University of Massachusetts Amherst

ScholarWorks@UMass Amherst

October 2018

IMPACT OF NATIVE NATURAL ENEMIES ON POPULATIONS OF THE INVASIVE WINTER MOTH (OPEROPHTERA BRUMATA L) IN THE NORTHEAST UNITED STATES

Hannah J. Broadley
University of Massachusetts Amherst

Recommended Citation

**IMPACT OF NATIVE NATURAL ENEMIES ON POPULATIONS
OF THE INVASIVE WINTER MOTH (*OPEROPHTERA BRUMATA* L)
IN THE NORTHEAST UNITED STATES**

A Dissertation Presented

by

HANNAH J. BROADLEY

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September 2018

Graduate Program in Organismic and Evolutionary Biology

IMPACT OF NATIVE NATURAL ENEMIES ON POPULATIONS
OF THE INVASIVE WINTER MOTH (*OPEROPHTERA BRUMATA* L)
IN THE NORTHEAST UNITED STATES

A Dissertation Presented

by

HANNAH J. BROADLEY

Approved as to style and content by:

_____
Joseph S. Elkinton, Chair

_____
Lynn S. Adler, Member

_____
John P. Burand, Member

_____
Benjamin B. Normark, Member

_____
Paige S. Warren, Program Leader
Organismic and Evolutionary Biology

_____
Patricia Wadsworth, Director
Interdepartmental Programs in Life Science

DEDICATION

for the trees and forests

ACKNOWLEDGMENTS

ABSTRACT

**IMPACT OF NATIVE NATURAL ENEMIES ON POPULATIONS
OF THE INVASIVE WINTER MOTH (*OPEROPHTERA BRUMATA* L)
IN THE NORTHEAST UNITED STATES**

SEPTEMBER 2018

HANNAH J. BROADLEY, B.S., BATES COLLEGE

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Joseph S. Elkinton

Invasive insects increasingly affect forested landscapes and have important
ecological and economic impacts. My dissertation focuses on population dynamics of
winter moth (*Operophtera brumata* L.), an invasive pest in the northeastern United
States. Native to Europe, this is the species' fourth accidental introduction to North
America. The Elkinton lab established the biological control agent *Cyzenis
albicans* across the range of winter moth in the northeastern U.S. Prior research indicates
that *C. albicans*' ability to control winter moth likely depends on additional mortality
from native natural enemies. My dissertation research evaluates the identity and role of
natural enemies already present in North America (predators, parasitoids, and pathogens)
on winter moth, and their interactions with mortality from *C. albicans*.

I found that in earlier years of the current North American introduction, predator
communities were saturated due to the abundance of pupae to consume; however, as
winter moth densities decreased to levels comparable to its native range (presumably due
to mortality added to the system by *C. albicans*) pupal predation's role on the population
dynamics has changed; pupal predation is now density dependent and helps stabilize the

winter moth populations. Furthermore, I detected parasitism on winter moth pupae by an ichenumonid wasp (*Pimpla* spp.), which causes additional mortality on winter moth pupae and rarely kills *C. albicans* puparia. While rates of mortality on winter moth from this parasitoid are lower than those from predation, parasitism is also density dependent and has a stabilizing effect on winter moth populations. Lastly, my research shows that mortality in larval and pupal stages is lower on winter moth than on the native congener, Bruce spanworm, *Operophtera bruceata* Hulst. Cadavers from both species had low rates of infection from nucleopolyhedroviruses. Each host had its own virus species and there were no cross-infectious between the two. Microsporidia were detected in Bruce spanworm, but not in winter moth.

I conclude that our biological control efforts have reduced winter moth populations to non-pest levels and I expect native natural enemies, along with *C. albicans*, will regulate population densities indefinitely.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

THE PHYLOGENETIC RELATIONSHIP AND CROSS-INFECTION OF NUCLEOPOLYHEDROVIRUSES BETWEEN THE INVASIVE WINTER MOTH (*OPEROPHTERA BRUMATA*) AND ITS NATIVE CONGENER, BRUCE SPANWORM (*O. BRUCEATA*)

1.1 Abstract

Disease can affect biological invasions by acting as either a synergist or antagonist. Disease-mediated invasions have important implications for understanding the spread of invasive insects, which cost billions of dollars in damages annually. One such non-native, destructive insect is the winter moth, *Operophtera brumata* L. (Lepidoptera: Geometridae), which causes defoliation and mortality of deciduous trees in its introduced range. In the northeastern United States, winter moth populations overlap with a native congener, Bruce spanworm, *Operophtera bruceata* Hulst. Nucleopolyhedrovirus (NPV), appears to be an important natural enemy in Bruce spanworm and there is some evidence that the NPV infection found in winter moth in the northeastern U.S. may originate from Bruce spanworm. By sequencing two viral genes (the polyhedrin and p74 genes) from field-collected larvae of both species, we found that the winter moth virus (OpbuNPV) is distinct from the virus from Bruce spanworm (OpbrNPV). However, the two viruses do constitute a clade within the Alphabaculovirus Group 2 NPVs, indicating that they are more similar to each other than they are to other lepidopteran viruses, even other geometrid-derived NPVs. As far as we know, this is the first report of sequences from an NPV from Bruce spanworm. Results from cross

infection trials suggest that cross infection is uncommon if it occurs at all. Our results show that these two closely related species have distinct viruses and, unlike previous suggestions, Bruce spanworm virus is not mediating the winter moth invasion.

1.2 Introduction

The role of disease (pathogen or parasite infections) in biological invasions is increasingly of interest because of the multifaceted ways in which disease can mediate invasions. Disease-mediated invasions have recently been reviewed, indicating widespread and far-reaching effects, which can either facilitate or hinder invasions of vertebrate, invertebrate, and plant taxa (Strauss et al., 2012). Whether facilitating or hindering the invasion, the role of disease-mediated invasions has important implications for our understanding and management of invasive species, which damage forests and agricultural systems and incur large ecological and economic costs.

In North America, the European winter moth, *Operophtera brumata* L. (Lepidoptera: Geometridae), is one such non-native insect causing widespread defoliation in rural and urban settings. Winter moth was accidently introduced to Nova Scotia in the 1950s (Embree, 1966) and to the Pacific Northwest in the 1970s (Roland, 1986). It was first noted in the northeastern United States in the 1990s, near Boston, MA (Elkinton et al., 2010). Since its introduction to the northeastern United States (New England), winter moth has been in almost continuous outbreak and has spread to coastal New Hampshire, Maine, Rhode Island, Connecticut, and New York (Elkinton et al., 2014). In Massachusetts, outbreak densities of 500 pupae/m2 have resulted in 50% defoliation and significant damage to blueberry, cranberry, and apple crops (Elkinton et al., 2015). With

repeated defoliation, winter moth has caused growth reduction and mortality of deciduous trees (Embree, 1967; Simmons et al., 2014), resulting in far-reaching effects on forest and agricultural landscapes.

The outbreaks by winter moth were initially assumed to be caused by a native geometrid, such as Bruce spanworm (*Operophtera bruceata*, Hulst) (Elkinton et al., 2010). Bruce spanworm was suspected because it is prone to outbreaks (though relatively localized and short-lived outbreaks), is indigenous to the area (Brown, 1962), and is difficult to distinguish morphologically from winter moth. Additionally, Bruce spanworm produce and respond to the same pheromone compound as winter moth (Roelofs et al., 1982) and can hybridize with winter moth (Troubridge and Fitzpatrick, 1993; Elkinton et al., 2010, 2014). The cause of the widespread and persistent defoliation in Massachusetts was suspected to be winter moth because it remained in continuous outbreak levels each year rather than subsiding due to natural enemies as Bruce spanworm population tend to do. Upon close examination of adult females and subsequent DNA analyses, the outbreaks were definitely identified as being caused by winter moth in December of 2003 (Elkinton et al., 2010). The close relationship but differences in population dynamics between these two species can provide insight into possible biological control methods for winter moth.

Baculoviruses, most notably nucleopolyhedroviruses (NPVs), are important natural enemies of many Lepidoptera and appear to be particularly important in species whose populations undergo cycles of outbreak and collapse (Cory, 2010; Myers and Cory, 2013). They may play a vital role in the suppression of outbreak populations in Bruce spanworm. Bruce spanworm populations rarely exhibit outbreaks, and when a

Bruce spanworm outbreak does occur, it is usually localized and only lasts for a few years before the population collapses (Brown, 1962; Rose and Lindquist, 1997). In Bruce spanworm outbreaks that have been reported, NPVs have been noted. Smirnoff (1964) reported finding NPVs in Bruce spanworm collected in Quebec, Canada in 1961 and suggested that NPV infection was responsible for the successful control of an outbreak of Bruce spanworm on maple trees. Similarly, a Bruce spanworm outbreak at the same time in Nova Scotia was found to have NPV, and NPV was thought to have caused the decline of this population (Neilson, 1965). A decade later in Alberta, Canada, an outbreak of Bruce spanworm was also suppressed by an NPV epizootic. In this outbreak, while trees were sprayed with NPV derived from the caterpillars collected from the Alberta population the previous year, control plots also experienced significant mortality from the virus and had a prevalence of up to 77% of larvae (Ives and Cunningham, 1980), indicating high levels of NPV naturally occurring at this site.

Winter moth, on the other hand, is known to continuously outbreak in its introduced range (Embree, 1965; Roland, 1990; Elkinton et al., 2015) and an NPV epizootic has never been noted. Prior to the introduction of parasitoids as biological control agents in Nova Scotia and British Columbia, winter moth persisted at outbreak levels (Embree, 1965; Roland, 1990), and while the biocontrol agent builds up in New England, populations remain high (Elkinton et al., 2015). Following the introduction of winter moth to Nova Scotia, Neilson (1965) conducted surveys of winter moth in that province from 1955 to 1960 and none were found to be infected with NPV. In 1961, a single larva was found with NPV-like symptoms. Similarly, Cunningham et al. (1981) surveyed winter moth larvae in British Columbia and found low (0–1%) incidences of

naturally occurring NPV. Further, in the most recent North American invasion of winter moth in Massachusetts, only low levels of NPV have been detected, and no viral epizootic has been noted (Burand et al., 2011; Elkinton et al., 2015).

On the other hand, Neilson (1965) reported substantially more NPV-infected individuals and more mortality in his survey plots in Nova Scotia in the years following the 1960 survey. This decline of winter moth occurred simultaneously and sympatrically with a Bruce spanworm epizootic, which he speculated might have crossed over into winter moth (Neilson, 1965; Murdoch et al., 1985). Neilson (1965) found, however, that the occlusion bodies isolated from the two species were different in shape. He called for further research to determine if they were the same virus and if they cross-infect. Gillespie et al. (1978) inadvertently tried cross-infection trails on an outbreak of geometrids was reported on southern Vancouver Island, B.C., that was thought to be Bruce spanworm. They tried to infect caterpillars from this outbreak with Bruce spanworm virus. Infection was not successful, and it was later confirmed that the insect outbreaks were of winter moth and not Bruce spanworm. This suggested that cross infection does not occur, but when this work was done, it was not known if the Bruce spanworm virus stock was still infectious.

Identifying the NPV infecting Bruce spanworm, and evaluating the relationship of this virus to winter moth's NPV and others', are necessary steps towards understanding the role that disease plays in the current winter moth invasion as well as the earlier Nova Scotia invasion. We need to know if disease from Bruce spanworm is mediating the winter moth invasion or, alternately, if disease from winter moth can harm Bruce spanworm. Cross infection, as was proposed by Neilson (1965) and piloted inadvertently

5

by Gillespie et al. (1978), warrants careful testing. Additionally, we now have molecular techniques to help elucidate the relationship between the NPV found in Bruce spanworm and that found in winter moth.

We build on these earlier observations to address two questions: (1) What is the is phylogenetic relationship between the NPV infecting Bruce spanworm and the NPV infecting winter moth, and (2) in a laboratory setting, can Bruce spanworm-derived NPV infect winter moth and can winter moth-derived NPV infect Bruce spanworm.

1.3 Methods

1.3.1 Collecting larvae and rearing

To acquire virus samples and live specimens to rear eggs from, Bruce spanworm and winter moth larvae were collected from the field in late May of 2013 and 2014. The larvae were reared to adulthood and mated to produce eggs used the following springs (2014 and 2015) for cross infection trials. Any mortality (cadavers) from the collections was noted and inventoried for disease prevalence. In 2013, Bruce spanworm larvae were collected from a small outbreak population near Millinocket, Maine (Township 2, Region 8 NWP). This is 150 km inland of the area where winter moth has been detected in Maine and in an area that only has Bruce spanworm (Elkinton et al., 2010). In 2014, Bruce spanworm larvae were collected in the Mohawk Trail State Forest, Charlemont, MA, which is 60 km from any detected winter moth. Both years, winter moth larvae were collected as fourth and fifth instars in long-term study sites in eastern Massachusetts, where winter moth is abundant and pure winter moth make up almost 100% of the

genetic stock (Elkinton et al., 2010, 2014). Larvae were reared in batches of 500 or fewer in ventilated 20 L (5 gallon) buckets with the foliage from the tree species on which they were found. Larvae collected from different sites were reared in separate buckets; in this way winter moth larvae were reared separately from Bruce spanworm larvae. The foliage was mounted in Oasis Floral Foam bricks (Smithers-Oasis North America). Foliage was replaced every other day and any cadavers were removed. Larvae were reared for a week or less, which is not enough time for any NPV transmitted within the buckets to spread between individuals (Wigley, 1976). Any larval cadavers that were found were placed individually in sterile 1.5 mL microcentrifuge tubes (Fisherbrand) and immediately frozen at -20 °C. When the larvae started to show signs of pupating (thickening body shape and rolling a leaf edge over themselves), sifted peat moss was added to the bottom of the buckets for pupation. The resulting pupae were sifted, sorted by sex, and stored at 10–12 °C until adult emergence at the end of October to beginning of November.

To produce eggs for the cross-infection trials, adult moths were mated with their conspecifics. Batches of approximately 50 adults were placed together in clear, 350 mL (12-oz) cups lined with paper, in an incubator (Percival) set to 10–12 °C with a 13.5/10.5 h LD cycle. Females laid eggs on the paper lining of the mating cups. The temperature in the incubator with the eggs was reduced gradually to 2 °C by mid-January.

1.3.2 DNA amplification and sequencing

Larval cadavers that had been set aside from the rearing process and stored frozen were thawed and homogenized with 200 μL molecular grade water. An aliquot from each individual was screened for NPV using light microscopy. DNA was extracted from potentially NPV-infected cadavers using 250 μL DNAzol (Invitrogen Life Technologies), 50 μL sample (cadaver homogenized with 200 μL molecular grade water), and an additional 50 μL water.

DNA was amplified from two different loci—the polyhedrin gene region (polh, associated with the envelope protein) and the p74 gene region (a *per os* infectivity factor gene, associated with the virus envelope). To amplify the polyhedrin gene of NPV from Bruce spanworm, the primer set Polyh-81F/Polyh-249R, a generalized NPV polyhedrin primer, was used. Initially, the primer set ObPol1 and ObPol2, designed to detect winter moth NPV (Graham et al., 2004) was tried, but it was not successful in amplifying the Bruce spanworm NPV. For PCR using Polyh-81F/ Polhy249R, a reaction of 12.5 μL Taq 2X Master Mix (New England Biolands) with 5.5 μL RNAse free water, 1.5 μL MgCl2, and 0.5 μL of each primer was mixed with 4 μL DNA template for each sample. Reactions were run on an Eppendorf Mastercycler ep. using the following temperature profile: 95 °C for 3 min; 36 cycles of 95 °C for 30 s, 49.4 °C for 1 min, and 72 °C for 1 min; and a final extension of 72 °C for 10 min.

The resulting PCR product was run on an agarose gel, as described in Burand et al. (2011), and the resulting NPV DNA fragments from four Bruce spanworm individuals from the 2013 Millinocket, ME collection (sample number #7 and #8) were excised

from the gel, purified (Qiagen gel purification kit), and pooled to render enough DNA for sequencing. PCR products were Sanger sequenced at the University of Massachusetts Genomic Resource Laboratory on ABI Model 3130XL and 3100 sequencers yielding clean (>60HQ%) sequences. The sequence pairs (forward and reverse-complement) were aligned and edited using Geneious 8.1.8 (Biomatters Ltd.) to produce a consensus sequence.

For the p74 gene, DNA was extracted and amplified from Bruce spanworm and winter moth cadavers collected in 2013 using generalized p74 primers for Bruce spanworm and winter moth specific p74 primers (Supplementary S1) using the following temperature profile: 95 °C for 3 min; 36 cycles of 95 °C for 30 s, the primer specific annealing temperature (50.8 °C for winter moth and 52.1 °C for Bruce spanworm) for 1 min, and 72 °C for 1 min; and a final extension of 72 °C for 10 min. The resulting PCR product was run on an agarose gel and the resulting DNA fragments were excised from the gel, re-amplified using the p74 PCR procedure described above, and sequenced, as was described for the polyhedrin gene. To extend the Bruce spanworm sequence, a new primer (Table 1) was designed using NCBI Primer BLAST to acquire the flanking sequences at the leading and trailing ends of the read. These new primers were used with the same PCR protocol described above to amplify the sequence ends for the Bruce spanworm DNA extraction.

1.3.3 Polyhedrin and p74 gene comparison

The resulting polyhedrin sequence for Bruce spanworm and p74 sequences for both species were aligned with published sequences acquired from GenBank (Table 2). The sequence previously submitted by Burand et al. (2011) (accession No. HQ663848.1) was used for the winter moth polyhedrin gene comparison. The sequences were aligned using Geneious 8.1.8 (Kearse et al., 2012), adjusted by eye, and then truncated to the length of the shortest included gene sequence. JModelTest was used in the CIPRES Science Gateway (Miller et al., 2010) to select the best base-pair substitution model for each locus. HKY was selected as the best substitution model for analysis of the polyhedrin gene fragment and GTR was selected as the best model for p74. To infer the phylogenic relationships, we conducted neighbor-joining, Maximum Likelihood, and Bayesian analyses. Neighbor-joining analyses were implemented in Geneious (Kearse et al., 2012) using a Jukes-Cantor genetic distance model for both genes and 1000 bootstrap replication. Maximum likelihood analyses were run using PhyML (Guindon et al., 2010) using the designated substitution model for each locus. Support for each node was estimated using 1000 bootstrap replication. Bayesian analyses were run using MrBayes 3.2.6 (Huelsenbeck and Ronquist, 2001) with the designated substitution model for each locus. A MCMC chain of 1,000,000 was used with a burn in length of 10%. MabrNPV (accession No. JQ798165.1), with the lowest percent pairwise identity with our sequences, was used as the outgroup for both trees. The computed output was visualized using FigTree Version 1.4.2 (Rambaut, 2014).

1.3.4 Cross infection trials

To test for the possibility of cross infection, in spring 2014 and 2015 larvae of both winter moth and Bruce spanworm were exposed to virus from the other species. We ran four different combinations: (1) Bruce spanworm larvae exposed to Bruce spanworm virus, (2) Bruce spanworm exposed to winter moth virus, (3) winter moth exposed to Bruce spanworm virus, and (4) winter moth exposed to winter moth virus. Controls, with the larvae not receiving a virus inoculum, were also run for each larval type. Replicates were not balanced across treatments due to differences in rearing outcomes.

Batches of eggs, still on the paper lining of the mating cups, were surface-sterilized using a 2% bleach solution wash, followed by three rinses with distilled water, then allowed to air dry. The paper with the eggs was stapled to the inside of surface of the diet cup lid. A standard, high wheat germ gypsy moth diet was used (Bell et al., 1981). The diet was made at the USDA Animal and Plant Health Inspection Service lab at Otis ANGB, MA. The eggs and diet were moved from 2 °C storage to a 15 °C incubator to temperature induce hatch. The neonates were allowed to feed for 8–12 days with diet refreshed once during this time. When they reached their third instar (body ~1 cm long, head capsule 2 mm wide), individual larvae were placed singly in 1.5 mL Eppendorf microcentrifuge tubes and starved overnight. After starvation, the caterpillars were given a 0.5 cm^3 cube of the same gypsy moth diet formulation treated with 8 μl of dilute NPV inoculum—the maximum volume the diet cube could absorb.

The inoculum was prepared by combining 3–10 NPV-positive larval cadavers with 500 μL dH20, crushing with a sterile pestle, and allowing to settle overnight. The top layer was removed so that only the NPV concentrate at the bottom of the tube

remained, and an additional 500 μL dH20 was added. Separate inoculum solutions were made from infected winter moth cadavers and from infected Bruce cadavers. The resulting occlusion body (OB) concentrations were counted using a hemocytometer (American Optical Spencer Bright-Line, 1/10 mm deep) and the resulting concentration was found to be approximately 2.0×10^8 OB/mL for the winter moth inoculum and 1.0×10^8 OB/mL for the Bruce spanworm inoculum. The winter moth inoculum was diluted to 1.0×10^8 OB/mL and stored at 4 °C.

Treated larvae were stored at 22 °C and monitored daily. If the infected diet was completely consumed, additional non-infected diet was added as needed. Any dead caterpillars were removed and immediately frozen at -20 °C. A subset of the resulting larval cadavers were tested for NPV using PCR and gel electrophoresis as described above. Prior to DNA extraction from the larval cadavers, the surfaces of the larvae were rinsed three times with distilled water to remove any superficial virus particles, such that only internal virus would be detected.

1.4 Results

1.4.1 Polyhedrin and p74 gene phylogenetic comparisons

Larval cadavers were recovered from both winter moth and Bruce spanworm field collections with a higher percent mortality evident in both years from Bruce spanworm (Table 3). Using light microscopy on the recovered cadavers, occlusion bodies characteristic of NPV were found in both the winter moth samples as well as the Bruce spanworm. No noticeable difference in the shape was the occlusion body was noted. The

polyhedrin gene segment from Bruce spanworm (OpbrNPV_polh) had only an 83.0%

sequence similarity to that of the Massachusetts winter moth (OpbuNPV_polh) strain

(Table 4). However, the OpbuNPV and OpbrNPV sequences were more similar to each

other than to the gene sequences of viruses from the other lepidopteran species included

in the gene tree (Figure 1). Aligned p74 sequences for winter moth (OpbuNPV_p74) and

Bruce spanworm (OpbrNPV_p74) were only 81.2% similar to one another (Table 5), but

when considered with other Lepidoptera NPVs, also separated into their own clade

(Figure 2). For both loci, OpbuNPV and OpbrNPV fell out with the Group II

Alphabaculoviruses.

