BIOLOGY AND ECOLOGY OF *SIREX*, *DELADENUS* AND *AMYLOSTEREUM* IN NORTH AMERICA

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By
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Abstract

Sirex noctilio is a woodwasp that attacks stressed or dying pine trees. It is native to Eurasia and North Africa but has been invasive in the southern hemisphere since the early 1900s. It was found for the first time in the United States in New York State in September 2004 and in Ontario in 2005. Since then it has spread to a total of seven northeastern US states. *S. noctilio* is more aggressive than the native pine specialist *Sirex nigricornis* and it is able to kill living pines by injecting a phytotoxic venom and its symbiotic fungus *Amylostereum areolatum* into tree trunks. The Kamona strain of the nematode *Deladenus siricidicola* has been extensively used as a biological control agent against invasive *S. noctilio* in the Southern Hemisphere, where it sterilizes female hosts by entering the eggs and making them inviable. In North America, a non-sterilizing (NS) strain of *D. siricidicola*, thought to have been introduced with *S. noctilio*, is commonly found parasitizing this invasive woodwasp. Species of *Deladenus* that parasitize *Sirex* have a parasitic form as well as a mycophagous form. Studies were conducted to understand 1. *Sirex* mating behavior and sexual receptivity, 2. the growth of two strains of *D. siricidicola,* Kamona and NS, with different strains and species of *Amylostereum* spp. for food, and 3. the use of *D. siricidicola* and *Deladenus proximus*, a native species of nematode in North America found associated with *S. nigricornis,* as biological control agents for *S. noctilio* as well as potential impacts caused by *D. siricidicola* on the native species of woodwasp *S. nigricornis*. When we conducted trials with 10 males per female in cages in the shade, sexual receptivity of *S. noctilio* females was mainly driven by temperature, with increased mating when it was warm. Mating always occurred in the section of the cage toward sunlight. Mycophagous forms of the two *D. siricidicola* strains displayed relatively similar production of offspring when feeding on most of

the *A. areolatum* strains found associated with *S. noctilio* in this continent, except for strain BD on which NS produced more offspring than the biological control strain Kamona. Growth of both nematodes was greater on the introduced versus the native *A. areolatum* isolates. When testing *D. siricidicola* Kamona and *D. proximus* for potential control of *S. noctilio*, very low infection with Kamona was found, and when females were infected with this nematode the percentage of egg sterilization was usually not high. None of the wasps of either species were infected with *D. proximus*. When *D. siricidicola* Kamona was tested against *S. nigricornis* none of the wasps emerging from treated logs were infected. These findings suggest that further studies on the use of nematodes to control *S. noctilio* in North America are necessary. Possibly studying new strains of *D. siricidicola* would result in discovery of a strain of this nematode with higher efficacy in controlling the invasive woodwasp *S. noctilio* in North America.

BIOGRAPHICAL SKETCH

Isis Lima Caetano graduated as an agronomist engineer from the University of São Paulo in Brazil in 2003. She focused her undergraduate studies on agro-ecology and organic agriculture.

After college, Isis worked for several years as an inspector of certified organic produce and traveled to different regions of Brazil visiting and inspecting organic farms, food industries and traders. This job provided her with extensive experience in organic crop management, and crop pest control. This experience sparked her interest in doing research on natural pest control.

In 2011, Isis started working as a volunteer at the Dr. Hajek's laboratory in the Department of Entomology at Cornell University, where she learned concepts of biological control and assisted in several different research projects. In fall 2012, Isis started studying the Sirex system, designing and executing an experiment on *Sirex noctilio* mating behavior, which helped to understand the main factors that influence mating success of this invasive pest. This experiment became the first chapter of her Master's thesis. In May 2013, Isis became a graduate student at Dr. Hajek's laboratory, focusing on *S. noctilio* biological control and potential environmental implications related to the use of *Deladenus siricidicola* Kamona in North America, testing this biological control agent against *S. noctilio* and the North American native wood wasp *Sirex nigricornis*.

Isis is mainly interested in sustainable agricultural development and biological control of crop and invasive pests. After she finishes her master's studies she wants to pursue a PhD program and to work on biodiversity in agricultural systems with a major focus on interactions between organisms and a minor focus on regulations for organic agriculture.

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Chapter 1

Mating Behavior and Sexual Receptivity of *Sirex noctilio* (Hymenoptera: Siricidae)

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Abstract

Sirex noctilio F. is a woodwasp that develops within stressed and dying pine trees, *Pinus* spp. It is native to Eurasia and North Africa but has spread to other continents and has become a pest of pine plantations in the Southern Hemisphere. In nature adults live for less than 2 weeks, during which time males form leks. We investigated environmental and biological conditions related to mating of these woodwasps. We hypothesized that temperature, light and size ratio of the sexes (male:female) influence mating receptivity. A total of 71 12-minute mating trials, with 10 males and 1 female in each trial, were conducted in outdoor cages. As a precursor to mating, a male would approach a female from behind and touch her with his antennae and, if the female was receptive, mating proceeded. *S. noctilio* mated more often at higher temperatures but the size ratio of sexes and degree of cloud cover did not influence female receptivity to mating. Females that mated often mated multiple times (2.6 ± 0.2, mean ± SE; range 1-7) and numbers of matings increased with increasing temperature and was greatest during the last third of the 12 minute.

Key Words: Mating, copulation, woodwasp, polyandry, multiple mating.

Introduction

Sirex noctilio F. is a woodwasp that develops within stressed or dying pine trees, *Pinus* spp. (Ryan and Hurley 2012). It is native to Eurasia and North Africa but has been invasive in the Southern Hemisphere since the early 1900s (Hurley et al. 2007). It was collected for the first time in the United States in New York State in September 2004 (Hoebeke et al. 2005) and in Ontario in 2005 (de Groot et al. 2006). Since then it has spread to a total of seven northeastern US states (Center for Environmental and Research Information [CERIS] 2014) but at present the distribution is not being monitored. When a female *S. noctilio* lays eggs in the trunk of a pine tree, also injects a phytotoxic venom (Bordeaux et al. 2014, Coutts and Dolezal 1969) and oidia of the symbiotic white rot fungus *Amylostereum areolatum* (Chaillet ex Fr.) Boidin (Gaut 1969, Nielsen et al. 2009) along with the eggs. The phytotoxic venom and the fungus work together to kill trees (Bordeaux and Dean 2012). *Amylostereum areolatum* is an obligate mutualistic fungus associated with *S. noctilio* that decays the wood around larval tunnels, acting as an external rumen for larval feeding (Thompson et al. 2014).

Many species of Hymenoptera exhibit complex mating systems and behaviors and these vary greatly among different species (Ayasse et al. 2001). Mating systems are poorly understood for most of the wasps within the hymenopteran suborder Symphyta, which includes the Siricidae, although in some genera of the family Diprionidae, females produce volatiles to attract males for mating (Ayasse et al. 2001). Hymenopteran sex ratios often tend to favor production of males (Ayasse et al. 2001), which also is evident in *S. noctilio* populations (Ryan and Hurley 2012), and male-biased sex ratios can lead to high competition among males during mating.

Gregarious behaviors in insects often function to bring sexes together for mating (Matthews and Matthews 2010). Shelly and Whittier (1997) proposed that when male insects make swarms they aggregate to increase signal range and attract more females. Early studies of the behavior of *S. noctilio* adults showed that this species is protandrous, with males emerging up to a few days before females (Dolezal 1967). After emerging, males fly to the upper branches of pine trees and then make periodic short flights, just above and around tree tops on sunny, warm days. When woodwasps are fairly abundant, groups of flying males appear as small swarms (Morgan and Stewart 1966; Morgan 1968). Dolezal (1967) described that males form swarms on brighter sides of cages, suggesting that they are attracted to light. In *S. noctilio*, a male-produced pheromone functions as an aggregation-sex pheromone that attracts both males and virgin females, leading them to form leks (Cooperband et al. 2012; Cardé 2014). Activity resulting in mating is initiated when a female passes near a male resting in the foliage (Morgan and Stewart 1966). Mating is more likely to occur when there are numerous males in the same location and they are disturbed by constantly contacting each other (Morgan and Stewart 1966). Adult females have monoalkenes on their cuticles that act as contact sex pheromones (Böröczky et al. 2009). A male senses the contact pheromones when he touches a female, and he then follows the female and attempts to mate. *Sirex noctilio* adults mate during the daytime, usually in the early morning and late afternoon (Dolezal 1967).

We investigated mating of *S. noctilio* to provide information toward improved detection and management as well as to learn more about siricid mating hehavior. In preliminary work, we tested methods to identify conditions triggering mating in *S. noctilio*. We tried paired matings indoors with wasps confined in 29 ml cups or 60 ml jars. Further trials indoors within larger enclosures and using varying temperature and lighting regimes were also largely unsuccessful.

We also tried varying the sex ratio with multiple males per female. Subsequent methods were developed based on these preliminary data and on observations reported by Morgan and Stewart (1966) and Dolezal (1967): the ratio of males:female was increased and trials were conducted outdoors in midsummer. This study tested the effects of temperature, cloudiness, and size ratio of the woodwasps (male pronotum width:female pronotum width) on sexual receptivity. The goal for these studies was to mate females and to observe the behaviors of males and females during the caged period.

