Title: Hardwood content impacts the parasitoid community associated with Eastern spruce budworm (Lepidoptera: Tortricidae)

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Abstract

A major pest of eastern North American forests is spruce budworm, *Choristoneura fumiferana* Clemens (Lepidoptera: Tortricidae), which outbreaks every 30–40 years and causes large scale tree mortality. Researchers have established that hardwood content reduces the defoliation and mortality of balsam fir and spruces during spruce budworm outbreaks. One mechanism posited to explain these patterns is that hardwood content positively impacts the parasitoids of spruce budworm. Researchers have found that parasitism of spruce budworm by individual parasitoids is impacted by hardwood content. Yet, more research is needed to understand how hardwood content impacts the parasitoid community as a whole. In this study, we used DNA barcoding and stable isotope analysis of Malaise trap sampled parasitoids to examine how hardwood content influenced parasitoid community composition, structure, and trophic interactions. We found that although composition did not significantly differ along a hardwood content gradient, phylogenetic community structure did differ. Furthermore, the trophic relationships between several parasitoids and caterpillars on balsam fir or hardwood trees changed over time. Our study highlights the importance of hardwood trees for spruce budworm dynamics through influencing the parasitoid community.

Keywords

Choristoneura fumiferana, *Abies balsamea*, hardwood, parasitoids, trophic relationships, food webs, community, stable isotopes, forest management

Introduction

Every 30–40 years, Spruce budworm, *Choristoneura fumiferana* Clemens (Lepidoptera: Tortricidae), have massive outbreaks in eastern North American forests (Royama et al. 2017). These outbreaks last about 5-15 years, severely defoliating balsam fir and spruce trees and causing high growth loss and tree mortality (Hennigar et al. 2008). Spruce budworm outbreaks have been known to damage millions of hectares of Canadian forests per outbreak and have large impacts on the forestry sector (Chang et al. 2012). Consequently, finding methods to reduce the severity of spruce budworm outbreaks is important to maximize forestry economic activity while minimizing losses of balsam fir and species of spruce.

Hardwood trees have long been thought to reduce the severity of spruce budworm outbreaks. Since the 1920s, the importance of tree diversity to spruce budworm control has been periodically brought up, unfortunately with little empirical testing (Miller and Rusnock 1993). More recently, researchers have evaluated the effectiveness of hardwood content on the growth, defoliation, and mortality of balsam fir and spruces. Spruce budworm-caused growth reductions of balsam fir during the 1972–1992 outbreak was significantly related to hardwood content (Campbell et al. 2008). Balsam fir defoliation was lower in mixed forest stands containing hardwood trees compared to balsam fir dominated stands during spruce budworm outbreaks (Su et al. 1996; Zhang et al. 2018, 2020). In contrast MacKinnon and MacLean (2003) found no effect of surrounding forest type on spruce budworm defoliation of balsam fir. Instead, MacKinnon and MacLean (2003) found that spruce budworm defoliation of white spruce was reduced in stands surrounded by mixed wood forest. Balsam fir mortality due to spruce budworm defoliation was greater in extensive conifer stands than fir stands surrounded by deciduous forest or on islands in the middle of a lake (Cappuccino et al. 1998). Researchers have also tested the effect of hardwood content on spruce budworm abundances and densities. Quayle et al. (2003) found that relative basal area of non-host tree species had a significant negative effect on the abundance of spruce budworm and Eveleigh et al. (2007) found lower peak spruce budworm densities in heterogeneous plots compared to homogeneous plots. Overall, the evidence points to a complicated yet important impact of hardwood content on spruce budworm outbreaks.

One proposed mechanism behind hardwood content impacting spruce budworm outbreaks is the insects that parasitize and then kill spruce budworm caterpillars (parasitoids). Among the natural enemies of spruce budworm, parasitoids have arguably the strongest impact on spruce budworm mortality causing between 30-90% mortality depending on the surrounding forest composition and the point in the spruce budworm cycle (Cappuccino et al. 1998; Royama et al. 2017). Several researchers

have examined how hardwood content impacts the parasitism of spruce budworm by individual parasitoid species finding that depending on the parasitoid species there was either no effect of tree composition or an increase in parasitism with higher diversity of trees (Simmons et al. 1975; Kemp and Simmons 1978; Quayle et al. 2003). However, these studies have examined parasitoid species individually. An important further research direction is how hardwood content influences the parasitoid community as a whole because we know the parasitoid community responds strongly to spruce budworm density with increases in diversity cascading up parasitoid trophic levels (the bird feeder effect) (Eveleigh et al. 2007) and the parasitoid community responds largely indiscriminately to changing spruce budworm and other caterpillar abundances on balsam fir (Greyson-Gaito et al. 2021). Indeed in an initial survey, Eveleigh et al. (2007) did find increased diversity and abundance of primary parasitoids in plots with greater proportions of hardwood trees. Marrec et al. (2018) also found that variation in spruce budworm parasitoid community structure was mostly explained by surrounding forest structure. Eveleigh et al.'s (2007) and Marrec et al.'s (2018) research show that examining how hardwood content influences the parasitoid community as a whole is a useful endeavour.

Two methods lend themselves well to examining how hardwood content impacts the parasitoid community associated with spruce budworm. The first method is DNA barcoding where a region of an organism's DNA is sequenced and compared to the same region in other organisms (Ratnasingham and Hebert 2007). One advantage of using DNA barcoding compared to exclusively morphological identification is that cryptic species can be identified, increasing the resolution of the community (Smith et al. 2011). Another advantage is that researchers can compare the phylogenetic structure of different communities which can illuminate processes that structure the community including environmental filtering and competition (Kembel and Hubbell 2006; Ricklefs 2006).The second method is stable isotope analysis which aims to deduce diets and identify trophic relationships (Boecklen et al. 2011). This method involves measuring the ratio of heavy to light isotopes of different chemical elements (often carbon and nitrogen). In fact, the ratio of heavy to light carbon isotopes in a consumer will be similar to that of the consumer's diet and the ratio of heavy to light nitrogen isotopes increases at each level of a trophic food chain. From this information, a food web of the different organisms measured can be elucidated. Importantly for this study, the ratio of heavy to light carbon isotopes differs between balsam fir and hardwood trees (Risk et al. 2009). Thus, we can use these techniques to examine how hardwood content influences the phylogenetic structure of the parasitoids and how parasitoids utilize caterpillars on balsam fir versus hardwoods.

In this study, we examined how the parasitoid community associated with spruce budworm differed along a hardwood gradient and how trophic relationships changed over time. First, using DNA barcoding of Malaise caught parasitoids in plots where spruce budworm were implanted, we tested whether the parasitoid community composition and phylogenetic community structure differed along a hardwood gradient. Second, using stable isotope analysis of Malaise caught parasitoids sampled immediately prior to and after a local spruce budworm peak, we identified how trophic relationships between different parasitoids and spruce budworm on balsam fir or other caterpillar species on hardwood trees changed within and between years. We found that hardwood content did impact the parasitoid community structure. We also found that the utilization of caterpillars on balsam fir or hardwood trees changed, depending on the type of parasitoid, within and between years.