1.4.2 Cross infection trials

In the cross-infection trials, the Bruce spanworm virus (OpbrNPV) was able to

infect Bruce spanworm larvae (26.7% infected) but was unable to infect winter moth

larvae (0%; Table 6). Similarly, the winter moth virus (OpbuNPV) was able to infect

winter moth larvae (41.1% infection) but was unable to infect Bruce spanworm larvae

(0%). In a few cases (2 of 86 tested; 2.3%), when winter moth larvae were exposed to

Bruce spanworm virus the larvae appeared to die from winter moth NPV infection, as

determined by sequencing the NPV in the resulting cadavers. When Bruce spanworm

larvae were inoculated with the same Bruce spanworm virus (OpbrNPV), no winter moth

virus (OpbuNPV) was detected in the resulting cadavers. No virus was detected in the

controls.

Table 1: Primers used for PCR and sequencing.

Primer	Author	Primer Sequence	Fragment Size	Purpose
ObPol1 ObPol2	Graham et al. 2004	5'-CGAAATGAACGGAGCCTATCG-3' 5'-TCACCTTCTCGATGAACTCTTCG-3'	482 bp	Specific primer for winter moth polyhedron
Polyh-81F Polyh-249R	Unknown	5'-TTATGGATGTCTACCTAACTG-3' 5'-AGGCTGATTCTGTATTCG-3'	168 bp	Generalized primer for NPV polyhedron (amplifies Bruce spanworm but not winter moth polyhedron region)
p74-F p74-R	Rodríguez et al 2011	5'-CGCGGGTGCGANAGCATG-3' 5'-GAAACTCGCGCGGAAACAT-3'	1186 bp	Generalized primer for p74
p74-3 p74-4	Kim et al. 2011	5'-ATCCGTTTCACGAGACGG-3' 5'-ATGCCAACACCAGATCGC-3'	1116 bp	Specific primer for winter moth p74
BswP74-FA BswP74-RB	Matt Boucher	5'-GCCCTTGTGTTCGGCATTTT-3' 5'-AACGATGGCAGTAGGGCTTC-3'	200+ bp	Outward sequencing of known ends of p74 for Bruce spanworm

Table 2: Lepidoptera species used to construct the NPV phylogenetic estimates.

Virus genus	Virus	Virus host	Host common name	Host family	p74 Accession No.	Portion from	Polyhedron Accession No.	Portion from
Alphabaculovirus Group I	AgMNPV	*Anticarsia gemmatalis*	Velvetbean moth	Noctuidae	DQ813662.2	Complete genome	KR815463.1	Complete genome
Alphabaculovirus Group I	AcMNPV	*Autographa californica*	Alfalfa looper	Noctuidae	M31301.1	Complete cds	JN674785.1	Partial cds
Alphabaculovirus Group I	BomaNPV	*Bombyx mandarina*	Wild silkmoth	Bombycidae	JQ071499.1	Complete genome	DQ483053.1	Complete cds
Alphabaculovirus Group I	BmNPV	*Bombyx mori*	Domesticated silkmoth	Bombycidae	L33180.1	Complete genome	KR139829.1	Complete cds
Alphabaculovirus Group I	CfMNPV	*Choristoneura fumiferana*	Eastern spruce budworm	Tortricidae	M97904.1	Complete cds	AY327402.2	Complete cds
Alphabaculovirus Group I	HycuNPV	*Hyphantria cunea*	Fall webworm	Arctiidae	AP009046.1	Complete genome	AF300872.1	Partial cds
Alphabaculovirus Group I	PlxyMNPV	*Plutella xylostella*	Diamondback moth	Plutellidae	DQ457003.1	Complete genome	DQ457003.1	Complete genome
Alphabaculovirus Group II	AdhoNPV	*Adoxophyes honmai*	Summer fruit tortrix	Tortricidae	AP006270.1	Complete genome	AP006270.1	Complete genome
Alphabaculovirus Group II	EcobNPV	*Ectropis obliqua*	Tea looper	Geometridae	DQ837165.1	Complete genome	KC960018.1	Complete genome
Alphabaculovirus Group II	BusuNPV	*Buzura suppressaria*	Tung oil geometrid	Geometridae	KM986882.1	Complete genome	KM986882.1	Complete genome
Alphabaculovirus Group II	LafiNPV	*Lambdina fiscellaria*	Hemlock looper	Geometridae	KP752043.1	Complete genome	KP752043.1	Complete genome
Alphabaculovirus Group II	LdMNPV	*Lymantria dispar*	Gypsy moth	Erebidae	AF081810.1	Complete genome	AF499687.1	Complete cds
Alphabaculovirus Group II	LyxyNPV	*Lymantria xylina*	Casuarina tussock moth	Lymantriidae	GQ202541.1	Complete genome	GQ202541.1	Complete genome
Alphabaculovirus Group II[a]	OpbuNPV	*Operophtera brumata*	Winter moth	Geometridae	KY064006	Partial cds	HQ663848	Partial cds
Alphabaculovirus Group II[a]	OpbrNPV	*Operophtera bruceata*	Bruce spanworm	Geometridae	KY064005	Partial cds	KY064007	Partial cds
Alphabaculovirus Group II	SeMNPV	*Spodoptera exigua*	Beet armyworm	Noctuidae	HG425347.2	Complete genome	GQ392064.1	Partial cds
Alphabaculovirus Group II	SfMNPV	*Spodoptera frugiperda*	Fall armyworm	Noctuidae	EF035042.2	Complete genome	KC845532.1	Partial cds
Alphabaculovirus Group II	TnSNPV	*Trichoplusia ni*	Cabbage looper	Noctuidae	DQ017380.1	Complete genome	JN674704.1	Partial cds
Alphabaculovirus Group II	MabrNPV	*Mamestra brassicae*	Cabbage moth	Noctuidae	JQ798165.1	Complete genome	AB198073.1	Complete cds

[a] Suggested placement.

Table 3: Larval collections and mortality.

Species	Year	Collection site	Number of larvae collected	Larval cadavers recovered	Percent mortality	Number of cadavers with NPV	Percent of cadavers with NPV
Bruce spanworm	2013	Millinocket, ME	433	173	40.0	8	4.6
Winter moth	2013	Across Massachusetts Sites	15677	203	1.3	56	27.6
Bruce spanworm	2014	Charlemont, MA	170	25	14.7	5	20.0
Winter moth	2014	Across Massachusetts Sites	17347	600	3.5	198	33.0

Table 4: Polyhedrin Gene Distance matrix.

	OpbuNPV	OpbrNPV	AgMNPV	AcMNPV	BomaNPV	BmNPV	CfMNPV	HycuNPV	PlxyMNPV	AdhoNPV	EcobNPV	BusuNPV	LafiNPV	LdMNPV	LyxyMNPV	SeMNPV	SfMNPV	TnSNPV	MabrNPV
OpbuNPV		83.0	73.7	80.1	68.4	68.4	68.4	80.1	80.1	73.7	74.3	74.3	76.0	68.4	67.3	73.1	73.7	75.7	77.8
OpbrNPV	83.0		70.8	77.8	69.0	69.0	68.4	77.8	77.8	77.8	79.5	80.1	81.3	71.3	71.3	73.7	70.8	76.9	75.4
AgMNPV	73.7	70.8		76.0	80.1	80.1	77.8	76.0	76.0	73.7	77.2	77.2	76.6	71.9	69.0	72.5	74.9	77.8	76.0
AcMNPV	80.1	77.8	76.0		71.3	71.3	77.2	100.0	100.0	80.1	80.7	75.4	77.2	72.5	69.6	76.0	78.9	86.0	84.2
BomaNPV	68.4	69.0	80.1	71.3		100.0	76.0	71.3	71.3	68.4	73.1	65.5	69.0	71.9	71.9	69.6	73.1	70.2	73.1
BmNPV	68.4	69.0	80.1	71.3	100.0		76.0	71.3	71.3	68.4	73.1	65.5	69.0	71.9	71.9	69.6	73.1	70.2	73.1
CfMNPV	68.4	68.4	77.8	77.2	76.0	76.0		77.2	77.2	74.9	80.1	71.9	73.1	71.3	69.0	71.9	74.3	72.5	74.3
HycuNPV	80.1	77.8	76.0	100.0	71.3	71.3	77.2		100.0	80.1	80.7	75.4	77.2	72.5	69.6	76.0	78.9	86.0	84.2
PlxyMNPV	80.1	77.8	76.0	100.0	71.3	71.3	77.2	100.0		80.1	80.7	75.4	77.2	72.5	69.6	76.0	78.9	86.0	84.2
AdhoNPV	73.7	77.8	73.7	80.1	68.4	68.4	74.9	80.1	80.1		80.7	80.7	74.3	70.2	68.4	77.2	78.4	79.8	79.5
EcobNPV	74.3	79.5	77.2	80.7	73.1	73.1	80.1	80.7	80.7	80.7		86.0	80.7	73.7	70.2	77.2	77.8	78.9	81.9
BusuNPV	74.3	80.1	77.2	75.4	65.5	65.5	71.9	75.4	75.4	80.7	86.0		80.1	66.7	66.7	81.9	74.9	78.1	77.2
LafiNPV	76.0	81.3	76.6	77.2	69.0	69.0	73.1	77.2	77.2	74.3	80.7	80.1		70.2	69.6	75.4	77.2	74.9	77.8
LdMNPV	68.4	71.3	71.9	72.5	71.9	71.9	71.3	72.5	72.5	70.2	73.7	66.7	70.2		91.7	67.8	72.5	71.1	71.3
LyxyMNPV	67.3	71.3	69.0	69.6	71.9	71.9	69.0	69.6	69.6	68.4	70.2	66.7	69.6	91.7		70.2	72.5	68.1	71.9
SeMNPV	73.1	73.7	72.5	76.0	69.6	69.6	71.9	76.0	76.0	77.2	77.2	81.9	75.4	67.8	70.2		84.2	76.0	83.0
SfMNPV	73.7	70.8	74.9	78.9	73.1	73.1	74.3	78.9	78.9	78.4	77.8	74.9	77.2	72.5	72.5	84.2		76.6	80.1
TnSNPV	75.7	76.9	77.8	86.0	70.2	70.2	72.5	86.0	86.0	79.8	78.9	78.1	74.9	71.1	68.1	76.0	76.6		79.8
MabrNPV	77.8	75.4	76.0	84.2	73.1	73.1	74.3	84.2	84.2	79.5	81.9	77.2	77.8	71.3	71.9	83.0	80.1	79.8	

Table 5: p74 Gene Distance matrix.

	OpbuNPV	OpbrNPV	AgMNPV	AcMNPV	BomaNPV	BmNPV	CfMNPV	HycuNPV	PlxyMNPV	AdhoNPV	EcobNPV	BusuNPV	LafiNPV	LdMNPV	LyxyMNPV	SeMNPV	SfMNPV	TnSNPV	MabrNPV
OpbuNPV		81.2	61.2	62.3	62.1	61.8	58.1	60.8	62.5	62.0	61.8	58.5	56.0	58.5	59.6	60.0	61.2	61.4	58.7
OpbrNPV	81.2		61.1	62.7	62.6	62.5	61.5	61.4	62.7	60.5	62.3	59.1	57.7	59.9	61.4	60.9	61.7	62.7	58.8
AgMNPV	61.2	61.1		73.7	73.6	73.2	76.6	77.7	73.7	58.3	62.0	61.0	61.1	61.9	63.5	61.6	60.3	58.1	59.1
AcMNPV	62.3	62.7	73.7		99.9	94.5	72.8	72.3	99.7	59.4	63.2	59.7	60.9	62.4	63.3	62.7	59.2	59.6	57.8
BomaNPV	62.1	62.6	73.6	99.9		94.6	72.7	72.2	99.6	59.3	63.3	59.9	60.8	62.4	63.3	62.8	59.1	59.5	57.9
BmNPV	61.8	62.5	73.2	94.5	94.6		72.1	70.9	94.6	60.1	63.2	59.6	61.6	61.4	62.7	62.7	59.2	60.2	58.5
CfMNPV	58.1	61.5	76.6	72.8	72.7	72.1		80.4	72.8	57.9	61.0	57.0	59.3	63.3	65.1	63.5	61.1	57.8	55.4
HycuNPV	60.8	61.4	77.7	72.3	72.2	70.9	80.4		72.3	58.5	60.3	57.4	57.9	61.5	62.1	61.2	58.9	58.6	55.9
PlxyMNPV	62.5	62.7	73.7	99.7	99.6	94.6	72.8	72.3		59.4	63.1	59.6	60.9	62.4	63.3	62.5	59.1	59.7	57.9
AdhoNPV	62.0	60.5	58.3	59.4	59.3	60.1	57.9	58.5	59.4		62.0	60.1	55.6	59.2	61.4	62.3	60.7	61.1	60.5
EcobNPV	61.8	62.3	62.0	63.2	63.3	63.2	61.0	60.3	63.1	62.0		69.4	63.6	65.1	66.0	65.9	62.3	65.6	62.8
BusuNPV	58.5	59.1	61.0	59.7	59.9	59.6	57.0	57.4	59.6	60.1	69.4		63.8	60.9	63.3	60.7	60.9	63.1	59.1
LafiNPV	56.0	57.7	61.1	60.9	60.8	61.6	59.3	57.9	60.9	55.6	63.6	63.8		60.4	59.4	61.0	58.4	58.3	58.5
LdMNPV	58.5	59.9	61.9	62.4	62.4	61.4	63.3	61.5	62.4	59.2	65.1	60.9	60.4		92.3	66.7	60.3	60.3	58.3
LyxyMNPV	59.6	61.4	63.5	63.3	63.3	62.7	65.1	62.1	63.3	61.4	66.0	63.3	59.4	92.3		65.8	60.9	60.9	58.9
SeMNPV	60.0	60.9	61.6	62.7	62.8	62.7	63.5	61.2	62.5	62.3	65.9	60.7	61.0	66.7	65.8		70.9	62.9	63.2
SfMNPV	61.2	61.7	60.3	59.2	59.1	59.2	61.1	58.9	59.1	60.7	62.3	60.9	58.4	60.3	60.9	70.9		61.2	62.5
TnSNPV	61.4	62.7	58.1	59.6	59.5	60.2	57.8	58.6	59.7	61.1	65.6	63.1	58.3	60.3	60.9	62.9	61.2		62.9
MabrNPV	58.7	58.8	59.1	57.8	57.9	58.5	55.4	55.9	57.9	60.5	62.8	59.1	58.5	58.3	58.9	63.2	62.5	62.9	

Table 6: Cross-infection trials larvae infection results.

Larvae exposed	Treatment	No. Larvae (N)	% shown to have OpbuNPV	% shown to have OpbrNPV
Bruce spanworm	OpbrNPV	15	0.0	26.7
	OpbuNPV	7	0.0	0.0
	Control	27	0.0	0.0
Winter moth	OpbrNPV	245	2.3	0.0
	OpbuNPV	140	41.1	0.0
	Control	8	0.0	0.0

OpbrNPV = virus from Bruce spanworm; OpbuNPV = virus from winter moth.
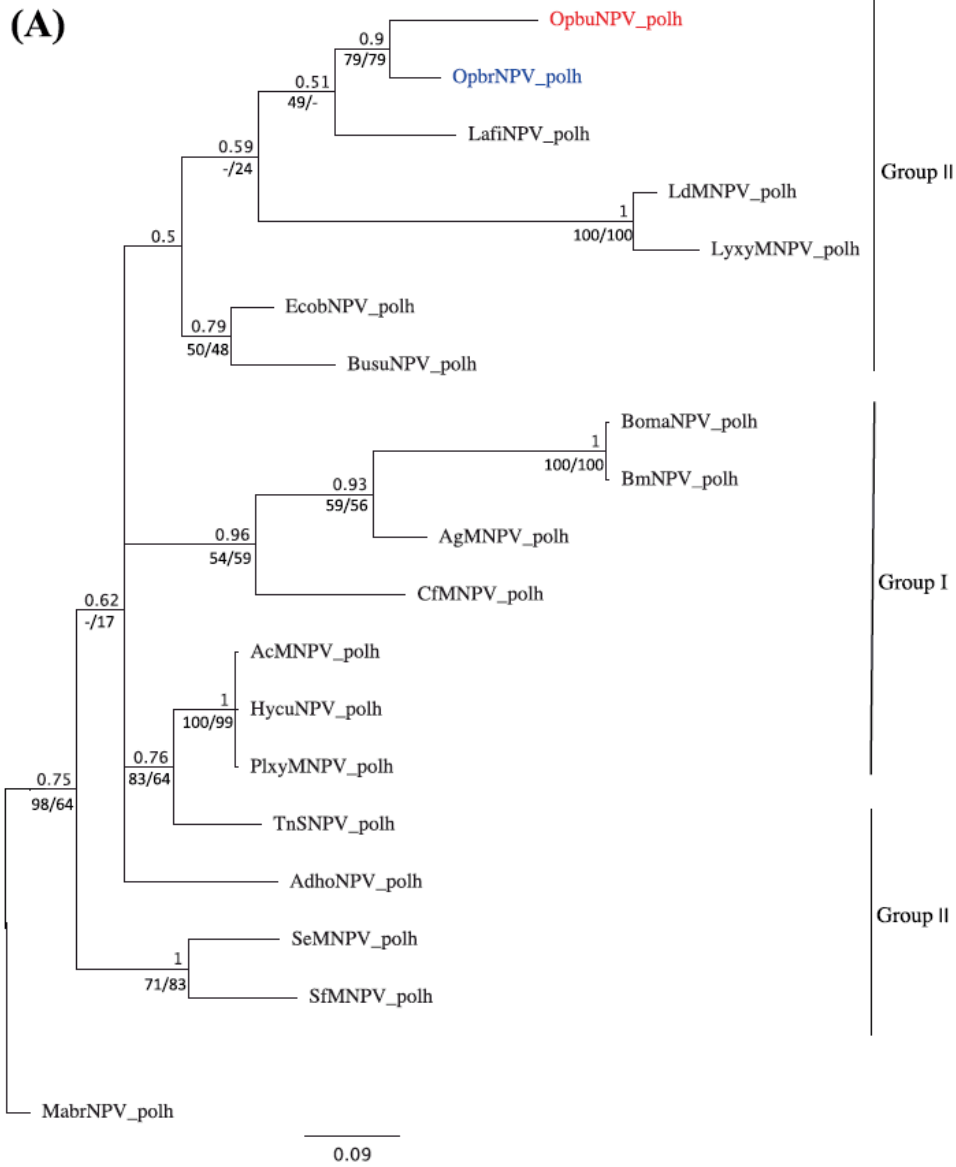
Figure 1: Phylogenetic tree of the NPV polyhedrin gene nucleotides. The sequences acquired from winter moth (OpbuNPV) are shown in red and those from Bruce spanworm (OpbrNPV) are shown in blue. Node support values are Bayesian posterior probability (probability out of 1) with Neighbor Joining/Maximum likelihood support (support out of 100) below.
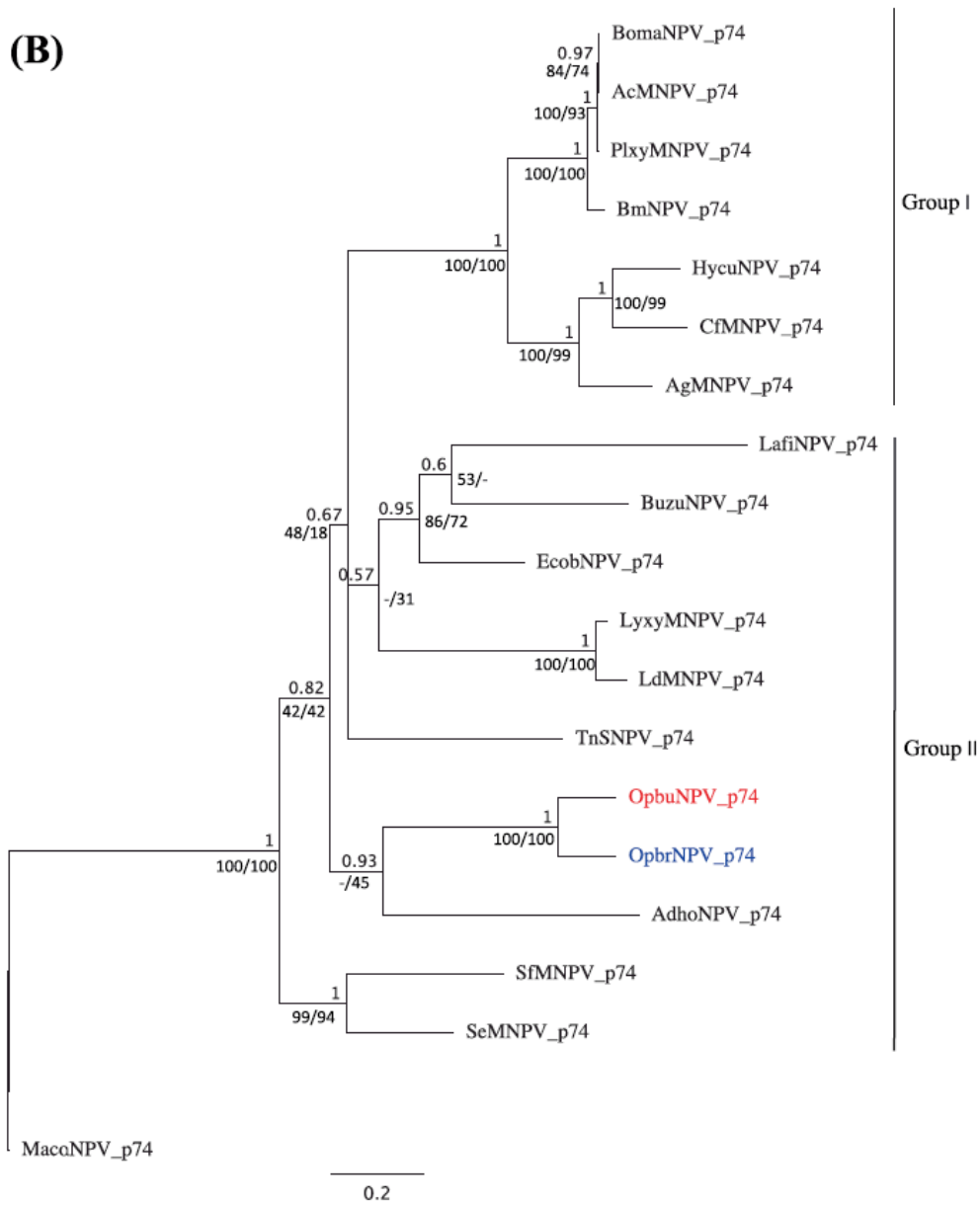
Figure 2: Phylogenetic tree of the p74 envelope protein gene. The sequences acquired from winter moth (OpbuNPV) are shown in red and those from Bruce spanworm (OpbrNPV) are shown in blue. Node support values are Bayesian posterior probability (probability out of 1) with Neighbor Joining/Maximum likelihood support (support out of 100) below.