Methods

Rearing Woodwasps

Sirex noctilio were reared from naturally infested red pine (*Pinus resinosa* Sol. ex Aiton) and Scotch pine (*Pinus sylvestris* L.) collected in north-central Pennsylvania, central New York and the southern Adirondack Mountains (approximately 250 m elevation) that were cut down in spring and early summer 2012 and 2013. Infested trees were chosen based on signs of *S. noctilio* infestation, such as resin beads along the trunk (Dodds et al. 2010). Infested portions of trees were cut into 70 cm long logs, ends of logs were waxed and logs were kept inside of fiber barrels (91 cm high x 61 cm diam) with window screening covers. Barrels were all kept in an unheated barn with windows and thus, were exposed to ambient. Barrels were checked daily for *S. noctilio* that had emerged, from early June to the end of September. *Sirex noctilio* identity was confirmed according to Schiff et al. (2012). Each wasp was placed individually in a 29 ml clear plastic cup, numbered, and the emergence date was recorded. Adult females of *S. noctilio* live up to 7 days and males live up to 12 days in nature (Madden 1974; Neumann and Minko 1981). To extend the

life spans of wasps, after emergence adults were maintained at 4 ± 1°C, with approximately ~9-10 h of fluorescent light per day, so they could be available over a longer period. *Sirex noctilio* adults maintained under the same conditions before successful use in flight tunnel studies (Sarvary et al. 2015, 2016).

Mating Behavior Trials

Mating trials were conducted outdoors, inside fabric-netting cages (60 x 60 x 60 cm) (BugDorm 2; Bioquip, Rancho Dominguez, CA). Preliminary studies with cages in direct sunlight resulted in rapid death of females. Therefore, mating trials were conducted in the shade under the open door of a garage. On one side the cages faced the dark interior of the garage and on the other side a glass greenhouse was 19 m away. The shaded garage opening was chosen because it provided contrasting light conditions on opposite sides of the cage: the greenhouse side provided reflected sunlight, the opposite side was dark, and the cage itself was in the shade. Wasps were always transported to the experimental site inside a cooler containing an ice pack but were removed from the cooler for at least 10 minutes before use in trials.

Males and females that were active at room temperature were randomly selected before they were used in trials. *Sirex noctilio* adults can vary extensively in size (Madden 1981) and it was observed from initial trials that the size ratio of females to males might have an effect on whether mating occurred. Therefore, the width of the widest part of the pronotum (mm) of each wasp was measured using a digital caliper; pronotum width has been used as a proxy for *S. noctilio* adult size by Madden (1981) and Haavik et al. (2016). Our studies included females with pronotum widths ranging from 2.04 mm to 5.29 mm and males ranging from 1.87 mm to 5.35 mm. Male pronota were painted with different colors of fingernail polish (Nail Art Pen, Sally

Hansen, New York) to identity individuals within a cage during each trial.

For each trial a set of ten males was placed inside of a cage. One virgin female was then placed in the cage for 12 minutes. After a female was released in the cage data about mating began being collected. The 12 minutes that each female spent in the cage was divided into three periods of 4 minutes each for recording activity. Mating was recorded whenever it occurred within each 4 minute period. Each of 71 females was used for only one trial. A total of 132 males were used in this study. Sets of males were used in multiple trials, with an average of 5.4 ± 0.2 trials per male; use of a male for increasing numbers of trials was not associated with an increase in percent trials in which that male mated (% trials with mating = -0.0254 * number of trials + 0.47864; $r^2 = 0.112$).

Mating trials were conducted between 11:38-15:35 (95.5% of trials occurred in the afternoon, between 13:00 and 15:35) and between 18 July and 12 September. During trials, the location for cages was shaded from direct sunlight. Trials were conducted on days with varying temperatures and cloudiness but never on rainy days. For each trial, sky coverage by clouds was categorized as sunny, partially cloudy, or cloudy. Sunny described skies that were clear or up to 20% covered by clouds, but the sun was not cloud-covered. Cloudy described skies that were >80% covered by clouds and the sun was always covered. Partially cloudy described skies with 20-80% cloud cover. Temperature inside the cage was recorded for every trial at the moment a new female was released in the cage. For a subsample of matings (n=105 out of the total 133 matings) we recorded whether mating occurred within the half of the cage toward the garage or toward the greenhouse.

Data Analysis

A general linear mixed model with a binomial distribution and logit link (SAS Institute 2013) was used to analyze whether females mated or not. Temperature, cloudiness, and male:female size ratio (male pronotum width: female pronotum width, mm) were fixed effects and the female identification number was a random effect in the model. As a posthoc test, Student's t-test was used to compare ambient temperatures for assays during which females mated or did not.

When analyzing the number of times females mated, a generalized linear model with a Poisson distribution and overdispersion for tests and intervals was used with cloudiness, temperature and size ratios as fixed effects. A one-way analysis of variance with repeated measures was used to compare the numbers of female matings in the 0-4, 4-8 and 8-12 minute intervals for the trials when mating occurred, Tukey HSD tests.

Results

Mating Behavior

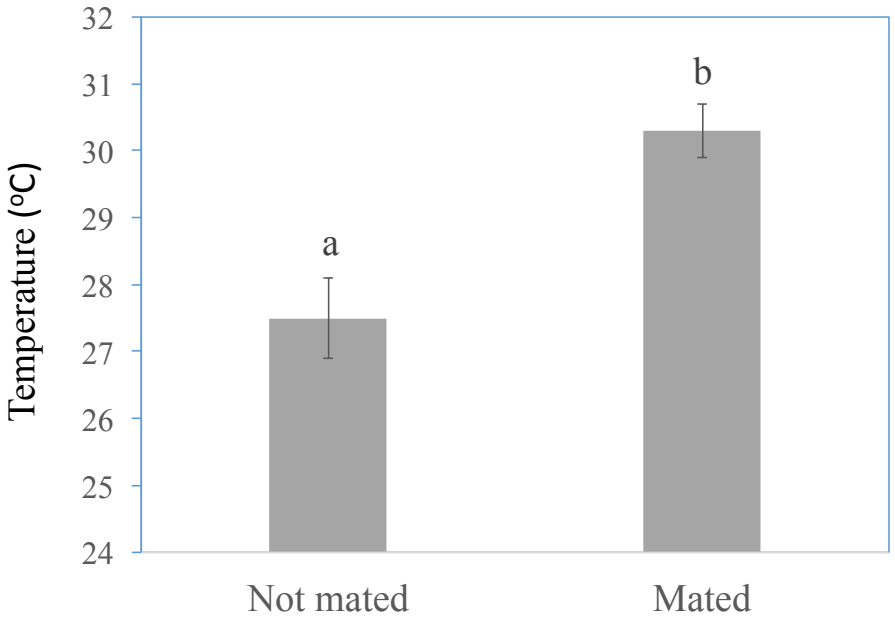
On sunny and partially cloudy days, mating mostly occurred when males and females were active and near each other in the corner of the cage facing toward the strong source of reflected light. Mating always occurred in the part of the cage toward the greenhouse and not toward the dark interior of the garage. Mating also always occurred when females and males were standing on the cage walls and a few times on the floor of the cage but never during flight, although limited flight could have occurred within the cage. For some of the females that did not mate at all, males showed no interest, although they contacted the female with their antennae. For other females that did not mate, males were clearly interested in mating but these females

were not receptive and hit the males with their wings and lifted their abdomens, not allowing the male to mount them. These latter females sometimes later changed their responses and were receptive later in the trial. When a female was receptive and mating occurred, a male approached from behind her and touched her abdomen with his antennae. The male then mounted the female, holding her abdomen or sometimes thorax and wings with his hind legs, bending his abdomen under one side of the female's abdomen and then copulation took place. Copulation usually lasted at least a few seconds, but occasionally a few minutes.

Sexual Receptivity of Females and Males

Among the 71 females tested, 71.8% mated during the 12-minute trials (133 matings were observed). The ambient temperature was positively associated with successful female mating; more trials conducted during periods of warmer temperatures resulted in mating ($F_{1,450} = 4.32$; $P = 0.0381$; Fig. 1). No significance was found for mating receptivity when comparing cloudiness categories ($F_{2,450} = 2.18$; $P = 0.1141$), or size ratios ($F_{1,450} = 0.12$; $P = 0.7317$).

Fig. 1. Ambient temperatures (mean ± SE) associated with trials during which *S. noctilio* mating occurred or not. Different letters above bars demonstrate significant differences.



Among males included in trials, 52.3% never mated and 25.0% of the total males only mated once. Four males each mated three times in one trial but it was much more common for males to mate only once per trial or not at all; out of the 710 male exposures, 13.5%, 1.7% and 0.6% resulted in 1, 2 and 3 matings per male, respectively.