Methods

Study sites

All sampling was done in the Acadia Research Forest (ARF) near Fredericton (66º25'W, 46º00'N). The ARF is a 9,000 ha (22,230 ac) experimental forest with a mixture of softwood, hardwood, and mixed wood stands (Figure 1). Spruce (*Picea* spp.) and balsam fir (*Abies balsamea* (L.) Mill.) are the most abundant trees (Swift et al. 2006). All plots sampled in this study were outside areas of aerial application of insecticides for spruce budworm control.

Parasitoid community differences along a hardwood gradient

Sampling

In 2014, nine 150 metre by 120 metre plots were selected, where three were balsam fir dominated (70% balsam fir), three were hardwood tree dominated (75% hardwood), and three had an even mixture of balsam fir and hardwood trees (40-60% balsam fir) (Figure 1). The nine plots were chosen using a forest cover map provided by the ARF, lidar maps, and ground truthing. In 2016 five balsam fir trees, at least 20 metres apart and with healthy crowns, were chosen within each plot in the ARF (45 trees total). In April of 2016, 2,000 2nd instar spruce budworm individuals were placed onto each of the 45 trees. Spruce budworm individuals were reared by Insect Production Services (IPS) at the Great Lakes Forestry Centre in Sault St Marie, Ontario on a bed of gauze, which were cut up into squares of about 250 caterpillars (Roe et al. 2018). We placed a total of eight squares on each of the 45 trees, with each square being pinned to the underside of single branch in the mid-crown layer that had new growth. Then, to examine the parasitoid community associated with spruce budworm between these three types of stands, on May 19th 2016 we placed a Malaise trap in every plot chosen above close to

one of the trees where spruce budworm individuals were seeded. The Malaise traps were taken down on August 11th 2016. The flying insects from the Malaise traps were sampled once a week during May and June, and once a month during July and August. We separated out individuals belonging to insect families that we knew contained species that attack spruce budworm. These families included Tachinidae, Sarcophagidae, Braconidae, and Ichneumonidae. We stored the collected parasitoids in 70% ethanol and in a refrigerator at 4°C, until they were barcoded.

DNA Barcoding

To quantify parasitoid diversity and phylogenetic structure, we used DNA barcoding. Tissue samples were taken using 1-6 legs and placed in 30 µL of 95% ethanol and stored at -20°C. Mitochondrial DNA from the cytochrome *c* oxidase I (COI) region (the standard animal DNA barcode locus) was amplified and sequenced at the Biodiversity Institute of Ontario (BIO; University of Guelph, Ontario). High resolution photographs were taken of wet specimens under a dissecting microscope using Leica Application Software V4.9. Sequences and photographs were uploaded to the Barcode of Life Data System (Ratnasingham and Hebert 2007). For diversity measurements, we used Barcode Index Numbers (BINs), a DNA-based delineation of species based on patterns of intra and interspecies variations outlined by Ratnasingham & Hebert (2013). We constructed a single-representative maximum likelihood tree in MEGA6 based on estimation of the best substitution models in MEGA6 (Nei and Kumar 2000; Tamura et al. 2013).

Statistical Analyses

Parasitoid community composition

To test whether the parasitoid community composition differed along the hardwood gradient, we ran an nMDS analysis using the Bray-Curtis dissimilarity measure (function metaMDS, R package vegan, version 2.5.2, (Oksanen et al. 2018)). We ran a perMANOVA between the balsam fir dominated plots, the mixed wood plots, and the hardwood dominated plots (function adonis, R package vegan version 2.5-6). In this perMANOVA, we used the Bray-Curtis dissimilarity measure

Phylogenetic community structure

To examine how hardwood content affected the phylogenetic community structure of spruce budworm parasitoids, we calculated the mean nearest taxon distance (MNTD) using maximum likelihood trees between the three forest types for the Malaise caught parasitoids. Maximum likelihood trees used a general time reversible model with discrete gamma distribution under the assumption that sites were evolutionarily invariable (Nei and Kumar 2000; Tamura et al. 2013). The standard effect size of the

MNTD was then calculated and phylogenetic clustering and dispersion assessed by performing 999 random permutations of hardwood content associations to simulate a distribution of MNTD for each community. The significance of the observed MNTD values for each community was examined with a two-tailed test of significance ($p = 0.05$) (function ses.mntd, R package Picante, version 1.7, (Kembel et al. 2010)).

As a further comparison of phylogenetic clustering in plots differing in hardwood content, we calculated the mean nearest taxon distance (MNTD) and assessed phylogenetic clustering and dispersion (function ses.mntd, R package Picante, version 1.7, (Kembel et al. 2010)) of reared parasitoids collected from the three plots in Eveleigh et al. (2007). These parasitoids were reared from both spruce budworm and other caterpillars found on the study's balsam fir trees. Eveleigh et al. (2007) compared the richness of reared parasitoids between three plots with differing tree compositions (tree basal area, Plot 1: balsam fir 98%, Spruce 1%, Hardwood 1%. Plot 2: balsam fir 77%, spruce 8%, hardwood 14%. Plot 3: balsam fir 50%, spruce 36%, hardwood 14%). Note, these three plots are different from the plots in Figure 1. A subset of these parasitoid species were preserved at -20ºC then DNA barcoded to explore how genetic estimates of isolation and species identification changed the estimates of food web connectance (connectance was reduced as the number of nodes increased) (Smith et al. 2011). However, Smith et al. (2011) did not report estimates of phylogenetic community structure for the parasitoids of these three plots, and so in this study we add an examination of phylogenetic community structure of parasitoids sampled in the 1980s and compare with phylogenetic clustering of parasitoids sampled along a hardwood gradient in 2016. For further details of the three plots and all sampling and rearing procedures, see Lucarotti et al. (2004), Eveleigh et al. (2007) (SI Materials and Methods) and Royama et al. (2017).

Parasitoid community balsam fir/hardwood trophic relationships

Sampling

All parasitoid sampling was performed in a single balsam fir dominated plot in ARF for the years of 1982, 1984, 1986, and 1987 (in this plot, spruce budworm peaked in 1985). This plot was 98% *Abies balsamea*, 1% *Picea rubens* Sarg., and 1% *Acer rubrum* L. by basal area (Lethiecq and Regniere 1988). Parasitoids were collected using modified 1 m^3 Malaise traps (Nyrop and Simmons 1982). A Malaise trap was placed with the open sides perpendicular to the tree trunk at the top, middle, and lower crown levels of three balsam fir trees separated by approximately 100 metres (i.e. 3 traps at each crown level, 9 traps in total). The Malaise traps were placed in the same trees every year beginning in May and ending in September. Flying insects were collected daily,

immediately stored in 70% ethanol, and frozen at -7°C until preparation for stable isotope analysis in 2017 (except insects collected in 1982 which were stored without ethanol but still in the freezer).