1.5 Discussion

The winter moth and Bruce spanworm have distinct NPV species, but the NPV are more closely related to each other than to viruses taken from other host species, including other Geometridae. The ObPol1/ObPol2 primer set, designed for winter moth, did not amplify Bruce spanworm virus DNA while the general polyhedrin primer, Polyh-81F/Polyh-249R, amplified Bruce spanworm virus DNA, but not that of winter moth. This was the first indication that the two sequences were distinct. The closest match to the resulting Bruce spanworm NPV polyhedrin sequence on Genbank was that of Massachusetts winter moth NPV strain (OpbuNPV_polhMA) as published by Burand et al. (2011). When compared to sequences obtained from a United Kingdom winter moth also published in Burand et al. (2011), the match was just slightly less similar. However, when compared to other Lepidoptera NPV sequences, including other NPVs derived from geometrids (*Ectropis obliqua, Buzura suppressaria*, and *Lambdina fiscellaria*), OpbuNPV matched OpbrNPV more closely than any other sequence. The overarching phylogenetic relationships of the viruses between the two trees are slightly different, but in both trees the winter moth and Bruce spanworm NPVs formed part of a clade within the Alphabaculovirus Group 2 sequences. Not surprisingly, the relationships are more resolved in the p74 tree than in the polh tree. The polh region has been traditionally used for simple molecular identification and has many published sequences available for comparison; however, its utility in resolving phylogenetic relationships has been questioned (Lange et al., 2004; van Oers et al., 2004). The p74 gene has been shown to be conserved and highly host specific, thus providing a more reliable phylogenetic tree (Haas-Stapleton et al., 2004). Overall, the trees show that the NPVs infecting these two

congeners are closely related Group 2 Alphabaculoviruses, but they have distinct viral lineages.

Previous studies examining cross-infectivity have indicated that the host range of NPVs is highly variable. Many tend to have narrow host ranges, confined to a single species or family, but a few (e.g. AfMNPV and AcMNPV) have broad host ranges extending across multiple families of Lepidoptera (Hostetter and Puttler, 1991; Cory and Myers, 2003). Our cross-infection trials show that despite the close phylogenetic relationship between Bruce spanworm and winter moth and the parallel relationship between their respective NPVs, the viruses cannot cross-infect and are specialized on a single host species. We know that the virus inoculum was viable; when Bruce spanworm larvae were given NPV derived from Bruce spanworm, some of the Bruce spanworm cadavers showed detectable levels of Bruce spanworm NPV. Similarly, when winter moth larvae were given winter moth derived NPV, some of the winter moth cadavers showed detectable levels of winter moth NPV. This shows that, contrary to previous suggestions (Neilson, 1965), winter moth does not appear to be vulnerable to Bruce spanworm NPV and thus, winter moth's invasion is not likely mediated by Bruce spanworm's virus. This also means that if winter moth virus were to be considered as a microbial control in North America, it is unlikely to affect Bruce spanworm populations.

Interestingly, some winter moth NPV was detected when winter moth was exposed to Bruce spanworm NPV. Covert infections can be triggered and can switch to overt infections (Murillo et al., 2011; Cory, 2015). It is possible that when the winter moth larvae were exposed to the Bruce spanworm virus, this stress served as the trigger inducing an overt expression of the previously covert (latent) OpbuNPV infection in the

19

eggs. Similar results have been found in other lepidopteran NPV studies (Jurkovicova, 1979; McKinley et al., 1981; Hughes et al., 1993; Cooper et al., 2003); these studies show that a host's covert infection can be activated when exposed to a similar but heterologous NPV. It is also possible that our results reflect contamination by the winter moth virus either in the egg sterilization process, during larval feeding, or in the inoculum; however, this is unlikely as no control samples showed contamination and when Bruce spanworm larvae were given the same inoculum, no winter moth NPV was detected any the resulting cadavers.

To compare the viruses for this study, winter moth and Bruce spanworm larvae were collected from the field. As was found in other studies (Elkinton et al., 2015), winter moth was abundant, easy to find across eastern Massachusetts, and maintained high density populations year to year. On the other hand, Bruce spanworm larvae could only be found in small, isolated populations which varied year to year. The Bruce spanworm collection site in Maine used in 2013 was checked the following year (2014) and Bruce spanworm densities were found to have declined precipitously, likely due to an epizootic (Brown, 1962; Rose and Lindquist, 1997). Similarly, in our rearing of field-collected larvae, Bruce spanworm experienced more mortality than winter moth. These observations reflect the difference in population dynamics between the two species.

While Bruce spanworm larvae experienced higher mortality rates, there was no notable trend in the percent of cadavers with NPV between the two species. The low rates of NPV infection in our winter moth larvae field collections are consistent with those found in other studies both in the introduced and native range of winter moth. In New England, where the current study was conducted, comparable incidences of larvae with

NPV have been noted (Burand et al., 2011; O'Donnell, 2015). In prior introductions in Nova Scotia and British Columbia, disease was also not noted as an important source of mortality in winter moth (Cunningham et al., 1981; Roland and Embree, 1995). In its native range, winter moth experiences NPV, but historically at low prevalence levels and seemingly without significant effects on its population dynamics (Feeny, 1970; Varley et al., 1973; Wigley, 1976). NPV caused mortality above 50% only in outbreaks of winter moth on heather in Orkney, U.K. (Graham et al., 2004) likely due to suboptimal food quality, which has been shown to correlate with incidences of NPV (Raymond et al., 2005; Raymond and Hails, 2007). For Bruce spanworm, the high larval mortality, but relatively low NPV infection may be because there is another dominant source of mortality in Bruce spanworm.

Overall, our results do not support the suggestion that previously reported epizootics of NPV in winter moth in Canada originated from Bruce spanworm. Similarly, in Massachusetts, Bruce spanworm virus is likely not mediating the winter moth invasion. However, it appears that Bruce spanworm larvae experience more mortality than winter moth larvae, thus further work should be conducted to study the role other pathogens and parasites on the populations of these two species.

CHAPTER 2

**IDENTIFICATION AND IMPACT OF HYPERPARASITOIDS AND
PREDATORS AFFECTING *CYZENIS ALBICANS* (TACHINIDAE), A
RECENTLY INTRODUCED BIOLOGICAL CONTROL AGENT OF WINTER
MOTH (*OPEROPHTERA BRUMATA* L.) IN THE NORTHEASTERN U.S.A.**

2.1 Abstract

The success or failure of an introduced biological control agent may depend on its rate of mortality from disease, predation, and hyperparasitism. *Cyzenis albicans* Fallén (Diptera: Tachinidae) was introduced to the northeastern U.S. as a biocontrol agent of the invasive species winter moth, *Operophtera brumata* L. (Lepidoptera: Geometridae). This study aimed to determine the rates of mortality from predation by generalist ground predators and hyperparasitism of *C. albicans* puparia, identify any hyperparasitoids, and assess the impact of predation and hyperparasitism on the potential success of *C. albicans* in controlling the winter moth. Mortality of *C. albicans* puparia was primarily due to predation, but there was also hyperparasitism. Predation and parasitism of *C. albicans* puparia were consistently high across the six study sites and two years of study, but somewhat lower than was reported from British Columbia, where successful establishment of *C. albicans* in the 1970s was followed by a decrease in winter moth densities. In this study, three genera of ichneumonid hyperparasitoids were detected and identified using a combination of morphological and molecular approaches: *Phygadeuon* (1 species), *Pimpla* (2 species), and *Gelis* (2 species), all of which contain species with broad host ranges and were likely acting as facultative hyperparsitoids. We conclude that

while total mortality of *C. albicans* puparia is high, it is unlikely to have a significant effect on biological control of winter moth in this system, although it may explain why *C. albicans* has been slow to establish in this region. Our study emphasizes the importance of assessing the mortality of introduced biological control agents caused by native predators and hyperparasitoids.

2.2 Introduction

High mortality from disease, predation, and hyperparasitism has the potential to interfere with introduced parasitoids in biological controlprograms and can reduce their efficacy against targeted pest species in a diversity of insect taxa (Ehler, 1979; Hajek, 2004; Kellogg et al., 2003; McDonald and Kok, 1991; McNeil and Rabb, 1973; Schooler et al., 2011; Strauss, 2012; Sullivan and Völkl, 1999). Natural enemy cultures established during foreign exploration are routinely screened for any hyperparasitoids prior to introduction (Goldson, et al., 2014; Van Driesche et al., 2008). However, whereas hyperparasitism may exist in a food web, it may or may not affect the effectiveness of the natural enemy (Flanders, 1963; Hassell, 1969, 1980; McNeil and Rabb, 1973; Nofemela, 2013). Although the impact of hyperparasitoids in biological control programs may be important, relatively few assess the impact of hyperparasitism and other sources of agent mortality during post-release monitoring (Mills and Gutierrez, 1996; Schooler et al., 2011).

Classical biological control has been implemented in the northeastern United States to manage outbreaks of winter moth, Operophtera brumata L., an invasive geometrid that was accidently introduced in the 1990s and since has been causing heavy

defoliation to hardwood trees in both urban and forest settings, as well as damage to commercial blueberry, apple, and cranberry crops (Elkinton et al., 2014a; Simmons et al., 2014). Since its initial introduction near Boston, MA, winter moth has spread west into central Massachusetts, south into Rhode Island and Connecticut, and north along coastal New Hampshire and Maine (Elkinton et al., 2010, 2014b). Following earlier successes using the tachinid fly *Cyzenis albicans* (Fallén) as a classical biological control agent in Nova Scotia and British Columbia, Canada (Murdoch et al., 1985; Roland and Embree, 1995), it was first introduced to the northeastern United States in 2004.

In the northeastern U.S., eggs of winter moth hatch at the time of bud-break of its host plant (Elkinton et al., 2014a). *Cyzenis albicans* lays microtype eggs on the edge of partially defoliated leaves in the spring, and a portion of the eggs are inadvertently ingested by late instar winter moth larvae (Embree and Sisojevic, 1965; Hassell, 1969, 1980). When a fly egg is consumed by a winter moth larva, it hatches and migrates to the larva's salivary glands where it remains until the caterpillar drops to the soil and pupates in mid-late May (Elkinton et al., 2014a). *Cyzenis albicans* develops fully inside its host pupa and emerges as an adult the following spring (Embree and Sisojevic, 1965; Hassell, 1980). Because *C. albicans* spend the majority of their lives (10–11 months) in the soil as puparia, they are highly vulnerable to pupal mortality by predation and parasitism (Hassell, 1969; Roland, 1990). In contrast, healthy winter moth pupate for 6–7 months, emerge as adults in early winter from late November through early January, and then overwinter as eggs (Elkinton et al., 2014a).

For the winter moth biological control program in the northeastern U.S., *C. albicans* flies were collected from Vancouver Island, British Columbia starting in 2004

and first released in the northeastern U.S. in spring 2005; subsequent collections from the same locations in British Columbia were released the following year and every spring until the final collection in 2014, at which time the flies had established at 11 sites (Elkinton et al., 2014a). Subsequent collections of flies were made from sites with high parasitism in Massachusetts for release in winter moth outbreak sites in Massachusetts, Connecticut, Rhode Island, and Maine. Analysis of the population dynamics of *C. albicans*, and the degree of winter moth population control it provides, are ongoing (Elkinton et al., 2014a).

Studies of pupal mortality in the winter moth-*C. albicans* system in the northeastern United States revealed the presence of hyperparasitoids (HJB, pers. observ.). Previous studies found hyperparasitism of *C. albicans* in invasive winter moth populations in British Columbia (Humble, 1985; Roland and Embree, 1995). Although hyperparasitism was found to be a cause of Cyzenis spp. mortality in British Columbia (Humble, 1985; Roland and Embree, 1995), subsequent control of winter moth by *C. albicans* was still deemed a success (Murdoch et al., 1985; Roland, 1990; Van Driesche et al., 2008). Hyperparasitism of *C. albicans* was not detected in Nova Scotia (Embree, 1965; MacPhee et al., 1988; Pearsall and Walde, 1994), which is closer in geographic proximity to the population studied here. However, the goal of the investigations in Nova Scotia was not necessarily detection of hyperparasitism, so any hyperparasitoids may have been missed. Hyperparastism was also noted in studies conducted on native populations of winter moth and *C. albicans* in England (Hassell, 1969, 1980). Little is known about hyperparasitoids of *C. albicans* in the northeastern U.S. other than that they are present.

In this study, we investigated hyperparasitism of *C. albicans* puparia by deploying sentinel winter moth cocoons parasitized by *C. albicans*. More specifically, this study aimed to (1) quantify the overall mortality and hyperparasitism of *C. albicans* puparia, (2) identify the species of hyperparasitoids present, and (3) infer the potential impact of hyperparasitism on biological control of winter moth by *C. albicans*.

2.3 Methods

2.3.1 Deployment of sentinel puparia

Pupae deployed as sentinels were reared from spring collections of larvae obtained from long-term study plots in eastern Massachusetts (Elkinton et al., 2014a). Larvae were reared in batches of up to 500 in ventilated 20 L (5 gallon) buckets with foliage from the collection tree species. When larvae showed signs of pupation (thickening body shape and rolling a leaf edge over themselves), sifted peat moss was added to the bottom of the buckets for pupation. Peat moss was pre-sifted through a screen (with 3mm x 3mm openings). This allows for later removing peat through the same screen, while not letting pupae pass through. The resulting pupae were non-destructively evaluated under a dissecting microscope (M5A Wild Heerbrugg stereo) for *C. albicans* parasitism. Winter moth pupae were determined as parasitized if the winter moth integument flaked away easily and thus revealed the darker, smoother integument of a *C. albicans* puparium within. The *C. albicans* puparia were set aside for the study.

Cyzenis albicans puparia were deployed in sets of 100 puparia at six sites across the northeastern U.S. from 2015 to 2016, and data on all hyperparasitism and mortality

26

were recorded. The study sites were chosen to coincide with winter moth long-term study sites and to reflect a range of *C. albicans* establishment (Elkinton et al., 2014a). This included sites that have *C. albicans* establishment, as well as sites that do not have *C. albicans* introduced yet. The study sites were all in mix hardwood forests dominated by red oak (*Quercus rubra*) and red maple (*Acer rubrum*). To estimate winter moth pupae density and percent *C. albicans* parasitism, 16 buckets (16 cm width x 28 cm length x 10 cm height), filled 3 cm deep with sifted peat moss, were placed under each study tree in late May before pre-pupal winter moth caterpillars began to spin down from the tree canopies at each site. Each bucket was placed at a randomly selected distance between the tree stem and the edge of the tree canopy along one of eight evenly spaced directions radiating from the tree stem as described in Varley et al. (1973) and Whited (2007). Parasitism rates on winter moth by *C. albicans* were estimated from collections of 100 to 500 late instar larvae collected from a range of host trees at each site. From these values, we calculated the corresponding *C. albicans* density for each plot except for the Kingston, RI site, from which no density estimates were taken.

The *C. albicans* puparia were deployed at six sites each year of the study (Table 7) in two or three consecutive rounds of deployments that ran from mid-summer to mid-autumn. In 2015, two rounds were completed, whereas in 2016 three rounds were completed. In 2015, the first round ran from 5 August until 18 September 2015, and the second round ran from 18 September until 31 October 2015. In 2016, the first round ran from 7 July until 8 August 2016, the second round ran from 8 August until 18 September 2016, and the third round ran from 18 September until 1 November 2016.

27

For each of the two or three consecutive deployments per year, the sets of 100 puparia each were deployed haphazardly under the drip line of a red oak (Quercus rubra L.) at each study site. The puparia were secured to burlap squares in their cocoons using beeswax (Elkinton et al., 1996; Whited, 2007). The purpose of the burlap was to enable us to relocate the deployed cocoons later and provide conclusive evidence that predators had removed a cocoon. Earlier experiments by Whited (2007) showed that predation rates of winter moth pupae deployed in this way were indistinguishable from those of cocoons buried directly in the soil without any manipulations. The cocoons containing the puparia on their burlap squares were buried 2.5 cm deep in the soil in sets of five tethered samples, meaning that five burlap squares with their puparia were secured approximately 0.5m apart along a nylon string and buried as a set. This depth was chosen to mimic natural winter moth pupae depths, which are known to be buried but within the upper 5 cm of soil (Embree, 1965; East, 1974; Holliday, 1977). After each round, the puparia were retrieved and stored at 12 °C in an incubator (Percival) in constant dark until analysis.

2.3.2 Examination and dissection of puparia

The remains of the *C. albicans* puparia were examined and scored as survived, diseased, preyed upon, or parasitized. Healthy, intact puparia were returned to their cocoon (original cocoon made by the winter moth during pupation) to protect them from jostling and maintain their moisture balance and then removed from their burlap square. To allow the fly or any hyperparasitoids to develop, intact puparia were stored in the Percival at 12 °C in batches of up to 50 individuals in sterile, 100mmÅ~15mm

polystyrene petri dishes (Fisherbrand) with a mesh lid for ventilation. All other burlap squares and puparia were discarded after being scored. The overwintering storage temperature of the puparia was lowered to 9.5 °C at the beginning of December, to 5 °C at the end of December, and to 2 °C in early January to parallel outdoor ground temperatures. During this time, they were watered once a month with water treated with sodium propionate to prevent growth of mold. Starting in late March, the storage temperature was increased again to 12 °C in increments of 4 °C until they were taken out of storage and kept at room temperature in April. The intact puparia were checked for hyperparasitoids (either emerged or pharate adults), and pupae scoring records for each deployment were updated to account for any additional parasitism that was noted. Any samples that had hyperparasitoid adults or larvae were stored at −20 °C for follow up morphological and molecular identification.

2.3.3 Mortality estimate

Mortality of puparia were estimated for each deployment of sentinel cocoons, and cumulative mortality was estimated for each year. The proportion of total puparia that did not survive was calculated by dividing the sum of those puparia that were preyed upon or parasitized for each deployment by the total number of pupae. Predation was inferred for cocoons and/or pupae that had been pulled off the burlap square, puparia with only the crushed cuticle remaining, puparia with holes chewed in them, and evidence of teeth or claw marks left in the wax. Parasitism was inferred for any puparia with distinct wasp emergence holes and puparia that yielded wasp adults or larvae. The total number of puparia that did not survive was tallied as the sum of samples eliminated by predation

29

and parasitism. For the purposes of this study, we excluded mortality due to unknown causes, including potentially diseased, moldy, or desiccated samples. Such puparia accounted for a small proportion of mortality ($< 6\%$) and possibly occurred as a result of rearing conditions.

The proportion of puparia that suffered predation was calculated by dividing the number of puparia with evidence of predation by the total number of puparia. Hyperparasitism was subsequently calculated as a proportion out of puparia that remained after predation (i.e. number of pupae that survived predation that were hyperparasitized divided by the number of pupae surviving predation). This way of calculating mortality sequentially was employed by Varley and Gradwell (1968) in their work on winter moth in Europe and was also inherent in the marginal rate calculations introduced by Royama (1981) and Elkinton et al. (1992). The calculations include the observation that parasitism rates can be obscured by predation rates because predation will always 'win' over parasitism. Therefore, if predators do not discriminate between healthy and parasitized *C. albicans* puparia, then predators can remove a proportion of the parasitized puparia before we have a chance to calculate parasitism rates. The marginal rate of hyperparasitism accounts for this.

Mortality rates were converted to survival rates (S_i) by subtracting the proportion dying from one ($1-M_i$). These then were standardized to a mean daily survival rate across 42 days (6 weeks) by taking the nth root of the proportion surviving, where n is the number of days deployed. The daily survival rates were then raised to an exponent of 42 to yield the expected survival over 42 days ($S_{42}=[(S_i)^{1/n}]^{42}$). This conversion enabled us to compare mortalities across years and months in the face of small differences in the

30

number of days (32–45) that puparia were deployed. The normalized mortality proportions (M_{42}) were calculated as $M_{42}=1-S_{42}$. Cumulative survivorship values were calculated as the product of successive normalized survivorships of each deployment (S_T=1st deployment, S_{42} x 2nd deployment, S_{42} x 3rd deployment, S_{42}). The cumulative predation, hyperparasitism, and mortality values were calculated by subtracting the cumulative survival of each respectively from 1 (e.g. $M_T=1-S_T$).

2.3.4 Statistical analysis

Mortality results were visualized using JMP Pro 12.1.0 (SAS Institution Inc.) and statistical analyses run using RStudio 1.0.136 and JMP Pro. With the exception of the analysis of hyperparasitism across the 2016 season (which used all three deployments), all statistical analyses used only the data from two overlapping 2015 and 2016 deployments (i.e. for consistency, the first deployment was not used from the 2016 dataset). The effect of predation and parasitism, *C. albicans* parasitism status, deployment period, and density of winter moth pupae and *C. albicans* puparia on cumulative total mortality, cumulative hyperparasitism, cumulative predation, and standardized hyperparasitism were tested using a logistic generalized linear regression model with a quasibinomial fit. When multiple years were included in the analysis, year was included in the model as an effect, and potential interactions between year and mortality were considered.

2.3.5 Morphological identification

Vouchers for hyperparasitoid species are deposited in the University of Massachusetts Insect Collection, Amherst, MA. Emerged adult wasps from the 2015 collection were tentatively divided into three groups based on morphological similarity, and 10 representative adult specimens were sent to the fourth author (RRK) for identification. One of the groups had only four specimens and, for this group, all were sent for morphological identification, and thus DNA molecular identification was not undertaken. Specimens from the three groups were examined using a Leica M205 A stereomicroscope with 10X and 25X oculars. They were identified to genus using keys in Townes (1969, 1970) and then sorted into morphospecies. The fourth author (RRK) attempted to identify one morphospecies to species using keys in Townes et al. (1960); the other morphospecies are in genera with high species richness that lack identification keys for the Nearctic Region (Yu et al., 2012).

