



Community Ecology

Spatial patterns of hyperparasitism along a latitudinal gradient of forest genus diversity: insights from the spruce budworm-parasitoids community

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High-order mobile predators are generally thought to increase ecosystem stability and resilience to natural perturbations. In many insect food-webs, higher trophic positions are occupied by parasitoids, which are themselves hosts for hyperparasitoids that can reduce primary parasitoids' efficiency in controlling insect pests. Hyperparasitoids can thus provide *ecosystem disservices* by facilitating pest outbreaks, or *ecosystem services* by stabilizing food web fluctuations over longer time periods. To better understand how hyperparasitism affects multitrophic forest systems, we examined for the first time spatial variations in hyperparasitism associated with the spruce budworm. We examined 2 common primary parasitoids of the spruce budworm during outbreaks (*Apanteles fumiferanae* and *Glypta fumiferanae*), and estimated their true and pseudohyperparasitism rates in 2014–2015 from 28 locations across a latitudinal gradient (over 450 km) of forest genus diversity. Hyperparasitoid cryptic diversity was also quantified using DNA-barcoding. We found that *A. fumiferanae* and *G. fumiferanae* share at least 2 of 5 common hyperparasitoid species, confirming the connected nature of the spruce budworm-parasitoid food web. Moreover, hyperparasitism is modulated by spatial context as we observed a positive correlation between forest genus diversity and hyperparasitism for *A. fumiferanae*, but not for *G. fumiferanae*. Further monitoring hyperparasitism holds significant potential to provide new insights into how forest composition affects multitrophic interactions and spatio-temporal outbreak dynamics.

Key words: insect outbreak, forest landscape, indirect interaction, food-web, DNA-barcoding

Introduction

Outbreaks of plant-feeding insects are an important large-scale natural disturbance that affects boreal and sub-boreal forest ecosystems throughout the Northern Hemisphere that can have dramatic ecological and economic consequences (Peltonen et al. 2002, Cooke et al. 2007, Netherer and Schopf 2010, Chen 2014, Schowalter 2017). For example, outbreaks can influence timber flow (Chang et al. 2012), the risk of wildfire (James et al. 2017), forest succession (Baskerville 1975, Bouchard et al. 2006), ecosystem service provisioning (Maguire et al. 2015), and rates of carbon uptake (Kurz et al. 2008, Dymond et al. 2010). Identifying and understanding the mechanisms that drive outbreaks, and alternatively, those that can promote resilience in forest ecosystems, is of paramount importance to ensure long-term sustainability of ecosystem services provided by forests (Loreau and de Mazancourt 2013).

Vegetational species diversity and mobile predators are generally thought to increase ecosystem stability and resilience to insect outbreaks (Staab and Schuldt 2020, Jactel et al. 2021). Natural enemies of insect pests (i.e., predators and parasitoids) have the capacity to modulate their foraging behavior (numerical and functional responses) and disperse to new areas with high local prey densities (i.e., the *birdfeeder effect*), which can, in turn, attenuate insects food-webs fluctuations and stabilize host densities in space and time (Eveleigh et al. 2007, McCann and Rooney 2009). Further, the *enemies hypothesis* (Root 1973) suggests that the abundance and species diversity of such natural enemies tend to be positively correlated with vegetational species diversity, with diverse habitats or landscapes providing a greater abundance and diversity of alternative prey species, microhabitats, and other resources (Staab and Schuldt 2020, Jactel et al. 2021).

In many insect food-webs, higher trophic positions are taken by secondary insect parasitoids (i.e., hyperparasitoids) whose larval development is carried out exclusively at the expense of a primary parasitoid (Brodeur 2000). As such, hyperparasitism can be an important factor in reducing the efficiency of primary parasitoids in controlling insect pests (Sullivan 1987, Rosenheim 1998), thus providing *ecosystem disservices* by facilitating pest outbreaks (Gagic et al. 2012). Indeed, theoretical models have demonstrated that the most probable outcome of hyperparasitism is an increase in the equilibrium density of the herbivore pest (Beddington and Hammond 1977, May and Hassell 1981, Hawkins 1994). Therefore, one would expect increased hyperparasitism to translate into more severe pest outbreaks. In contrast, hyperparasitoids may provide *ecosystem services* by acting as dampening agents of local food web fluctuations by preventing primary parasitoids from overexploiting their host populations, which could result in the demise of the primary parasitoids themselves (Muldrew and Ives 1984, Tougeron and Tena 2019).

Due to their relatively complex, cryptic life cycles, the role of hyperparasitoids in food web dynamics and insect outbreaks remain poorly understood, and little empirical information is available on their ecology. Specifically, we know little about how hyperparasitism rates influence outbreak dynamics, how these rates vary through space and time, and how climate change might affect their relative abundance and influence within insect food-webs (Sullivan 1987, Sullivan and Völkl 1999, Tougeron and Tena 2019). For example, it is unclear if vegetational species diversity, which often promotes primary parasitism (Jactel et al. 2021), is positively or negatively associated with hyperparasitism. Empirical studies examining the effect of vegetational species diversity or landscape complexity on hyperparasitism in insect food-webs are rare, and most focus on aphid communities in agricultural landscapes. These studies generally agree that landscape complexity (i.e., vegetational species diversity) positively influences hyperparasitoids diversity and hyperparasitism rates (Gagic et al. 2011, Jonsson et al. 2012, Rand et al. 2012), but it is difficult to generalize these results to other ecosystems such as forests.

The spruce budworm (*Choristoneura fumiferanae* (Clem.); Lepidoptera: Tortricidae) is the most important insect defoliator of fir and spruce forests in Northeastern North America (Volney and Fleming 2007, Sturtevant et al. 2015). Areas defoliated by this indigenous species can easily reach tens of millions of hectares during epidemic phases (Blais 1983, Sturtevant et al. 2015) and often result in widespread tree mortality that negatively impact the forest industry (Ostaf and MacLean 1989, Chang et al. 2012). Spruce budworm outbreaks are often spatially synchronous at the sub-continental scale, with cycles' periodicity averaging 30–40 yr since the last millennia (Jardon et al. 2003, Boulanger and Arseneault 2004).

The length of spruce budworm population cycles have been attributed to the relatively weak density-dependent response of the primary parasitoid community to increasing host densities during outbreaks (i.e., the population densities of the primary parasitoids cannot increase fast enough; Royama et al. 2017). During spruce budworm outbreaks, many primary parasitoids are believed to shift their foraging activity and congregate to areas of high spruce budworm densities, a phenomenon referred to as the *birdfeeder effect* (Eveleigh et al. 2007). However, most of these primary parasitoid species are themselves hosts for many hyperparasitoid species (Eveleigh et al. 2007, Smith et al. 2011). It is hypothesized that hyperparasitism may reduce the rate of population increase in the primary parasitoid community as a whole, through the means of hyperparasitoid-mediated apparent competition between primary

parasitoids (Eveleigh et al. 2007, Smith et al. 2011). As a result, spruce budworm populations are allowed to continuously increase over time, resulting in long outbreak cycles (Royama et al. 2017).