Multiple Mating by Males and Females

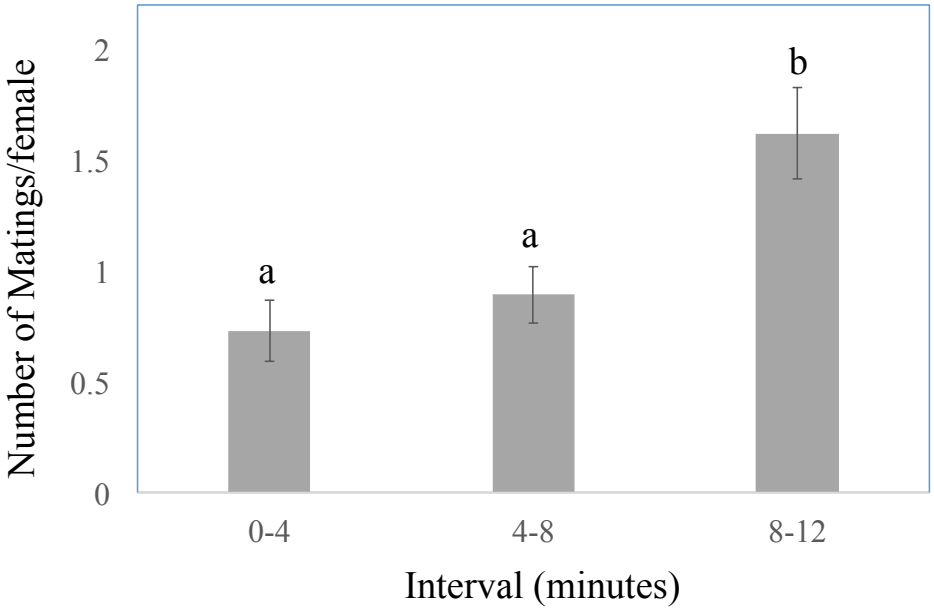
For 72.5% of the 51 trials during which mating occurred, females mated more than once. The overall number of matings per female within the 12-minute trials averaged 2.6 ± 0.2 and ranged from 1-7. When a female mated twice (n = 16), mating occurred with two different males

in 75% of the time. When a female mated more than twice (n = 24), she always mated with more than one individual male, but an individual female never mated with the same male more than three times.

We found a positive association between the number of matings per female and temperature ($F_{1,450} = 5.34$, $P = 0.0213$). However no association was found between number of matings per female and cloudiness ($F_{2,450} = 2.31$, $P = 0.3154$). Size ratios between pairs of wasps did not influence the number of matings ($F_{1,450} = 0.01$, $P = 0.9077$). The average ratio of male:female pronotum widths for mating pairs was 0.97 ± 0.03, demonstrating that the males and females were usually similar in size. However, size ratios during trials for mating pairs ranged from 0.5-2.06 and at extremes, particularly when males were much larger than females, difficulties were evident during copulation.

For females that mated multiple times, the receptivity to mating increased with time; more matings occurred during the 8-12 minute interval than the 0-4 or 4-8 minute intervals ($F_{2,100} = 8.04$; $P = 0.0006$) (Fig. 2).

Fig. 2. For females mating more than once, numbers of matings per female (mean ± SE) occurring during the three time intervals within 12 minute mating trials. Different letters above bars demonstrate significant differences.



Discussion

Temperature was positively associated with both initial female sexual receptivity and the number of times that a female would mate. Morgan and Stewart (1966) reported that temperatures above 21ºC would stimulate *S. noctilio* to mate. Our study evaluated temperatures with a mean of 29.5 ± 0.4ºC and range of 23-35ºC, and we found a positive association between temperature and both receptivity and number of matings within this temperature range. Both

Morgan and Stewart (1966) and Dolezal (1967) mentioned wing buzzing by males as part of mating behavior. However, during this study, buzzing was mostly observed by males on colder days and we did not observe that this was associated with mating behavior.

Sirex noctilio adults are attracted to light (Dolezal 1967, Sarvary at al. 2015) and we observed this in our study. During sunny conditions males and females tended to move towards a strong light source, in our case the brightness of the glass greenhouses across from the shaded location of the cages, and both males and females crowded in the brightest corner of the cage. However, counter to our predictions, we did not find significance for the influence of visible light in initial sexual receptivity or the number of matings by females. However, mating always occurred in the brightest portion of the cage.

Sirex noctilio adults vary greatly in size, which is thought to be based on growth of larvae being dependent on the area colonized by the symbiotic fungus that was deposited by the mother during oviposition (Madden 1981). Haavik et al. (2016) recently reported that the largest *S. noctilio* females were ≥ 2.5x larger than the smallest females in Ontario; results were comparable in this study. We hypothesized that size ratios would be one of the factors that drives copulation success, but this effect was not statistically significant. However, sometimes during our trials, when a male was much larger than a female, it appeared that the male had difficulty bending his abdomen enough to reach the female's genital opening.

Both females and males remained receptive to further mating after their first copulation, and they might or might not copulate more than once with the same or different individuals. Most of the times when a female was receptive to mating, she copulated more than once during the 12-minute mating assays, most often with more than one male. Few studies have reported on symphytan mating but females of the diprionid *Neodiprion sertifer* (Geoffroy) also mated several

times (Östrand and Anderbrant 2001). Monandrous species, such as the hymenopteran parasitoid *Diaeretiella rapae* Stary, are thought to optimize female fitness through heightened selectivity allowing females to choose the best partner (Kant and Minor 2016). In contrast, polyandrous species, such as *S. noctilio*, are thought to maximize fitness by repeated copulation, which occurred up to 7 times within 12-minute intervals during our trials. While we are uncertain whether sperm was transferred during each copulation event recorded in this study, we know from dissections that sperm was transferred during a subsample of copulations evaluated for confirmation (I.A.L.C., unpublished data).

The standard response of female to male insects is a reluctance to mate, with stimulation by males, diet, ovarian development and hormones influencing the change to sexual receptivity (Ringo 1996). When *S. noctilio* females were placed into cages with 10 males, sometimes mating occurred fairly soon but often, with increasing time in the cage, a female became more receptive to mating, i.e., females mated more frequently during the 8-12 minute interval. In nature, after emergence, females fly to the tops of trees (Morgan and Stewart 1966) where swarms of males occur and where we hypothesize that multiple matings with numerous males naturally occur. Adult females of *S. noctilio* only live up to 7 days (Neumann and Minko 1981), so they have a short time to mate, find a suitable tree and lay eggs. It is generally thought that after adult females emerge from trees they go to the upper canopy of trees, attracted by sunlight (Madden 1988). Males have already aggregated in the upper canopy and this is where mating takes place. After mating, the initially photopositive female response changes to a host-location response (Madden 1988) and subsequently, females locate appropriate trees and oviposit.

Our preliminary trials to optimize conditions for mating demonstrated that mating regularly occurred under specific conditions, including higher temperatures, indirect sunlight,

and high male:female ratios (I.A.L.C., unpublished data). Being hymenopteran, *S. noctilio* can successfully reproduce without mating but then, based on haplodiploid sex determination, only male offspring are produced. Therefore, if conditions are not conducive for a female to mate but she still eventually finds acceptable host trees and oviposits, her progeny will then all be males. Field studies have reported *S. noctilio* sex ratios that were extremely male-biased in decreasing populations (Morgan and Stewart 1966) or when invasive populations of *S. noctilio* are spreading into new areas (Ryan and Hurley 2012), e.g., an extreme of 32:1 (males:females) was reported from Brazil (Iede et al. 1998). Such highly male-biased sex ratios would decrease fitness locally and this could lead to Allee effects, i.e., declines in population growth rate associated with low density populations that, in this case, would be caused by highly male-biased sex ratios (Liebhold and Tobin 2008). Alternatively, sex ratios have been reported as closer to 1:1 where *S. noctilio* populations are increasing (Morgan and Stewart 1966) or where *S. noctilio* are native (Spradbery and Kirk 1978, Lombardero et al. 2016); in these situations, successful mating must more commonly occur. Our results suggest that when female *S. noctilio* are sexually receptive, they often mate multiple times, which could result in greater genetic diversity among offspring. Studies across different taxa have reported that multiple matings can result in increased fitness (see Li et al. 2014). We encourage future studies to investigate whether the occurrence of multiple matings in *S. noctilio* leads to a positive impact on fitness.

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Chapter 2

Growth of the *Sirex*-parasitic nematode *Deladenus siricidicola* on the white rot fungus *Amylostereum*

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Abstract

The Kamona strain of the nematode *Deladenus siricidicola* has been extensively used as a biological control agent against invasive woodwasp *Sirex noctilio* in the Southern Hemisphere, where it sterilizes female hosts. In North America, a non-sterilizing (NS) strain of *D. siricidicola*, thought to have been introduced with *S. noctilio*, is commonly found parasitizing this invasive woodwasp. Species of *Deladenus* that parasitize *Sirex* have a parasitic form, as well as a mycophagous form. The mycophagous form feeds on *Sirex* fungal symbionts in the genus *Amylostereum*. The goal of this study was to compare reproduction of NS and Kamona *D. siricidicola* when feeding on four isolates of *Amylostereum areolatum* (three introduced and one native in North America) and one native strain of *Amylostereum chailletii* isolated from *Sirex nigricornis*. Mycophagous forms of the two *D. siricidicola* strains displayed relatively similar production of offspring when feeding on most of the *A. areolatum* found associated with *S. noctilio* in this continent, except for strain BD on which NS produced more offspring than the biological control strain Kamona. Growth of both nematodes was greater on the introduced versus the native *A. areolatum* isolates.

Keywords:

Biological control, Invasive arthropod, *Sirex noctilio,* White rot fungus, Fungal-feeding nematode

Introduction

The nematode *Deladenus siricidicola*, Kamona strain has been used for decades as a biological control agent against invasive *Sirex noctilio* woodwasps that attack pines in the Southern Hemisphere. *S. noctilio* and its symbiotic white rot fungus *Amylostereum* areolatum were first collected from an established population in North America in 2004 (Hoebeke et al., 2005). While the Kamona strain of *D. siricidicola* acts to sterilize adult female *S. noctilio*, in North America, a non-sterilizing (NS) strain of this nematode species, thought to have been introduced with *S. noctilio*, is also now commonly found (Kroll et al., 2013; Yu et al., 2009). Native *Sirex* and *Deladenus* species also occur in North America. *Deladenus* are mainly fungal feeding and can live for many generations feeding on *Amylostereum*, but change to the infective form when near *Sirex* larvae (Bedding, 1972).