Authoritatively determined specimens of *Phygadeuon dumetorum* Gravenhorst and *Phygadeuon subfuscus* Cresson in the Smithsonian Institution National Museum of Natural History (USNM) were examined. The former has been reported from *C. albicans* in England (Hassell, 1969), and the latter has been reported from four tachinid species in the Nearctic Region (Yu et al., 2012). Authoritatively determined specimens of *Pimpla contemplator* (Müller), *Pimpla disparis* (Viereck), *Pimpla turionellae* (L.), and *Pimpla hesperus* (Townes) in the USNM were also examined. *Pimpla contemplator*, *P. disparis*, and *P. turionellae* have been introduced into North America to control winter moth and other lepidopteran pests (Graham, 1958; Yu et al., 2012; Quicke, 2015). In particular, *P.*

turionellae was introduced to Nova Scotia as a biological control agent of winter moth (Graham, 1958) and has also been reported from the tachinid *Compsilura concinnata* (Meigen) (Sharov and Izhevskiy, 1987). *Pimpla hesperus* has been reported as a parasitoid of winter moth and its native congener Bruce spanworm Hulst (*Operophtera bruceata*) in British Columbia (Humble, 1985). Lastly, four species of *Gelis* (i.e., Gelis areator Panzer*, Gelis acarorum* L., *Gelis discedens* Förster, and *Gelis rufogaster* Thunberg) have been reported as associated with winter moth in Europe (Sechser, 1970; Yu et al., 2012); comparisons were made with specimens in the USNM identified as each of those species. One puparium each associated with a *Phygadeuon* and *Pimpla* wasp, and two puparia associated with *Gelis* wasps, were dissected thoroughly for remnants of parasitism, particularly host remains.

2.3.6 Molecular identification

All remaining hyperparasitoid specimens from the 2015 collection and all of the 2016 collection were prepared for DNA sequencing. These samples represented individuals from all wasp taxa, both life stages (larvae and adult), and spanned all the study sites.

DNA was extracted following the QIAGEN DNeasy Blood and Tissue Kit protocol for purification of total DNA from animal tissues with the following modifications: the DNA was eluted twice in 100 µl Buffer AE instead of 200 µl (Step 7). The DNA extractions were stored at −20 °C. A master mix was prepared using the following amounts per sample: 17.3 µl nuclease free water, 0.5 µl dNTPs, 5 µl GoTaq Buffer, 0.2 µl GoTaq, and 0.5 µl of both the front and reverse primer. The CO1 primers

LCO/HCO (Folmer et al., 1994) were used with the temperature profile outlined by

Hebert et al. (2003). Samples that produced bands of the expected fragment size when

run on an agarose gel were prepared for sequencing. A master mix of Exonuclease 1

(Thermo Scientific) and Thermolabile Recombinant Shrimp Alkaline Phosphatase (New

England BioLabs) was prepared following the ThermoScientific protocol. The resulting

product was submitted to Yale University's DNA Analysis Facility on Science Hill for

Sanger sequencing.

The sequences obtained were visualized and forward and reverse sequences were

aligned using Geneious R8.1.8 (Biomatters Ltd.). The ends were trimmed so that all

sequences were high quality (> 90% high quality sequences). Consensus sequences were

created from clusters of sequences that had identical sequences.

We used the National Center for Biotechnology Information (NCBI) Basic Local

Alignment Search Tool (GenBank BLAST) and the Barcode of Life Database (BOLD) to

locate the closest sequence matches to each of our consensus *Pimpla* and *Phygadeuon*

sequences. For the *Phygadeuon* phylogenetic comparisons, we used both repositories to

download representative sequences for the ichneumonids *Phygadeuon*, *Mastrus*, and

Buathra. These three genera were searched as Humble (1985) reported Phygadeuon sp.

from undetermined *Cyzenis* (i.e. either *C. albicans* or *Cyzenis pullula*, Townsend),

Mastrus sp. from ichneumonid primary parasitoids of winter moth, and *Buathra*

dorsicarinata (Pratt) as a primary parasitoid of winter moth in British Columbia. Multiple

representatives of each genus and species were detected, but we chose the first listing of

each replicate. Following morphological work, additional *Phygadeuon* sequences were

added to the analysis. We then ran a Geneious multiple alignment with our consensus

sequences and the downloaded sequences and trimmed the ends to the shortest sequence. A *Tryponinae* sequence (accession JX833193.1) was included as the outgroup. We looked for evidence of nuclear mitochondrial DNAs (NUMTs) or pseudogenes by considering the translation (Using transl_table 5 for Invertebrate Mitochondrial DNA) of our CO1 fragment sequences.

JModelTest was used in the CIPRES Science Gateway (Miller et al., 2010) to select the best base-pair substitution model for each locus. HKY+G was selected as the best substitution model for analysis. We conducted neighbor-joining, Maximum Likelihood, and Bayesian analyses to assess genetic distance and phylogenetic relationships. Neighbor-joining analyses were implemented in Geneious (Kearse et al., 2012) using 1000 bootstrap replication and a majority rule (50%) consensus threshold. Maximum likelihood analyses were run using PhyML (Guindon et al., 2010) with 100 bootstrap replications. Bayesian analyses were run using MrBayes 3.2.6 (Huelsenbeck and Ronquist, 2001) with a MCMC chain length of 1,000,000 and a burn in length of 10%. The computed outputs were visualized using FigTree Version 1.4.2 (Rambaut, 2014).

2.4 Results

2.4.1 Pupal mortality

Cyzenis albicans experienced consistently high mortality throughout the study (Figure 3). Cumulative mortality was not significantly different between years (df=2,1, $\chi 2$=0.001, p=0.98), but there was a significant difference (df=2,1, $\chi 2$=6.62, p=0.037)

between mortality types (mortality due to predation, or mortality due to hyperparasitism). In both years, predation was the largest contributor to total mortality and was not significantly different from the total mortality ($\chi 2=0.90$, p=0.34). Mortality due to hyperparasitism was significantly lower than predation ($\chi 2=6.5$, p=0.011), but was observed even at sites where *C. albicans* has not yet been introduced or established as a biocontrol agent. While there was no significant difference (df=1,22, $\chi 2=0.25$, p=0.61) in hyperparasitism at sites where *C. albicans* was present or absent, there was a trend toward higher hyperparasitism in sites where *C. albicans* was present (Figure 4). Sentinel puparia deployed earlier in the season (July-August) had the highest percent hyperparasitism, but there was no significant difference (df=2,15, $\chi 2=2.5$, p=0.29) in hyperparasitism among deployment periods (Figure 5). Neither winter moth pupae nor *C. albicans* pupal densities were significant predictors of cumulative total mortality (df=1,8, $\chi 2=0.0001$, p=0.99 and df=1,9, $\chi 2=0.018$, p=0.89, respectively) or hyperparasitism (Figure 6, df=1,8, $\chi 2=0.62$, p=0.43 and df=1,9, $\chi 2=0.48$, p=0.49, respectively) and in all fit models, year did not have a significant effect (p > 0.44). While there was no significant effect of density, there was a trend of more hyperparasitism at sites with high pupal and *C. albicans* densities, and the site with the highest hyperparasitism in the 2016 data (Yarmouth) was also the site with the highest *C. albicans* density.

2.4.2 Hyperparasitoid identification by morphology

Three ichneumonid wasp genera were identified using morphological features: *Phygadeuon* (Cryptinae), *Pimpla* (Pimplinae), and *Gelis* (Cryptinae). All three genera

contain generalist parasitoids with few records of parasitism on tachinids; the species here likely were facultative or accidental hyperparasitoids of *C. albicans*.

Records of *Phygadeuon* attacking *Cyzenis* are limited to *P. dumetorum* and *P. elegans* (Förster) parasitizing *C. albicans* in Europe (Hassell, 1969; Sechser, 1970) and an undetermined species of *Phygadeuon* reported from *Cyzenis* spp. (either *C. albicans* or *C. pullula*) in British Columbia (Humble, 1985). However, other species of *Phygadeuo*n have been reported as parasitoids of tachinids in other genera, including *P. subfuscus* in the Nearctic Region (Yu et al., 2012). There are no specimens of *P. elegans* in the USNM and only one male specimen of *P. dumetorum*. Based on examination of morphological features, the male *Phygadeuon* specimens we obtained were similar to, but probably not conspecific with, *P. dumetorum*. However, our *Phygadeuon* specimens were morphologically similar to specimens of *P. subfuscus* in the USNM. There are also two female specimens in the USNM identified as *P. subfuscus* that are potentially a species different than the other six. Thus, our *Phygadeuon* specimens are possibly *P. subfuscus*, but unequivocal identification is not possible at this time due to the aforementioned ambiguity in the USNM specimens. Dissection of a puparium associated with a *Phygadeuon* wasp yielded only the wasp cocoon and remains of a flattened late fly pupa located between the wasp cocoon and fly puparium confirming hyperparasitism and suggesting no evidence that a host other than the fly was parasitized.

Species of *Pimpla* have been previously reported from winter moth as primary parasitoids (Sechser, 1970; Humble, 1985; Yu et al., 2012) but not as hyperparasitoids. *Pimpla hesperus* (previously *Coccygomimus hesperus*) was reported by Humble (1985) as a parasitoid of winter moth and Bruce spanworm in British Columbia and *P.*

turionellae, *P. contemplator*, and *P. disparis* were all introduced into Canada and the U.S. to control winter moth and other lepidopteran pests (Graham, 1958; Quicke, 2015; Yu et al., 2012). However, their potential as hyperparasitoids of *Cyzenis* is unknown. The specimens reared in this study were not considered conspecific with specimens in the USNM determined as *P. hesperus*, *P. contemplator*, *P. disparis*, and *P. turionellae* based on examination of specimens in the USNM identified as those species. Dissection of a puparium associated with a *Pimpla* specimen yielded only the wasp cocoon and remains of a flattened late fly pupa located between the wasp cocoon and fly puparium confirming hyperparasitism and suggesting no evidence that a host other than the fly was parasitized.

There are no records of any *Gelis* spp. as parasitoids of tachinids in Yu et al. (2012), but Sechser (1970) found *G. areator* and an unknown *Gelis* sp. attacking other parasitoids of winter moth in Europe, but not *C. albicans*. Additionally, there are records of three other *Gelis* spp. (*G. acarorum*, *G. discedens*, and *G. rufogaster*) associated with winter moth in Europe (Yu et al., 2012). The four *Gelis* specimens recovered from puparia in this study were compared with specimens in the USNM identified as each of those four species, but none of these appeared to be conspecific with the two species reared in this study. A puparium associated with each of the *Gelis* species was dissected; both contained a wasp cocoon and the remains of a flattened late fly pupa located between the wasp cocoon and fly puparium. One puparium also contained a piece of hardened, desiccated tissue that was possibly the remains of a wasp larva. It is possible that the hardened tissue is the remains of a host attacked by *Gelis*; thus, the *Gelis* larva may have attacked a parasitoid of the fly rather than the fly itself.

2.4.3 Hyperparasitoid identification by molecular techniques

One hundred and thirty-seven samples (82.5% of the total sequenced) yielded high quality CO1 sequences (> 95% high quality sequences, after trimming the ends). Comparison of the sequences from our samples to those available from GenBank and from BOLD supported the morphological identifications of *Phygadeuon* and *Pimpla*. Of our sequences, 129 were *Phygadeuon* (94.2%) and eight (5.8%) were *Pimpla*. Molecular analyses were not conducted on specimens of *Gelis* because the few specimens reared were all used for morphological identification. The *Gelis* specimens identified using morphological features (n=4) were considered two species.

After aligning and trimming the ends of the sequences, the *Phygadeuon* sequences were 609 bp long and fell into two clades (Table 8, Figure 7). One group (referred to here as Clade 1; GenBank Accession numbers MG491041 – MG491040) had 79 samples, and the other (referred to here as Clade 2; GenBank Accession numbers MG490987 – MG491034) had 48 samples. Clade 2 had 100% sequence identity within the samples, whereas Clade 1 could be further broken down into two subgroups – one with 73 samples and one with 6 samples – each with 100% sequence identity within the subgroups and a distance of 0.3% between the two subgroups (Clade 1.1 and Clade 1.2) or two base pairs. The two clades (Clade 1 and 2) had 1.1 to 1.5% sequence difference between each other representing a difference of 7–9 base pairs. However, analysis of corresponding ecological data (collection year, site, time period, life stage) between individuals from the two different clades yielded no trends to predict assignment to either *Phygadeuon* clade.

The subset of *Phygadeuon* specimens identified using morphological features (n=8) were considered one species.

The closest sequence matches to our *Phygadeuon* sequences were unidentified Hymenoptera species and *Cryptinae* sp. available from GenBank (Figure 7). These were all matches of between 98.8% and 100% (Table 8). Our *Phygadeuon* Clade 1.1 consensus sequence had a 100% sequence match to an unidentified Hymenoptera sequence (GenBank Accession KM997587.1) and a *Cryptinae* sp. (KR782755.1) both collected in Ontario. There were no 100% matches to the consensus sequence for *Phygadeuon* Clade 1.2. The *Phygadeuon* Clade 2 consensus sequence matched a *Cryptinae* sp. from Nova Scotia (KR784654.1) and an unidentified Hymenoptera species from Ontario (KM997587.1). Our sequences clearly were not conspecific with any previously published sequences of *Buathra* or *Mastrus* available from GenBank or BOLD.

After trimming the ends, the *Pimpla* CO1 sequences (eight sequences) were 620 bp long. These eight sequences represented eight different individuals collected from five different sites (from samples collected in 2016 from Kingston, RI, Hingham, MA, Wellesley, MA, and Hanson, MA). Of these, five sequences were very similar to each other (sequence identity of 99.2–100%, representing 0–5 bp differences) and were homologous (99–100% identity) to the first 100 samples available from NCBI GenBank and BOLD, which were all identified as *Pimplinae* sp. (e.g. GenBank Accession KR409035.1 and KJ167171.1) or *Pimpla* sp. (e.g. KR809211.1 and KR932958.1). The next closest homologous sequences listed were *Pimpla nuda* Townes (BOLD Accession numbers: CNFNR3907-14, CNGBG1989-14, CNGSF433-15, CNGSF436-15) and *Pimpla stricklandi* (Townes) (BOLD Accession numbers: BBHYK694-10, GMODL399-

15) but with a sequence identity of 92.6–94.3%. The remaining three *Pimpla* sequences

(from samples collected in Kingston, RI and Hanson, MA in 2016) were identical to each

other (sequence identity of 100%) and homologous (97.9–100%) to samples in the

database labeled *Pimpla aequalis* Provancher (e.g. accessions AF146681.1, KR791821.1,

KJ445028.1). The sequence identity between our two clades of hyperparasitic *Pimpla*

wasps (the five individuals that most closely matched sequences in GenBank and BOLD

that were labeled Pimplinae sp./*Pimpla* sp. versus the three sequences that most closely

matched sequences labeled *P. aequalis*) was 90.0–90.3% (or 60–62 bp difference). The

subset of *Pimpla* specimens identified using morphological features (n=3) were

considered one species. No tree showing these comparisons is presented in this

manuscript as it will be presented in a subsequent manuscript focusing on the identity,

life history, and role of pimpline wasps in the winter moth system.

2.4.4 Hyperparasitoid abundance

Identification of hyperparasitoid specimens using a combination of morphological

and molecular techniques was successful in identifying a large proportion of wasp

samples (75%) leaving only a small percentage, mostly larval samples, unidentified

(Table 9). In 2015, *Phygadeuon* sp. was the most common hyperparasitoid among

identified samples (65.3% overall; 63.2% of adults and 69.7% of larvae). In 2015, *Pimpla*

samples were observed almost equally as adults and larvae, and *Gelis* samples were

observed only as pharate adults. In 2016, *Phygadeuon* sp. was the only confirmed

hyperparasitoid present. A small percentage of samples each year (4% in 2015 and 2.5%

in 2016) were identified as *C. albicans* due to sequencing picking up host DNA instead of

hyperparasitoid DNA.

Table 7: Locations and coordinates for study sites where sentinel puparia of *Cyzenis albicans* were deployed in 2015 and 2016. The X-mark indicates each year the site was sampled.

Site	GPS Coordinates	2015	2016
Centennial Park, Wellesley, MA	42.308444, −71.266778	X	X
Garden In the Woods, Framingham, MA	42.340833, −71.427667	X	
Co-op Extension, Hanson, MA	42.048889, −70.873806	X	X
Maquan St., Hanson, MA	42.060694, −70.844167	X	X
Wompatuck SP, Hingham, MA	42.208333, −70.853056	X	X
Parkwood, Drive, Kingston, RI	41.475250, −71.529444	X	
Pondview Dr., Falmouth, MA	41.626417, −70.580417		X
Route 6, Yarmouth, MA	41.686167, −70.287722		X

Table 8: *Phygadeuon* spp. CO1 gene distance matrix corresponding to the phylogentic analysis presented in Figure 7. The table is split into two to fit on the page, but the second half could have been placed to the right of the first half in the matrix.

	Accession number	Phygadeuon sp Clade 1.1	Phygadeuon sp Clade 1.2	Phygadeuon sp Clade 2	Hymenoptera sp Ontario	Cryptinae sp Ontario	Cryptinae sp Ontario	Hymenoptera sp Ontario	Cryptinae sp Nova Scotia	Cryptinae sp Manitoba	Cryptinae sp Alberta	Phygadeuon cephalotes	Phygadeuon variabilis	Phygadeuon nanus
Phygadeuon sp Clade 1.1	MG491041 - MG491113		99.8	98.8	100	100	100	98.8	98.8	98.8	99.6	89.1	90.4	87.5
Phygadeuon sp Clade 1.2	MG491035 - MG491040	99.8		98.9	99.8	99.8	99.8	98.9	98.9	98.9	99.8	89.3	90.6	87.7
Phygadeuon sp Clade 2	MG490987 - MG491034	98.8	98.9		98.8	98.8	98.8	100	100	99.6	98.8	89.5	90.6	87.3
Hymenoptera sp Ontario	KM998016.1	100	99.8	98.8		100	100	98.8	98.8	98.8	99.6	89.1	90.4	87.5
Cryptinae sp Ontario	KR782755.1	100	99.8	98.8	100		100	98.8	98.8	98.8	99.6	89.1	90.4	87.5
Cryptinae sp Ontario	KR927250.1	100	99.8	98.8	100	100		98.8	98.8	98.8	99.6	89.1	90.4	87.5
Hymenoptera sp Ontario	KM997587.1	98.8	98.9	100	98.8	98.8	98.8		100	99.6	98.8	89.5	90.6	87.3
Cryptinae sp Nova Scotia	KR784654.1	98.8	98.9	100	98.8	98.8	98.8	100		99.6	98.8	89.5	90.6	87.3
Cryptinae sp Manitoba	KR806223.1	98.8	98.9	99.6	98.8	98.8	98.8	99.6	99.6		98.8	89.8	90.6	87.5
Cryptinae sp Alberta	KR791419.1	99.6	99.8	98.8	99.6	99.6	99.6	98.8	98.8	98.8		89.5	90.7	87.9
Phygadeuon cephalotes	ASAHY202-13	89.1	89.3	89.5	89.1	89.1	89.1	89.5	89.5	89.8	89.5		91.1	89.1
Phygadeuon variabilis	BOLD BCHYM3983-14	90.4	90.6	90.6	90.4	90.4	90.4	90.6	90.6	90.6	90.7	91.1		86.6
Phygadeuon nanus	BOLD AGAKL2275-17	87.5	87.7	87.3	87.5	87.5	87.5	87.3	87.3	87.5	87.9	89.1	86.6	
Phygadeuon pegomyiae	HPPPB153-13	86.6	86.5	86.8	86.6	86.6	86.6	86.8	86.8	86.8	86.6	86.8	86.3	88.9
Phygadeuon fumator	BOLD GRAFW1680-12	90.7	90.6	90.7	90.7	90.7	90.7	90.7	90.7	90.9	90.7	90.9	90.2	92.2
Phygadeuon forticornis	ASWAY304-08	90.7	90.6	90.9	90.7	90.7	90.7	90.9	90.9	90.7	90.7	89.8	90	90.9
Phygadeuon exiguus	ASWAT873-08	89.8	89.7	90	89.8	89.8	89.8	90	90	90.2	89.8	89.7	88.9	89.1
Phygadeuon laeviventris	ASWAU676-08	89.7	89.5	88.9	89.7	89.7	89.7	88.9	88.9	89.1	89.7	89.7	87.5	87.7
Phygadeuon trchops	BOLD ASWAV271-08	89.7	89.8	90.4	89.7	89.7	89.7	90.4	90.4	90.6	90	89.7	89.5	88.9
Phygadeuon rugulosus	ASWAV311-08	87.5	87.5	88.1	87.5	87.5	87.5	88.1	88.1	88.2	87.7	86.6	86.3	85
Mastrus sp	GU086930.1	84.7	84.5	84.5	84.7	84.7	84.7	84.5	84.5	84.8	84.7	84.8	84.7	84.5
Phygadeuon melanopygus	ASGLE023-10	86.5	86.6	86.5	86.5	86.5	86.5	86.5	86.5	86.6	86.8	86.5	86.6	86.6
Buathra laborator	KF604335.1	83.4	83.6	84	83.4	83.4	83.4	84	84	84.3	83.8	84.7	84.7	84
Cryptus arcticus	KF604497.1	85.7	85.9	86.1	85.7	85.7	85.7	86.1	86.1	86.5	86.1	86.5	85.4	83.6
Phygadeuon solidus	BOLD ASWA147-08	86.1	86.3	86.5	86.1	86.1	86.1	86.5	86.5	86.5	86.5	86.6	87.3	86.3
Tryphoninae sp	JX833193.1	81.8	81.8	81.8	81.8	81.8	81.8	81.8	81.8	81.8	82	82.5	83.1	81.3