In 2017, as an initial attempt to understand how parasitoids with different life cycles utilize caterpillars on balsam fir and hardwood trees, we separated the 1980s Malaise caught parasitoids into three groups (see Table S1): Group 1, univoltine parasitoid species that attack one type of caterpillar within a year and do not require an alternate caterpillar (to spruce budworm) in which to overwinter (Elliott et al. 1987; O'Hara 2005); Group 2, parasitoid species that attack spruce budworm and likely other caterpillars on hardwoods within a year; and Group 3, multivoltine parasitoid species that require an alternate caterpillar (to spruce budworm) in which to overwinter (Thireau and Régnière 1995; O'Hara 2005). These three groups were then further split into three periods to capture the phenology of the parasitoid emergences from spruce budworm and other caterpillars: May/June, July, and August/September. When there were fewer than 50 total individuals in a group and sampling period, all individuals were used for stable isotope analysis. When there were more than 50 total individuals in a group and sampling period, we randomly selected 50 individuals and ensured the proportions of selected individuals of each species matched the proportions of total number of individuals for each species (within the group and sampling period). We removed legs and wings from all individuals, keeping the mass of legs and wings approximately constant between individuals and species. Legs and wings were combined for each group and sampling period and were dried at 60°C for at least 48 hours. We used legs and wings because many parasitoids as adults consume non-host nutrient sources, and legs and wings have a slower turnover rate compared to other body parts (Gratton and Forbes 2006; Benelli et al. 2017)

In stable isotope analysis, carbon and nitrogen stable isotopes are measured in samples from basal resources plus any intermediate consumers of each resource compartment (food chain). From these measurements, called baselines, researchers can deduce the trophic relationships of the focal organisms. Our baselines consisted of balsam fir and hardwood foliage, and caterpillars from these sampled foliage. In 2017 beginning on May 30th and ending on June 27th, once a week we sampled one metre long, mid-canopy branch from 5 balsam fir trees in each of the nine plots studied in **Parasitoid community along a hardwood gradient** (one branch per tree, five trees per plot, 45 branches per week). Each week, we also sampled one metre long branch from multiple hardwood tree species in each plot. These multiple hardwood species were the most abundant in each plot as found by the original plot ground truthing. On the 17th July and on the 4th August, we randomly sampled a single balsam fir branch

from each plot, and we sampled branches from the same hardwood species as we sampled in June (a branch per species in each plot). We sampled foliage without any noticeable herbivory damage from all branches. This foliage was rinsed with distilled water and dried at 60°C for at least 48 hours. We ground the foliage and ensured that the combination of different hardwood species in each plot's ground sample matched the proportions of hardwood trees found in each plot. This was repeated for June, July and August. From the balsam fir branches and the hardwood branches, we collected all caterpillar individuals and separated them into caterpillars from balsam fir or hardwoods and by plot and by sampling period. The caterpillar samples were dried at 60°C for at least 48 hours. All parasitoid, caterpillar and foliage samples were analyzed for carbon and nitrogen isotope ratios at the University of Windsor GLIER (Windsor, ON, Canada) laboratories.

Statistical Analyses

Normal practice when using stable isotopes is to use mixing models, where both δ13C and δ15N are included to establish the trophic levels and percentage of diet from multiple resource pathways (Phillips et al. 2014). However, the δ13C of the parasitoid samples were enriched by 16% compared to the foliage and caterpillar baselines probably because the parasitoid samples were stored in ethanol and frozen for about 30 years whereas the foliage and caterpillars were sampled in 2017 (Jesus et al. 2015). Because mixing models are unable to account for this enrichment, we were not able to use mixing model analyses with both δ13C and δ15N. Instead, we used δ13C only by comparing δ13C between years, sampling periods, and groups because we knew that there were consistent differences in δ13C between hardwood and softwoods which were transferred to the caterpillars (Balsam fir and hardwood foliage Welch t-test: $t = 2.813$, $df = 40.219$, $P = 0.00756$. Balsam fir caterpillars and hardwood caterpillars Welch t-test: $t = 3.161$, $df = 39.161$, $P = 0.00303$). Note, from the three sampling periods above (May/June, July, August/September), we simplified the periods into two sampling periods, May/June and July/August/September, by averaging the δ13C values of the July and August/September periods. We ran a generalized least squares regression to test the effects of year, sampling period (May/June or July/August/September), parasitoid group, and all interactions on the δ13C of sampled parasitoid legs and wings (function gls, R package nlme, version 3.1-137, (Pinheiro et al. 2018)). We added a varIdent variance structure to account for the different variation in the residuals between the sampling periods. We fitted the full model using maximum likelihood estimation and then used backwards selection with log likelihood ratio tests to select the final fixed effects. We refitted the final model using restricted maximum likelihood estimation to give unbiased maximum likelihood predictors (Zuur et al. 2009) .

Figure 1 Map of the Acadia Research Forest of plots used in the 2016 Malaise trapping of parasitoids. Plots 1,2,3 are balsam fir dominated, plots 4,5,6 are mixed wood plots, and plots 7,8,9 are hardwood dominated. Hardwood areas have greater than or equal to 70% of hardwood trees by species %. Softwood areas have greater than or equal to 70% of softwood trees by species %. Hardwood – softwood areas have greater percentage of hardwood trees than softwood trees but both make up less than 70% individually. Softwood – hardwood areas have greater percentage of softwood trees than hardwood trees but both make up less than 70% individually.

Results

Parasitoid community differences along a hardwood gradient

Parasitoid community composition

Although qualitatively, hardwood dominated plots do appear to harbour different parasitoid communities compared to balsam fir dominated plots and mixed wood plots, there was no significant difference in parasitoid community composition between balsam fir dominated plots, mixed wood plots, and hardwood dominated plots ($F = 1.170$, $P = 0.207$, 999 permutations, perMANOVA, Figure 2).