Based on sequencing of the intergenic spacer region (IGS), previous studies have suggested that *A. areolatum* IGS BD and D were introduced with *S. noctilio* to North America, and *A. areolatum* IGS BE is native to this continent (Nielsen et al., 2009). *S. noctilio* has often been found carrying *A. areolatum* IGS BD and D, and rarely carrying *Amylostereum chailletii* (Wooding et al., 2013), which is usually found associated with native species of *Sirex*.

To develop a successful and environmentally safe biological control program, it is important to study how associated organisms will interact within a given system. Since *Sirex* larvae are surrounded by *Amylostereum* in the xylem, and *Deladenus* nematodes feed on this fungus, species and strains of fungi could influence both presence and size of *D. siricidicola* colonies, thus impacting whether *S. noctilio* become parasitized. It is now known that *Sirex* species are less tightly associated with fungal strains than previously thought (Hajek et al., 2013;

Wooding et al., 2013) and recent work has shown that *D. siricidicola* Kamona reproduction is affected by fungal strain (Morris et al., 2012, 2014). However, no similar studies have been conducted on the NS strain. This study compares the fitness of the recently discovered NS strain of *D. siricidicola* with *D. siricidicola* Kamona by quantifying the ability of the nematode strains to develop and reproduce when feeding on different isolates of *Amylostereum* associated with *Sirex* in North America.

Materials and methods

Nematode and fungal strains

To obtain *D. siricidicola* NS, *S. noctilio* collected from infested trees were dissected and nematodes were reared in petri dishes containing 1⁄2PDAh (Morris et al., 2012) inoculated with *A. areolatum*. Nematode strain was determined via molecular characterization of the mtCO1 gene, which was compared to *D. siricidicola* mtCO1 sequences in GenBank (JQ241275, Leal et al., 2012). *D. siricidicola* Kamona was imported in 2014 from Ecogrow Environment (Westgate, NSW), the Australian commercial producer of this nematode for biological control of *S. noctilio*. A colony of the Kamona strain was maintained in a quarantine facility and all experiments with this strain were conducted under quarantine.

Isolates of *Amylostereum* used had been obtained from siricids collected in North America (Table 1). Four isolates are *A. areolatum*, commonly found associated with invasive *S. noctilio*, and one isolate is *A. chailletii*, a fungal species which is usually associated with native *Sirex*. One of the *A. areolatum* strains included is thought to be native to North America (BE) while the other three strains of *A. areolatum* are thought to have been introduced with *S. noctilio*

(Nielsen et al., 2009). *S. noctilio* and *S. nigricornis* can co-infest the same tree and thus *A. chailletii* used in this study was isolated from *S. nigricornis*.

Bioassays

Petri dishes containing 1⁄2PDAh were inoculated with one of the five fungal isolates and incubated for five days. All bioassays were conducted at 23 C without light. Nematodes and eggs were removed from colony dishes of *D. siricidicola* Kamona or NS by flooding the dishes with autoclaved water + gentamicin (6.25 mg/L). The resulting suspension was filtered through a 60 lm filter (Swinnex, Millipore) to eliminate juvenile and adult nematodes. The resulting egg suspension was adjusted to 167 eggs/20µll. Three 20µll drops of the final suspension were transferred to prepared fungal dishes, placing drops close to the edge of the dish and equidistant from each other. Therefore, each dish received approximately 500 eggs.

After 27d, three dishes of each treatment (Table 1) were thoroughly rinsed with tap water to remove all nematodes and eggs, creating a separate suspension from each dish. Nematodes and eggs within five 20µll drops of the suspension from each plate were counted and the number of nematodes plus eggs/plate was calculated.

Each treatment was replicated 2–4 times. To compare the production of nematodes and eggs on different fungal isolates after 27d, we used analysis of variance with main effects of nematode strain, fungal isolate and the interaction between fungal isolate and nematode strain. Means were separated using Tukey HSD posthoc tests.

Table 1. Strains of *Amylostereum* included in nematode reproduction bioassays.

Isolate ID	**Species**	**Isolation from**	**Original host**	**Date isolated**	**IGS strain**
BE	*A. areolatum*	Warrensburg, NY	*S. nigricornis*	19 Sep 2009	BE
BD	*A. areolatum*	Fulton County, NY	*S. noctilio*	19 Feb 2008	BD
Dni	*A. areolatum*	Oswego County, NY	*S. nigricornis*	19 Sep 2007	D
Dno	*A. areolatum*	Oswego County, NY	*S. noctilio*	Jan 2006	D
Ch	*A. chailletii*	New Haven, NY	*S. nigricornis*	21 Sep 2007	G

Results and Discussion

The interaction between nematode and fungal treatment was highly significant ($F_{4,89}$ = 7.182; P < 0.001). *D. siricidicola* Kamona grew best on *A. areolatum* strains D_{no}, D_{ni} and BD, which are assumed to have been introduced to North America with *S. noctilio* (Nielsen et al., 2009) (Fig. 4). Growth was intermediate on the native strain *A. areolatum* BE. The NS strain of *D. siricidicola* displayed greatest reproduction when grown on *A. areolatum* BD, followed by both *A. areolatum* D strains. Reproduction of *D. siricidicola* NS on *A. areolatum* BE was significantly less than BD and D_{no}. Neither nematode strain increased in numbers when offered *A. chailletii* (Ch). Comparing nematode strains, the only significant difference was seen for *A. areolatum* BD on which *D. siricidicola* NS increased to higher densities than Kamona (P < 0.001). Both nematode strains grew faster on introduced isolates of *A. amylostereum* (BD, D_{no} and D_{ni}) than on the native isolate (BE) (contrast statement results: NS: $F_{1,86}$ = 27.0868, P < 0.0001; Kamona: $F_{1,86}$ = 35,1503, P < 0.0001).

When developing biological control methods, understanding interactions between the organisms involved within a system is important. Knowing the reproductive performance of these nematode strains when feeding on different isolates of *A. areolatum* may help with decisions about which fungus to use to mass-produce the nematode for biological control. Morris et al. (2012, 2014) and Hurley et al. (2012) tested growth of *D. siricidicola* on different strains and species of *Amylostereum*. Results from our study differ from those of bioassays with Kamona by Morris et al. (2014) in which highest reproduction was seen on *A. areolatum* BE and reproduction was very poor on *A. areolatum* D. While our study tested these same fungal strains, surprisingly Kamona reproduced best on *A. areolatum* D_{no} and reproduction on *A. areolatum*

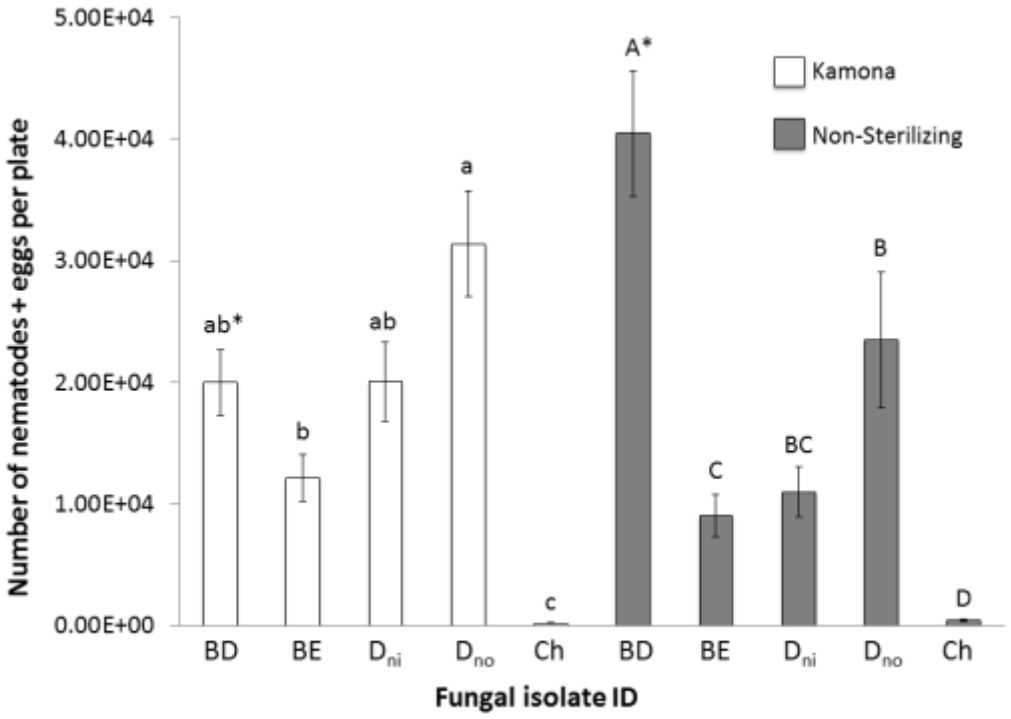
BE was intermediate.

This study provides a better understanding of how these two nematode strains could potentially interact in nature in North America. The mycophagous forms of both *D. siricidicola* strains displayed relatively similar production of offspring when feeding on most of the *A. areolatum* found associated with *S. noctilio* in this continent, except for strain BD on which NS nematodes reproduced more than the biological control strain Kamona.