In this study, we aim to provide additional empirical support for the hypothesized role of hyperparasitoids in outbreak dynamics. We describe for the first time spatial variations in hyperparasitism rates associated with the common primary parasitoids *Apanteles fumiferanae* Viereck (Hymenoptera: Ichneumonidae) and *Glypta fumiferanae* (Viereck) (Hymenoptera: Ichneumonidae) within 6 different areas in Québec, Canada, during a spruce budworm outbreak (Fig. 1). We also sought to identify the relative influences of multiple ecological factors on hyperparasitism rates for these 2 parasitoid species. Specifically, using a series of beta regressions, we verified if hyperparasitism increases with estimates of spruce budworm outbreak age, spruce budworm population density, and/or primary parasitoid population density (Eveleigh et al. 2007). Based on the results of Eveleigh et al. (2007) and the *enemies' hypothesis* (Root 1973), we expected that the primary parasitoid species we examined are in hyperparasitoid-mediated apparent competition, and that hyperparasitoid species diversity and hyperparasitism rates would decrease along a latitudinal gradient of over 450 km of decreasing vegetational genus diversity (from ~7 to 1; Legault and James 2018).

Methods

Study Organisms

This study focuses on hyperparasitism related to 2 species of early larval parasitoids of the spruce budworm: *A. fumiferanae* and *G. fumiferanae*. While many other parasitoid species that are part of the spruce budworm food web also experience hyperparasitism (Eveleigh et al. 2007, Smith et al. 2011), we concentrated our sampling efforts on the early larval component of the food web because of the prominent role of *A. fumiferanae* and *G. fumiferanae* during past and present spruce budworm outbreaks within our study area (Blais 1960, McLeod 1977, Bradette 1994, Legault and James 2018, Marrec et al. 2018).

Both species of primary parasitoid have similar life cycles and share several species of true hyperparasitoids and pseudohyperparasitoids. True hyperparasitoids are secondary parasitoids that lay their eggs in the larvae of the primary parasitoid while they are still developing inside their host. In contrast, pseudohyperparasitoids lay their eggs in the pupae of primary parasitoids once they have emerged from their host (Poelman et al. 2022). Both primary parasitoid species are univoltine: in late summer, females of both species lay their eggs within the first 2 larval instars of the spruce budworm, in which their immature progenies overwinter (Brown 1946a, 1946b). The following spring, parasitoid larvae resume their development within the feeding larval host. During this period, they are susceptible to true hyperparasitoids such as *Stictopisthus* sp. (Bennett 2008, Legault and James 2018). *Apanteles fumiferanae* larvae egress from their hosts a bit earlier than *G. fumiferanae*: mature larvae of *A. fumiferanae* egress from the third to fifth^h larval instars of their hosts, while *G. fumiferanae* egress from the fourth to sixth instars (McGugan 1955). Parasitoid larvae then weave their cocoons and initiate their metamorphosis into adults. During the 9–12-day period spent in this immobile form (Brown 1946a, 1946b), both species are susceptible to various species of pseudohyperparasitoids (Miller 1959, 1960, Eveleigh et al. 1994, 2007).

Insect Sampling, Rearing, and Identification

In 2014, we sampled spruce budworm larvae with the goal of evaluating hyperparasitism of *A. fumiferanae* and *G. fumiferanae*

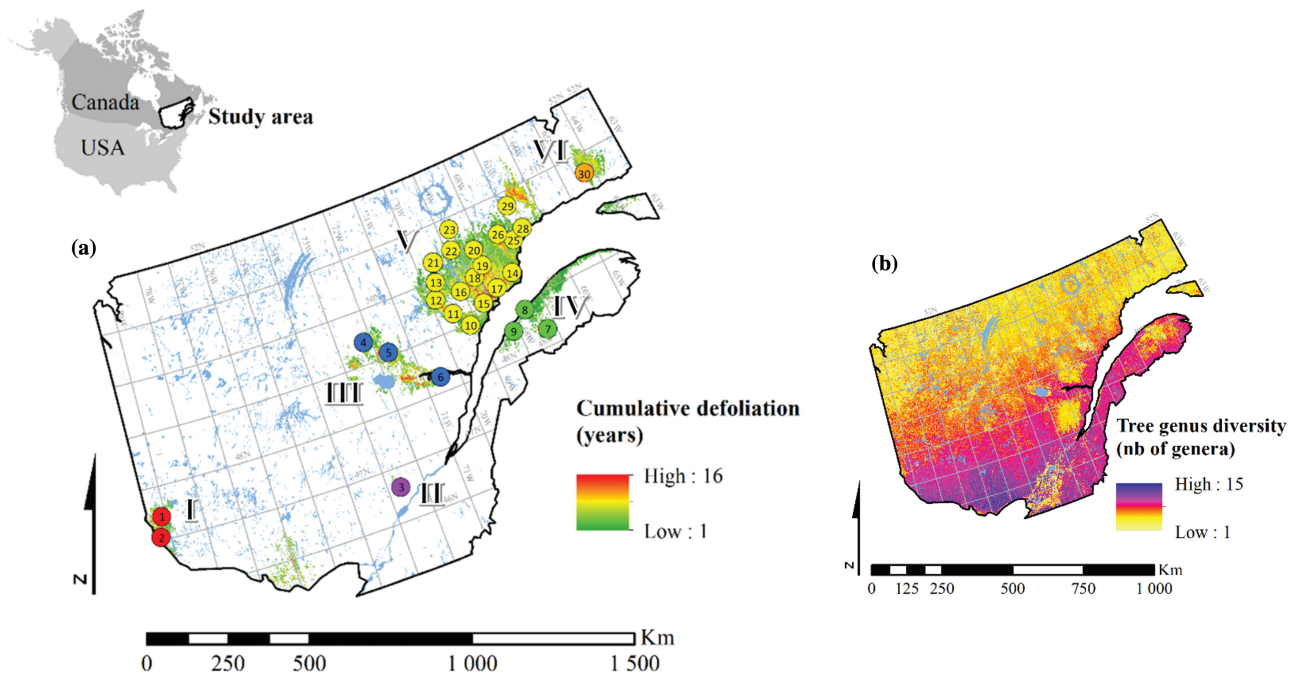


Fig. 1. a) Study area in continental context showing the locations of the 28 sampling sites within 6 different outbreak areas (Roman numerals) in the province of Québec (Canada) with extents of spruce budworm cumulative defoliation (CDEF) as of 2014. b) Map showing a latitudinal gradient of forest diversity represented as the number of tree genera (tree genus diversity; TGD). Spatial datasets (CDEF and TGD) are from [Beaudoin et al. \(2014\)](#) and the Ministère des Forêts, de la Faune et des Parcs du Québec (see text and [Legault and James \[2018\]](#) for details).

in 28 sites in areas affected by spruce budworm defoliation in the province of Québec (Canada) ([Fig. 1](#); [Table 1](#)). Sampling was conducted during 3 distinct phenological periods to match the life cycles of the primary parasitoid species: (i) at the peak of the fourth larval instar of the spruce budworm, when immature parasitoids of both species are still inside the spruce budworm larvae, (ii) at the peak of the sixth larval instar of the spruce budworm, when most *A. fumiferanae* parasitoids are at the pupal stage outside their host and most *G. fumiferanae* parasitoids are still inside the spruce budworm larvae, and (iii) at the peak of the pupal stage of the spruce budworm when both primary parasitoid species are at the pupal stage outside their host ([Brown 1946a, 1946b, McGugan 1955](#)). Because primary parasitoid sample sizes to estimate pseudohyperparasitism were relatively low in 2014, we re-sampled 10 sites in 2015 during the third (iii) phenological period ([Table 1](#)). For each site and phenological period, timing of sampling was informed using the phenological model BioSIM ([Régnière and Saint-Amant 2013](#)), such that we sampled for hyperparasitism within comparable phenological windows.