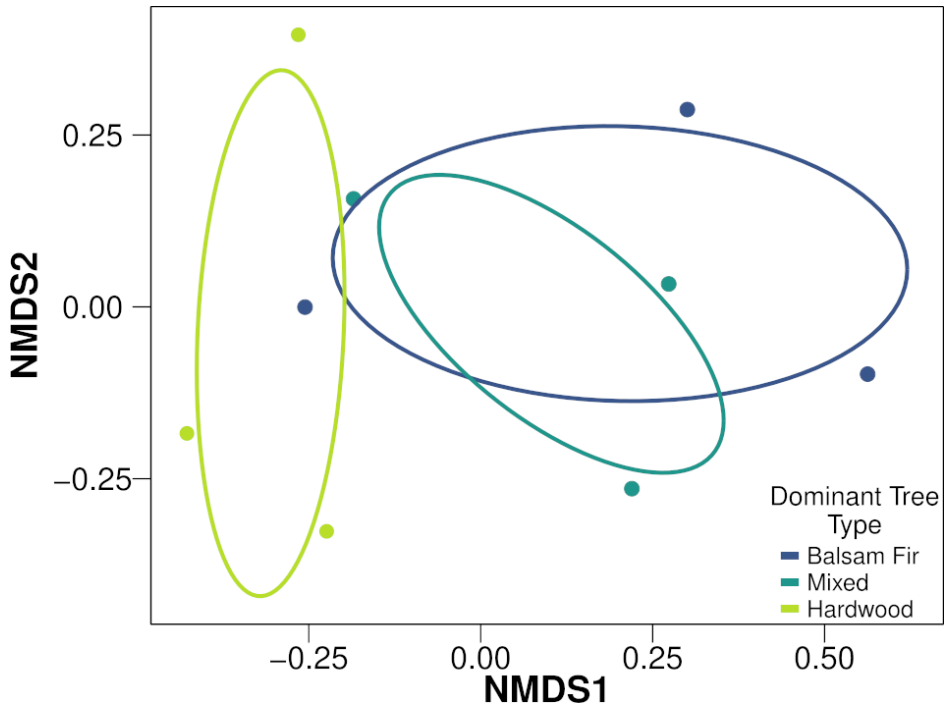

Figure 2 nMDS of parasitoid community caught in Malaise traps in 2016 in balsam fir dominated, mixed wood, and hardwood dominated plots. Each point is a single plot. Each ellipse is a covariance ellipse for the plot type. 20 iterations. Final stress of 0.191 Instability for preceding 10 iterations was 0.038.

Phylogenetic clustering

Plots dominated by balsam fir were consistently phylogenetically clustered. Phylogenetic clustering was found in the balsam fir dominated plots with Malaise caught parasitoids from 2016 (Balsam Fir: MNTD z =-2.502, P = 0.009. Figure 3a). Neither phylogenetic clustering nor dispersion were found in the mixed forest plots and the hardwood dominated plots with Malaise caught parasitoids from 2016 (Mixed: MNTD z = 1.135, P = 0.877. Hardwood: MNTD z = -1.368, P = 0.087. Figure 3a). Phylogenetic clustering was tentatively found in Plot 1 from the 1980s (MNTD z = -1.601, p = 0.055, Figure 3b). Neither phylogenetic clustering nor dispersion were found in the two other plots from the 1980s (Plot 2: MNTD z = -1.497, p = 0.075. Plot 3: MNTD z = -0.518, p = 0.303. Figure 3b).

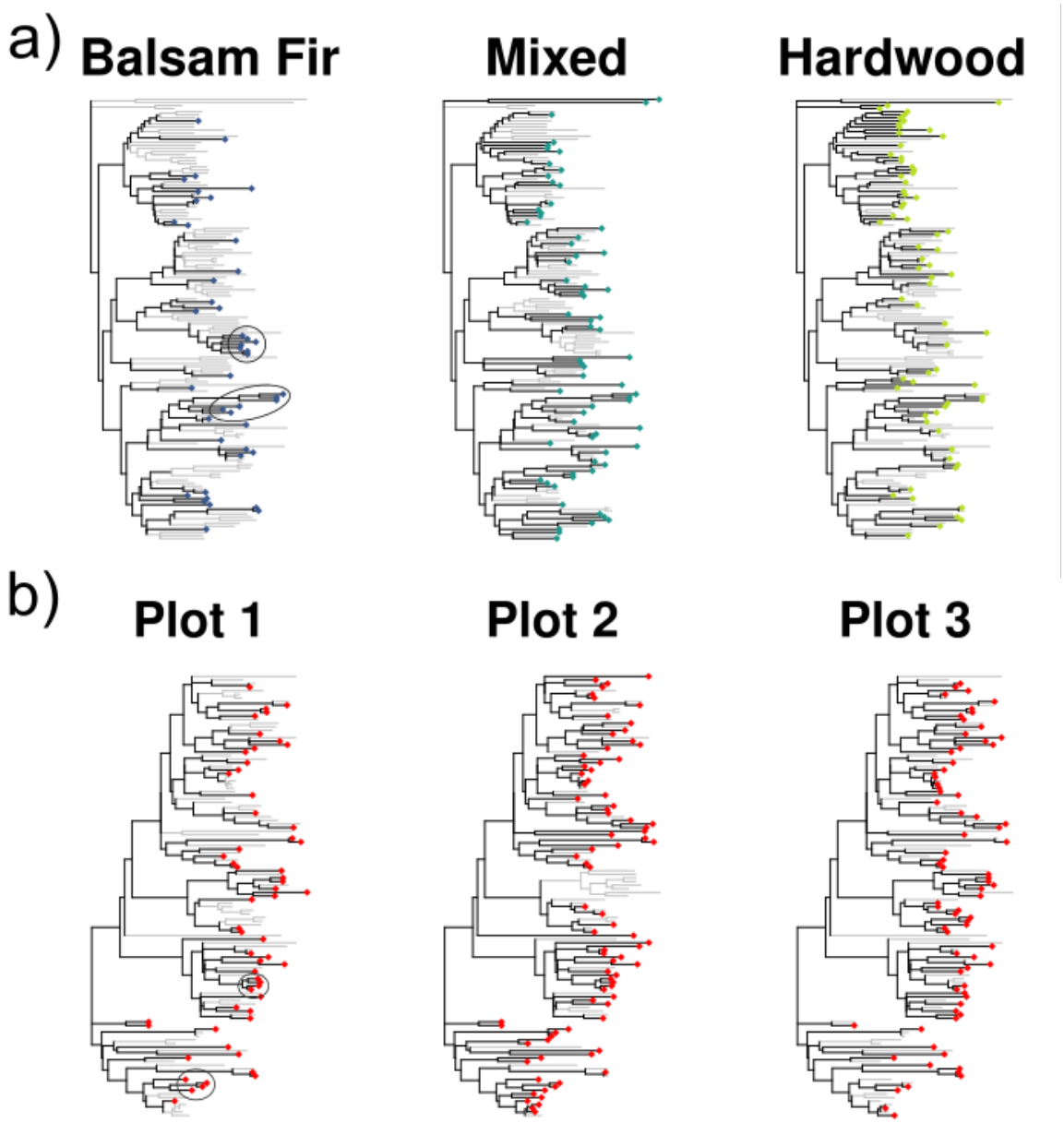

Figure 3: a) Phylogenies of Malaise caught parasitoid communities with presence denoted by diamonds and black branches in three balsam fir dominated plots, three mixed wood plots, and three hardwood dominated plots in Acadia Research Forest in 2016. b) Phylogenies of reared parasitoid communities with presence denoted by diamonds for Plots 1, 2 (Acadia Research Forest), and 3 (Saint-Quentin) for all years sampled (1983-1995). Tree basal area, Plot 1: balsam fir 98%, Spruce 1%, Hardwood 1%. Plot 2: balsam fir 77%, spruce 8%, hardwood 14%. Plot 3: balsam fir 50%, spruce 36%, hardwood 14%. Examples of clusters of species are indicated with ellipses. Absence of parasitoid taxa are denoted by grey branches.

