. Pathol. 113(2), 177-183.
- 605 Nahrung, H.F., Ramsden, M., Griffiths, M., 2015. *Sirex* woodwasp range expansion in Australia:
606 performance and parasitism on two commercial pine species. Forestry 0: 1-6,
607 doi:10.1093/forestry/cpv039
- 608 Neumann, F.G., Morey, J.L., McKimm, R.J., 1987. The *Sirex* wasp in Victoria. Dept. Conserv.
609 For. Lands, Victoria. Bull. No. 29, 44 pp.
- 610 NSCC, 2002. National *Sirex* control strategy. Operations worksheets. Unpublished manuscript
611 of the National *Sirex* Coordinating Committee.
- 612 Nuttall, M.J., 1980. *Deladenus siricidicola* Bedding. Nematode parasite of *Sirex*. Forest and
613 Timber Insects in New Zealand No. 48, NZ Forest Service. Reprinted and updated in
614 <http://www.nzffa.org.nz> .
- 615 Rawlings, G.B., 1948. Recent observations on the *Sirex noctilio* population in *Pinus radiata*
616 forests in New Zealand. New Zealand J. For. 5, 1-11.
- 617 Ryan, K., de Groot, P., Nott, R.W., Drabble, S., Ochoa, I., Davis, C., Smith, S.M., Turgeon, J.J.,
618 2012. Natural enemies associated with *Sirex noctilio* (Hymenoptera: Siricidae) and *S.*
619 *nigricornis* in Ontario, Canada. Environ. Entomol. 41, 289-297.

- 620 Ryan, K., Hurley, B.P., 2012. Life history and biology of *Sirex noctilio*. In: The Sirex
621 woodwasp and Its Fungal Symbiont: Research and Management of a Worldwide Invasive
622 Pest. B. Slippers et al. (Eds), Springer-Verlag, New York, pp. 15-30.
- 623 SAS Institute Inc., 2012. What's new in SAS 9.3[®] and SAS[®] Analytical Products 12.1. SAS
624 Institute Inc., Cary, NC,
- 625 Schlotzhauer, S.D., 2009. Elementary Statistics using SAS. SAS Institute, Cary, NC.
- 626 Shields, L., 2009. Update on *Sirex noctilio* in Canada. In: Proceedings. 19th U.S. Department of
627 Agriculture Interagency Research Forum on Invasive Species. Gen. Tech. Rep. NRS-P-36.
628 U.S.D.A. Forest Service, Newtown Square, Pennsylvania.
- 629 Slippers, B., Hurley, B.P., Mlonyeni, X.O., de Groot, P., Wingfield, M.J., 2012. Factors affecting
630 the efficacy of *Deladenus siricidicola* in biological control systems. In: The Sirex woodwasp
631 and Its Fungal Symbiont: Research and Management of a Worldwide Invasive Pest. B.
632 Slippers et al. (Eds). Springer-Verlag, New York, pp. 119-139.
- 633 Taylor, K.L., 1967. The introduction, culture, liberation and recovery of parasites of *Sirex*
634 *noctilio* in Tasmania 1962-67. Tech. Paper Div. Entomol. CSIRO Aust., No.8.
- 635 Taylor, K.L., 1976. The introduction and establishment of insect parasitoids to control *Sirex*
636 *noctilio* in Australia. Entomophaga 21, 429-440.
- 637 Tribe, G.D., 1995. The woodwasp *Sirex noctilio* Fabricius, a pest of *Pinus* species, now
638 established in South Africa. African Entomol. 3, 215-217.

- 639 Thompson, B.M., Bodart, J., McEwen, C., Gruner, D.S., 2014. Adaptations for symbiont-
640 mediated external digestion in *Sirex noctilio* (Hymenoptera: Siricidae). Ann. Entomol. Amer.
641 107, 453-460.
- 642 Villacide, J.M., Corley, J.C., 2008. Parasitism and dispersal potential of *Sirex noctilio*:
643 implications for biological control. Agric. For. Entomol. 10, 341-345.
- 644 Wermelinger, B., Thomsen, I.M., 2012. The woodwasp *Sirex noctilio* and its associated fungus
645 *Amylostereum areolatum* in Europe. In: The Sirex woodwasp and Its Fungal Symbiont:
646 Research and Management of a Worldwide Invasive Pest. B. Slippers et al. (Eds.), Springer-
647 Verlag, New York, pp. 65-80.
- 648 Williams, D.W., Zylstra K.E., Mastro, V.C., 2009. Biological control of *Sirex noctilio* in North
649 America by *Deladenus siricidicola*: 2008 update. In: Proceedings 20th U.S. Department of
650 Agriculture Interagency Research Forum on Invasive Species. Gen. Tech. Rep. NRS-P-51
651 U.S.D.A. Forest Service, Newtown Square.
- 652 Williams, D.W., Zylstra, K.E., Mastro, V.C., 2012. Ecological considerations in using *Deladenus*
653 (= *Beddingia. siricidicola*) for the biological control of *Sirex noctilio* in North America. In:
654 The Sirex woodwasp and Its Fungal Symbiont: Research and Management of a Worldwide
655 Invasive Pest. B. Slippers et al. (Eds.), Springer-Verlag, New York, pp. 135-148.
- 656 Ye, W., Giblin-Davis, R.M., Davies, K.A., Purcell, M.F., Scheffer, S.J., Taylor, G.S., Center,
657 T.D., Morris, K., Thomas, W.K., 2007. Molecular phylogenetics and the evolution of host
658 plant associations in the nematode genus *Fergusobia* (Tylenchida: Fergusobiinae) Mol.
659 Phylogenet. Evol. 45, 123-141.
660