	Accession number	Phygadeuon pegomyiae	Phygadeuon fumator	Phygadeuon forticornis	Phygadeuon exiguus	Phygadeuon laeviventris	Phygadeuon trchops	Phygadeuon rugulosus	Mastrus sp	Phygadeuon melanopygus	Buathra laborator	Cryptus arcticus	Phygadeuon solidus	Tryphoninae sp
Phygadeuon sp Clade 1.1	MG491041 - MG491113	86.6	90.7	90.7	89.8	89.7	89.7	87.5	84.7	86.5	83.4	85.7	86.1	81.8
Phygadeuon sp Clade 1.2	MG491035 - MG491040	86.5	90.6	90.6	89.7	89.5	89.8	87.5	84.5	86.6	83.6	85.9	86.3	81.8
Phygadeuon sp Clade 2	MG490987 - MG491034	86.8	90.7	90.9	90	88.9	90.4	88.1	84.5	86.5	84	86.1	86.5	81.8
Hymenoptera sp Ontario	KM998016.1	86.6	90.7	90.7	89.8	89.7	89.7	87.5	84.7	86.5	83.4	85.7	86.1	81.8
Cryptinae sp Ontario	KR782755.1	86.6	90.7	90.7	89.8	89.7	89.7	87.5	84.7	86.5	83.4	85.7	86.1	81.8
Cryptinae sp Ontario	KR927250.1	86.6	90.7	90.7	89.8	89.7	89.7	87.5	84.7	86.5	83.4	85.7	86.1	81.8
Hymenoptera sp Ontario	KM997587.1	86.8	90.7	90.9	90	88.9	90.4	88.1	84.5	86.5	84	86.1	86.5	81.8
Cryptinae sp Nova Scotia	KR784654.1	86.8	90.7	90.9	90	88.9	90.4	88.1	84.5	86.5	84	86.1	86.5	81.8
Cryptinae sp Manitoba	KR806223.1	86.8	90.9	90.7	90.2	89.1	90.6	88.2	84.8	86.6	84.3	86.5	86.5	81.8
Cryptinae sp Alberta	KR791419.1	86.6	90.7	90.7	89.8	89.7	90	87.7	84.7	86.8	83.8	86.1	86.5	82
Phygadeuon cephalotes	ASAHY202-13	86.8	90.9	89.8	89.7	89.7	89.7	86.6	84.8	86.5	84.7	86.5	86.6	82.5
Phygadeuon variabilis	BOLD BCHYM3983-14	86.3	90.2	90	88.9	87.5	89.5	86.3	84.7	86.6	84.7	85.4	87.3	83.1
Phygadeuon nanus	BOLD AGAKL2275-17	88.9	92.2	90.9	89.1	87.7	88.9	85	84.5	86.6	84	83.6	86.3	81.3
Phygadeuon pegomyiae	HPPPB153-13		88.8	88.6	89.1	85.9	87.3	85	84.5	84.5	83.4	85.2	87	79.5
Phygadeuon fumator	BOLD GRAFW1680-12	88.8		93	90.7	90	89.5	85.7	84	85.9	84.3	85.6	86.8	80
Phygadeuon forticornis	ASWAY304-08	88.6	93		91.4	88.6	90.4	85.9	85	87.3	85.7	85.2	87	79.3
Phygadeuon exiguus	ASWAT873-08	89.1	90.7	91.4		89.5	89.8	85.2	85.4	85.2	83.4	85.4	87	80.7
Phygadeuon laeviventris	ASWAU676-08	85.9	90	88.6	89.5		89.3	87	83.4	84.3	83.8	85.4	85.9	81.3
Phygadeuon trchops	BOLD ASWAV271-08	87.3	89.5	90.4	89.8	89.3		88.1	84.3	84.5	85.9	85.7	86.1	81.1
Phygadeuon rugulosus	ASWAV311-08	85	85.7	85.9	85.2	87	88.1		84.3	85	84.3	86.1	86.3	82
Mastrus sp	GU086930.1	84.5	84	85	85.4	83.4	84.3	84.3		88.1	83.1	85.9	85.9	80.9
Phygadeuon melanopygus	ASGLE023-10	84.5	85.9	87.3	85.2	84.3	84.5	85	88.1		84	86.1	85.4	81.6
Buathra laborator	KF604335.1	83.4	84.3	85.7	83.4	83.8	85.9	84.3	83.1	84		88.8	86.1	79.9
Cryptus arcticus	KF604497.1	85.2	85.6	85.2	85.4	85.4	85.7	86.1	85.9	86.1	88.8		88.2	83.2
Phygadeuon solidus	BOLD ASWA147-08	87	86.8	87	87	85.9	86.1	86.3	85.9	85.4	86.1	88.2		82.2
Tryphoninae sp	JX833193.1	79.5	80	79.3	80.7	81.3	81.1	82	80.9	81.6	79.9	83.2	82.2	

43

Table 9: Hyperparasitoid specimens reared from *C. albicans* puparia in 2015 and 2016 across all sites, indicating wasp taxon and life stage obtained as a proportion of all adult specimens, all larval specimens, and total specimens per year.

ID	2015			2016		
	Adult (n = 33)	Larvae (n = 68)	Total (n = 101)	Adult (n = 19)	Larvae (n = 61)	Total (n = 80)
Phygadeuon	0.70	0.63	0.65	0.84	0.66	0.70
Pimpla	0.12	0.10	0.11	0.00	0.00	0.00
Gelis	0.09	0.00	0.03	0.00	0.00	0.00
Unknown	0.09	0.27	0.21	0.16	0.34	0.30
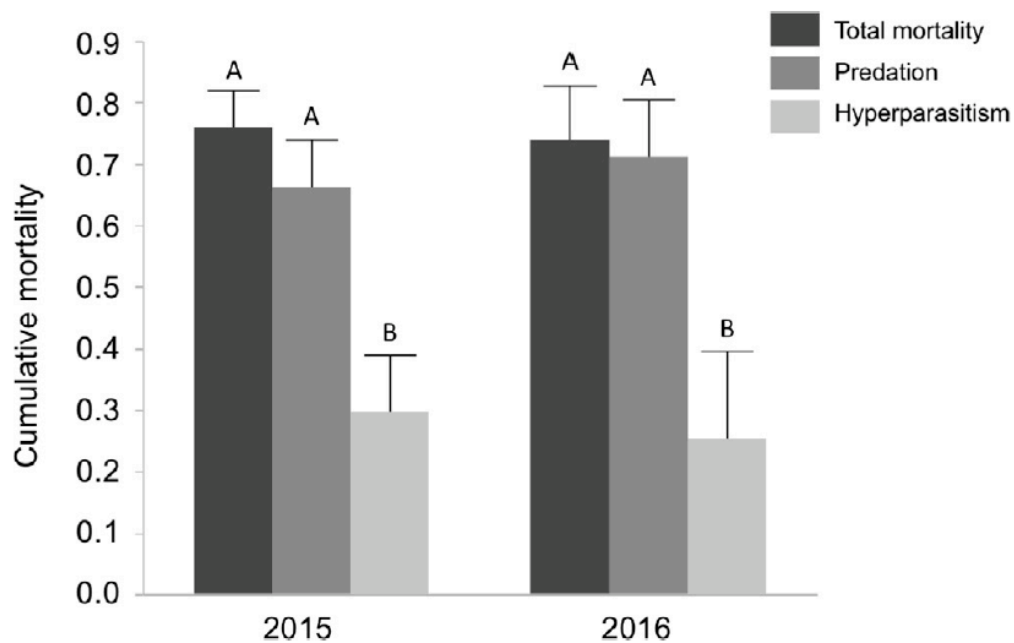
Figure 3: Mean (+SE) annual cumulative total mortality of *Cyzenis albicans* puparia and the proportional contribution of predation and hyperparasitism across the various sites and field deployments (August–September and September–October) in 2015 and 2016. Hyperparasitism values were calculated from pupae that survived predation, so survivorships of predation and hyperparasitism do not add up to the total survivorship. In both years, proportional mortality due to predation was higher than that due to hyperparasitism, but not significantly different from the total mortality (GLM with a logit link function and overdispersion parameter, $\alpha=0.05$).
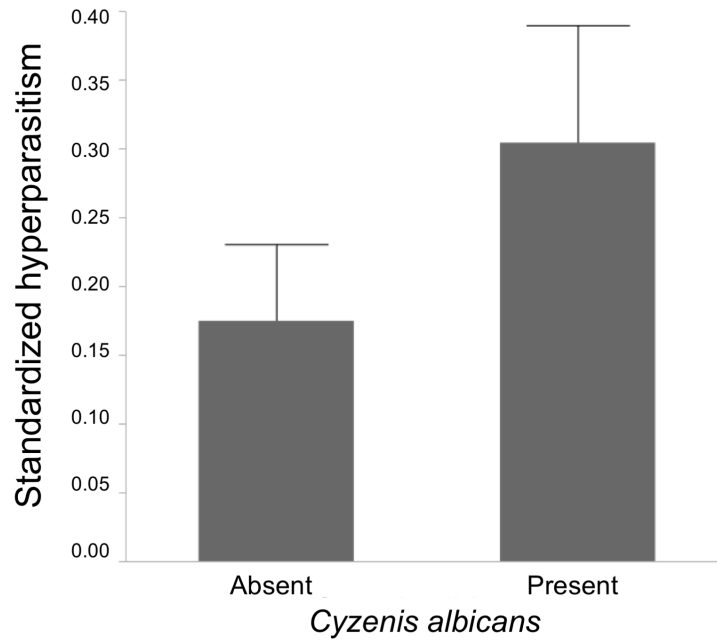
Figure 4: Mean (+SE) hyperparasitism of *Cyzenis albicans* **puparia across all years, sites, and sentinel deployments plotted according to the presence/absence of** *Cyzenis albicans***. Hyperparasitism here is the average of each deployment, which was normalized to 42 days (6 weeks) to represent average deployment length. Differences were not significant (GLM with a logit link function and overdispersion parameter, $\alpha > 0.05$)**
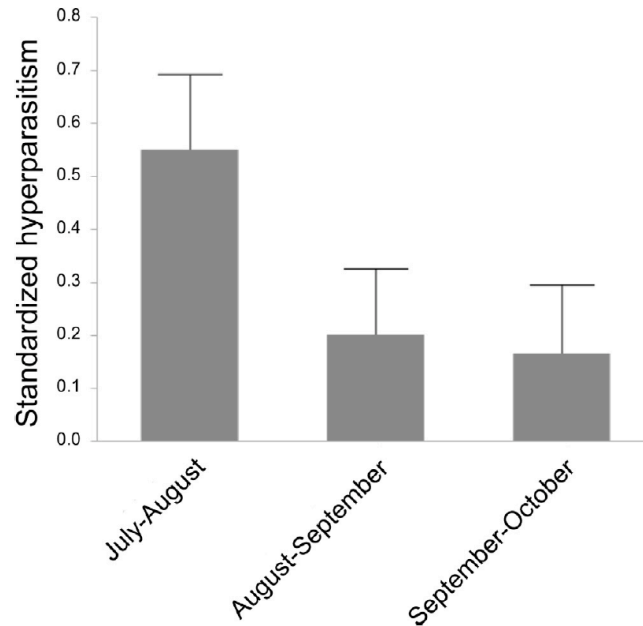
Figure 5: Mean (+SE) hyperparasitism of *Cyzenis albicans* puparia during 2016 sentinel deployments. Only 2016 is shown as only two deployments were made in 2015, rather than three. Rates of hyperparasitism are normalized to 42 days (6 weeks) to represent the average deployment period. Differences were not significant among time periods (GLM with a logit link function and overdispersion parameter, $\alpha > 0.05$).
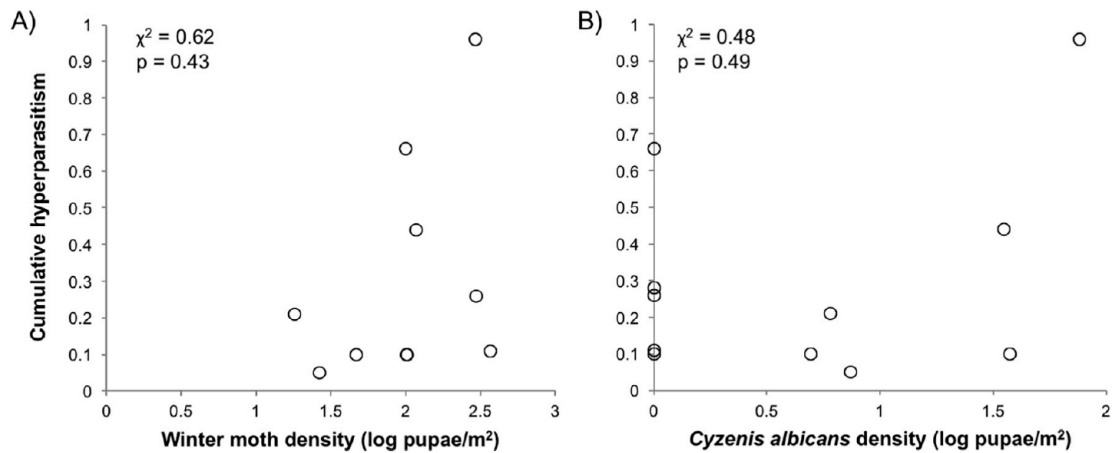
Figure 6: Mean (+SE) annual cumulative hyperparasitism of *Cyzenis albicans* puparia for each site and year by A) the corresponding log10-transformed density of winter moth and B) *Cyzenis albicans* pupal density (log pupae/m2) at each site. The fit and p-value represent the GLM with a logit link function and overdispersion parameter.
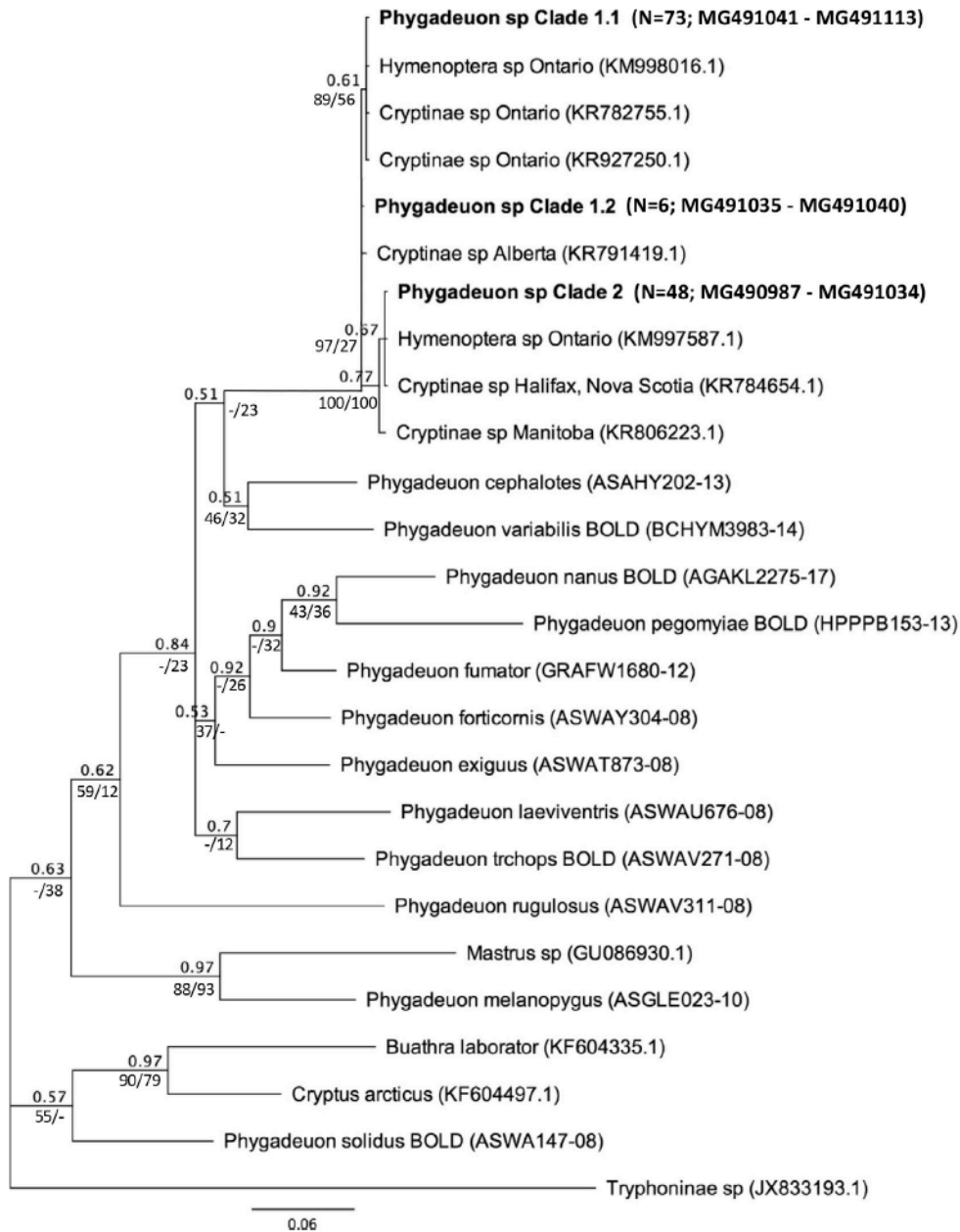
Figure 7: *Phygadeuon* spp. phylogenetic tree obtained using a 710 bp fragment of the CO1 gene. The base tree was generated using a Bayesian analysis with corresponding bootstrap proportion values (out of 1) presented with the Neighbor Joining/Maximum Likelihood values (out of 100) presented below or to the right. Instances where there were no equivalent branches in models are indicated with a dash. Scale bar shows 0.06 substitutions per site. The *Phagadeuon* sp. sequences generated from this study are bolded and fit with representative sequences from NCBI GenBank and BOLD.

2.5 Discussion

Cyzenis albicans has been established as a classical biological control agent to manage invasive winter moth populations at four separate locations in North America: Nova Scotia in the 1950s (Embree, 1965), Oregon and British Columbia in the 1970s (Roland and Embree, 1995), and the northeastern U.S. beginning in 2005 (Elkinton et al., 2014a). The biological control programs in Canada were deemed successful in reducing outbreak winter moth populations and are often cited as an example of biological control success (Hassell, 1980; Murdoch et al., 1985; Roland, 1990; Van Driesche et al., 2008). However, prior studies in this and other pest systems have found that hyperparasitism can affect biological control success (Ehler, 1979; McDonald and Kok, 1991; McNeil and Rabb, 1973; Schooler et al., 2011; Strauss, 2012; Sullivan and Völkl, 1999) or, alternatively, can be present with little or no effect on biocontrol outcomes (Flanders, 1963; Hassell, 1969, 1980; Humble, 1985; McNeil and Rabb, 1973). Evaluating the extent of hyperparasitism in the most recent winter moth biocontrol program is thus pertinent.

This study is the first report of hyperparasitism of *C. albicans* following its introduction into northeastern U.S. Following an earlier introduction of *C. albicans* in British Columbia, Humble (1985) reported that 12% of a sample of 33 *Cyzenis* puparia were hyperparasitized by *Phygadeuon* and Villa (Hemipenthes) catulina. However, this did not prevent winter moth densities from declining, and parasitism levels by *C. albicans* remained high (Roland, 1986; Roland and Embree, 1995). As far as we know, no work was done to assess hyperparasitism following the releases in Oregon. In Nova Scotia, studies assessed mortality factors acting on winter moth pupae, including

parasitism by *C. albicans* (Embree, 1965; MacPhee et al., 1988; Pearsall and Walde, 1994), but did not report on hyperparasitism of *C. albicans*. Likely, this is because *C. albicans* sentinel puparia were not deployed and retrieved, and hyperparasitoids may have been overlooked. Hassell (1969) reported hyperparasitism of *C. albicans* by *P. dumetorum* in low-density populations of winter moth in England, but his detailed analyses (Hassell, 1969, 1980) suggested that hyperparasitism played an insignificant role in *C. albicans* population dynamics. In Austria, one *Gelis* species was considered a potential threat to a wasp introduced as a biological control agent of an invasive planthopper (Strauss, 2012). Additional rearing and biological study is necessary to elucidate the role of *Gelis* species in the winter moth food web in the northeastern U.S. Unidentified tissue found in one *C. albicans* puparium parasitized by Gelis is possibly the remains of a host other than *C. albicans*. *Gelis* is a genus of approximately 300 species with host ranges spanning multiple insect orders, even extending to spiders. Some species of *Gelis* have a broad host range*; G. areator*, has been reported from nearly 200 host species. Further, the genus contains both primary and secondary parasitoids. Thus, it is conceivable that the *Gelis* spp. observed could attack either the fly or its primary parasitoids. Similarly, little is known about the role of *Pimpla* sp. on *C. albicans*; however, Elkinton et al. (2014a) presented evidence that *C. albicans* was beginning to suppress outbreak populations of winter moth in the northeastern U.S. despite the presence of the hyperparasitoids we report here.

  Cummulative mortality of *C. albicans* puparia was consistently high in this study, averaging more than three quarters of the total puparia deployed. This mortality was due to a combination of predation and hyperparasitism, with predation the

larger contributor. This mortality is comparable to that reported on winter moth pupae in Nova Scotia (60–95%, Embree, 1965; 79%, MacPhee et al. 1988; 49–96%, Pearsall and Walde, 1994). The main predators of pupae are likely carabid beetles, staphylinid beetles, and small mammals (Frank, 1967; East, 1974; Holliday, 1985; Roland, 1990). Pupal predators are unlikely to distinguish between parasitized and unparasitized pupae as the *C. albicans* puparia are contained within the cocoon casing and the integument of the winter moth pupae, and both are buried in the soil. The mortality of *C. albicans* puparia in our study, however, was somewhat lower than the 77–98% reported in British Columbia by Horgan and Myers (2004).

In British Columbia, winter moth densities declined following the introduction of *C. albicans* (Roland, 1986), and parasitism of winter moth by that species has remained at high levels (~50%) for several decades (Horgan and Myers, 2004; Roland, 1988). The relative role of predation on moth pupae versus parasitism by *C. albicans* in the decline of winter moth densities at these sites, and possible synergism between these factors, has been debated by previous researchers (Hassell, 1980; Roland, 1988; Roland and Embree, 1995). At our multiple study sites in the northeastern U.S., parasitism by *C. albicans* has been steadily increasing and is coupled with a decline in winter moth densities (Elkinton et al., 2014a), despite the high parasitoid mortality we report here. However, this mortality on *C. albicans* is likely delaying subsequent parasitism by *C. albicans* on winter moth for several years because the year-to-year increase in *C. albicans* densities following each release is relatively small.

The present study provides evidence that hyperparasitism of *C. albicans*

50

is unrelated to either winter moth or primary parasitoid densities. The hyperparasitoids discovered – *Phygadeuon* sp., *Pimpla* spp. and *Gelis* spp. – are all common in lepidopteran systems (Sullivan, 1987). All three may act as generalist parasitoids and facultative or accidental hyperparasitoids, switching between various hosts depending on their availability, suggesting that their densities are not strongly linked to densities of *C. albicans* (Hassell,1969; Fitton et al., 1988; Harvey, 2008; Hassell, 1969; Strauss, 2012; Yu et al., 2012). Further, some *Pimpla* spp. are likely facultative or accidental hyperparasitoids and have been documented infrequently as hyperparasitic (Yu et al., 2012). This is supported in the winter moth system; pimpline parasitoids of winter moth in the northeastern U.S. appear to be attacking winter moth at higher rates than *C. albicans* (HJB, pers. observ.). In addition, winter moth females lay 150–200 eggs, whereas *C. albicans* females lay 1000–2000 (Hassell, 1980; Varley and Gradwell, 1970). This may allow *C. albicans* populations to keep pace with those of winter moth despite the five additional months spent underground where predation is high. These results have important population dynamics implications. They suggest that none of these facultative hyperparasitoids would likely reduce equilibrium densities of *C. albicans* to levels that would limit parasitism of winter moth.