For each site and phenological period, spruce budworm larvae and/or parasitoid cocoons were collected haphazardly from lower and mid-crown branches of budworm-attacked balsam fir (*Abies balsamea*) and spruce (*Picea glauca* and/or *P. mariana*) trees. Samples were brought back to the laboratory in large paper bags. Spruce budworm larvae were then reared individually on artificial diet ([Hervet et al. 2016](#)). Emerging parasitoid cocoons were transferred to clean 1 oz. plastic cups. After emergence from reared spruce budworm larvae, immature parasitoids identified as *A. fumiferanae* or *G. fumiferanae* ([Brown 1946a, 1946b, Eveleigh et al. 2003](#)) were also transferred in clean 1 oz. plastic cups until the emergence of adult parasitoids or hyperparasitoids.

Upon emergence, adult hyperparasitoids were stored in 70% ethanol, and afterward identified to the species level using keys based

on morphological characteristics ([Huber et al. 1996, Bennett 2008, Fernández-Triana and Huber 2010](#)). Remaining primary parasitoid cocoons were examined or dissected under a binocular microscope. Empty *A. fumiferanae* cocoons were categorized as parasitized or not, based on morphology of the emergence hole(s). Unparasitized cocoons of *A. fumiferanae* present a typical emergence hole with a cap at the apex of the cocoon, while parasitized cocoons present one or many irregular emergence hole(s) that can be found anywhere else on the cocoon ([Eveleigh et al. 2003; Fig. 2a](#)). These cocoons were afterward categorized as parasitized by unknown hyperparasitoids.

Upon final dissections of *G. fumiferanae* cocoons during the winter of 2014–2015, 26 showed the presence of hyperparasitoid larvae that were still alive, but had not yet matured ([Fig. 2c](#)). To identify this hyperparasitoid species that appears to overwinter within *G. fumiferanae* cocoons at the larval stage, we placed similar specimens ($N = 21$) that were reared from 2015 samples in an outdoor shelter located near the municipality of Ferme-Neuve, Quebec, Canada (46.8391; -75.4948). Thirteen (62%) of these hyperparasitoids successfully emerged as adults during the spring of 2016, and were identified as *Scambus* sp.

Finally, to confirm specimen identifications based on morphology, and assess the presence of cryptic hyperparasitoid species that cannot be distinguished morphologically ([Smith et al. 2011](#)), ~45% of the reared hyperparasitoid specimens were identified using DNA barcodes (COI gene) by following the methodology described in [Smith et al. \(2011\)](#). DNA-barcoded specimens were then associated with a Barcode Index Numbers (BIN) using the BOLD Identification System for COI sequences ([Ratnasingham and Hebert 2007](#)) ([Table 2](#)).

Hyperparasitism Rates

To assess true hyperparasitism rates at each site (i.e., hyperparasitism occurring on primary parasitoid larvae while they are located

Table 1. Sites coordinates, cumulative spruce budworm defoliation (CDEF) and tree genus diversity (TGD), and estimated population densities at each site for spruce budworm larvae (SBW_d), the primary parasitoids *A. fumiferanae* (APA_d) and *G. fumiferanae* (GLY_d), and the combined densities of potential primary parasitoids (PRIM_d) for the hyperparasitoid species identified in this study. CDEF and TGD are expressed as the averaged values for all spatial scales (1–20 km). All population densities estimates are expressed as the average number of individuals per 100 buds. Outbreak areas and site numbers and colours are as in Fig. 1.

Outbreak Area	Site	Long.	Lat.	CDEF	TGD	SBW _d	APA _d	GLY _d	PRIM _d
I. Abitibi-Témiscamingue	1 ●	-79.11	47.42	2.78	6.47	16.58	2.49	0.24	2.73
	2* ●	-79.27	47.05	1.76	7.19	19.94	2.39	0.29	2.68
II. Mauricie	3* ●	-72.65	46.62	0.13	7.03	6.95	0.28	0.20	0.48
	4* ●	-72.23	49.40	2.80	5.03	24.65	1.73	<0.01	1.74
III. Saguenay-Lac-Saint-Jean	5 ●	-71.66	49.06	2.51	5.66	26.95	1.35	1.78	3.13
	6* ●	-70.54	48.28	2.59	6.74	10.75	1.29	0.34	1.63
IV. Bas-St-Laurent	7 ●	-68.14	48.52	0.00	6.18	23.48	2.35	0.54	2.89
	8 ●	-67.24	48.30	0.48	6.22	9.57	2.11	0.41	2.52
V. Côte-Nord	9 ●	-67.60	48.81	0.65	6.52	22.14	3.99	0.56	4.55
	10 ●	-69.19	48.96	2.55	5.94	31.12	2.49	2.62	5.11
V. Côte-Nord	11 ●	-69.53	49.28	2.88	5.46	30.38	2.73	1.94	4.67
	12 ●	-69.84	49.65	2.77	4.36	47.48	2.85	6.83	9.68
	13 ●	-69.64	49.95	1.67	4.10	22.50	1.13	2.76	3.89
	14 ●	-67.49	49.54	2.05	5.45	74.69	10.46	10.79	21.25
	15 ●	-68.60	49.24	3.45	5.46	32.08	0.96	2.27	3.23
	16* ●	-69.08	49.61	3.07	3.81	43.27	0.87	2.60	3.47
	17 ●	-68.07	49.39	5.11	5.16	16.84	1.18	1.46	2.64
	18 ●	-68.54	49.75	3.71	5.00	22.16	0.89	0.80	1.69
	19 ●	-68.20	49.90	4.07	4.98	41.47	2.07	1.60	3.68
	20 ●	-68.24	50.23	1.11	3.72	41.53	2.08	4.49	6.57
	21* ●	-69.49	50.32	0.21	4.16	26.90	0.81	0.93	1.74
	22* ●	-68.85	50.39	0.18	3.66	10.51	0.11	0.45	0.56
	23* ●	-68.66	50.77	0.26	4.18	6.96	0.28	0.51	0.79
	25 ●	-67.05	50.09	3.77	4.24	34.44	0.69	4.66	5.35
	26 ●	-67.40	50.31	2.57	3.65	27.93	0.28	1.51	1.79
VI. Basse-Côte-Nord	28* ●	-66.66	50.22	0.90	3.89	37.06	0.37	6.68	7.05
	29 ●	-66.80	50.72	0.61	3.95	49.45	5.44	8.44	13.88
VI. Basse-Côte-Nord	30* ●	-64.30	50.62	2.22	3.20	19.34	0.58	3.09	3.67

*Sites that were also sampled in 2015 during the third phenological period.