- 661 Yu, Q., de Groot, P., Leal, I., Davis, C., Ye, W., Foord, B., 2009. Characterization of *Deladenus*
 662 *siricidicola* (Tylenchida: Neotylenchidae) associated with *Sirex noctilio* (Hymenoptera:
 663 Siricidae) in Canada. Intl. J. Nematol. 19, 23-32.
- 664 Yu, Q., de Groot, P., Leal, I., Ye, W., Foord B., 2011. First report and characterization of
 665 *Deladenus proximus* (Tylenchida: Neotylenchidae) associated with *Sirex nigricornis*
 666 (Hymenoptera: Siricidae) in Canada. Intl. J. Nematol. 21, 139-146.
- 667 Yousuf, F., Carnegie, A.J., Bedding, R.A., Bashford, R., Nicol, H.I., Gurr, G.M., 2014. Effect of
 668 temperature on woodwasp development and parasitism by the entomopathogenic nematode,
 669 *Deladenus siricidicola*. Biological Control 79, 67-74.
- 670 Zondag, R., 1975. A non-sterilising strain of *Deladenus siricidicola*. Forestry Research Institute
 671 Report 1974, Rotorua, pp. 51-52.
- 672 Zylstra, K.E., Mastro, V.C., 2012. Common mortality factors of woodwasp larvae in three
 673 northeastern United States host species. J. Insect Science 12:83 available online:
 674 insectscience.org/12.83

(FIGURE CAPTIONS)

Fig. 1. Nematode release locations in New York and Pennsylvania in 2007 (A), 2008 (B), 2009 (C), 2010 (D), 2011 (E), and 2012 (F). *Sirex noctilio* was first trapped in Fulton, New York (☆) in 2004. See Table 1 for abbreviations of site names.

Fig. 2. Comparison of the density of *S. noctilio* (A) and the *D. siricidicola* infection rate (B) in two *Pinus* species at sites treated with the Kamona strain during 2007-2009. Bars and brackets are means and standard deviations. Numbers inside the bottoms of the bars are the sample sizes.

- 1 Table 1. Characteristics of study sites in New York and Pennsylvania for controlled release experiments with the Kamona strain of *D.*
- 2 *siricidicola* during 2007-2012, and total numbers of *S. noctilio* emerging from tree samples, average dbh of the study trees, and the
- 3 mean densities of *S. noctilio* in the study sites.

Year	Site	Map Code	<i>Pinus</i> species ¹	Total no. of trees	No. of control trees	Percent trees without <i>S. noctilio</i>	Percent trees with <i>S. noctilio</i> only	Percent trees with <i>S. noctilio</i> and nemas	dbh (cm)	Total no. of <i>S. noctilio</i> emerged from bolts	<i>S. noctilio</i> density ²
2007	Pompey NY	P	<i>Ps</i>	17	0	11.8	17.6	70.6	15.2 ±1.9	702	1266.3 ±795.8
2007	Richland NY	R	<i>Ps</i>	40	0	30.0	47.5	22.5	10.1 ±2.4	602	1366.2±1729.8
2007	Spafford NY	S	<i>Ps</i>	18	0	5.6	16.7	77.7	14.9 ±2.0	584	1092.1±855.9
2007	Highland For NY	HF	<i>Pr</i>	22	0	22.7	36.4	40.9	17.0 ±3.4	162	204.9 ±191.1
2008	Rodman NY	RM	<i>Ps</i>	14	0	42.9	21.4	35.7	18.1 ±3.9	416	775.2±741.8
2008	Rte 69 NY	RT	<i>Ps</i>	27	0	29.7	33.3	37.0	14.3 ±2.7	424	588.8±592.1
2008	W Monroe NY	W	<i>Ps</i>	7	0	14.2	42.9	42.9	18.2 ±2.1	208	573.7±792.9
2008	Zink NY	Z	<i>Ps</i>	11	0	27.3	18.2	54.5	17.0 ±4.9	85	315.0±290.7
2008	Hills Creek PA	H	<i>Pr</i>	26	0	34.6	15.4	50.0	12.1 ±3.4	340	937.9±716.2
2009	Richland NY	R	<i>Ps</i>	36	12	66.7	19.4	13.9	11.4 ±2.5	39	189.2±198.5
2009	Trenton NY	T	<i>Ps</i>	24	8	33.3	29.2	37.5	16.3	131	205.6±178.2