Parasitoid community balsam fir/hardwood trophic relationships

The final model explaining δ13C included year, group, sampling period (May/June or July/August/September), and the interactions of year with group (year: group interaction, L = 13.230, P = 0.0013, df = 1, log likelihood ratio test, Figure 4) and group with sampling period (group: sampling period interaction, L = 28.900, P < 0.0001, df = 1, log likelihood ratio test, Figure 4, see Table 1 for ANOVA output of model). Group one parasitoids became slightly more negative by approximately 0.5% each year, and group one parasitoids caught when spruce budworm were absent had more negative δ13C values by 2.4% compared to group one parasitoids caught when in May/June. δ13C values for group two parasitoids became less negative overtime by approximately 1.6% each year. Group three parasitoids showed a difference of 12.2% in δ13C between May/June and July/August/September. In May/June, group three parasitoids had more negative δ13C values. In July/August/September, group three parasitoids had less negative δ13C values. In comparison to the difference in δ13C between May/June and July/August/September, δ13C for group three parasitoids changed little with no noticeable trend between years.

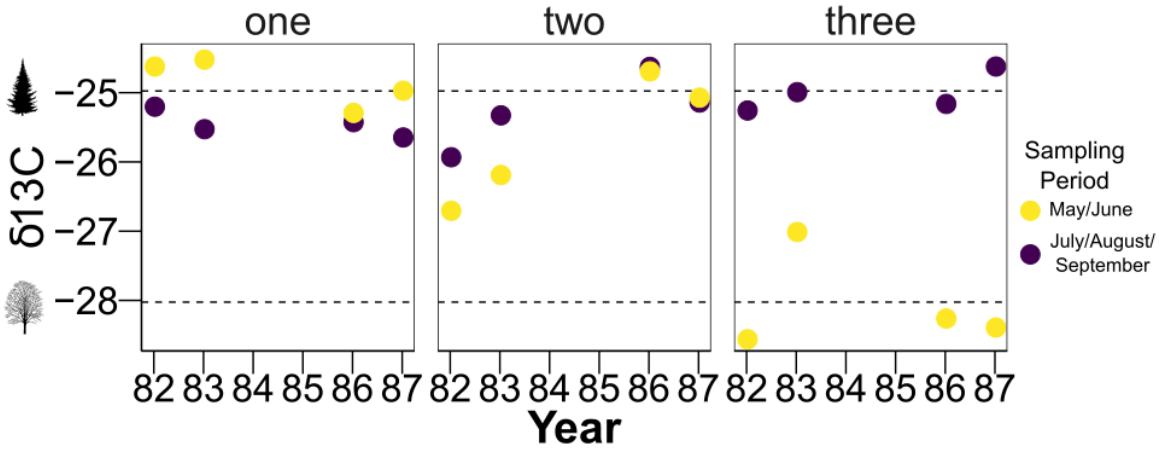

Figure 4 δ13C for three groups of parasitoid species: group one parasitoids are univoltine species that attack one type of caterpillar within a year (left plot); group two parasitoids attack spruce budworm and likely other caterpillars on hardwoods within a year (centre plot); and group three parasitoids are multivoltine species that require an alternate caterpillar in which to overwinter (right plot). Spruce budworm populations peaked in 1985. δ13C was measured on parasitoids captured in the sampling periods of May/June and July/August/September. Dashed lines depict the average δ13C value for the group three parasitoids in May/June and July/August/September (used as estimates for the balsam fir and hardwood foliage δ13C values). See Figures S1, S2, S3 for time series of the proportions of the parasitoids in each group. Balsam fir and red maple images shown on the y-axis are publicly available from Natural Resources Canada, Canadian Forest Service.

Table 1 ANOVA output for model with δ13C from 1980s Malaise caught budworm parasitoids as the response variable and Year, Sampling Period, Parasitoid Group, Year:Parasitoid Group, Group:Sampling Period as explanatory variables.

Predictor variables	**df**	**F value**	**P value**
Intercept	1	115952.08	**<0.0001**
Year	1	3.15	0.0964
Sampling Period	1	28.14	**0.0001**
Parasitoid Group	2	2.50	0.1159
Year:Parasitoid Group	2	5.50	**0.0162**
Group:Sampling Period	2	36.67	**<0.001**

Discussion

Our study has shown that the spruce budworm-associated parasitoid community was impacted by hardwood trees. Using Malaise caught parasitoids, we found that although the parasitoid community composition was not significantly different along the hardwood gradient, the phylogenetic community structure of the parasitoid community was consistently clustered in balsam fir dominated plots. From comparing the stable isotopes of parasitoids during a spruce budworm outbreak, we found that the trophic relationships between several parasitoids and caterpillars on balsam fir or hardwood trees changed over time. Taken together, our study highlights the need to include hardwood trees when examining spruce budworm dynamics and the need to carefully consider the scale of hardwood tree placement when attempting to reduce spruce budworm outbreaks.

The hardwood content of the stands did appear to impact the spruce budworm-associated parasitoid community. Although using our three plots per stand type did not identify any statistical difference in parasitoid community composition differences along the hardwood gradient, qualitatively hardwood dominated stands did appear different from mixed and balsam fir dominated stands. We suspect that increasing the replication would find a statistical difference, and we encourage future researchers to continue this examination of parasitoid community composition along the hardwood gradient. In terms of how hardwood content influenced phylogenetic structure of the parasitoid community, we found that the balsam fir dominated plots exhibited phylogenetic clustering in 2016 and in the 1980s. When examining phylogenetic structure, researchers test for either phylogenetic clustering, where communities are made up of closely related species, or overdispersion, where communities are made up of distantly related species (Webb et

al. 2002). Identifying clustering or overdispersion can illuminate processes including filtering and competition that establish these communities. We know that closely related parasitoid species are more likely to share host species or search within the same plant species than distantly related parasitoid species (Ives and Godfray 2006), thus in our study, the observed phylogenetic clustering suggests that environmental filtering is more important than competition (Webb et al. 2002). We speculate that the environmental filtering is likely due to the differences in caterpillar composition maintained by balsam fir dominated stands compared to stands with greater hardwood content. Potentially, balsam fir dominated plots host a subset of caterpillar species thus filtering closely related parasitoid species. Similarly, Marrec et al. (2018) found environmental filtering to be important in shaping spruce budworm parasitoid communities. One caveat to our environmental filtering pattern is that our sampling does not differentiate between primary parasitoids and hyperparasitoids. Because hyperparasitoids may be key in driving spruce budworm outbreaks (Nenzén et al. 2018), examining the differential impacts of hardwood content on primary parasitoids and hyperparasitoids is critical. Overall, hardwood content impacts the spruce budworm-associated parasitoid community likely through influencing the caterpillar communities. Further research should extensively sample caterpillar communities on all tree types along a hardwood gradient as well as sample and differentiate between primary parasitoids and hyperparasitoids.