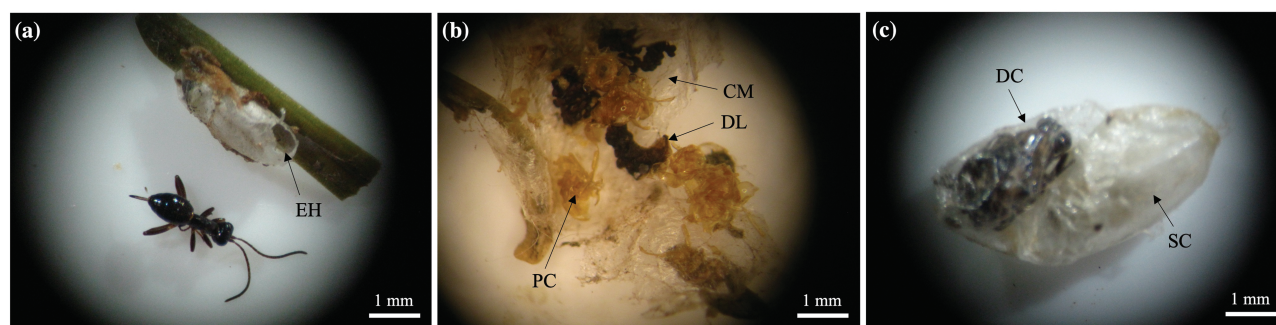


Fig. 2. a) Cocoon of *A. fumiferanae* showing the emergence hole (EH) created by the wingless hyperparasitoid *Gelis* sp. b) Dissected cocoon of *G. fumiferanae* showing the cocoon membrane (CM), the pupal cases (PC), and a dead larva (DL) of the pseudohyperparasitoid *Mesopolobus verditer*. c) Cocoon of *G. fumiferanae* parasitized by a larvae of the pseudohyperparasitoid *Scambus* sp. located within a secondary cocoon (SC) beside the dead carcass (DC) of its primary parasitoid host. Photos from S. Legault.

within spruce budworm larvae), we pooled spruce budworm larval samples from the first 2 sampling periods in 2014. For both *A. fumiferanae* and *G. fumiferanae*, true hyperparasitism rates were obtained by dividing the number of true hyperparasitoids emerging from *A. fumiferanae* or *G. fumiferanae* by the number of spruce budworm larvae parasitized by each primary parasitoid species (Legault and James 2018).

Pseudohyperparasitism rates (i.e., hyperparasitism occurring on primary parasitoids after they emerged from spruce budworm

larvae) for each site were estimated by pooling primary parasitoid cocoons collected during the last 2 sampling periods in 2014 and 2015. We removed from our datasets cocoon samples that resulted in the emergence of *Stictopisthus* sp., which is a known true hyperparasitoid that emerge from primary parasitoid pupae (Bennett 2008). Pseudohyperparasitism rates were obtained by dividing the number of pseudohyperparasitoids emerging from *A. fumiferanae* or *G. fumiferanae* cocoons, by the total number of *A. fumiferanae* or *G. fumiferanae* cocoons directly sampled from infested tree branches.

Table 2. List of hyperparasitoids species identified in this study using morphological taxonomic keys (Huber et al. 1996, Bennett 2008) and DNA-barcoding. Known primary parasitoid host species are included in the last column (parasitoid species codes are the same as in SI Table 3 of Eveleigh et al. [2007]).

Hyperparasitoid species (ID based on morphology)	Reared individuals (N ^a)	DNA-barcoded individuals (N)	Barcode Index Number(s) (N)	Known primary parasitoid hosts ^b
True hyperparasitoids²				
<i>Stictopisthus</i> sp. (93)	40♦; 4◆	6◆	AAA5519 (6◆) 9 other BINs	9 9, 10, 11, 18, 19, 21, 25, 49, 71
Pseudohyperparasitoids				
<i>Acrolyta</i> sp. (96)	1◆	1◆	AAE8196 (1◆)	9
<i>Baryscapus coeruleus</i> (Ashmead) (85)	4◆	4◆	AAA4401 (4◆)	9, 11, 17, 18, 19, 81, 83; 98
<i>Gelis</i> sp. (79)	9◆; 2◆	9◆; 2◆	AAA6276 (1◆) AAA6278 (3◆) AAA6279 (1◆) AAA6280 (3◆; 1◆) AAA6283 (2◆)	10, 80 9, 17 9, 11, 17, 19 9, 10, 11, 17, 18, 19, 71 9, 11, 71
<i>Mesopolobus verditer</i> (Norton) (83)	13◆; 68◆	13◆; 27◆	AAA4053 (12◆; 27◆) AAA4055 (1◆)	4, 5, 9, 10, 11, 18, 71, 80, 93 9, 11, 18
<i>Pteromalus phycidis</i> Ashmead (82)	1◆	1◆	AAA8289 (1◆)	9, 10, 11, 18, 71
<i>Scambus</i> sp. (80)	50◆	23◆	AAB0893 ^c (2◆) AAB0894 ^d (21◆)	10, 11 10

^aSymbols' color indicates the primary parasitoid hosts: ◆= from *A. fumiferanae*; ♦= from *G. fumiferanae*.
^bParasitoid species codes are as in Eveleigh et al. (2007); codes in bold represents hyperparasitoids: 4-*Eumea Caesar* (Aldrich); 5-*Nemorilla pyste* (Walker); 9-*Apanteles fumiferanae* Viereck; 10-*Glypta fumiferanae* (Viereck); 11-*Meteorus trachynotus* Viereck; 17-*Apanteles petrovae* Walley; 18-*Dolichogenidea absona* (Muesebeck); 19-*Apanteles morrissi* Mason; 21-*Orgilus* sp. 25-*Tranosema tenuifemur* (Walley); 49-*Microgaster* sp.; 71-*Glyptapanteles* sp.; 80-*Scambus* sp.; 81-*Elasmus atratus* Howard; 83-*Mesopolobus verditer* (Norton); 93-*Stictopisthus* sp.; 98-*Elachertus cacoeciae* (Howard).
^cOur rearings indicate that this species emerges as adults at the end of summer.
^dOur rearings indicate that this species overwinters as mature larvae within *G. fumiferanae*' cocoons.

Spruce Budworm and Primary Parasitoid Densities

In this study, we sought to identify the relative influences of multiple ecological factors on true and pseudohyperparasitoids attacking *A. fumiferanae* and *G. fumiferanae*. Specifically, we used beta regressions (see below) to measure the effects of (i) spruce budworm and/or primary parasitoid population densities, (ii) outbreak age, and (iii) forest genus diversity on hyperparasitism rates.