If both strains of nematode occurred in the same tree and were located within the same fungal colonies, potential interactions between the two nematode strains could include food resource competition as well as potential strain hybridization, as Akhurst (1975) has shown hybridization among *D. siricidicola* strains to be possible.

Based on our results, if a *Sirex* larva was surrounded by *A. chailletii*, *D. siricidicola* NS and Kamona might not infect that larva because both nematode strains do not readily feed on *A. chailletii*. If a *S. noctilio* larva was surrounded by the introduced *A. areolatum* strains that are currently present in North America, then either of the nematode strains might occur within that fungal culture and would possibly infect the larva. We included *A. areolatum* BE because it is native and has historically been associated with native *Sirex*. We wanted to evaluate whether NS would grow well on BE in order to determine whether NS would be in the vicinity of native *Sirex* surrounded by symbiotic BE. Both nematode strains grew better on introduced *A. areolatum* rather than the native *A. areolatum*, although there was still growth on the native fungus. We found that growth of the two *D. siricidicola* strains was equal on *A. areolatum* BE, suggesting that either strain of nematode has an equal chance of being near native *Sirex* larvae having BE as a symbiont.

Fig. 3. The number of nematodes and eggs of *D. siricidicola* produced per plate by 27 days (white = Kamona; gray = NS). Lower case letters compare the growth of *D. siricidicola* Kamona when feeding on different fungal isolates. Uppercase letters compare the growth of *D. siricidicola* NS when feeding on different fungal isolates. Asterisks indicate differences between the two nematode strains feeding on the same fungal isolate. BD, BE, D_{ni}, D_{no} = Strains of *A. areolatum*; Ch = Strain of *A. chailletii*.



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Chapter 3

Use of *Deladenus siricidicola* Kamona to control *Sirex noctilio* and the potential for non-target effects on *Sirex nigricornis*

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Abstract

Sirex noctilio is an invasive pest in North America that kills pine trees (*Pinus* spp.). The nematode *Deladenus siricidicola* Kamona, which can sterilize some strains of *S. noctilio* females, can also be mycophagous and free living or parasitic. We tested *D. siricidicola* Kamona against *S. noctilio* to investigate how effective it can be at controlling this woodwasp in North America. In North America, another strain of *D. siricidicola* that does not sterilize *S. noctilio* is already present, presumably introduced with this invasive. In 2013 *Deladenus siricidicola* Kamona was reared on two different strains of the white rot fungus *Amylostereum areolatum*, one native to North America (strain BE) and another (strain Aussie) used commercially to mass-produce the nematode in Australia. We inoculated Kamona, reared on both strains of *A. areolatum*, into logs infested with *S. noctilio* larvae. In 2014 we inoculated logs with Kamona grown on *A. areolatum* BE and *Deladenus proximus*, a native nematode species that is usually found parasitizing the native woodwasp *Sirex nigricornis*. In this same year 6 logs infested with *S. nigricornis* were injected with *D. siricidicola* Kamona. All woodwasps that emerged from treated and control logs in the summers following inoculation, were dissected and nematode samples were taken from infected wasps. To identify the nematodes collected from wasps we sequenced the mtCO1 gene. In the results from the woodwasps emerged in 2014 we found that only two were infected with the Kamona strain of *D. siricidicola*: one (of 46) from the Kamona + Aussie treatment was sterile, and the other, from the Kamona + BE treatment, was partially sterile. Forty *S. noctilio* emerged from control logs and none were infected with nematodes. None of the *S. noctilio* or *S. nigricornis* that emerged from 2014-2015 assays were infected with either Kamona or *D. proximus*. However, a very high rate of infection by *D. siricidicola* NS was found among *S. noctilio* for all treatments,

and all *S. nigricornis* were found not to be parasitized by nematodes. These studies did not show that the *D. siricidicola* strain Kamona is capable of efficiently parasitizing and sterilizing *S. noctilio* in North America. The reasons for the lack of infection are not yet well understood and further studies are necessary. Also, tests with new strains of *D. siricidicola* should be undertaken in North America.

Introduction

Sirex noctilio is native to Eurasia and North Africa but has been invasive in the southern hemisphere since the early 1900s. Since *Sirex noctilio* was found in the United States in September 2004 (Hoebeke et al. 2005), it has spread to seven US states (CERIS 2014). It is important to find a method to control this invasive woodwasp as it spreads towards areas with extensive pine forests. Parasitoid wasps such as *Ibalia leucospoides ensiger*, *Rhyssa lineolata* and *Megarhyssa nortoni* can naturally provide some control of *S. noctilio*. It has been reported that these parasitoids can kill up to 21 to 28% of *S. noctilio* (Long et al. 2009, Zylstra and Mastro 2012; Foelker et al., 2016), but this level of mortality will not be enough to stop the spread of *S. noctilio*.

A successful biological control program using the nematode *Deladenus siricidicola* Kamona was developed and implemented in Australia and New Zealand, where pine trees and *S. noctilio* are not native. According to Bedding (2009) the success of *D. siricidicola* controlling *S. noctilio* depends on the density of the woodwasps. When more nematode-parasitized wasps attack susceptible trees, it leads to higher percentage parasitism of larvae parented by unparasitised wasps that laid eggs in the same trees in the treated area and the percentage of parasitized wasps increases over the years when nematodes are applied to a *S. noctilio* infested area in consecutive years.

In North America, where many species of pine trees are native and support very diverse communities, implementing a classical biological control program is not simple due to the possibility of impacts caused to non-target species. Native siricid species are present in North America, especially including *Sirex nigricornis*, which can coinhabit the same trees as *S. noctilio*

and could be impacted by treatments used to control the invasive wasp (Williams et al. 2012). This native species is not considered a pest and instead it is part of the decomposer community in the forest (Spradbery and Kirk 1978).

This study was conducted to test *D. siricidicola* Kamona for possible *S. noctilio* control. Evaluating the potential impacts of this nematode on the native woodwasp, *S. nigricornis*, that also attacks North American pines was another goal of this study. To conduct this study, mated female wasps ware caged to lay eggs into tree trunks that were previously prepared, and, after winter incubation, these tree trunks were cut and inoculated with *D. siricidicola* Kamona or *D. proximus*, while control logs were not inoculated with nematodes. Final results demonstrated levels of nematode parasitism resulting from the inoculated nematodes.

Methods

Two cycles of bioassays were conducted, the first one began in summer 2013 and ended in summer 2014 and the second cycle began in summer 2014 and ended in summer 2015. For both cycles, the wasps that were used for ovipositing into experimental trees were reared from naturally infested trees, and a new culture of *D. siricidicola* Kamona was imported from Australia (Ecogrow Environment, Westgate, NSW). Details for each procedure are described below.

Rearing woodwasps for oviposition in experimental logs

In spring and early summer of 2013 and 2014, portions of infested trees were cut into logs about 70 cm long, all logs had both ends waxed to reduce loose of moisture, and they were

kept inside fiber barrels with window screening covers. Numbers were given to each tree, and barrels were labeled with a code that identified sites where the trees originated and the tree number. Barrels were checked daily from late in June to the end of September and adults of the invasive wood wasp *S. noctilio* and also the native *S. nigricornis* were collected from barrels and used for different experiments.

Insects that emerged from the logs in the barrels were kept in 29 ml cups at 4 ± 1ºC until they were used for studies, therefore extending the insects' life spans. Cups were labeled with a number, which was the insect identification, information on the barrel that the woodwasp originated from and the date when it was collected. Later, some of the wasps that were used to lay eggs in experimental trees were dissected to determine whether nematodes were present within these woodwasps.

Mating females

Sirex spp. are haplodiploid insects and, to test the efficacy of *D. siricidicola* in sterilizing female wasps we needed to obtain female offspring. So, for both cycles of assays, 2013-14 and 2014-15, female wasps were mated before they were taken to the forest to lay eggs into experimental trees. For this purpose, healthy males and females were chosen, we used wasps that had intact legs, intact antennae, no defective wings and seemed healthy when warmed up for a few minutes to room temperature.

Females were mated in cages (60 x 60 x 60 cm) (BugDorm 2; Bioquip, Rancho Dominguez, CA) using the methods described in chapter 1, with cages placed in a shaded spot close to bright greenhouses. Each cage contained ten males and one female. For these studies females were gently pushed close to males, to stimulate them to mate sooner, and they were

removed as soon as they were mated. Then mated females were replaced at 4ºC in their original containers for at least 24 hours before they were taken to lay eggs in the trees prepared for biological control trials.

Assay set up, 2013-2014

In 2013 twenty-four mature red pines, *Pinus resinosa*, were selected at Arnot Teaching and Research Forest. To make these trees attractive to the wasps, they were weakened by injection with the herbicide, Banvel (49.4% diluted in water 1:1). Holes were drilled into the trees trunks 50 cm above ground level, and about 5 cm deep, at a 45 º angle. Holes were made 10 cm apart from each other around the circumference of the tree trunk (Zylstra et al. 2010) and 1 ml of the herbicide solution was injected into each hole. Half of these trees were used for tests with *S. noctilio* and half for tests with *S. nigricornis*. Trees used for *S. noctilio* tests were prepared on 3 July 2013 and the trees used for *S. nigricornis* on 8 August 2013. The herbicide procedure was conducted a few weeks before the predicted beginning of the flight season of each *Sirex* species used in our assays at the same location.

Figure 4: Cage around a tree trunk in which female wasps could lay eggs into experimental trees.