From our study, our three groups of parasitoids differed in how they utilized caterpillars on balsam fir and hardwood trees over time. The parasitoids that must alternate between attacking spruce budworm on softwoods and caterpillars usually on hardwoods within a single year (group three) provide us with the clearest comparison of trophic relationships between balsam fir and hardwood. The δ13C of group three parasitoids sampled in May/June was more negative than the δ13C of group three parasitoids sampled in July/August/September. Our sampled hardwood foliage was similarly more negative in δ13C compared to our sampled balsam fir foliage (hardwood foliage = -30.222 δ13C, balsam fir foliage = -29.521 δ13C). This correspondence of the differences between group three in the two sampling periods and the differences in balsam fir and hardwood δ13C matches what we know of the life history of group three parasitoids because, in May/June, group three parasitoids emerge from other caterpillar species often on hardwood trees to attack spruce budworm, and in July/August/September, group three parasitoids emerge from spruce budworm to attack other caterpillars. Therefore, we suggest any comparable changes in δ13C for the other groups should be due to the parasitoids changing their attack rates on spruce budworm on balsam fir and other caterpillar species on hardwoods.

The parasitoids that attack only spruce budworm within a year (group one) seemingly did not change their relative utilization of spruce budworm and other caterpillars on hardwoods within a year nor between years. Group one parasitoids not changing relative utilization within a year is unsurprising because these parasitoids are univoltine. Group one parasitoids not changing utilization between years as spruce budworm densities change is consistent with other studies that concluded that these parasitoids attack spruce budworm more than other caterpillar species (O'Hara 2005; Cossentine et al. 2007). Furthermore, the populations of group one parasitoids are supported by other caterpillar species that feed on balsam fir as suggested by *Apanteles fumiferana* Vier. (Hymenoptera: Braconidae) and *Glypta fumiferana* Vier. (Hymenoptera: Ichneumonidae) attacking other caterpillar species on balsam fir (Greyson-Gaito et al. 2021). In contrast to group one parasitoids, the parasitoids that likely attack both spruce budworm and other caterpillars within a year (group two) exhibited greater change in δ13C between years, from more to less negative, suggesting that these parasitoids likely attacked other caterpillars on hardwoods when spruce budworm had lower densities and then attacked spruce budworm on balsam fir when spruce budworm had higher densities. With these findings in mind, we hope that future researchers measure the stable isotopes of individual parasitoid species again within a year and between years to increase the resolution of how parasitoids utilize spruce budworm on softwoods and other caterpillars on hardwoods. We also recommend that researchers include understory plants as stable isotope baselines because parasitoids gain nutrients from non-host sources including nectar from understory plants (Benelli et al. 2017).

Interestingly, the pattern that groups two and three parasitoids exhibited could be classed as coupling, an important stabilizing ecological mechanism (McCann et al. 2005). Coupling usually occurs when a generalist consumer attacks prey from two or more spatially separate subgroups of a larger food web (resource compartments) (McCann et al. 2005). In the spruce budworm food web, the parasitoids collectively may be attacking other caterpillars on hardwoods when spruce budworm are rare and attacking spruce budworm on balsam fir when spruce budworm are plentiful, thus coupling the softwood and hardwood resource compartments. The lack of information on hardwood feeding alternative hosts for budworm parasitoids limits our future ability to assess this coupling pattern as well as restricts our fundamental understanding of the spruce budworm system. While this lack of information exists, we argue that further use of stable isotope analysis in spruce budworm research is beneficial. We also recommend the use of fatty acid analysis because the fatty acid compositions differ between softwoods and hardwoods more than δ13C (Mueller et al. 2012). Another promising method for evaluating this softwood/hardwood coupling pattern is by using the qPCR approach to determine whether and by what a spruce budworm larvae has

been parasitized (Nisole et al. 2020). So far this method is limited to 20 common natural enemies of spruce budworm as a compromise between time/costs and broad applicability. To examine the coupling pattern, we suggest that DNA libraries of spruce budworm parasitoids be expanded to include representation from hardwood forest parasitoid communities. Overall, comprehensive sampling of parasitoids and caterpillars on softwoods and hardwoods throughout the spruce budworm cycle is required to evaluate the contribution of hardwoods to parasitoid population maintenance and softwood/hardwood coupling. Stable isotope analysis, fatty acid analysis and qPCR would all be highly complementary techniques.

A full reckoning of how hardwood content influences the spruce budworm-associated parasitoid community requires careful consideration of both spatial and temporal scale. Hosts and parasitoids disperse, aggregate, and are influenced by landscape structure at different spatial scales often larger than a few hundred metres (Cronin and Reeve 2005). Indeed, Legault and James (2018) found that the parasitism rate of spruce budworm by *Apanteles fumiferena* was positively correlated with tree diversity at 3km, and the parasitism rate of spruce budworm by *Glypta fumiferana* was negatively correlated with non-host tree density at 15km. Legault and James (2018) suggest that the different dispersal abilities of parasitoids impact how parasitoids respond to forest diversity at the landscape scale; *A. fumiferana* would be affected by tree composition at smaller scales than G. fumiferana because *A. fumiferana* is smaller than *G. fumiferana* (~3.5mm compared to ~8.0mm in length) and likely disperses smaller distances than *G. fumiferana*. Interestingly, Zhang et al. (2020) did not find any difference in parasitism rate of spruce budworm across a hardwood gradient, but Zhang et al.'s (2020) plots were 500m^2, much smaller than the determining scale found in Legault and James (2018). Our study similarly examined a relatively small scale (plots were 150m by 120m and the ARF is 90km^2, Figure 1) compared to the large distribution of spruce budworm outbreaks. Yet, phylogenetic clustering in balsam fir dominated plots was consistently found even with small plot scales. A complication to our phylogenetic structure findings is that communities were sampled while overall spruce budworm densities were relatively low even with spruce budworm implanting and were combined for all seasons within a year. In contrast, Marrec et al. (2018) compared spruce budworm parasitoid communities between seasons within a year while spruce budworm were at outbreak densities. Marrec et al. (2018) found that dispersal limitation was likely most important in spruce budworm's early larvae and pupae stages, and environmental filtering was likely most important in the late larvae stage. We agree with Marrec et al.'s (2018) assessment that the dominance of different processes structuring the parasitoid community will change over time as spruce budworm develop and as spruce budworm densities fluctuate, an assessment similar to the birdfeeder pattern outlined in Eveleigh

et al. (2007). Clearly, scale is important in the spruce budworm system and therefore to better understand the parasitoid community's response to hardwood content, future research will require greater replication over a variety of spatial and temporal scales.