First, to test our hypothesis that hyperparasitism increases with spruce budworm population density and/or primary parasitoid density, we calculated an index of population density for spruce budworm larvae (SBW_d), *A. fumiferanae* (APA_d), *G. fumiferanae* (GLY_d), and the combined densities of potential primary parasitoids ($PRIM_d$) for the hyperparasitoid species identified in this study. In Legault and James (2018), we provided details on how we calculated, for each sampling locations, an index of local spruce budworm larval density (mean number of spruce budworm larvae per 100 annual shoots, measured on ten 45-cm branches of the mid-crown of host trees; SBW_d), and how we estimated parasitism rates by *A. fumiferanae* and *G. fumiferanae*. In this study, we multiplied for each site SBW_d values by parasitism rates by *A. fumiferanae* or *G. fumiferanae* to provide an estimate of primary parasitoid population densities (APA_d and GLY_d ; Table 1). In addition to *A. fumiferanae* and *G. fumiferanae*, potential primary parasitoids included *Eumea caesar*, *Nemorilla pyste*, *Meteorus trachynotus*, *Dolichogenidea absona*, *Apanteles morrissi*, *Orgilus* sp., and *Tranosema tenuifemur* (Table 2). Details on extensive primary parasitoid sampling (i.e., from early larvae, late larvae, and pupae of the spruce budworm), and the calculation of primary parasitoid densities for the remaining species are provided in Marrec et al. (2018). For each site, SBW_d , APA_d , GLY_d , and $PRIM_d$ values are expressed as the average number of individuals found per 100 annual shoots (Table 1).

Spatial Data at Different Spatial Scales

To test our prediction that hyperparasitism increases with vegetational diversity, we used MODIS imagery (circa 2000–2006; 250 m; Beaudoin et al. 2014) to calculate a simple metric of forest genus diversity (i.e., richness; alpha diversity) in a rasterized format that represent the number of tree genera in each 250m pixel (i.e., tree genus diversity; TGD ; Fig. 1b). While the main focus of this study was to better understand spatial variation in hyperparasitism, we also attempted to take into account potential temporal variation in hyperparasitism linked with outbreak stage. To do so, we used in our analyses provincial monitoring datasets from 1993 to 2014 (Ministère des Forêts de la Faune et des Parcs 2014) to create a rasterized map of cumulative spruce budworm defoliation, expressed in years ($CDEF$; Fig. 1a). Only moderate (35–69%) or severe (>69%) defoliation were considered. More details on TGD and $CDEF$ rasters' creation can be found in Legault and James (2018).

As different insect parasitoid (and hyperparasitoid) species are known to perceive and respond to landscape structure and composition at different spatial scales (Roland and Taylor 1997, Cronin and Reeve 2014, Legault and James 2018, Marrec et al. 2018), spatial datasets ($CDEF$ and TGD) were summarized using 8 concentric circular buffers (1, 2, 3, 5, 7.5, 10, 15, and 20 km radius) centered on each of the 28 sites. All procedures on spatial datasets were completed using ArcMap 10.3.1.

Statistical Analyses

The relative influences of local spruce budworm population density (SBW_d), primary parasitoid population density APA_d , GLY_d , or $PRIM_d$, and landscape factors ($CDEF$ and TGD) on hyperparasitism rates associated with *A. fumiferanae* and *G. fumiferanae* were examined using a series of beta regressions as implemented in the

betareg package (Cribari-Neto and Zeileis 2010) for R. We choose this approach as it is suited to model hyperparasitism rates as they are (0,1) bounded (Cribari-Neto and Zeileis 2010). All explanatory variables were standardized to zero mean and unit variance prior to analyses so that their relative effects on hyperparasitism rates could be compared. Also, because sampling effort varied among sites and periods (true hyperparasitism vs. pseudohyperparasitism), each site was weighted in each model by the number primary parasitoids examined. Separate series of models were built for: (i) true hyperparasitism (2014) and pseudohyperparasitism (2014–2015), (ii) primary parasitoid hosts (i.e., through *A. fumiferanae*, *G. fumiferanae*), and (iii) total vs. species-specific hyperparasitism (i.e., the most common species: *M. verditers* and *Scambus* sp.).

Models of hyperparasitism rates were built in 2 steps. In the first step, we identified the spatial scale (1–20 km radius) at which each spatial metric (*CDEF* and *TGD*) was the most related to hyperparasitism using beta regressions. We then retained scale-specific predictors associated with the model with the higher weight of the Akaike's information criterion corrected for small sample sizes (*AICc w*) using the *AICcmodavg* package (Mazerolle 2017) in R. Landscape factors were not retained for the second modeling step (see below) if none of the *AICc w* were higher than the *AICc w* of the intercept model (Legault and James 2018).

In the second step, we modeled hyperparasitism rates as a function of different combinations of both scale-specific landscape factors retained from the first step, and population density predictors measured at the local scale (*SBW_d*, *APA_d*, *GLY_d*, and *PRIM_d*). When factors were highly correlated, only one of them was retained in the list of candidate models (Table 3). *TGD* and latitude were always correlated (Pearson's correlation = −0.90 to −0.93). Spruce budworm larval density (*SBW_d*) and estimated population density of primary parasitoids (*APA_d*, *GLY_d*, and *PRIM_d*) were also correlated (0.59–0.84). Also, because latitude and longitude associated with our sampling locations were correlated (Pearson's correlation = 0.70 to 0.81), we chose not to include longitude in the analyses, and focused on latitude as this factor best fits our research questions regarding the effects of latitudinal gradients of vegetational genus diversity (Fig. 1b) on hyperparasitism associated with the spruce budworm. Finally, we retained the model with the higher *AICc w*.

Results

True Hyperparasitism of Primary Parasitoid Larvae

To estimate true hyperparasitism rates associated with spruce budworm larvae in 2014, we examined 856 and 1,448 larvae parasitized by *A. fumiferanae* and *G. fumiferanae*, respectively. From these, 40 hyperparasitoid individuals emerged from *A. fumiferanae* (4.67%), and only 4 from *G. fumiferanae* (0.28%). Based on morphological identification keys (Bennett 2008), true hyperparasitism for both primary parasitoids was attributed to only one species, *Stictopisthus* sp. (Supplementary Table S1). Although all of the 6 barcoded specimens belonged to the same BIN (AAA5519; Table 2), we suspect that the few specimens emerging from *G. fumiferanae* might be from a different cryptic species (i.e., AAA5517; Smith et al. (2011)). For this reason (and because true hyperparasitism was very low for *G. fumiferanae*), we only attempted to model true hyperparasitism rates for *A. fumiferanae*.

True hyperparasitism rates of *A. fumiferanae* larvae by *Stictopisthus* sp. ranged from 0 to 100% between sites (Fig. 1, Supplementary Table S1), with a mean value of 13.11% (SD = 22.93%). True hyperparasitism was higher in outbreak areas

V (34/331 = 10.27%) and VI (4/4 = 100.00%). Inversely, true hyperparasitism was low in area III (2/99 = 2.02%) and null in the southernmost areas I (0/94), II (0/38) and IV (0/229) (Supplementary Table S1).

The best candidate model explaining true hyperparasitism of *A. fumiferanae* by *Stictopisthus* sp. included *TGD* measured at 3 km and *PRIM_d* (*AICc w* = 1.00, *pseudo R*² = 0.662) (Fig. 3a). For this model, the predictor with the highest coefficient was *TGD*, with a significant negative relationship with hyperparasitism rates (−1.09; SE = 0.03; *P* < 0.001), while the coefficient associated with *PRIM_d* was also negative and significant, but much weaker (−0.16; SE = 0.03; *P* < 0.001), and its significance was probably influenced by only 2 data points in outbreak areas V and VI (Fig. 3a).