Cages made out of window screening were built around the trunks of the trees (cages were about 1 m long and 50 cm in diameter) (Figure 4). Two sets of *S. noctilio* females that had previously mated (see "*Mating females*" section), were placed in each cage for oviposition. These woodwasps had intact legs, antennae, and no defective wings and seemed healthy after being warmed up. Wasps released in the same cage had their pronota painted different colors to identify individual wasps.

The first set of *S. noctilio*, in 2013, was released in the cages 4 weeks after the herbicide had been injected into the trees, on 30 July, and on this date, we placed two females in each cage. This set of wasps was kept in cages for three days. A second set of *S. noctilio* females with two females per cage, was released in the cages on 6 August and this set remained in the cages for four days. After being in the cages, wasps of both sets were brought back to the lab and dissected to check for nematode infection.

In this same year, *S. nigricornis* were taken to cages in October. Due the low number of *S. nigricornis* available in 2013, only one set of one wasp per cage was used. Females were released in cages on 2 October. *Sirex nigricornis* remained in cages for four days, and were then retrieved.

Standing experimental trees were left to overwinter in the forest and were cut in mid-April of 2014. The portions of the trunk that had been caged were brought back to the lab for nematode injection late in the same month.

Treatments, 2013-2014

In 2013-2014, the efficacy of *D. siricidicola* Kamona grown on two different strains of *A. areolatum* was tested: *A. areolatum* Aussie which is used to commercially mass produce *D. siricidicola* Kamona in Australia (Ecogrow Environment, Westgate, NSW) and *A. areolatum* BE (Chapter 2, Table 1) which is native in North America. For this bioassay six treatments were implemented. Four logs were used for each treatment.

1. *D. siricidicola* Kamona grown on *A. areolatum* Aussie against *S. noctilio*
2. *D. siricidicola* Kamona grown on *A. areolatum* BE against *S. noctilio*
3. Control *S. noctilio*
4. *D. siricidicola* Kamona grown on *A. areolatum* Aussie against *S. nigricornis*
5. *D. siricidicola* Kamona grown on *A. areolatum* BE against *S. nigricornis*
6. Control *S. nigricornis*

Table 2. Details of 2013-2014 treatments including numbers of the trees, treatments, numbers of wasps released in cages and dates of the procedures

Sirex species	Treatment	Tree number	Tree preparation	Date of tree preparation	Dates first set of females were in cages for oviposition	Number of females laying eggs - First set	Dates second set of females were in cages for oviposition	Number of females laying eggs - Second set	Inoculation date*
S. noctilio	Control	4	Herbicide - Banvel	3-Jul-13	7/31 - 08/02/2013	2	8/6 - 08/09/2013	2	4/30/14
S. noctilio	Control	5	Herbicide - Banvel	3-Jul-13	7/31 - 08/02/2013	2	8/6 - 08/09/2013	2	4/30/14
S. noctilio	Control	12	Herbicide - Banvel	3-Jul-13	7/31 - 08/02/2013	2	8/6 - 08/09/2013	2	4/30/14
S. noctilio	Control	15	Herbicide - Banvel	3-Jul-13	7/31 - 08/02/2013	2	8/6 - 08/09/2013	2	4/30/14
S. noctilio	*D. siricidicola* Kamona on *A. areolatum* BDF	6	Herbicide - Banvel	3-Jul-13	7/31 - 08/02/2013	2	8/6 - 08/09/2013	2	4/30/14
S. noctilio	*D. siricidicola* Kamona on *A. areolatum* BDF	17	Herbicide - Banvel	3-Jul-13	7/31 - 08/02/2013	2	8/6 - 08/09/2013	2	4/30/14

S. noctilio	*D. siricidicola* Kamona on *A. areolatum* BDF	21	Herbicide - Banvel	3-Jul-13	7/31 - 08/02/2013	2	8/6 - 08/09/2013	2	4/30/14
S. noctilio	*D. siricidicola* Kamona on *A. areolatum* BDF	22	Herbicide - Banvel	3-Jul-13	7/31 - 08/02/2013	2	8/6 - 08/09/2013	2	4/30/14
S. noctilio	*D. siricidicola* Kamona on *A. areolatum* BE	10	Herbicide - Banvel	3-Jul-13	7/31 - 08/02/2013	2	8/6 - 08/09/2013	2	4/30/14
S. noctilio	*D. siricidicola* Kamona on *A. areolatum* BE	14	Herbicide - Banvel	3-Jul-13	7/31 - 08/02/2013	2	8/6 - 08/09/2013	2	4/30/14
S. noctilio	*D. siricidicola* Kamona on *A. areolatum* BE	20	Herbicide - Banvel	3-Jul-13	7/31 - 08/02/2013	2	8/6 - 08/09/2013	2	4/30/14
S. noctilio	*D. siricidicola* Kamona on *A. areolatum* BE	24	Herbicide - Banvel	3-Jul-13	7/31 - 08/02/2013	2	8/6 - 08/09/2013	2	4/30/14

S. nigricornis	Control	1	Herbicide - Banvel	8-Aug-13	10/2 - 10/05/2013	1	-	-	4/30/14
S. nigricornis	Control	2	Herbicide - Banvel	8-Aug-13	10/2 - 10/05/2013	1	-	-	4/30/14
S. nigricornis	Control	8	Herbicide - Banvel	8-Aug-13	10/2 - 10/05/2013	1	-	-	4/30/14
S. nigricornis	Control	13	Herbicide - Banvel	8-Aug-13	10/2 - 10/05/2013	1	-	-	4/30/14
S. nigricornis	*D. siricidicola* Kamona on *A. areolatum* Aussie	7	Herbicide - Banvel	8-Aug-13	10/2 - 10/05/2013	1	-	-	4/30/14
S. nigricornis	*D. siricidicola* Kamona on *A. areolatum* Aussie	18	Herbicide - Banvel	8-Aug-13	10/2 - 10/05/2013	1	-	-	4/30/14
S. nigricornis	*D. siricidicola* Kamona on *A. areolatum* Aussie	19	Herbicide - Banvel	8-Aug-13	10/2 - 10/05/2013	1	-	-	4/30/14
S. nigricornis	*D. siricidicola* Kamona on *A. areolatum* Aussie	23	Herbicide - Banvel	8-Aug-13	10/2 - 10/05/2013	1	-	-	4/30/14

S. nigricornis	*D. siricidicola* Kamona on *A. areolatum* BE	16	Herbicide - Banvel	8-Aug-13	10/2 - 10/05/2013	1	-	-	4/30/14
S. nigricornis	*D. siricidicola* Kamona on *A. areolatum* BE	3	Herbicide - Banvel	8-Aug-13	10/2 - 10/05/2013	1	-	-	4/30/14
S. nigricornis	*D. siricidicola* Kamona on *A. areolatum* BE	9	Herbicide - Banvel	8-Aug-13	10/2 - 10/05/2013	1	-	-	4/30/14
S. nigricornis	*D. siricidicola* Kamona on *A. areolatum* BE	11	Herbicide - Banvel	8-Aug-13	10/2 - 10/05/2013	1	-	-	4/30/14

* Inoculation of nematodes into experimental logs.

Origin of nematode and fungal cultures

In January of 2014 cultures of *Deladenus siricidicola* Kamona were imported from Ecogrow Environment (Westgate, NSW), the Australian commercial producer of this nematode for biological control of *S. noctilio*. Colonies of this nematode strain were kept in a quarantine facility under USDA APHIS permits (see Chapter 2) and were cultured in 100 mm diameter petri dishes contained *Amylostereum areolatum* Aussie, the fungal strain used to commercially mass-produce *D. siricidicola* for biological control. Half of the imported nematodes were transferred to petri dishes containing ½ PDAh (Morris at. al. 2012) and *Amylostereum areolatum* BE (see Table 1 for source of fungal strain), a fungal strain found in North America. Nematodes transfer followed methods described in the Bioassays section of Chapter 2. Nematodes were allowed to grow and reproduce in Petri dishes and were increased in number by transferring them into new Petri dishes containing ½ PDAh every two weeks, until we obtained at least 30 well-colonized plates of each nematode/fungus combination.

Nematode Inoculation

Suspensions of nematodes were prepared by rinsing nematodes with autoclaved water from culture plates into 50 ml centrifuge tubes. Nematodes were counted under a dissecting scope at a magnification of 60x in drops of 20µl and volumes were adjusted to obtain 40 nematodes per drop of 20µl, which resulted in an average of 2000 nematodes per milliliter. Then 0.5 g of AquaRock (polyacrylamide gel) was added to each 50 ml tube containing nematodes in suspension (Williams and Hajek, in review).

Following methods of Bedding (2009), a punch hammer, which is a punch mounted in a hammer, was used to punch holes (1 cm wide by 1 cm deep) into the logs that had been caged

the previous summer/fall. Two rows of holes along the length of the log were made in opposite sides of each log; these holes were placed 20 cm apart within a row and each of the holes was filled with 1 ml of the suspension of nematodes planned for each log.

Logs that were inoculated with a suspension of *D. siricidicola* Kamona were kept in a quarantine facility and control logs were kept in an unheated barn located about 1.6 km from the quarantine.

After inoculation, treated logs were stored inside fiber barrels with screening covers, and during the following summer (2014) these barrels were checked daily and specimens were collected and stored at 4ºC until each adult *S. noctilio* was dissected to check for nematode infection (see below).

Assay set up, 2014-15

Most of the protocols used on 2014-15 assays were similar to the previous year. This section includes a description of the changes in protocols and treatments.