Hardwood trees in forest stands have long been thought to be important to reducing the severity of spruce budworm outbreaks. Although several studies have examined how hardwood content impacts spruce budworm directly, relatively few studies have examined how hardwood impacts the parasitoids of spruce budworm. In this study, we used DNA barcoding and stable isotope analysis of Malaise trap sampled parasitoids to examine how hardwood content impacts the spruce budworm-associated parasitoid community. We found that hardwood content influenced the phylogenetic structure of parasitoid communities and several parasitoids could be coupling the hardwood and softwood resource compartments. Our results combined with other researcher's results indicate that having hardwoods on the landscape would be beneficial. However, a major obstruction to using hardwood trees to manage spruce budworm outbreaks is that we don't know how much hardwood content would be required nor the spatial arrangement of hardwood trees within host stands or in the surrounding landscape. Further research should examine how the scale of hardwood content influences spruce budworm dynamics in general and the spruce budworm-associated parasitoid communities specifically. With this information, forest managers would have better information on the quantities and placement of hardwood trees. Taken together, we have provided further evidence that hardwood trees are important in spruce budworm dynamics but understanding how scale mediates this hardwood-spruce budworm relationship is critical to effectively reduce the severity of spruce budworm outbreaks.

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Author contributions

ESE designed the initial studies. ESE, WM, GF, RL, CJGG, and SJD did the field and laboratory work. CJGG did the statistical analyses with assistance from ESE, MAS, SJD, and KSM. CJGG wrote the first draft and all authors contributed to editing the manuscript.

Data accessibility

All sequences and photographs are publically available at http://dx.doi.org/10.5883/DS-ASNBPAR. All data and code (v2.0) to reproduce the reported results are publicly available on GitHub (https://github.com/cgreysongaito/SpruceBudworm_Parasitoids_Hardwood) and have been archived on Zenodo (https://doi.org/10.5281/zenodo.4432484).

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Supporting Information

For "Hardwood content impacts parasitoid community associated with Eastern spruce budworm (Lepidoptera: Tortricidae)"

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Table 1: List of Malaise caught parasitoid species in each group. This list refers to the species caught in the 1980s. Previous names of species are provided in brackets if applicable. Group 1 are univoltine parasitoid species that attack one type of caterpillar within a year and do not require an alternate caterpillar (to spruce budworm) in which to overwinter. Group 2 are parasitoid species that attack spruce budworm and likely other caterpillars on hardwoods within a year. Group 3 are multivoltine parasitoid species that require an alternate caterpillar in which to overwinter

Group	Species	Spruce Budworm Stage Attacked
1	*Apantales fumiferanae* Vier. (Hymenoptera: Braconidae)	Early instar larvae
1	*Glypta fumiferanae* Vier.(Hymenoptera: Ichneumonidae)	Early instar larvae
1	*Lypha fumipennis* (*Lypha setifacies*) Brooks (Diptera: Tachinidae)	Late instar larvae
1	*Smidtia fumiferanae (Winthemia fumiferanae)* Tothill (Diptera: Tachinidae)	Late instar larvae
2	*Actia interrupta* Curran.(Diptera: Tachinidae)	Late instar larvae
2	*Agria affinis* (*Psuedosarcophaga affinis*) Fallén (Diptera: Sarcophagidae)	Late instar larvae
2	*Compsilura concinnata* Meigen (Diptera: Tachinidae)	Larvae
2	*Eumea caesar* Aldrich (Diptera: Tachinidae)	Late instar larvae
2	*Hemisturmia parva* (*Hemistermia tortricis*) Bigot (Diptera: Tachinidae)	Late instar larvae
2	*Nilea erecta* (*Pseudoperichaeta erecta*) Coquillett (Diptera: Tachinidae)	Late instar larvae
2	*Sarcophaga aldrichi* Parker (Diptera: Sarcophagidae)	Pupae
2	*Tachinomyia nigricans* Webber (Diptera: Tachinidae)	Unknown
3	*Ceromasia auricaudata (Ceromasia aurifrons)* Townsend (Diptera: Tachinidae)	Late instar larvae
3	Madremyia saundersii Williston (Diptera: Tachinidae)	Late instar larvae
3	*Meteorus trachynotus Vier* (Hymenoptera: Braconidae)	Late instar larvae
3	*Nemorilla psyte* Walker (Diptera: Tachinidae)	Late instar larvae
3	*Phryxe pecosensis* Townsend (Diptera: Tachinidae)	Late instar larvae

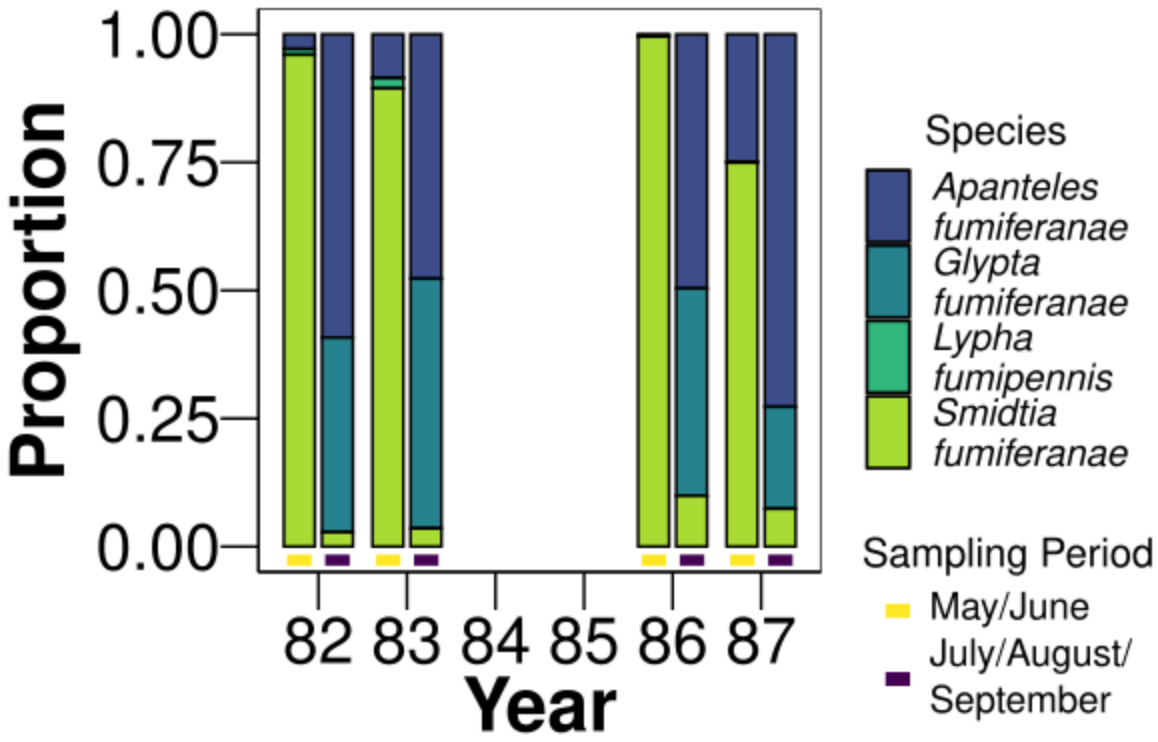

Figure S1 Proportion of each parasitoid species within group one that were Malaise caught in May/June or July/August/September for the years 1982, 1983, 1986, and 1987. To access the data behind this figure, please contact Eldon Eveleigh (eldon.eveleigh@canada.ca).