Pseudohyperparasitism of Primary Parasitoid Cocoons

In 2014–2015, we examined 171 and 728 parasitoid cocoons of the primary parasitoids *A. fumiferanae* and *G. fumiferanae*, respectively, that were directly sampled from foliage to estimate pseudohyperparasitism (Supplementary Tables S2 and S3). Overall we found higher pseudohyperparasitism rates for *A. fumiferanae* (28.07%; Supplementary Table S2) and *G. fumiferanae* (17.17%; Supplementary Table S3) than for true hyperparasitism (Supplementary Table S1).

Based on morphological identification keys, we identified at least 6 different species of pseudohyperparasitoids attacking *A. fumiferanae* and/or *G. fumiferanae*. Two pseudohyperparasitoid species attacked both *A. fumiferanae* and *G. fumiferanae*: *Mesopolobus verditers* was the most commonly reared species from both primary parasitoid cocoons (*A. fumiferanae*: 11.11%; *G. fumiferanae*: 9.33%), while *Gelis* sp. was commonly found in *A. fumiferanae* cocoons (4.71%), but only sporadically found in *G. fumiferanae* cocoons (0.27%) (Fig. 4). Three species attacked only *A. fumiferanae*: *Acrolyta* sp. (0.52% of cocoons), *Baryscapus coeruleus* (2.09%), and *Pteromalus phycidis* (0.52%) (Fig. 4). One species, *Scambus* sp., attacked only *G. fumiferanae* (6.86%) (Fig. 4). Relative to

Table 3. List of candidate models considered to describe variations in rates of true hyperparasitism or pseudohyperparasitism through the primary parasitoids *A. fumiferanae* or *G. fumiferanae*. LAT = Latitude; *TGD* = Tree genus diversity; *CDEF* = cumulative defoliation; *SBW_d* = Spruce budworm larval density; *APA_d* = Estimated population density of *A. fumiferanae*; *GLY_d* = Estimated population density of *G. fumiferanae*; *PRIM_d* = Estimated population density of potential primary parasitoid hosts (see Table 2).

Model	Parameters
1	Intercept model
2	LAT
3	<i>TGD</i>
4	<i>SBW_d</i>
5	<i>APA_d</i> or <i>GLY_d</i>
6	<i>PRIM_d</i>
7	<i>CDEF</i>
8	LAT + <i>SBW_d</i>
9	LAT + <i>APA_d</i> or <i>GLY_d</i>
10	LAT + <i>PRIM_d</i>
11	LAT + <i>CDEF</i>
12	<i>TGD</i> + <i>SBW_d</i>
13	<i>TGD</i> + <i>APA_d</i> or <i>GLY_d</i>
14	<i>TGD</i> + <i>PRIM_d</i>
15	<i>TGD</i> + <i>CDEF</i>

morphological identification, DNA-barcoding revealed a more diverse community of hyperparasitoids, with 5 cryptic species for *Gelis* sp., 2 for *Mesopolobus verditer*, and 2 for *Scambus* sp. (Table 2). Based on our rearing data, one *Scambus* species (AAB0893) emerges from *G. fumiferanae* cocoons at the end of the season, while the other (AAB0894) overwinters as a larvae within its primary host's cocoon (Fig. 2c).

Most of these cryptic species have been previously identified as generalists by Smith et al. (2011) (i.e., they were reared from at least 2 different primary parasitoid species). *Baryscapus coeruleus*, *Gelis* sp., and *M. verditer* have also been identified as tertiary parasitoids (i.e., attacking a secondary parasitoid) in some occasions (Eveleigh et al. 1994, 2007, Smith et al. 2011).

Using Spearman correlations, we found a positive and significant relationship between the diversity of pseudohyperparasitoids (number of species based on morphological identification keys or DNA-barcoding) and pseudohyperparasitism rates for *A. fumiferanae* cocoons (Fig. 5a and b). However, we found no significant relationship for pseudohyperparasitoids of *G. fumiferanae* (Fig. 5c and d).

Using beta regression, we found significant predictors to explain the spatial variations in pseudohyperparasitism rates for *A. fumiferanae* cocoons (Fig. 3b–d), but not for *G. fumiferanae* (results not shown). For *A. fumiferanae* cocoons, the best model explaining

total pseudohyperparasitism included TGD measured at 3 km and SBW_d (model 11: $AICc w = 1.00$, $pseudo R^2 = 0.314$) (Fig. 3b). For this model, the relationship between TGD measured at 3 km and pseudohyperparasitism rates was significant and negative (-0.45 ; $SE = 0.05$; $P < 0.001$), as was the relationship between SBW_d and pseudohyperparasitism rates (-0.32 ; $SE = 0.04$; $P < 0.001$) (Fig. 3b). However, we found that each of these 2 predictors impacted different components of the pseudohyperparasitoid food web attacking *A. fumiferanae* cocoons. Indeed, when modeling only pseudohyperparasitism rates caused by *M. verditer*, we found that the best model (model 3: $AICc w = 0.54$, $pseudo R^2 = 0.174$) included only a negative relationship between SBW_d and pseudohyperparasitism (-0.41 ; $SE = 0.05$; $P < 0.001$) (Fig. 3c). In contrast, when modeling pseudohyperparasitism rates caused by the less abundant species (i.e., *Acrolyta* sp., *B. coeruleus*, *Gelis* sp., and *P. phycidis*), we found that the best model (model 2: $AICc w = 0.59$, $pseudo R^2 = 0.333$) included only a negative effect of TGD measured at 1 km (-0.56 ; $SE = 0.05$; $P < 0.001$) (Fig. 3d).

Discussion

Hyperparasitism is an important yet poorly understood and seldom documented process in forest ecosystems due to the relatively cryptic life cycles of hyperparasitoid. In this study we described for the very