									±5.5		
2009	Hills Creek PA	H	<i>Pr</i>	30	10	23.3	60.0	16.7	14.1 ±3.7	204	379.2±273.4
2010	Armenia Mt PA	A	<i>Ps</i>	18	6	72.2	27.8	0.0	14.6 ±2.4	4	40.1±17.8
2010	Bell Hill NY	B	<i>Pr</i>	15	7	26.7	46.6	26.7	15.4 ±3.0	60	223.3±170.3
2010	Ole Bull PA	O	<i>Pr</i>	15	5	66.7	20.0	13.3	15.8 ±2.5	6	42.0±7.0
2010	Hills Creek PA	H	<i>Pr</i>	32	16	34.4	56.2	9.4	17.2 ±4.5	278	431.3±496.4
2011	Fr Slocum PA	F	<i>Pr</i>	16	8	50.0	43.7	6.3	13.8 ±2.0	25	143.5±90.5
2011	L Harrison PA	L	<i>Pr</i>	12	6	83.3	16.7	0.0	11.2 ±0.6	6	184.0±177.3
2011	Hills Creek PA	H	<i>Pr</i>	24	12	45.8	33.4	20.8	14.3 ±2.8	83	212.1±251.0
2012	Fr Slocum PA	F	<i>Pr</i>	15	7	33.3	53.4	13.3	16.2 ±2.2	78	308.5±306.8
2012	Hypocrite Tr PA	HY	<i>Pr</i>	30	15	43.3	30.0	26.7	18.0 ±3.7	210	323.6±450.8
2012	L Harrison PA	L	<i>Pr</i>	5	2	20.0	60.	20.0	17.7 ±3.8	16	177.3±264.8
2012	Hills Creek PA	H	<i>Pr</i>	10	5	20.0	70	10.0	21.0 ±1.6	45	297.8±370.6

Sum	464	119				4,708
Mean			36.4	35.5	28.1	
S.D.			19.7	16.3	20.5	

5

6 ¹*Pr* = *Pinus resinosa*; *Ps* = *sylvestris*.

7 ²Mean numbers of *S.noctilio* emerging per m³ of wood.

8

Table 2. Mean infection rates of *S. noctilio* by nematodes in 2007 and 2008 before controls (i.e., gel without nematodes) were sampled.

Year	Site	Number of trees infested with <i>S. noctilio</i>	Mean percent infection of all <i>S. noctilio</i> per tree	S.D.	Percent Kamona strain ¹
2007	Pompey	15	29.4	26.0	—
2007	Richland	28	7.7	18.5	—
2007	Spafford	17	43.6	30.7	—
2007	Highland	17	16.8	25.9	—
	All	77	21.8	28.0	
2008	Rodman	8	33.4	29.2	0.0
2008	Rte 69	19	18.5	23.2	0.0
2008	W Monroe	6	12.6	18.2	0.0
2008	Zink	8	43.7	40.0	16.7
2008	Hills Creek	17	15.9	17.8	48.6
	All	58	22.7	26.4	

¹ Note that %Kamona + %non-sterilizing = 100.0

29

30 Table 3. Comparison of nematode infection rates in *S. noctilio* in trees inoculated with the

31 Kamona strain in gel versus control trees inoculated only with gel.

Year	Site	Trees treated with Kamonas		Control trees (gel only)		Z	p	Percent Kamona ¹
		Number of infested trees sampled	Median percent nematode infection	Number of infested trees sampled	Median percent nematode infection			
2009	Hills Creek	9	0.0	7	0.0	-1.196	.232	16.7
2009	Richland	6	37.5	5	0.0	-0.201	.841	0.0
2009	Trenton	11	16.7	5	8.3	-0.237	.813	22.2
2010	Bell Hill	6	41.7	5	0.0	-1.167	.243	0.0
2010	Hills Creek	9	0.0	12	0.0	0.700	.484	0.0
2011	Hills Creek	6	27.0	5	0.0	-2.395	.017*	0.0
2012	Hills Creek	3	0.0	4	0.0	.866	.387	100.0 ²
2012	Hypocrite	8	15.5	9	0.0	1.615	.106	33.3

32

33

34 ¹ Note that %Kamona + %non-sterilizing = 100.035 ² One sample only

Table 4. Sterilization of *S. noctilio* eggs in nematode-treated versus control trees.

Release year	Treated with Kamona nematodes		Control (gel only)	
	Total <i>S. noctilio</i> females infected	No. of sterile <i>S. noctilio</i> females	Total <i>S. noctilio</i> females infected	No. of sterile <i>S. noctilio</i> females
2007	112	15	—	—
2008	93	24	1	0
2009	12	0	3	0
2010	1	0	12	0
2011	10	5	0	—
2012	0	0	4	0