Because species identities for the hyperparasitoid families obtained here are largely unknown, and identification of larval specimens (a large proportion of our dataset) is extremely difficult, we used CO1 gene sequences combined with morphological examinations to aid taxonomic identification. This not only aids identification, but also provides sequence-specimen voucher information for future research reference (Andersen and Wagner, 2016). From the CO1 sequence data, the

Phygadeuon specimens fell into two distinct clades that coexist attacking the same hosts at the same sites. The distinct clades suggest there may be restriction of gene flow between them, but the differences in sequences between the two clades is only 1.1 and 1.2% (Table 8) suggesting they may not be different species (Ball et al., 2005). Further clarification of their taxonomic status would require further molecular, morphological, and life history studies. All the closest matches to our *Phygadeuon* sequences were collected from Ontario, Nova Scotia, Manitoba, or Alberta. In Canada, Nova Scotia and British Columbia is known to have winter moth, but winter moth is not known from the other locations (Elkinton et al., 2010; Elkinton et al., 2014a). However, Bruce spanworm, a native congener of winter moth, is known to exist in these other provinces (Brown, 1962; Elkinton et al., 2010; Ives and Cunningham, 1980; Rose and Lindquist, 1997) and is closely related to winter moth (Havill et al., 2017). This indicates that the *Phygadeuon* sp. we detected is associated with other lepidopteran species besides winter moth and may also attack Bruce spanworm.

In this study, we report facultative hyperparasitism of *C. albicans* by three different ichneumonid genera and high levels of predation on *C. albicans* puparia. These findings are consistent with those reported elsewhere in North America, particularly in British Columbia. In British Columbia, this mortality has not prevented *C. albicans* from causing high levels of parasitism on winter moth and the consequent decline of winter moth densities that have persisted in non-outbreak levels for several decades. Thus, we do not expect these agents to prevent *C. albicans* from causing significant mortality to winter moth in the northeastern U.S. and the subsequent decline of winter moth densities as a result. The high mortality of *C. albicans*, due to both

predators and hyperparasitoids, may explain why 2–6 years elapse between release of *C. albicans* and its subsequent recovery from winter moth at various release sites and for more years to elapse before onset of high levels of parasitism in winter moth. These lag times occurred in Nova Scotia in the 1950s (Embree, 1965; Roland and Embree, 1995) and in the northeastern U.S. (Elkinton et al., 2014a). The findings reported here contribute to our effort to understand the population dynamics of both winter moth and *C. albicans* as part of our effort to evaluate the success of the biological control program directed at winter moth.

CHAPTER 3

RECRUITMENT OF NATIVE ICHNEUMONID WASPS TO POPULATIONS OF

THE INVASIVE WINTER MOTH, *OPEROPHTERA BRUMATA* L., IN THE

NORTHEAST U.S.

3.1 Abstract

Ecological communities may be resistant to invasive species through a combination of top-down and bottom-up mechanisms, including predation, competition, parasitism, and disease. In particular, natural enemies that cross over from native species to use newly introduced non-native species as hosts can influence invasive species population dynamics and may slow down invasions. We used the model species winter moth (*Operophtera brumata*) to study the effect of recruitment of native parasitoids on an invasive population of winter moth in the northeast United States. We deployed sentinel pupae over four years across this population's range, identified recovered parasitoids, and measured the rate of parasitism by native sources across years, seasons, invasion history, and host densities. Native *Pimpla* wasps (Hymenoptera: Ichneumonidae) inflicted 98% percent of the parasitism detected, resulting in an annual average of 15-40% mortality on pupae not depredated. *Pimpla* were present across all years, seasons, and sites. Parasitism was greatest at the leading edge of the winter moth population spread and when winter moth pupal density was high (positive density dependence). The parasitoid wasps were morphologically identified as *Pimpla aequalis*; however, using a multilocus genetic comparison approach, they were determined to comprise two cryptic species. Overall, this study shows that recruitment of these native wasps to the invasive winter

54

moth population is likely playing a significant role in regulating population outbreaks and is likely aiding in classical biological control efforts.

3.2 Introduction

The introduction of non-native species to new communities is creating novel and altered predator-prey and parasite-host interactions (Faillace et al. 2017; Garnas et al. 2016; Hobbs et al. 2009; Pearson et al. 2018; Shea and Chesson 2002; Strauss et al. 2012). The species richness of an ecological community can predict the chances that an invasive species will successfully establish. This hypothesis (known as the biotic resistance hypothesis) holds that communities may resist invasions through a combination of factors including predation, competition, parasitism, and disease (Elton 1958; Jeschke et al. 2012; Levine et al. 2004; Maron and Vilà 2001; Sakai et al. 2001; Shea and Chesson 2002). Natural enemies of native species that cross over to use non-native species can influence invasions in complex ways (Dearborn et al. 2016; Faillace et al. 2017; Grabenweger et al. 2010; Strauss et al. 2012) and have the potential to slow down invasions and aid in biological control (Dearborn et al. 2016; Kenis et al. 2008; Maron et al. 2001; Vindstad et al. 2013). These interactions are particularly strong for non-native species with sympatric native congeners and confamilials (Dearborn et al. 2016; Grabenweger et al. 2010; Strauss et al. 2012; Vindstad et al. 2013). Further, these interactions may especially affect invasive insects because they are typically r-selected (Sakai et al. 2001), and their population dynamics are closely related to natural enemies, predominantly parasitoids (Hassell 2000; Myers 2018; Waage and Greathead 1985).

The European winter moth, *Operophtera brumata* L. (Lepidoptera: Geometridae), is an invasive defoliator of forest and shade trees with multiple invasive populations established in North America, including a recent (circa late 1990s) introduction in the northeastern U.S. (Elkinton et al. 2015). Following successful biological control of winter moth in Nova Scotia and British Columbia using the tachinid fly *Cyzenis albicans* (Fallén) (Diptera: Tachinidae) (Embree 1966; Murdoch et al. 1985; Roland and Embree 1995), similar efforts have been initiated in this most recent invasion (Elkinton et al. 2015). While classical biological control of winter moth in the northeast U.S. has shown promising results (Elkinton et al. 2015), as previously found in Canada (Roland 1988; Roland 1990), the overall success will likely depend on additional mortality from native natural enemies. Parasitoid recruitment from related native species can have significant effects on invasive populations in other insect study systems (Duan et al. 2014; Duan et al. 2013; Grabenweger et al. 2010; Matosevic and Melika 2013; Schonrogge et al. 1995; Zappala et al. 2012). This is especially true if the parasitoids can respond to the invasive population in a density dependent manor (Holling, 1973) or if the parastioids can parasitize at high rates at the invasion front such that they can slow or stop the spread of invasive population.

Non-native species with native congeners in the introduced range may be less likely to establish an invasive population than species introduced to a range without a native congener; these invasive species face top-down pressure from the natural enemies of their congener (Callaway et al. 2013; Carrillo-Gavilan et al. 2012; Diez et al. 2008; Keane and Crawley 2002; Richardson and Pysek 2006). The native congener, Bruce spanworm (*Operophtera bruceata* Hulst), is a potential source of native parasitoid

recruitment to the winter moth population in its invasive range in North America. Bruce

spanworm is present in all regions winter moth has invaded. In addition to having similar

life-cycle dynamics, these two congeners use similar hosts, are present at similar times of

the year, and can hybridize in the field (Elkinton et al. 2010; Gwiazdowski et al. 2013;

Havill et al. 2017). Thus, it is likely that native natural enemies that parasitize Bruce

spanworm could use winter moth as a host. Additionally, the life histories of winter moth

and Bruce spanworm make their populations particularly vulnerable to pupal mortality by

predation and parasitism from native natural enemies. Both winter moth and Bruce

spanworm have a long pupation period (6–7 months during the summer, representing the

vast majority of its life span) and pupates in the top layer of soil or leaf litter. Together

this renders both species particularly vulnerable to pupal mortality by predation,

parasitism, and disease.

Parasitoid wasps in the genus *Pimpla* Fabricius (Hymenoptera: Ichneumonidae:

Pimplinae) might be an important source of mortality for invasive populations of winter

moth in North America. *Pimpla* is a diverse and widespread group of parasitoids; they

are typically idiobiont endoparasitoids of Lepidoptera prepupae and pupae (Bennett 2008;

Carlson 2009; Gauld 1991; Goulet and Huber 1993), particularly on geometrid

(Lepidoptera: Geometridae) and noctuid (Lepidoptera: Noctuidae) pupae concealed in

moss or soil (Fitton et al. 1988). Nineteen extant species of *Pimpla* are known for the

Nearctic region including both native and non-native species (Yu et al. 2012; Yu et al.

2016). Over the last century, several species of *Pimpla* have been introduced into North

America for biological control of winter moth and other lepidopterans (Graham 1958;

Quicke 2015; Yu et al. 2012). Further, a species of *Pimpla* in Maine was more abundant

at sites with high winter moth infestation than moderately infested sites (Morin 2015, unpublished) and species of *Pimpla* have been reported in the northeast U.S. as hyperparasitoids of *C. albicans* (Broadley et al. 2018), an introduced biological control agent of winter moth. These latter two studies revealed an association of *Pimpla* with winter moth in the northeast U.S., but neither study directly assessed species of *Pimpla* as primary parasitoids of winter moth. The assemblage and origin of *Pimpla* species associated with invasive winter moth populations in the northeast U.S. is unknown, as well as their prevalence, role in causing winter moth mortality, and potential to regulate winter moth densities.

In this study we aimed to (1) quantify parasitism by *Pimpla* on winter moth pupae and *C. albicans* puparia across a spatial and temporal gradient, (2) test for a density dependent effect of pupal parasitism, and (3) identify the *Pimpla* spp. using morphological and molecular characteristics. We discuss our findings in relation to their implications for understanding the role and origin of this understudied parasitoid in the control of an introduced lepidopteran pest.

3.3 Methods

3.3.1 Pupal deployment

From 2014 to 2017, we collected winter moth larvae from long-term study sites across eastern Massachusetts (Elkinton et al. 2015). Larvae were reared in batches of 500 or fewer individuals in ventilated 20 L (5 gallon) buckets with the foliage from the tree species on which they were found. Mortality from viruses, other diseases, and larval

parasitism in these collections was minimal (Broadley et al. 2017). When the larvae showed signs of pupating (thickening body shape), sifted peat moss was added to the bottom of the buckets for pupation. All winter moth pupae were non-destructively checked under a dissecting microscope (Wild Heerbrugg M5 stereo) for parasitism by *C. albicans* or other larval parasitoids.

To study parasitism by native parasitoids, winter moth pupae were then deployed at sites in eastern Massachusetts, Rhode Island, and Connecticut in 2014 to 2017 (Table 10). The study sites were chosen to coincide with winter moth long-term study sites and to reflect a range of winter moth and *C. albicans* establishment histories (Elkinton et al. 2015; Elkinton et al. 2014). The study sites were all in mixed hardwood forests dominated by red oak (*Quercus rubra*). The pupae were deployed in three to five consecutive rounds from mid-June until the end of October with five deployments (one every three weeks) in 2014 and three deployments (one every six weeks) in 2015-2017. Each deployment consisted of placing 100 winter moth pupae attached to small burlap squares with beeswax, as was done in a study of predation on gypsy moth pupae in Elkinton et al. (1996). In total 12,420 winter moth pupae were deployed. In 2014 to 2016, an additional 3,400 *C. albicans* puparia (winter moth pupae that were parasitized by *C. albicans*) were also deployed in three to five consecutive rounds at a subset of sites (Table 10). The burlap squares with pupae were then buried 2.5 cm below the soil surface haphazardly under the drip line of a red oak tree. This depth was chosen to mimic natural pupa depths, which is within the upper 5 cm of soil (East 1974; Embree 1965; Holliday 1977).

3.3.2 Pupal dissections, incubation, and parasitoid collection

After each pupal deployment, we retrieved the sentinel pupae and characterized their fate as alive (ostensibly intact) or dead (consumed by predators, parasitized, or diseased). The intact pupae were stored in an incubator (Percival) until the following spring to allow further parasitoid development. Pupae were stored at 12°C until the beginning of December when the temperature was lowered to 9.5°C. At the end of December, the temperature was adjusted to 4°C. The pupae were kept in dark with no day/night cycle, and once a month they were sprayed with a sodium propionate solution (5 g sodium propionate/L of water) to prevent mold. Starting in late March, the temperatures were gradually increased in increments of 4°C until April when pupae were taken out of storage and kept at room temperature. Parasitoids (either emerged adults, dissected sub-adults, or larvae) were then identified to family using Goulet and Huber (1993) and Triplehorn and Johnson (2005) and then stored in 95% ethanol at -20°C for molecular or morphological identification. All but four of the parasitized winter moth pupae and *C. albicans* puparia appeared to be parasitized by one species of Ichneumonidae.

A set of 302 of the ichneumonid wasps (289 from winter moth and 13 from *C. albicans* puparia) reared from all collection sites, seasons, and years were used for identification. Wasps had an even representation of males and females, and included both sentinel pupae and lab-reared wasps (see methods section 2.6). All wasps were identified as *Pimpla* using keys in Townes (1969; 1970).

3.3.3 Monthly and annual parasitism rate estimates

Across all sites and years, 242 *Pimpla* wasps were recovered from the 6,580 pupae and puparia retrieved after predation. However, this ratio does not represent the true parasitism rate because it does not include pupae that had wasp emergence holes and does not account for the annual (or season-long) cumulative rate of parasitism; the ratio reflects the observed parasitism rate rather than the underlying attack rate, or marginal attack rate. The method of using the marginal attack rate to calculate percent parasitism when contemporaneous sources of morality are acting on the system has been employed by Varley and Gradwell (1968), Royama (1981), Van Driesche (1983), Buonaccorsi and Elkinton (1990), and Elkinton et al. (1992) among others. It incorporates the observation that parasitism rates can be obscured by predation rates, which typically occurs on the pupae whether or not they were parasitized and thus aims to estimate the true underlying mortality rate of each source in the system. To test for any relationship between the rates of *Pimpla* parasitism and predation, we regressed the proportion parasitized by the proportion lost to predation and no trend was detected; thus, here we apply the marginal attack rate method for contemporaneous mortality with the assumption that predators do not discriminate between parasitized and unparasitized pupae.

We calculated monthly and annual mortality. We included both pupae that still had developing wasps as well as those with wasp emergence holes in our estimate of parasitism. We performed all analyses with both the standardized monthly and annual cumulative parasitism estimates. For both these estimates of mortality, the proportion of winter moth pupae parasitized by *Pimpla* for each deployment was calculated by dividing

the total number of *Pimpla* wasps (adult and larvae) collected and number of pupae with

distinct wasp emergence holes by the total number of intact pupae retrieved from the field

(i.e. number of pupae retrieved excluding the number of pupae that were lost due to

predation). To calculate the standardized mortality from parasitism for all deployments

to 31 days (one month), we subtracted the proportion parasitized for each deployment

from 1 for the survivorship from parasitism ($S_p = 1 - M_p$) then raised this to the nth root,

where n is the true number of days the pupae were deployed (which ranged from 19 to 45

days with a mean of 31 days). The survivorship from parasitism (S_p) was then raised to

an exponent of 31 to yield the expected survivorship over a standardized 31 days

($S_{31} = [(S_p)^{1/n}]^{31}$). To calculate the cumulative (life stage-long) parasitism rate, we

converted the parasitism rates for each deployment to survival rates (S_{p1}, S_{p2}, S_{p3}, etc.) by

subtracting the proportion of parasitized pupae from one (e.g. $1 - P_{p1}$). Next, cumulative

survivorship-from-parasitism was calculated as the product of successive survivorships of

each deployment (e.g. $S_c = S_{p1} \times S_{p2} \times S_{p3}$). Lastly, the cumulative parasitism values (P_c)

were calculated by subtracting the cumulative survival from 1 (e.g. $P_c = 1 - S_c$).

3.3.4 *Pimpla* host selection

In 2016, we deployed an additional set of 2,000 winter moth pupae to study

parasitism depth, host searching behavior, and host choice of *Pimpla*. Pupae were

assigned to one of two treatments to evaluate parasitism soil depth. The two treatments

were (1) pupae with only a thin layer of leaves over top and (2) pupae with 2.5 cm of soil

then leaves over top. In both treatments, the pupae were spread one layer thick across the base of the wire mesh cages over three consecutive rounds for 35 days each at two of our sites—Wellesley and Hanson, Massachusetts. The first deployment was from 7 July – 8 August, the second was from 8 August – 9 September, and the final deployment was from 9 September – 11 October. To keep large predators out, pupae were placed in in wire mesh cages (mesh size: 6.4 mm sides with a 17 mm mesh lid). After 35 days, pupae were retrieved, sifted from the soil and stored at room temperature with natural light cycling to allow wasps to complete their development and emerge. Every second or third day, the pupae were sprayed with water, and wasp emergence was recorded. With this comparison, we tested for an effect of soil and leaf coverage on the emergence counts using a generalized regression with a negative binomial fit.

To study development time of the wasps and overwintering behavior, *Pimpla* wasps that emerged were reared in the laboratory in two cloth mesh cages (BugDorm Insect Rearing Cage, 47.5 cm^3) with winter moth pupae for five to six days in 12 rounds of 100 to 500 pupae from 15 August until 17 October 2016. For the first six rounds, we reared only 100 to 200 pupae; for the seventh round set up 16 September 2016, we increased the number of pupae to 500 to build a lab colony for future experiments. The emerged wasps were divided between the two cages such that there were approximately 30 wasps per cage, and the ratio of males to females was distributed evenly. At night, cages were stored in an incubator in full dark at 12˚C, while during the day they were taken out and exposed to room temperature (23˚C) and ambient light. The wasps were sprayed with water twice a day and given honey water to simulate natural feeding on honeydew and nectar (Fitton et al. 1988; Leius 1960).

63

We used two approaches to assess hyperparasitism rates on *C. albicans* puparia. First, in the laboratory, while most wasps were given winter moth pupae only, a subset of wasps were reared in a mesh cage with two dishes, one with winter moth pupae and the second with *C. albicans* puparia in a choice test. These trials had 100 winter moth pupae with 120 *C. albicans* puparia exposed to parasitoids 1 – 6 September, 115 winter moth pupae with 100 *C. albicans* puparia exposed 6 – 12 September, and 200 winter moth pupae with 220 *C. albicans* puparia exposed 12 – 16 September. The exposed pupae and puparia were then stored in the same conditions described above (Methods 2.5) and monitored for subsequent wasp emergence. Second, using the long-term data, we compared the monthly parasitism rates from pupae and puparia deployed in the main study plots that received *C. albicans* puparia (Table 10, sites A – F) and only in years 2014 – 2016. We used a quasibinomial logistic ANOVA of monthly parasitism rates or cumulative mortality estimates with pupal type (winter moth *C. albicans* pupa) as the main effect and year, site, and deployment as fixed effects.

3.3.5 Parasitism seasonality, overwintering, and year-to-year variation

To assess seasonality in parasitism rates, we used parasitism rates from the long-term study of field-deployed pupae (2014 – 2017) and wasp counts from additional bulk deployments conducted in 2016. For the long-term study data, we used a logistic regression to analyze the monthly rate of parasitism on winter moth pupae weighted by the total pupae analyzed against the main effect of deployment date and included year and site. We used only data from the main study sites (Table 10, sites A – H). Similarly,

to assess seasonality from the 2016 bulk sentinel pupae, fall wasp emergence counts were evaluated by their date of exposure to parasitism using a negative binomial generalized linear model (GLM).

Both the 2016 bulk field-exposed pupae and lab-exposed pupae were monitored for emergence until 7 October when they were moved to chill conditions. They were acclimated by lowering the temperature by 2°C per day until a final temperature of 4°C was reached. On 17 May 2017, pupae were moved from their overwintering storage of 4°C to 12°C and on 17 June, they were brought back to room temperature. Samples were monitored for wasp emergence starting 23 June and were checked every two to three days for a month.

To assess yearly changes in pupal parasitism, we again used the parasitism rates from the winter moth deployed across all years and sites (Table 10, sites A – H). A logistic model (generalized linear model using the binomial family) was applied using both our estimation of monthly and annual parasitism rates weighted by the total pupae analyzed. For our analysis of monthly parasitism, we included year, site, and deployment as fixed effects. Four our analysis of annual cumulative parasitism, we weighted the logistic regression by the average number of pupae analyzed across the deployments and used year and site as a fixed effect.

3.3.6 Parasitism with winter moth spread and density

To compare *Pimpla* parasitism on winter moth pupae between sites infested by winter moth (the first eight sites in Table 10, to the east of the yellow line in Figure 8)

compared to sites on the edge of the infestation or outside the heavy-infestation area (the last six sites listed in Table 10, to the west of the yellow line in Figure 8), we used a logistic regression to analyze the monthly rate of parasitism weighted by the total pupae analyzed against the main effect of infestation status, and included year and site as fixed effects. We also analyzed the cumulative rate of parasitism weighted by the average number of pupae analyzed across the deployments against the main effect of infestation status and included year and site as fixed effects. Only years 2015 and 2016 were used for these comparisons as they included both heavily infested sites and sites outside the heavily infested area. The designation of a site as winter-moth-infested was determined from the winter moth spread analysis by Elkinton et al. (2014) and Elkinton et al. (2015).

To estimate winter moth pupal density at each long-term study plot (Table 10 sites A – H), 16 plastic buckets (16 cm width X 28 cm length X 10 cm height) filled 3 cm deep with sifted peat moss and rainwater drainage holes were placed under each study tree in late May before pre-pupal winter moth caterpillars began to spin down from the tree canopies. Each bucket was placed at a randomly selected distance between the tree stem and the edge of the tree canopy along one of eight evenly spaced directions as described in Varley et al. (1973a) and Whited (2007). We analyzed *Pimpla* parasitism by winter moth pupae density to test for any effects of density dependence. We used a logistic regression of monthly parasitism rates weighted by the total pupae analyzed regressed against the corresponding density of winter moth pupae (log-transformed) with site, year, and deployment as fixed effects. We also analyzed the cumulative, annual parasitism against the log-transformed pupal density with year and site effects.

Deployment effects were not included explicitly as the survivorships from each deployment were multiplied in the calculation of cumulative mortality.

3.3.7 Wasp development time and sex ratio

With a subset of pupae parasitized by *Pimpla* from the laboratory study, we estimated development duration of the wasp by noting the first event of parasitism and the subsequent date of wasp emergence. During the first day of exposure to the wasps, we monitored the set-up for evidence of parasitism. When we saw a female oviposit into a pupa, we moved the pupae singly to a tube (15 mL Falcon centrifuge tube with a ventilation hole at the top). A total of 120 tubes each with one potentially parasitized pupa were set up. The exposed pupae were stored at room temperature in ambient light and monitored for subsequent wasp emergence. Of these pupae, 42 wasps emerged. The average and standard deviation of wasp development time was calculated. The sex of wasps that emerged from the bulk field-exposed pupae and lab-exposed pupae was noted. The proportion of females that emerged was evaluated by treatment (field or lab pupae), by date of exposure to parasitism, and by season of emergence (fall or spring) using binomial GLM.