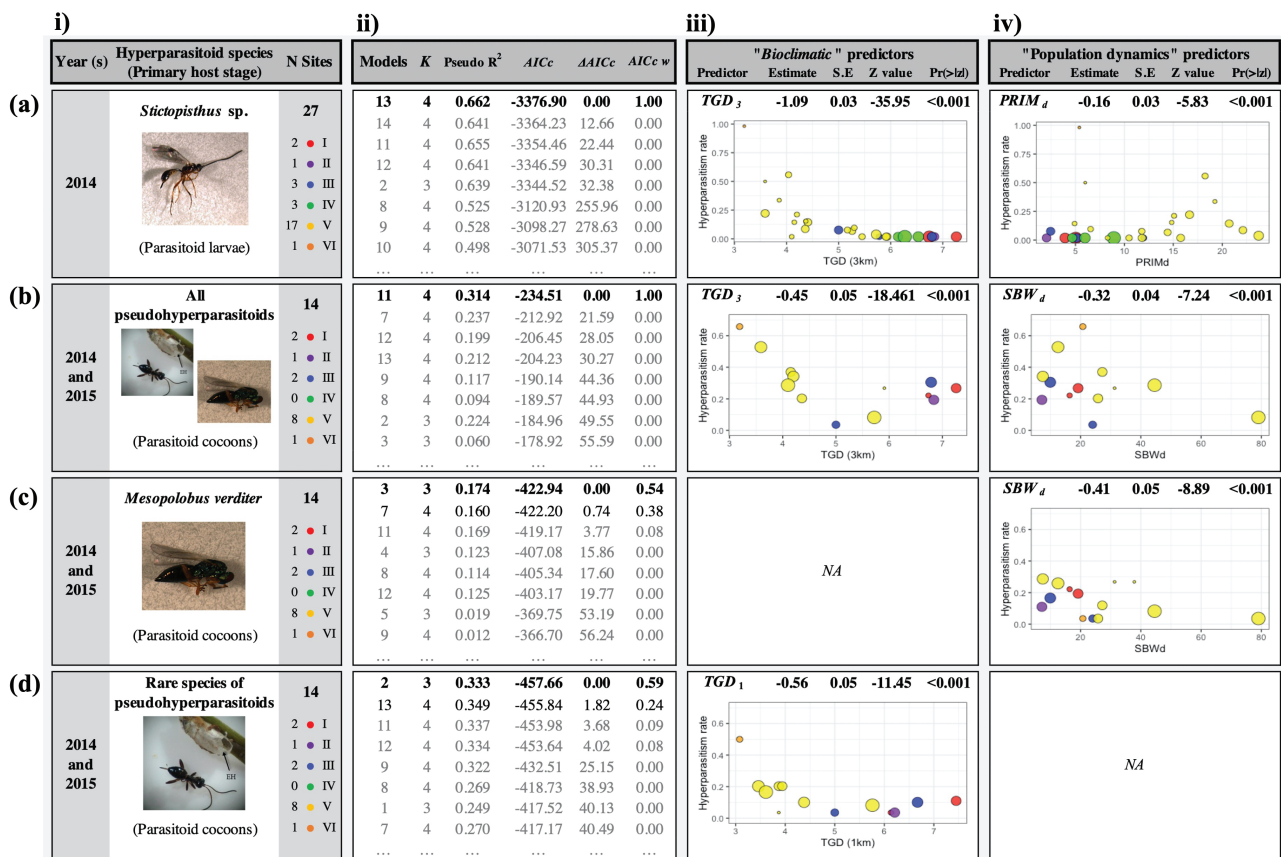


Fig. 3. Beta regression models describing variation in rates of hyperparasitism of *A. fumiferanae* larvae a) and cocoons b–d) in 2014 and 2015. From left to right we show: i) representative specimens and the number of sites per outbreak areas from which data is available; ii) lists of candidate models (see Table 3) and their relative support during the second step of modeling (models are ordered according to $\Delta AICc$ and $AICc w$); iii) and iv) beta regression parameter estimations and standard error (SE) for the selected model, and associated biplots. Color of points corresponds to the different outbreak areas (Fig. 1), and size of points are proportional to the number of *A. fumiferanae* individuals examined for hyperparasitism (N; Supplementary Tables S1 and S2). TGD = Tree genus diversity; PRIMd = Potential primary parasitoid population density; SBW_d = Spruce budworm larval density. For TGD, the spatial scale in km selected during the first step of modeling is indicated by a subscript. Biplots not shown can be found in Supplementary Materials.

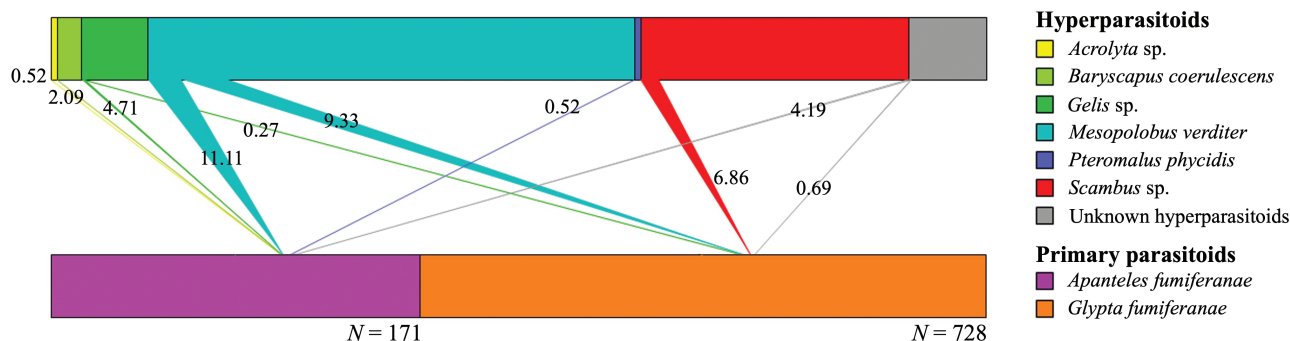


Fig. 4. Graphical representation of trophic interactions between the primary parasitoids *A. fumiferanae* and *G. fumiferanae*, and their pseudohyperparasitoids across our study area in 2014–2015. Numbers below primary parasitoid bars indicate the total number of cocoons examined for pseudohyperparasitism (N). The width of primary parasitoid bars is proportional to their respective averaged population densities across all sampled sites (Table 1). Numbers above trophic links represents total pseudohyperparasitism rates (%) for each primary parasitoid species.