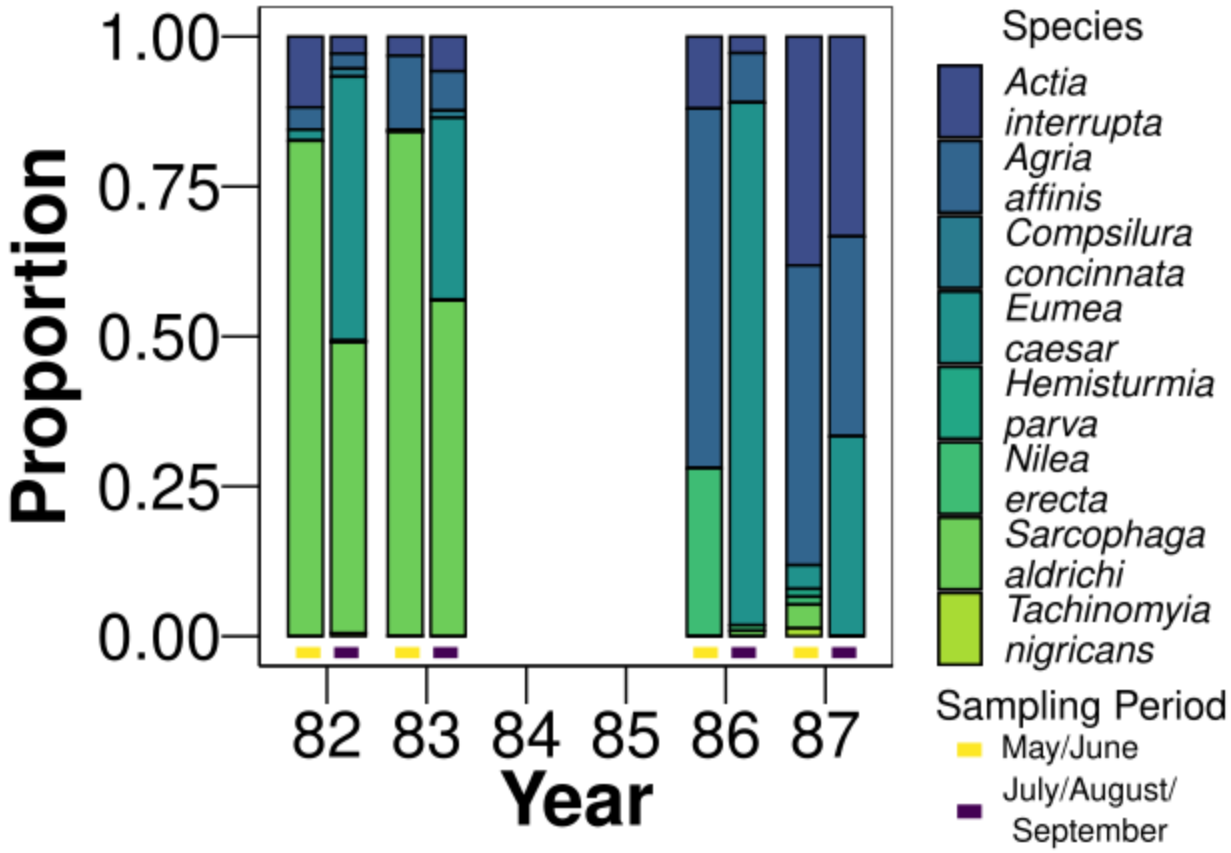

Figure S2 Proportion of each parasitoid species within group two that were Malaise caught in May/June or July/August/September for the years 1982, 1983, 1986, and 1987. To access the data behind this figure, please contact Eldon Eveleigh (eldon.eveleigh@canada.ca).

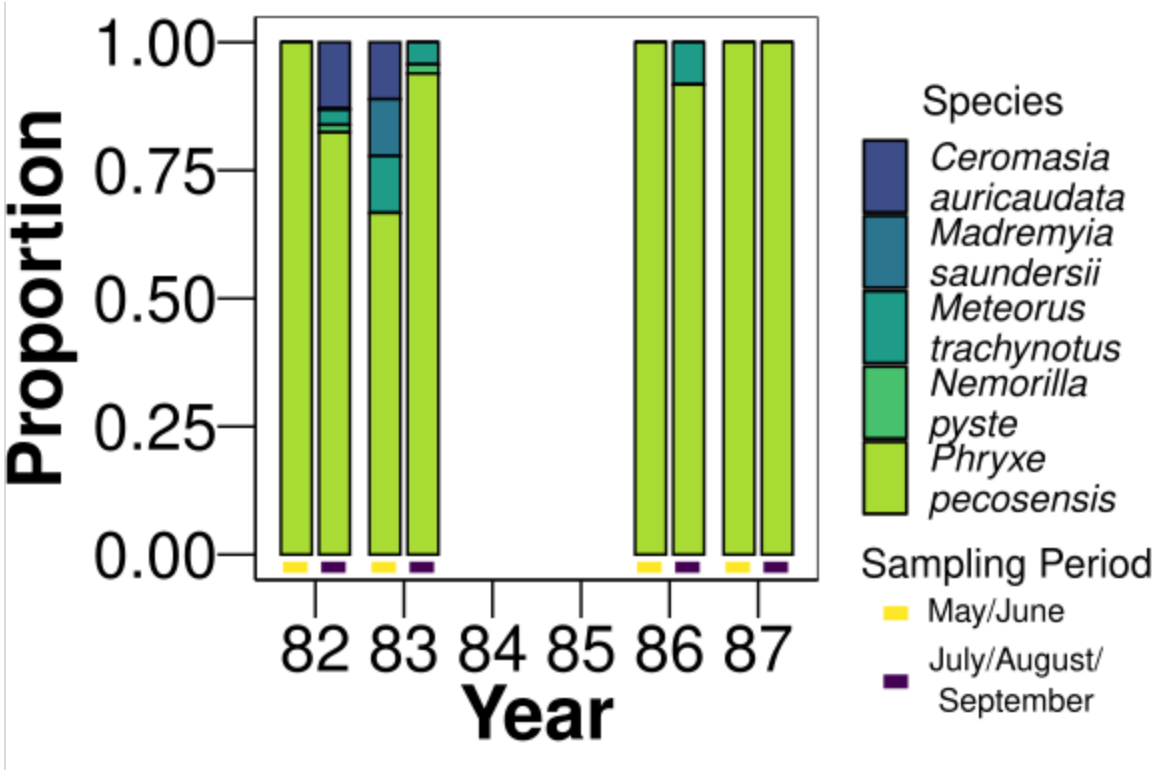

Figure S3 Proportion of each parasitoid species within group three that were Malaise caught in May/June or July/August/September for the years 1982, 1983, 1986, and 1987. To access the data behind this figure, please contact Eldon Eveleigh (eldon.eveleigh@canada.ca).