3.3.8 Statistical analyses

All analyses were performed in R 3.4.4 (RCoreTeam 2013) using RStudio, version 1.1.442 (RstudioTeam 2015). For each analysis, the full model was always run initially (including site, year, and deployment effects, etc.), the model was evaluated for evidence skew in the residuals or outliers, and any insignificant predictors were dropped sequentially until the best fit model was selected using AIC comparisons. We checked for overdispersion, and when evidence of overdispersion was noted, we applied a quasibinomial or quasipoisson distribution. Quasibinomial and quassipoisson analyses do not generate AIC values; thus, to select the best fit model, we compared the residual deviance of the fit model to that of the null model. A pseudo-R^2 was calculated by comparing the residual deviance of the fit model against the null model (deviance null model – deviance fit model / deviance null model). All graphical data was displayed using ggplot2 (Wickham 2009).

3.3.9 Morphological comparative analyses

Following initial identification of our specimens as *Pimpla* using keys in Townes (1969; 1970), further morphological and molecular identification was conducted. Townes et al. (1960) was used to identify the specimens to species. Specimens were also compared with authoritatively determined specimens of *Pimpla turionellae* L., *Pimpla contemplator* Müller*, Pimpla disparis* Viereck, and *Pimpla hesperus* Townes in the Smithsonian Institution National Museum of Natural History (USNM). All but the last species have been recorded as attacking winter moth in its native range (Wylie 1960) and

were introduced into Canada and the U.S. to control winter moth and other lepidopteran

pests in North America (Graham 1958; Humble 1985; Quicke 2015; Yu et al. 2012).

Pimpla hesperus is native to North America and has been reported as a parasitoid of

winter moth and Bruce spanworm in British Columbia (Humble 1985). We also

compared our specimens to the lectotype for *Pimpla aequalis* Provancher from the

Université Laval, Quebec City, Quebec, Canada (ULQC). Vouchers for parasitoid

species are deposited in the University of Massachusetts Insect Collection, Amherst, MA.

3.3.10 Molecular comparative analyses

A subset of the *Pimpla* wasps that emerged or were dissected from winter moth or

C. albicans pupae were selected for molecular analyses. When possible, three adult

samples and one larval sample were selected for each location and study year, otherwise

up to four wasps of any life stage were selected for a total of 77 field-collected

individuals and 20 laboratory-reared wasps. Since morphological identifications and

pilot molecular work found that the majority of wasps were *Pimpla*, it was assumed that

all wasp larvae were *Pimpla*; subsequent molecular work confirmed this. DNA was

extracted using the QIAGEN DNeasy Blood and Tissue Kits following the company

protocol with the following modifications: for larvae, individuals were destructively

sampled by grinding with a mortar and pestle; for adults, DNA was extracted from a

single leg removed from the specimen; for both life stages, DNA was eluted twice in 100

µl Buffer AE instead of once with 200 µL. All DNA extractions were stored at -20°C for

subsequent analysis.

A portion of the mitochondrial locus cytochrome c oxidase subunit I (CO1) was amplified using standard PCR techniques. For a subset of individuals collected across years, sites, life stages, and life history fragments from three additional nuclear gene regions were amplified: the carbomoylphosphate synthase domain (Cadherin, rudimentary, CAD), elongation factor 1-α (EF1-α), and the D2 and D3 expansion segments of the large subunit ribosomal RNA gene (28S).

For each locus, a master mix was prepared using the following ratios of reagents per sample: 17.3 μl nuclease free water, 0.5 μl dNTPs, 5 μl 5X GoTaq Buffer (Promega), 0.2 μl GoTaq (Promega), 0.5 μl of both the forward and reverse primer (10 μM each), and 1 μl of eluted DNA. To amplify a 710 bp fragment of CO1, the primer set LCO (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994) were used with the thermocycler profile outlined by Hebert *et al.* (2003). A 537 bp region of CAD was amplified using the primer pair CADf-CADKlopF (5'-AGCGTCGGTGAGGTAATGGC-3') and CADr-CADKlopR (5'-CCTATAACCATCGTGTAATTTCC-3') using the same temperature profiles as outlined for EF1-α (S. Klopfstein, unpublished). A 450 bp region of EF1-α was amplified using the following primer pair EF1r-EF1aKlo11F (5'-AGATGGGYAARGGTTCCTTCAA-3') and EF1r-EF1aKlo11R2 (5'-AACATGTTGTCDCCGTGCCATCC-3') (S. Klopfstein, unpublished) and the following touchdown temperature profile: 94°C for 2 min for initial denaturing, 2 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 1 min, followed by 2 cycles of 94°C for 30 s, 52°C for 45 s, for 72°C for 1 min, followed by 36 cycles of 94°C for 30 s, 52°C for 45 s, and 72°C for 1 min with a final extension of 72°C for 5 min and 4°C hold. Lastly, to amplify

70

a 824 bp region of the 28S region, the primer pair 28Sf-s3660 (5'-GAGAGTTMAASAGTACGTGAAAC-3') and reverse primer 28Sr-28b (5'-TCGGARGGAACCAGCTACTA-3') (Morse and Normark 2006; Whiting et al. 1997) with the touchdown PCR thermocycler protocol described in Morse and Normark (2006).

All PCR reactions were run on a BioRad T100 thermocycler, and the resulting PCR products were visualized on an 1.5% agarose gel stained with SYBERsafe (Invitrogen, Carlsbad, CA) to verify amplification. Samples that produced bands of the expected fragment size for each locus were then cleaned prior to sequencing using Exonuclease 1 (ThermoScientific) and Thermolabile Recombinant Shrimp Alkaline Phosphatase (New England BioLabs). The resulting products were submitted to The Yale University DNA Analysis Facility on Science Hill for Sanger sequencing in both sense and anti-sense orientations.

The resulting sequences were then visualized, the forward and reverse sequences were aligned, and sequences were edited using Geneious R8.1.8 and R9 (Biomatters Ltd.). The ends of the aligned sequences were trimmed by hand to remove primer sequences and so that all sequences had a high-quality score (>90% HQ nucleotide reads). The presence of heterozygous sites was determined by Geneious and encoded using the appropriate IUPAC-IUB ambiguity codes. All ambiguous regions were subsequently inspected by eye. Additionally, for our CO1 fragment sequences, we looked for evidence of nuclear mitochondrial DNAs (NUMTs) or pseudogenes by examining for the presence of stop codons based on translation with Invertebrate Mitochondrial DNA genetic code.

To compare our *Pimpla* CO1 sequences to other published *Pimpla* CO1 sequences, we searched both the National Center for Biotechnology Information (NCBI) GenBank database and the University of Guelph Centre for Biodiversity Genomics's Barcode of Life Data Systems (BOLD). We initially downloaded triplicate sequences from each *Pimpla* species available across the two repositories, but when the triplicates were identical to each other or nearly-identical (>99% identical), then we retained one representative sequence for each *Pimpla* species available. From the BOLD sequences, we prioritized sequences acquired from *Pimpla* samples in the hymenopteran collection of the Canadian Natural Collection of Insects, Arachnids and Nematodes (Agriculture and Agri-Food Canada) accessed by A. Bennett (accession numbers start with 'BOLD HYCNG'). These sequences included 57 *Pimpla* specimens representing 13 North American species [of 19 extant described species in the region, (Yu et al. 2016)]. When no representative sequence was available for a particular *Pimpla* species by the Canadian National Collection, we then searched GenBank for a representative sample, followed by any other sequences available in BOLD. This added an additional six species of *Pimpla*; however, many of these were from outside the Nearctic region.

We included five additional sequences identified as *P. aequalis* and nine additional published sequences referred to as *Pimpla* sp. from Carpenter and Wheeler (1999); Hebert et al. (2016); International Barcode of Life (iBOL) unpublished; and Dewaar, Telfer, and Young unpublished (accession numbers listed in Figure 12). These were the closest matches using the Basic Local Alignment Search Tool (BLAST) blastn suite to sequences of our each of our two *Pimpla* clades.

We used the Geneious alignment algorithm in Geneious v. 8.1.8 (Kearse et al. 2012) to generate a sequence alignment of all sequences generated in this study and those obtained from public databases. The alignment was truncated to the length of the shortest included sequence. To reduce the number of identical sequences included in analyses, if any of our sequences were identical we only included a single representative haplotype.

JModelTest was used to select the best substitution model for nucleotide evolution, as implemented in the CIPRES Science Gateway (Miller et al. 2010). Based on these results, we then performed neighbor-joining, maximum likelihood, and Bayesian reconstructions using the GTR substitution model. Neighbor-joining analyses were run in Geneious using 1,000 bootstrap replications and a majority rule (50%) consensus threshold. Maximum likelihood analyses were run using PhyML (Guindon et al. 2010) with 100 bootstrap replications. Bayesian analyses were run using MrBayes 3.2.6 (Huelsenbeck and Ronquist 2001) with a MCMC chain length of 1,000,000 and a burn in length of 10%. The resulting gene trees were then visualized using FigTree Version 1.4.2 (Rambaut 2014).

To determine whether specimens identified as *P. aequalis* might be members of a cryptic species complex, we used a multilocus genealogical concordance approach (Andersen et al. 2010; Avise and Wollenberg 1997; Baum and Shaw 1995; Dettman et al. 2003; Dettman et al. 2006; Groeneveld et al. 2009; Menkis et al. 2009; Starrett and Hedin 2007) to estimate the number of species present in our dataset. This method considers lineage sorting in multiple, independent loci and has become a common approach for species delineation. For these analyses, we created separate alignments for each gene fragment including each specimen from which all target loci were successfully amplified.

In addition, we included publicly available sequences from *Labena grallator* (Say) (Hymenoptera: Ichneumonidae) as the outgroup for each alignment. Individual gene trees were then estimated for each locus using the methods described above. The congruence of the topologies of the reconstructed gene trees were then visualized by inferring a majority-rule consensus tree using PAUP (Swofford 2003).

3.4 Results

3.4.1 Parasitoid collection

Of the 6,580 retrieved pupae and puparia that escaped predation in the field (5,009 winter moth pupae and 1,571 *C. albicans* puparia, Table 10) over four years of study (2014 – 2017), 342 had evidence of pimpline wasp parasitism. This included specimens with *Pimpla* larvae or adults still developing in pupae or puparia (206 from winter moth pupae and 36 from *C. albicans* puparia), and an additional 100 sentinel pupae had a distinct wasp emergence hole. Note that the ratio of total wasps per total retrieved pupae does not reflect cumulative percent parasitism values (see Methods 2.3). Besides the pimpline wasps, only four puparia (deployed in Kingston, Rhode Island from 5 Aug - 18 Sept 2015) were parasitized by a cluster of diapriid wasps (Hymenoptera: Diapriidae), and two winter moth pupae (deployed from 24 Jun - 5 Aug. 2015, one in Wellesley, MA and one in Pawckatuck, CT) were parasitized by a species of *Cratichneumon* (Hymenoptera: Ichneumonidae). The rest of the wasps were determined to be *Pimpla*. Of the 242 *Pimpla* wasps recovered from field-deployed sentinel pupae and puparia, 112 were adults (46%) and 130 were larvae (54%).

3.4.2 Parasitism seasonality, overwintering, and year-to-year variation

Monthly parasitism on winter moth pupae varied from 0 to 52%. Deployment was marginally significant with parasitism rates from early August to mid-September slighter higher than those of either late June to early August or mid-September to late October (df=100, pseudoR^2= 0.34, p=0.06). From the bulk pupae at Wellesley, MA and Hanson, MA in 2016, deployment was significant (df=8, pseudoR^2= 0.30, p=0.0018). We found that only pupae exposed to wasps after 8 August had wasps that overwintered, and most of the overwintering wasps (91%) were from winter moth pupae that were in the field from 9 September until 11 October. The cumulative *Pimpla* parasitism ranged from an average across years as low as 54% for Hingham, MA to 92% for Sunderland, MA (Table 10). *Pimpla* wasps were recovered from all years and sites, though some sites had a year without recoveries. There was a significant effect of year but not site or deployment date when analyzing monthly parasitism rates (df=57, pseudoR^2=0.40, p=0.034) and a site effect was evident when analyzing the annual cumulative parasitism rates (Figure 9; df=13, pseudoR^2=0.72, p=0.009). 2015 had significantly higher parasitism rates.

3.4.3 Parasitism with winter moth spread and density

No significant difference was found in percent *Pimpla* parasitism on winter moth pupae that were deployed in sites on the edge of the current winter moth infestation area

compared to the heavy-infestation area (Figure 8). No difference was found either for monthly parasitism or annual cumulative parasitism. However, there was a trend toward higher percent parasitism at the edge of the winter moth infestation. Pupae deployed in sites at the edge of the winter moth infestation had a mean ± SE parasitism rate of 0.53 ± 0.08, while it was 0.32 ± 0.03 in the infested sites. Pupal density of the study sites had a significant effect on the monthly (df=62, pseudoR^2= 0.25, p=0.039) and annual cumulative parasitism (df=19, pseudoR^2= 0.39, p=0.036), with a significant effect of year in both models (Figure 10).

3.4.4 Wasp development time and sex ratio

From pupae parasitized in the laboratory, 39 wasps developed and emerged as adults. On average, it took 21.2 ± 0.6 SE days from the date of parasitism to adult wasp emergence. No wasp emergence was noted after 9 October until spring. Thus, if we assume that *Pimpla* parasitize between 1 June and 1 October, then they have 122 days in which to parasitize and develop. And if wasps take 21 days from oviposition to emergence, then we could expect up to 5 generations per season (including some time for eclosion and mating) and a final 6th overwintering generation under laboratory rearing conditions. This estimate is from individuals held at a constant 23˚C temperature; however, in the field average temperatures for this time would have been slightly cooler (~19˚C), so development may be slower in field conditions. Across studies (bulk field deployed pupae and laboratory rearing), there were 305 *Pimpla* females and 238 *Pimpla* males; this is 56.2% female or roughly a 1:1 ratio.

3.4.5 *Pimpla* host selection

From the three deployments of bulk pupae placed in Wellesley, MA and Hanson, MA in 2016, there was a significant effect of soil treatment (df=8, pseudoR^2=0.57, p=0.0043) and deployment (p=0.0018). More wasps emerged from the pupae that were covered only by a layer of leaves (109 wasps ± 39.0 SE) than from the pupae that were buried under 1 cm of soil and a layer of leaves (32.3 wasps ± 14.3 SE). In the host-choice study, we did not observe any wasps attempting to oviposit in the pupae parasitized by *C. albicans*, and no wasps emerged from these trials. However, from the field studies (Table 10, Figure 11) and earlier studies (Broadley et al. 2018), we know that *C. albicans* puparia can be parasitized by *Pimpla* spp. but typically at a lower rate than winter moth. We compared parasitism rates on winter moth pupae and *C. albicans* puparia (Figure 11). *Cyzenis albicans* puparia experienced significantly lower parasitism rates than winter moth pupae for both models that included monthly parasitism rates (df=83, pseudoR^2=0.39, p=0.00012) and annual cumulative parasitism rates (df=22, pseudoR^2= 0.64, p= 0.0018).

3.4.6 Molecular and morphological comparative analyses

The specimens in this study key to *P. aequalis* using Townes et al. (1960), are consistent with the description of that species in Townes (1960), and the lectotype for *P. aequalis* fits within the morphological concept of *P. aequalis* discerned for our *Pimpla* specimens. Parasitism of the *C. albicans* puparia by *Pimpla* spp. is reported here, while

parasitism by other non-pimpline hyperparasitoids was the focus of an earlier study (Broadley et al. 2018).

We acquired high quality CO1 loci sequences for 74 individuals (50 adults and 24 larvae). These individuals represented all sites and years from which *Pimpla* wasps were detected. Based on reconstruction of the phylogeny using the CO1 gene fragment, our samples separated into two distinct clades that exhibited 9.7 to 10.1% sequence divergence (Figure 12). All nucleotide differences between the two clades represented third-codon substitutions and thus likely represent genetic differences accumulated due to genetic drift and not selection. Both *Pimpla* clades included wasps acting as primary parasitoids and hyperparasitoids (49 primary and 26 hyperparasitoids). Based on the CO1 data, sequences from individuals in one of the clades were most similar to those labelled *P. aequalis* in public databases. However, another sample identified as *P. aequalis* (BOLD HYCNG2310-12) appeared in a different clade. Our other sequences were most similar to sequences labelled in public databases simply as Hymenoptera sp. or *Pimpla sp.* (e.g. HQ978834.1 or KR877820.1) and had no matches to any sequences with species identifications which were publicly available at the time of this publication. We also detected no matches with *Pimpla* CO1 sequences from the hymenopteran collection of the Canadian Natural Collection of Insects, Arachnids and Nematodes (BOLD HYC sequences).

Additionally, we obtained high quality sequences for fragments of CAD, EF1-α, and 28S from 26 *Pimpla* wasps (19 *Pimpla aequalis* species 1 individuals and 7 *Pimpla aequalis* species 2 individuals as from the CO1 analyses). For CAD, sequences from the two *Pimpla* clades identified based on the CO1 analyses were 1.5-3.4% different from

78

each other, with six base-pair differences fixed between the two *Pimpla* clades (Figure 13). Similarly, for EF1-α the two clades were between 0.7-1.6% different with 3 base-pairs consistently fixed between clades consistently different. For 28S, there were no differences between individuals from the two CO1 clades, with all but two individuals (#876 and #997) having identical sequences; the two individuals (#876 and #997) differed from the other by a single base-pair substitution. Because 28S was invariant, it was left out of the multilocus analyses. The trees constructed from each of these three loci (Figure 13) and the majority rule consensus tree all supported the presence in our samples of two cryptic *Pimpla* species that morphologically fit the description of *P. aequalis*. For EF1-α, sequences from both *Pimpla* clades were 99% similar to sequences published by Heraty et al. (2011) and Klopfstein and Ronquist (2013) identified as *P. aequalis*. For 28S, there was no variation, and all sequences we generated matched (99% similarity) sequences in GenBank published by Quicke et al. (2009) for several species of *Pimpla* including *Pimpla* sp. (EU378837.1), *P. disparis* (EU378831.1), and *Pimpla mahalensis* Gribodo (EU378835.1).

Based on these comparisons using a morphological and molecular approach, we conclude that we have two distinct species of *Pimpla*. It is unclear which of the clades is *P. aequalis*. The second author (RRK) and another ichneumonid systematist (B. Santos, Smithsonian Institution NMNH) considered the *Pimpla* specimens in this research one species (i.e. *P. aequalis*) based morphological features; the lectotype for *P. aequalis* fits within the morphological concept. While one of the clades is *P. aequalis*, the other might represent an undescribed species. However, discerning the identities of these clades requires more extensive examination of *Pimpla* species from the Nearctic and Palearctic

regions, as some species of *Pimpla* have a Holarctic distribution (e.g., *Pimpla aquilonia* Cresson). Presumably, these two species of *Pimpla* are native to North America. Morphologically, both match *P. aequalis* and do not match any Palearctic species available to us that are known to attack winter moth in Europe. Further, the sequences generated from our specimens do not match any published sequences available for other *Pimpla* species, which includes 13 or the 19 extant described species in the region, (Yu et al. 2016) and an additional six species of Pimpla from outside the Nearctic region. The only molecular matches are for other unknown *Pimpla* species collected from the northeast U.S. and southeast Canada.

Table 10: Annual cumulative percent parasitism by *Pimpla* for each study site and each year of study. The parasitism estimate includes all signs of *Pimpla* parasitism, including recovered adult or larval wasps, as well as pupae with prominent wasp emergence holes for sentinel winter moth pupae and pupae parasitized by *Cyzenis albicans*. The total number of pupae examined to determine this proportion (i.e. the denominator) is included in parentheses. The first eight sites (A-H) are long-term study sites and are used for analyses of parasitism across the years of study and by pupal density, while the sites marked in gray were used for an evaluation of *Pimpla* parasitism within and outside the main winter moth infestation area.