In 2014, sixteen trees, at the same location of the previous year, were injected with herbicide, on 16 July 2014, to be used for experiments with *S. noctilio*. Herbicide injections followed the same protocol used in 2013. Another four trees were cut down on 27 August, instead of being injected with herbicide, to be used in the experiment with *S. nigricornis*.

For *S. noctilio*, the window screening cages built on tree trunks were similar to cages used in 2013. However, because the trees used for *S. nigricornis* had been cut down, the cage design was adjusted because the trees were lying on the forest floor in horizontally.

Two sets of mated *S. noctilio* female that seemed healthy, with intact legs, antennae, and no defective wings, were taken to the cages for oviposition. Wasps released in the same cage had their pronota painted different colors to identify individual wasps. The first set of *S. noctilio* in 2014, was taken to the cages 7 August. Three females were placed in each cage and they were kept in cages for five days. A second set of *S. noctilio* females, with two females per cage, were released in the cages on 19 August and remained in cages for 6 days. After removing all females that had been in cages for oviposition, they were brought back to the lab and dissected to check for nematode infections. In 2014, *Sirex nigricornis* females were placed in cages on 1 October. One set of two wasps was used per cage, females remained in cages for six days, and were then retrieved.

Experimental trees were left to overwinter in the forest and the caged portions of the experimental trees were cut in mid-April of 2015. Parts of trees that had been caged were brought to our facilities for nematode inoculations late in the same month. Logs treated with Kamona were inoculated and maintained at the quarantine lab and logs for treated with *D. proximus* and controls were inoculated and maintained at a nearby unheated barn.

Treatments, 2014-2015

In 2014-2015 three different treatments were set up for *S. noctilio*. Although we had planned to conduct an equal number of treatments with *S. nigricornis*, due to the restricted number of available *S. nigricornis*, only four experimental logs were used for this species of woodwasp and they were all treated with *D. siricidicola* Kamona grown on *A. areolatum* BE. For *S. noctilio* the three treatments were:

1. *D. siricidicola* Kamona grown on *A. areolatum* BE against *S. noctilio* (six trees)

2. *D. proximus* grown on *A. chailletii* against *S. noctilio* (five trees)

3. *S. noctilio* control (five trees)

4. *D. siricidicola* Kamona grown on *A. areolatum* BE against *S. nigricornis* (four trees)

Table 3. Details of 2014-2015 treatments including numbers of trees, treatments, numbers of wasps released in cages and dates of the procedures.

Sirex species	Treatment	Tree number	Tree preparation	Date of tree preparation	Date females were caged for oviposition -- First set	Number of females laying eggs - First set	Date females were caged for oviposition - Second set	Number of females laying eggs - Second set	Inoculation date*
S. noctilio	Control	1	Herbicide - Banvel	16-Jul-14	8/7 - 08/11/2014	3	08/19 - 08/25/2014	2	28/04/2015
S. noctilio	Control	15	Herbicide - Banvel	16-Jul-14	8/7 - 08/11/2014	3	08/19 - 08/25/2014	2	28/04/2015
S. noctilio	Control	18	Herbicide - Banvel	16-Jul-14	8/7 - 08/11/2014	3	08/19 - 08/25/2014	2	28/04/2015
S. noctilio	Control	26	Herbicide - Banvel	16-Jul-14	8/7 - 08/11/2014	3	08/19 - 08/25/2014	2	28/04/2015
S. noctilio	Control	27	Herbicide - Banvel	16-Jul-14	8/7 - 08/11/2014	3	08/19 - 08/25/2014	2	28/04/2015
S. noctilio	*D. proximus* on *A. chailletii*	17	Herbicide - Banvel	16-Jul-14	8/7 - 08/11/2014	3	08/19 - 08/25/2014	2	28/04/2015
S. noctilio	*D. proximus* on *A. chailletii*	23	Herbicide - Banvel	16-Jul-14	8/7 - 08/11/2014	3	08/19 - 08/25/2014	2	28/04/2015
S. noctilio	*D. proximus* on *A. chailletii*	24	Herbicide - Banvel	16-Jul-14	8/7 - 08/11/2014	3	08/19 - 08/25/2014	2	28/04/2015

S. noctilio	*D. proximus* on *A. chailletii*	28	Herbicide - Banvel	16-Jul-14	8/7 - 08/11/2014	3	08/19 - 08/25/2014	2	28/04/2015
S. noctilio	*D. proximus* on *A. chailletii*	29	Herbicide - Banvel	16-Jul-14	8/7 - 08/11/2014	3	08/19 - 08/25/2014	2	28/04/2015
S. noctilio	*D. siricidicola* Kamona on *A. areolatum* BE	2	Herbicide - Banvel	16-Jul-14	8/7 - 08/11/2014	3	08/19 - 08/25/2014	2	29/04/2015
S. noctilio	*D. siricidicola* Kamona on *A. areolatum* BE	3	Herbicide - Banvel	16-Jul-14	8/7 - 08/11/2014	3	08/19 - 08/25/2014	2	29/04/2015
S. noctilio	*D. siricidicola* Kamona on *A. areolatum* BE	5	Herbicide - Banvel	16-Jul-14	8/7 - 08/11/2014	3	08/19 - 08/25/2014	2	29/04/2015
S. noctilio	*D. siricidicola* Kamona on *A. areolatum* BE	7	Herbicide - Banvel	16-Jul-14	8/7 - 08/11/2014	3	08/19 - 08/25/2014	2	29/04/2015
S. noctilio	*D. siricidicola* Kamona on *A. areolatum* BE	8	Herbicide - Banvel	16-Jul-14	8/7 - 08/11/2014	3	08/19 - 08/25/2014	2	29/04/2015

S. noctilio	*D. siricidicola* Kamona on *A. areolatum* BE	10	Herbicide - Banvel	16-Jul-14	8/7 - 08/11/2014	3	08/19 - 08/25/2014	2	29/04/2015
S. nigricornis	*D. siricidicola* Kamona on *A. areolatum* BE	9	Felled	27-Aug-14	10/01/2014 - 10/06/2014	2	-	-	29/04/2015
S. nigricornis	*D. siricidicola* Kamona on *A. areolatum* BE	30	Felled	27-Aug-14	10/01/2014 - 10/06/2014	2	-	-	29/04/2015
S. nigricornis	*D. siricidicola* Kamona on *A. areolatum* BE	31	Felled	27-Aug-14	10/01/2014 - 10/06/2014	2	-	-	29/04/2015
S. nigricornis	*D. siricidicola* Kamona on *A. areolatum* BE	32	Felled	27-Aug-14	10/01/2014 - 10/06/2014	2	-	-	29/04/2015

* Inoculation of nematodes into experimental logs.

Origin of nematode and fungal cultures

In January 2015 new cultures of *Deladenus siricidicola* Kamona were imported from Ecogrow Environment (Westgate, NSW), as in the previous year. Colonies of this nematode strain were kept in a quarantine facility and were transferred to 100 mm diameter petri dishes containing ½ PDAh (Morris et al. 2012) inoculated with *A. areolatum* BE. Nematode transfer followed methods described in the Bioassays section of Chapter 2. Nematodes were allowed to grow and reproduce in Petri dishes and were multiplied by transferring them into new Petri dishes containing ½ PDAh every two weeks until we obtained at least 30 well-colonized plates of each fungal culture.

To obtain *D. proximus*, a specimen of *S. nigricornis* caught in a live trap on 30 October 2014 in Morgantown, WV, was dissected and nematodes collected from it were cultured on 31 October at Dr. Hajek's laboratory at Cornell University. The colony of *D. proximus* was reared in petri dishes containing 1⁄2PDAh (Morris et al. 2012) inoculated with *A. chailetii* (see Table 1 for source of fungal strain). Nematode strain was confirmed via molecular characterization of the mtCO1 gene (Morris et al, 2013), and comparison to *D. proximus* mtCO1 sequences in GenBank (X10427). *Deladenus proximus* was grown and multiplied in petri dishes as described for *D. siricidicola*.

Nematode Inoculation

A suspension of each nematode species was prepared following the same protocols described for the previous year. Following methods described by Bedding (2009) a punch hammer was used to punch holes (1cm wide by 1 cm deep) into the caged logs. Two rows of holes were made in opposite sides of each log; holes were placed 20 cm apart within a row and

they were filled with 1 ml of the one of the gel suspensions, based on as the treatments planned for each log: *D. siricidicola* Kamona grown on *A. areolatum* BE, *D. proximus* grown on *A. chailletii* or controls. Table 3 shows the treatments used in each log in 2014-2015.

Logs that were inoculated with a suspension of *D. siricidicola* Kamona were kept in a quarantine facility, logs that were inoculated with a suspension of *D. proximus* or control gel were kept in an unheated barn located about 1.6 km from the quarantine. After inoculation, treated logs were stored inside fiber barrels with window screening covers, and, during following summer (2015), these barrels were checked daily, and specimens were collected and stored in a refrigerator at 4ºC until they were dissected to check for nematode infections.

Wasp dissections

The same protocol for wasp dissections were used in both years, 2014 and 2015. Dissections were conducted in a sterile laminar flow cabinet, under a dissecting microscope at a magnification of 60x, for both mothers and offspring. A glass petri dish was used to hold wasps during dissection. All tools used were cleaned before every new dissection by being dipped in ethanol for ten minutes and then flamed. All wasps were brushed with a paintbrush soaked in ethanol before they were dissected.