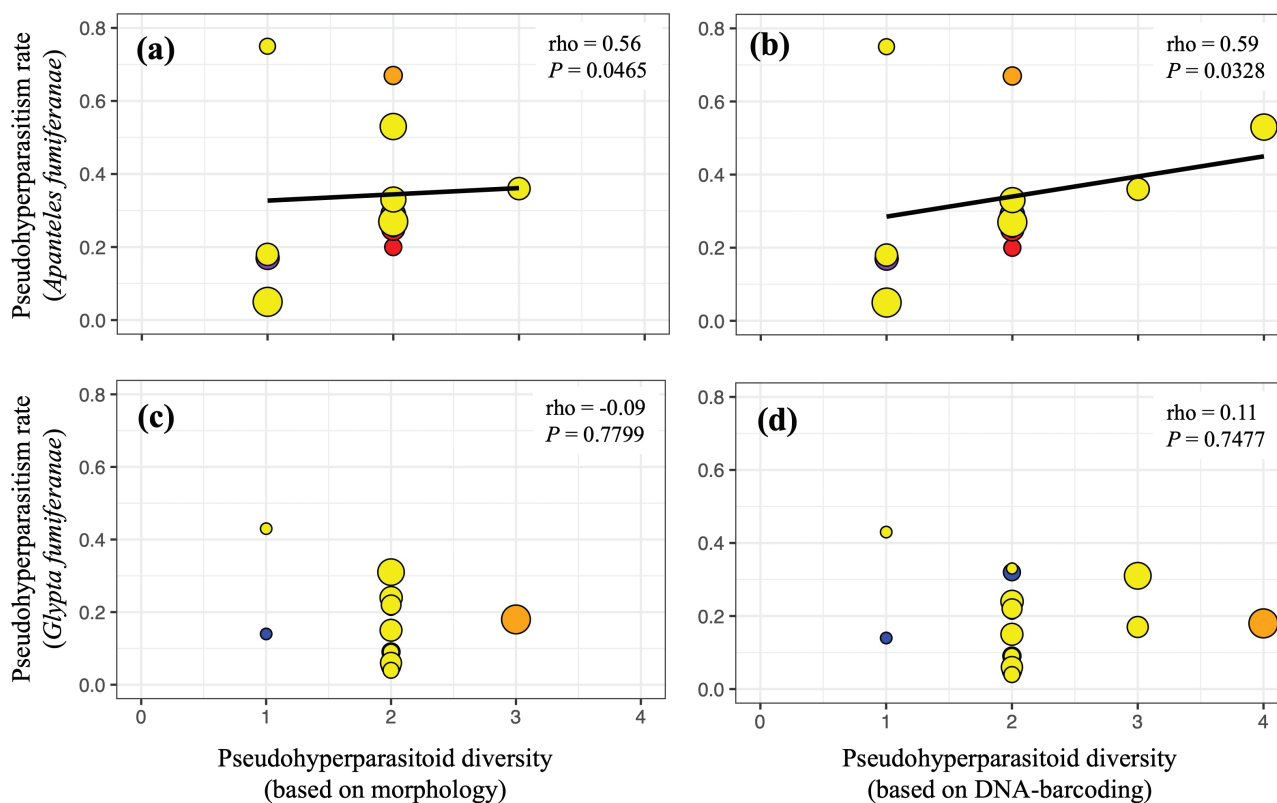


Fig. 5. Spearman's rank correlations between the number of species of pseudohyperparasitoids identified using morphological identification keys (a, c) or DNA-barcoding (b, d) for each site, and associated pseudohyperparasitism rates for *A. fumiferanae* (a, b) and *G. fumiferanae* (c, d). Color of points corresponds to the different outbreak areas (Fig. 1), and size of points are proportional to the number of cocoons examined for hyperparasitism (N; Supplementary Tables S1 and S2).

first time spatial variations in hyperparasitoid species (cryptic) diversity and hyperparasitism rates associated with the common primary parasitoids *Apanteles fumiferanae* and *Glypta fumiferanae*, within different areas affected by spruce budworm defoliation in Québec, Canada. We sought to identify the relative influence of multiple ecological factors at different scales on hyperparasitism rates with the goal of providing new perspectives in the study of large-scale insect outbreaks in forest landscapes. Furthermore, our study provide a methodological framework involving DNA-barcoding to resolve hyperparasitoids host associations and sampling the different life stages of primary parasitoids.

We found that the primary parasitoid species we examined can experience relatively high hyperparasitism rates. For example, *Stictopisthus* sp., based on DNA-barcoding, appears to be host specialist and can cause significant mortality to *A. fumiferanae*. This conform with previous suggestions that hyperparasitism can be viewed as an *ecosystem disservice* and reduce the impact of primary parasitoids during spruce budworm outbreaks (Eveleigh et al. 2007, Smith et al. 2011, Royama et al. 2017). In the central and southern parts of the spruce budworm outbreak range, *A. fumiferanae* was by far the most important parasitoid of the spruce budworm in terms of parasitism rates during previous outbreak cycles (Jaynes and Drooz

1952, McGugan and Blais 1959, Blais 1960, McLeod 1977, Eveleigh and Johns 2014, Royama et al. 2017). However, in the northern parts of the spruce budworm outbreak range, we found that *A. fumiferanae* experiences high rates of hyperparasitism in forests that contain fewer genera of tree (Fig. 1b). This result somewhat contradicts the *enemies' hypothesis* (Root 1973) which suggests that hyperparasitism would increase with vegetational diversity (Eveleigh et al. 2007). Hyperparasitism thus can partly explain why previous work has found that primary parasitism rates of spruce budworm larvae by *A. fumiferanae*, as well as genetic diversity of *A. fumiferanae*, decreases with decreasing forest diversity (Legault and James 2018, Legault et al. 2021). As such, high hyperparasitism of *A. fumiferanae* might contribute to higher intensities of spruce budworm outbreaks in northern, homogeneous coniferous forests (Bouchard and Auger 2014, Robert et al. 2018).

Other hyperparasitoid species, such as *Mesopolobus verditer*, are host generalists (Table 2, Eveleigh et al. 2007, Smith et al. 2011) and attacked both *A. fumiferanae* and *G. fumiferanae* (Fig. 4). This result conform with the hypotheses that (i) primary parasitoids such as *A. fumiferanae* and *G. fumiferanae* can coexist in forest landscapes through hyperparasitoid-mediated apparent competition (Legault and James 2018), and (ii) that generalist hyperparasitoids provide an *ecosystem service* by promoting the coexistence of numerous primary parasitoid species and stabilizing insect food web fluctuations in space and time (Eveleigh et al. 2007, McCann and Rooney 2009, Tougeron and Tena 2019).

To fully test this second hypothesis, more empirical datasets on hyperparasitism of other common spruce budworm primary parasitoids recorded during outbreaks are needed. Other common primary parasitoids likely affected by generalist hyperparasitoids such as *M. verditer* include the late larval parasitoids *Meteorus trachynothus* and *Smidtia fumiferanae*, and the pupal parasitoids *Dirophanes maculicornis*, *Itopectis conquisitor*, and *Apecthis ontario* (Marrec et al. 2018). Furthermore, while we attempted to take into account potential temporal variation in hyperparasitism linked with outbreak stage, the available datasets we used might not have enough power to identify meaningful temporal patterns (CDEF is a coarse estimate of outbreak stage that does not necessarily reflect population dynamics). To better understand the role of hyperparasitoids during the different phases of an outbreak cycle, datasets covering longer periods of time are needed, particularly during endemic phases. In these contexts, we suggest the use of sentinel hosts, i.e., primary parasitoid cocoons reared in the laboratory (Rappaport and Page 1985), to monitor hyperparasitism when spruce budworm populations are very low. This approach could allow testing the hypothesis that a gradual increase in hyperparasitism toward the end of endemic phases can trigger the onset of spruce budworm outbreaks (Nenzén et al. 2018).

Our study represents a preliminary, yet important first step in elucidating the role of hyperparasitism in spruce budworm spatio-temporal dynamics. Our methodological framework can be used to collect and analyze larger empirical datasets on spatio-temporal variations of true and pseudohyperparasitism associated with forest defoliators. Improving our ecological knowledge of hyperparasitoids dynamics during and between insect outbreaks is important if we aim at understanding the role of biodiversity at higher trophic levels in stabilizing ecological processes influencing our forest ecosystems.

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Author Contributions

Simon Legault (Conceptualization [Lead], Data curation [Lead], Formal analysis [Lead], Investigation [Lead], Methodology [Lead], Software [Lead], Validation [Lead], Visualization [Lead], Writing—original draft [Lead], Writing—review & editing [Equal]), and Patrick M. A. James (Conceptualization [Supporting], Data curation [Supporting], Formal analysis [Supporting], Funding acquisition [Lead], Investigation [Supporting], Methodology [Supporting], Project administration [Lead], Resources [Lead], Software [Supporting], Supervision [Lead], Validation [Equal], Visualization [Supporting], Writing—original draft [Supporting], Writing—review & editing [Equal])

Supplementary material

Supplementary material is available at *Environmental Entomology* online.

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