For males, abdomens were removed and cut open. Two drops of deionized water were added to the abdominal contents and internal organs were mashed and spread on the dish. When nematodes were present they were collected with disposable sterile pipettes and stored in 1.5 ml tubes containing 95% ethanol, at -20ºC until molecular characterization of the nematodes was conducted.

For females, abdomens were removed, cut lengthwise on both sides and dorsal sclerites were removed, exposing internal organs. Three drops of deionized water were added to the abdomen. Eggs were spread apart in the dissecting dish and, when nematodes were present, they were collected along with eggs using a disposable sterile pipette and stored in 1.5ml centrifuge tube containing 95% ethanol. Eggs were later spread in a 35 mm diameter Petri dish, and placed on an inverted microscope to quantify the percentage of egg sterilization. Two different magnification levels were used to evaluate egg sterilization: 60X and 200X.

Molecular characterization of the nematodes

To determine the species and strains of the nematodes collected from experimental wasps, molecular characterization of the mtCO1 gene was done by extracting DNA using a DNeasy micro kit (Qiagen, Valencia, CA) and amplifying DNA using CO1F and CO1R primers (Morris et al. 2013). The sequences of PCR products were obtained in both directions using CO1R and CO1_NEMA_INTf, designed by Tonya Bittner (5'- ATG TTA GGT TGT CCT GAT AT -3'). Sequencing was conducted at the Core Laboratory Center (CLC) at Cornell University. Results were aligned on Geneious (version 8.1.8) and compared to mtCO1 sequences in GenBank (X10427; JQ24127).

Results

2013-14

The logs used for *S. nigricornis* in 2014 did not produce any offspring but a total of 104 *S. noctilio* were collected from experimental barrels: 57 of these emerged from control logs, 46

from logs treated with *D. siricidicola* Kamona grown in *A. areolatum* Aussie and only one from logs treated with *D. siricidicola* Kamona grown in *A. areolatum* BE (Table 4). None of the wasps that emerged from control logs in 2014 were infected with nematodes.

Table 4. 2013-2014 percentage of offspring infected with strains of *Deladenus siricidicola* per treatment

Treatment	Total offspring	% offspring infected with NS[1]	% offspring infected with Kamona	% of non-infected offspring	% non-conclusive DNA analysis
Control	57	0	0	100	0
D. siricidicola Kamona on *A. areolatum* Aussie against *S. noctilio*	46	45.7	2.2	45.7	6.5
D. siricidicola Kamona on *A. areolatum* BE against *S. noctilio*	1	0	100	0	0

[1]NS= *Deladenus siricidicola* Non-sterilizing

Forty six woodwasps emerged from logs treated with *D. siricidicola* Kamona grown on *A. areolatum* Aussie and, of these, 23 were females. Only one of these females was infected with *D. siricidicola* Kamona and, it had 100% percent of the eggs sterilized. Surprisingly, females emerging from this treatment that were found to be infected with *D. siricidicola* NS (n=9) had an average of 17.6±10 of their eggs sterilized. (Table 5).

From all logs treated with *D. siricidicola* Kamona grown on *A. areolatum* BE, only one woodwasp emerged. It was female and was infected with *D. siricidicola* Kamona and had only 80% of eggs sterilized (Table 5).

Non-conclusive results from molecular characterization, were found for nematodes infecting two of the females emerged from logs treated with *D. siricidicola* Kamona grown in *A. areolatum* Aussie. This result indicates that hybridization of the two strains of nematodes happened, or there was a mixture of both stains in each female. However, those two wasps still had high percentages of their eggs sterilized (Table 5).

Table 5. 2013-2014 Percentage of sterilized eggs in infected females.

Treatment	Nematode strain	number of infected females	Average Number of eggs per female	Average % of sterilized eggs per female
D. siricidicola Kamona grown on *A. areolatum* Aussie	*D. siricidicola* Kamona	1	68	100
D. siricidicola Kamona grown on *A. areolatum* Aussie	*D. siricidicola* NS[2]	9	35.9±10.1	17.6±10
D. siricidicola Kamona grown on *A. areolatum* Aussie	inconclusive	2	39.5±21.5	75.5±14.5
D. siricidicola Kamona grown on *A. areolatum* BE	*D. siricidicola* Kamona	1	25	80

[2]NS= *Deladenus siricidicola* Non-sterilizing

2014-15

In 2015, for the 4 logs infested with *S. nigricornis* and inoculated with *D. siricidicola* grown on *A. areolatum* BE, forty wasps emerged, but none of them were infected with any nematodes. Many wasps (n = 215) were collected from *S. noctilio* control logs in 2015 and, over half of them (61.9%) were infected with *D. siricidicola* NS. Fairly high proportions of *S. noctilio* emerging from treated logs were infected with *D. siricidicola* NS: 83.3% of the wasps emerging from *S. noctilio* logs injected with *D. siricidicola* Kamona, and 42.4% of the *S. noctilio* emerging

from logs injected with *D. proximus* (Table 6). None of the wasps emerging in 2015 were infected with *D. siricidicola* Kamona or *D. proximus*.

Table 6. 2015 *Sirex* emergence and infections.

Treatment	**Total number of offspring**	**Number of males**	**% of males infected with *D. siricidicola* NS[3]**	**Number of females**	**% of females infected with *D. siricidicola* NS[3]**	**% of sterilized eggs**	**Total % of wasps infected with *D. siricidicola* NS[3]**
D. siricidicola Kamona on *A. areolatum* BE against *S. nigricornis*	40	33	0	7	0	0	0
S. noctilio control	215	138	61.6	9	50.6	0	61.9
D. siricidicola Kamona on *A. areolatum* BE against *S. noctilio*	7	6	66.6	1	100	0	83.3
D. proximus on *A. chailletii* against *S. noctilio*	66	61	41	5	60	0	42.4

[3]NS= *Deladenus siricidicola* Non-sterilizing

Discussion

This study was conducted with the objective of testing a biological control agent that is already successfully controlling *S. noctilio* in Oceania and South America (Slippers et al. 2012), against this invasive wasp in North America. This was a difficult study to conduct (approximately 1.5 years for each experiment) and results were disappointing. We frequently had few *S. noctilio* or *S. nigricornis* emerging from experimental wood, and nematode infection levels (of the strains inoculated into the wood) were low. A long-term study, similar to this one, was conducted by David Williams and Ann Hajek (in review), in which they used trees that were naturally infested with *S. noctilio*. These trees were cut down and injected with *D. siricidicola* Kamona in the fall and experimental trees were left on the ground in the woods until the following spring, when portions of trees were cut and brought to the lab, where they were kept in screen-covered barrels to collect wasps emerging from them the next summer. In the present study mated female wasps laid eggs into herbicided experimental trees, that were standing in the woods until the following April when they were inoculated with nematodes. We have not conducted experiments to figure out which of the differences between our study and the Williams and Hajek (in review) studies would be most important toward optimizing further studies of this type. However, we feel that the method used by Williams and Hajek (in review) of inoculating nematodes in autumn (instead of April) was more successful at infecting *S. noctilio* with *D. siricidicola* Kamona, although the infection rates obtained were also low.

An additional difference between our experiments and those of Williams and Hajek (in review) is that for the majority of our studies, *Sirex* were caged on standing stressed trees and trees were then standing all winter and early spring while for Williams and Hajek, infested trees

were felled for the winter. For our studies the only time that we felled trees in fall, we had much better success with oviposition by *S. nigricornis* so any further studies should consider this approach. An additional problem with our studies could be that the *Sirex* that we used had sometimes been in the refrigerator for some time. In nature *S. noctilio* females only live for up to 5 days (Ryan and Hurley 2012) so perhaps the females that we used were no longer as active as would be optimal.

The biological control program in Australia and New Zealand using *D. siricidicola* Kamona to control *S. noctilio* was very successful, as well as in South America, but similarly to North America, in South Africa tests using this nematode did not result in high infection rates or high rates of sterilization in females (Hurley et al. 2008). While we also did not have high nematode infection rates, a hypothesis is that part of the cause of poor performance in South Africa was due to low moisture levels in trees during winter. However, this weather pattern does not apply in northeastern North America.

In six years of bioassays, injecting nematodes in September and October in northeastern areas of the US, Williams and Hajek (in review), also found very low infection rates of *D. siricidicola* Kamona in *S. noctilio*: approximately 21%. Therefore, we hypothesize that this method of biological control would not work efficiently to reduce *S. noctilio* population in North America. We speculate that lack of success may occur due to competition between the non-sterilizing and Kamona strains of *D. siricidicola*, in which NS outcompetes Kamona. However, in addition *D. siricidicola* Kamona may not be able to infect the eggs of the strain of *S. noctilio* found in North America, which differs from the strain in Australia (Boissin et al., 2012; Bittner et al., in revision), and/or these two strains of nematodes may hybridize (Akhurst 1975), weakening the ability of Kamona strain to sterilize female wasps.

Bedding studied many different strains of *D. siricidicola* collected from various locations in Europe. He tested them against *S. noctilio* in Australia and New Zealand before the strain Kamona was chosen to be used as the biological control agent to use for control of *S. noctilio* in Australia (Slippers et al. 2012). Although data are not available, Bedding (2009) found great variability in infection and sterilization among the strains he evaluated, depending both on the nematode and *S. noctilio* strains. Future studies using other isolates of *D. siricidicola* could reveal a new strain that is capable of sterilizing the strain of *S. noctilio* found in North America.

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