Site	GPS location	Winter moth pupae deployments				*C. albicans* puparia deployments		
		2014	2015	2016	2017	2014	2015	2016
A. Co-op Extension, Hanson, MA	42.048889, -70.873806	00.0 (266)	24.2 (102)	11.5 (76)	15.1 (200)	-	48.2 (76)	00.0 (97)
B. Maquan St., Hanson, MA	42.060694, -70.844167	20.8 (204)	41.1 (120)	24.6 (159)	31.8 (171)	11.4 (65)	12.6 (93)	00.0 (166)
C. Pondview Dr., Falmouth, MA	41.626417, -70.580417	13.4 (366)	-	15.2 (107)	3.1 (167)	-	-	00.0 (150)
D. Centennial Park, Wellesley, MA	42.308444, -71.266778	33.6 (303)	50.2 (156)	23.2 (141)	19.1 (159)	11.1 (116)	44.9 (123)	00.0 (116)
E. Wompatuck SP, Hingham, MA	42.208333, -70.853056	13.3 (229)	39.4 (113)	23.8 (167)	7.3 (188)	-	12.0 (146)	00.0 (183)
F. Route 6, Yarmouth, MA	41.686167, -70.287722	1.3 (343)	-	43.3 (35)	00.0 (10)	-	-	00.0 (47)
G. Center St, W. Bridgewater, MA	42.020806, -70.982306	27.5 (331)	-	-	-	-	-	-
H. Bare Cove Park, Hingham, MA	42.238222, -70.913389	5.3 (221)	-	-	-	-	-	-
I. Cook Rd, Cumberland, RI	42.012278, -71.421361	-	35.8 (97)	75.2 (119)	-	-	-	-
J. Parkwood, Drive, Kingston, RI	41.475250, -71.529444	-	38.2 (85)	29.7 (100)	-	-	47.5 (78)	-
K. Broad St., Pawcatuck, CT	41.376194, -71.843528	-	42.6 (88)	-	-	-	-	-
L. Garden in the Woods, Framingham, MA	42.340833, -71.427667	-	47.9 (118)	-	-	-	6.8 (115)	-
M. Cadwell Forest, Pelham, MA	42.365500, -72.437667	-	-	60.1 (53)	-	-	-	-
N. Mt Toby SF, Sunderland, MA	42.502361, -72.530111	-	-	91.8 (15)	-	-	-	-
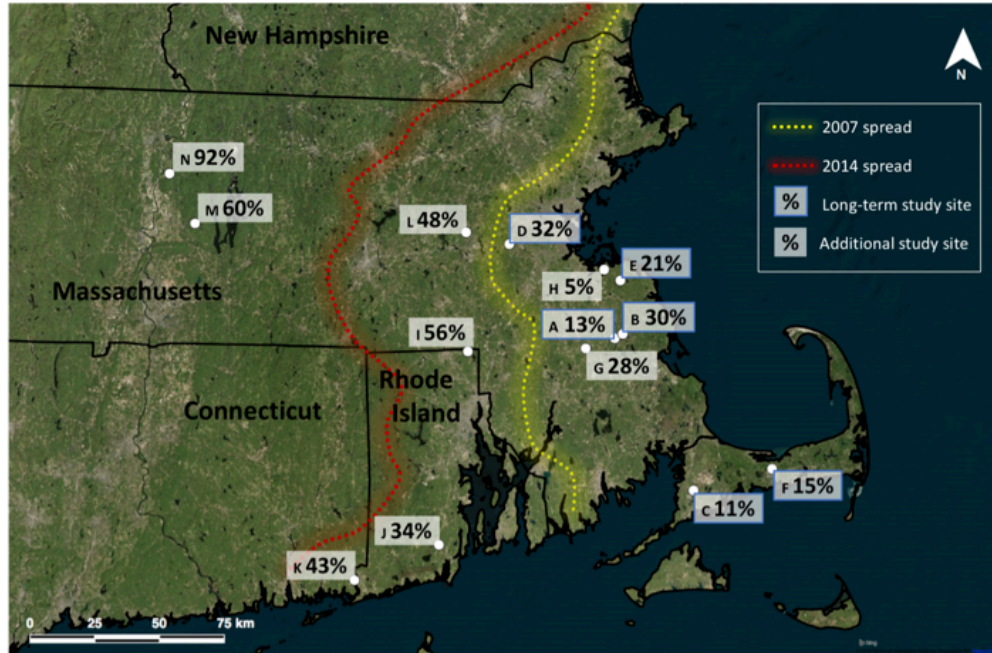
Figure 8: Average (2014 – 2017) cumulative percent parasitism by *Pimpla* on winter moth pupae across the pupal deployment sites. The letters for each study site correspond to Table 1. The area on the map to the right of the dashed lines approximates the winter moth infestation area for 2007 and 2014 (Elkinton et al. 2014a; Elkinton et al. 2014b). The six main study sites (sites A-F) are noted in blue.
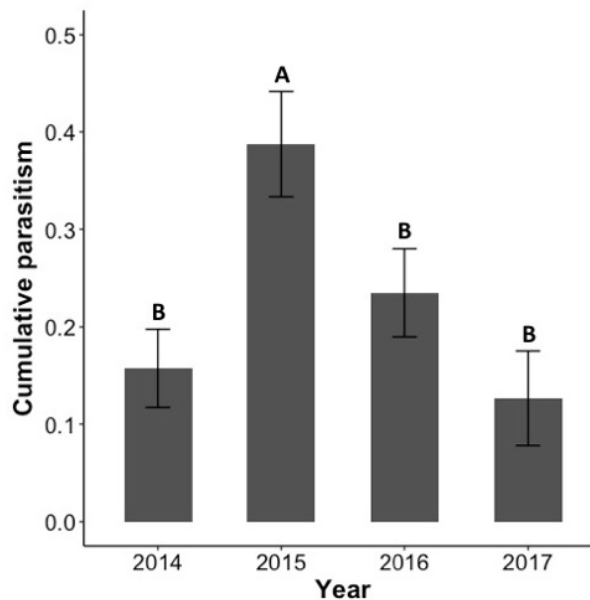
Figure 9: Mean (±SE) annual cumulative parasitism by *Pimpla* on winter moth pupae by year across sites. Only the six long-term study sites are included (the first six study sites listed in Table 1).
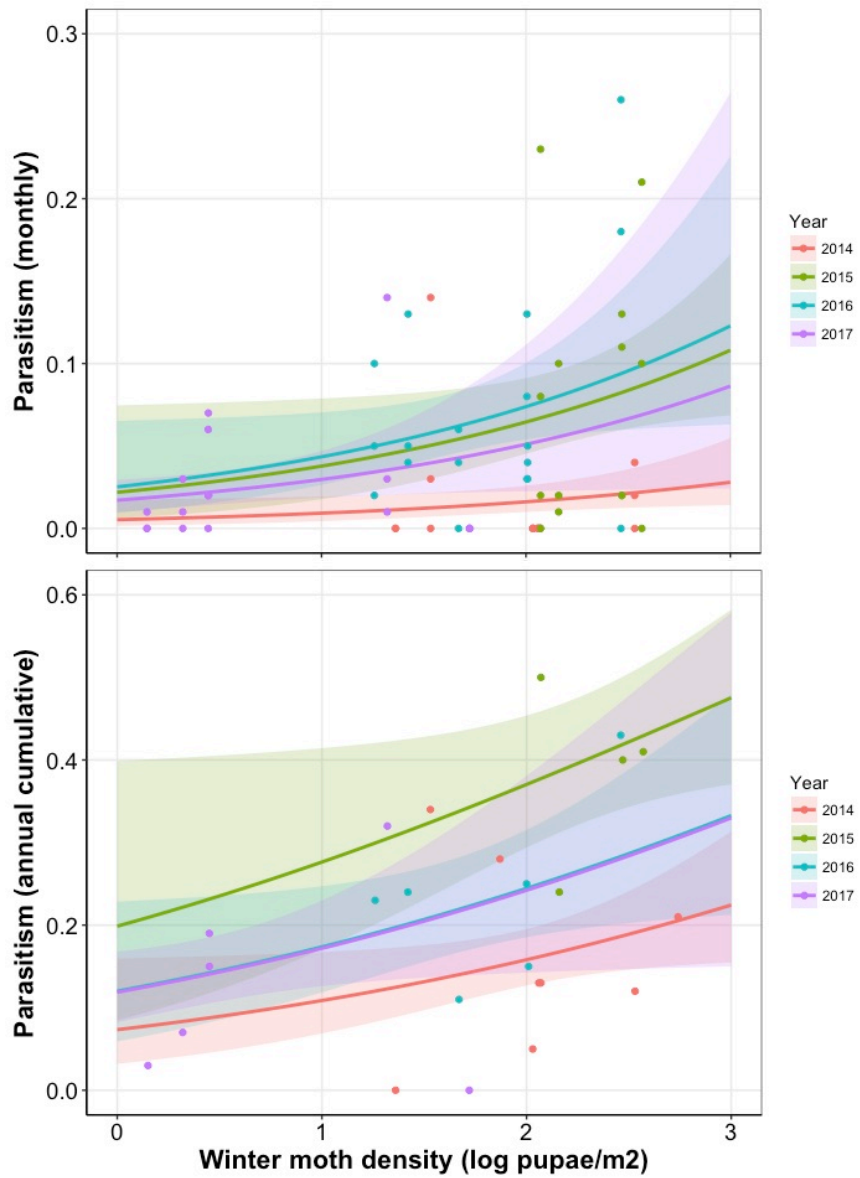
Figure 10: Logistic relationship between monthly (top panel) and annual cumulative (bottom panel) *Pimpla* parasitism on pupae by winter moth pupal density across sites (the six main study sites). The model shows year (2014 – 2017) ±SE.
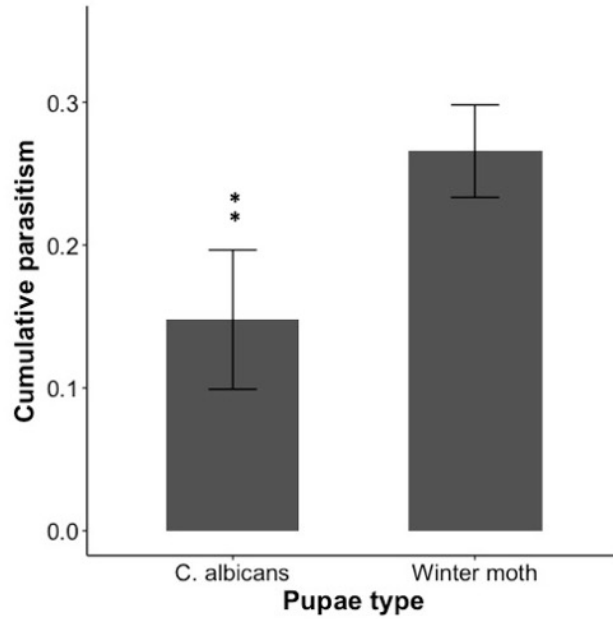
Figure 11: Cumulative annual *Pimpla* parasitism on winter moth pupae as compared to *Cyzenis albicans* puparia. The figure shows the mean ±SE for study sites and years that included both pupae types (sites A-L, years 2014 -2016). The *C. albicans* puparia received experienced significantly less mortality (df=22, pseudoR^2= 0.64, p= 0.0018).
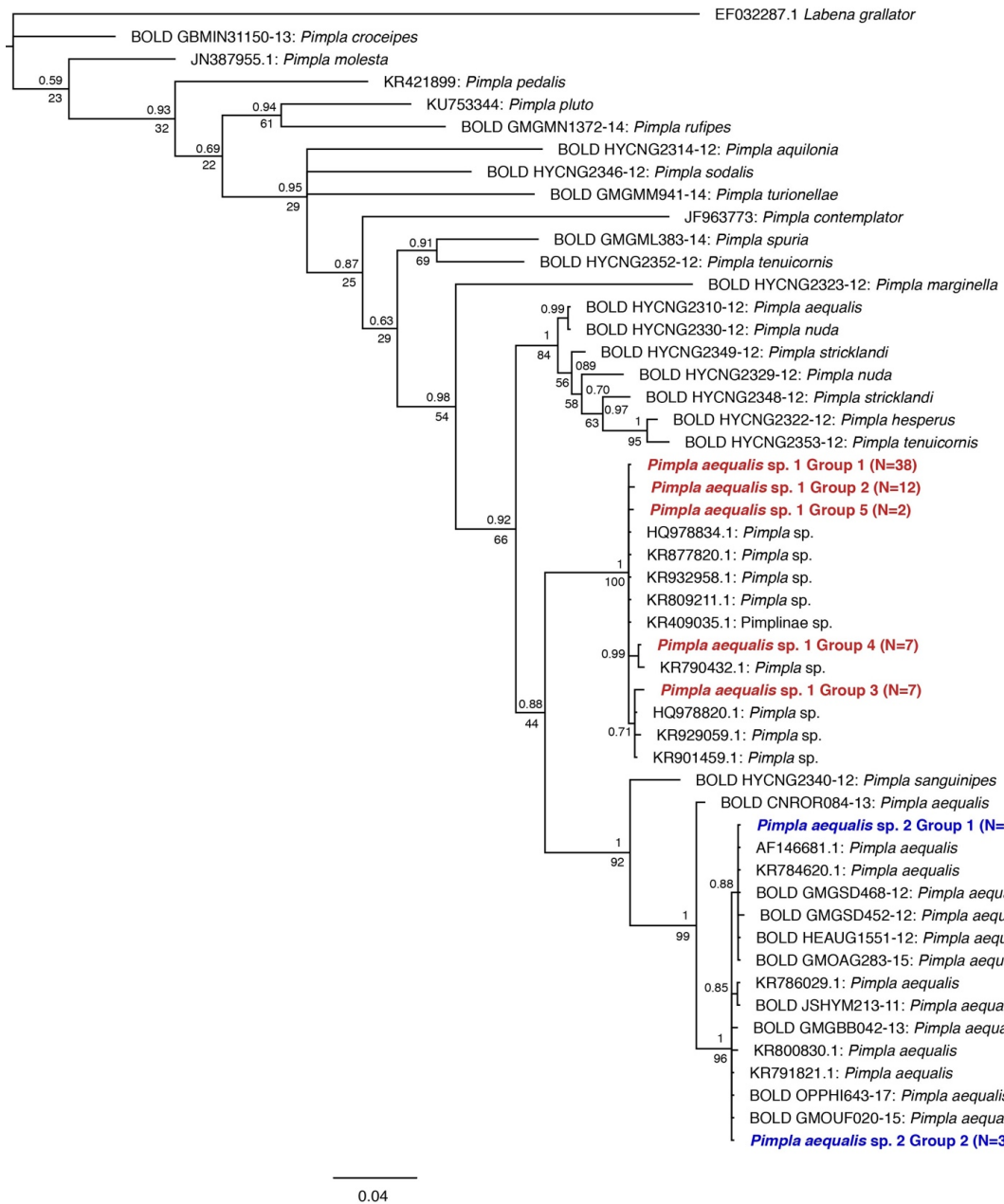
Figure 12: Bayesian tree of a 604 bp region of the CO1 loci with the sequences from our *Pimpla* samples (bolded) and representative sequences from NCBI GenBank and BOLD. Where our sequences were identical, the groups were collapsed as outlined in Table S2. *Pimpla aequalis* sp. 1 are noted with red and sp. 2 with blue. Branch lengths are drawn proportional to the rate of range observed. The number to the left each node represents the bootstrap support value for the branch (Bayesian over the Maximum Likelihood).
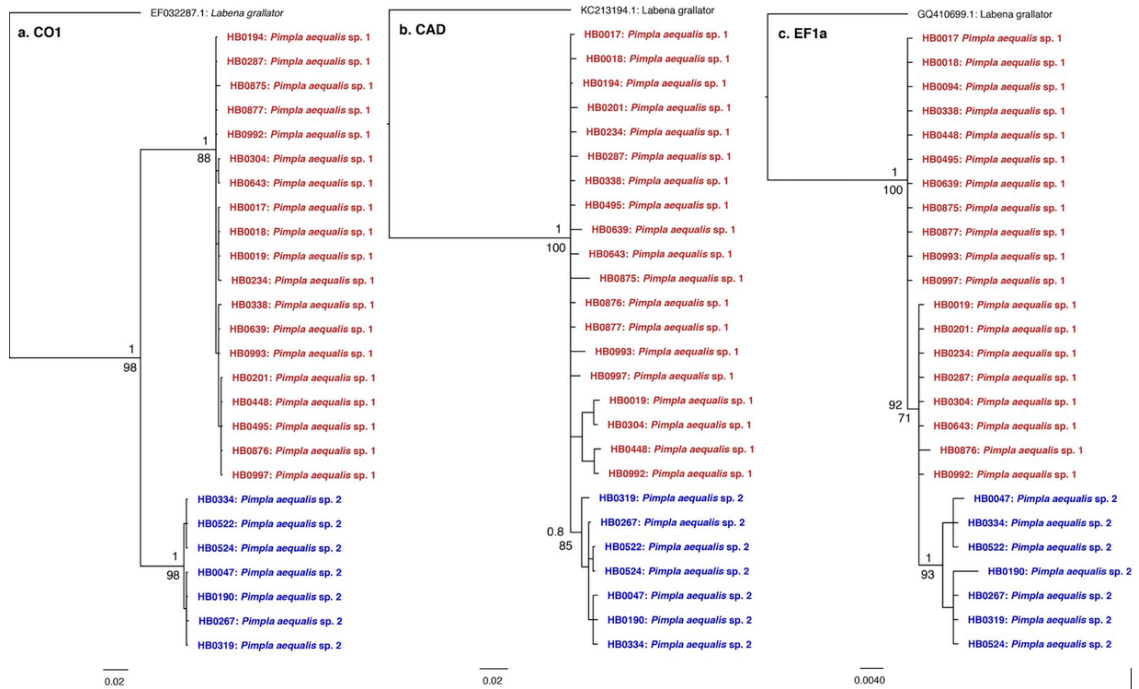
Figure 13: Phylogentic inferences using (a) CO1, (b) CAD, and (c) EF1-α gene regions for only our *Pimpla aequalis* sp. 1 (red) and sp. 2 (blue) samples. Branch lengths are drawn proportional to the rate of range observed. The number to the left each node represents the bootstrap support value for the branch (Bayesian over the Maximum Likelihood).

3.5 Discussion

Biotic resistance is the process by which native natural enemies spill over from native species to attack an invasive species and reduce the success of that invader (Diez et al. 2008; Elton 1958; Levine et al. 2004; Sakai et al. 2001; Shea and Chesson 2002). We found evidence of biotic resistance to invasive winter moth populations in the northeast U.S.; in this most recent invasion, winter moth populations are sustaining heavy, density dependent parasitism by two cryptic species of *Pimpla*. One of the *Pimpla* wasps is almost certainly *P. aequalis*, while the other appears to be a related unknown, and potentially undescribed, species. As far as we know, both wasp species are native, and parasitism of winter moth is likely the result of natural enemy spillover from the native congener Bruce spanworm or other geometrid or noctuid species in the area. While the association of these *Pimpla* wasps with winter moth is relatively recent, the impact of *Pimpla* is notable; based on wasp collections alone, estimates of parasitism across our study sites were as high as 56% on winter moth pupae in infested areas and were found across all of our study plots. Further, *Pimpla* parasitism responded to winter moth pupal density in a positive density-dependent manner and thus has the potential to be regulatory (Holling 1973). Finally, parasitism rates were highest at the leading edge of the winter moth infestation, suggesting that the wasp may help to control winter moth spread.

The ability of a native predator or parasitoid to respond functionally or numerically to a primary host population, while also using an alternative host when the primary host is at low densities, may result in particularly effective suppression of

87

populations of non-native invader by a native species (Necols et al. 1992; Shea and Chesson 2002). In this way, the native natural enemy has the ability to build up using the novel host but can also use other host species when this new host is less abundant. As a result, the natural enemy is maintained in the community and can aggregate or respond numerically when the invasive alien population outbreaks or spreads. This pattern of host use is common for a number of generalist predators, parasitoids, and pathogens (deRivera et al. 2005; Hassell and Rogers 1972; Holling 1973; Murdoch 1969; Oaten and Murdoch 1975; Schenk and Bacher 2002; Strauss et al. 2012).

Parasitism by *Pimpla* in this study exhibited both of these characteristics. While *Pimpla* may build up using winter moth as a host species and respond to high densities with higher rates of parasitism, it also can use other host species when winter moth populations are not at high densities. We detected a positive density dependent relationship of parasitism to winter moth density, suggesting that *Pimpla* may have a regulatory effect on the winter moth population densities, and since *Pimpla* is multivoltine, the population of *Pimpla* may be able to build up in outbreak populations in a numerical response. Such a numerical response would be density dependent and would have the potential to control an outbreaking population (Holling 1973). However, we did not detect increased parasitism rates over either season or years and were not able to test for a numerical response to densities. In the absence of a documented numerical response, it is likely that the density dependent response we found may arise from a Type III functional response driven by *Pimpla* host-switching behavior (Holling 1959; Murdoch 1969). While pimplines typically have a preferred host species (niche specialization), they are facultative generalists and can use a relatively broad host range

spanning multiple lepidopteran families (Bennett 2008; Fitton et al. 1988; Krombein et al. 1979). *Pimpla* exhibited particularly high parasitism 95 km beyond the nearest high-density winter moth infestation (i.e. Framingham, MA) and 22 km from the nearest capture of winter moths in pheromone traps (i.e. Orange, MA). This supports the hypothesis that *Pimpla* population densities do not depend on the presence of winter moth and are already high in the community using other hosts, especially at the leading edge of the winter moth infestation. In this way, *Pimpla* may provide a biotic resistance barrier to the spread of winter moth. This may be helping to hold back the spread of winter moth, which was documented by Elkinton et al. (2014).

If *Pimpla* acts as a hyperparasitoid (a parasitioid of a parasitoid) of any native or introduced parasitoids (biological control agents), then it has the potential to reduce population control of the invasive species by inflicting more mortality on the biological control agent than on the invasive species itself. However, if a parasitoid inflicts more mortality on the invasive species than on any introduced biological control agent, then it aids in controlling the pest population (Brodeur 2000; Necols et al. 1992; Sullivan 1987). While we found that *Pimpla* wasps can hyperparasitize, as is typical of many pimpline wasps (Bennett 2008; Fitton et al. 1988) and was found previously (Broadley et al. 2018), they appear to do so only facultatively. From our laboratory host range study, when given a choice of unparasitized winter moth pupae or winter moth pupae parasitized by *C. albicans*, the wasps only parasitized the winter moth pupae. From our field study, both species of *Pimpla* wasps can act as hyperparasitoids, but they do so at rates significantly lower than their rate of primary parasitism. Further, in 2016, we found *Pimpla* attacking winter moth but did not find any evidence of hyperparasitism. Overall, hyperparasitism

89

on *C. albicans* at these sites was primarily caused by wasps from the genus *Phygadeuon* rather than *Pimpla* (Broadley et al. 2018). Together, this further demonstrates that *Pimpla* wasps contribute toward controlling winter moth populations.

We detected two species of *Pimpla* parasitizing winter moth pupae; one appears to be *P. aequalis*; the other is unknown and might be an undescribed cryptic species. For our estimates of percent parasitism, we did not distinguish between the two species of *Pimpla* but from the molecular work, one clade is better represented than the other; the majority (88%) of the randomized samples we tested belonged to *P. aequalis* sp. 1. No life history difference was detected between the two species. Both species were found across study site, season, and year, and both species acted as both primary and hyperparasitoids. No statistically difference between the two groups in these life history parameters was detected. While both species are consistent with the description of *P. aequalis*, we were not able to aquire DNA from the lectotype to definitely determine which of our species was consistent with the lectotype. Future research is needed comparing DNA from the lectotype and our samples or the relationship can be approximated by comparing to DNA samples collected from the same location and date that the lectotype was collected.

Winter moth has been extensively studied in its native range in Europe (Klemola et al. 2008; Myers and Cory 2013; Tenow et al. 2013; Varley et al. 1973a; Vindstad et al. 2011; Vindstad et al. 2013), as well as in the prior accidental introductions to North America (Embree 1965; Roland 1990; Roland and Embree 1995); however, this is the first report of *Pimpla* as an important parasitoid of winter moth. To our knowledge none of the studies of winter moth pupal mortality in Nova Scotia recorded parasitism by

Pimpla, although native parasitoids were noted (Embree 1965; Graham 1958; Macphee et al. 1988; Pearsall and Walde 1994; Roland 1990). In British Columbia, *Coccygomimus* (=*Pimpla*) *hesperus* was recorded from *Operophtera* spp. pupae by Humble (Humble 1985) but was not recorded in later studies by Roland (Roland 1990; Roland and Embree 1995). This suggests that the wasps were accidently overlooked in the Nova Scotia studies or that *Pimpla* now shows more host-switching to winter moth than when winter moth was first introduced to North America in the 1930s. Surveys were conducted in winter moth infested sites in coastal Maine, and pimplines, likely *P. aequalis* and possibly *P. aquilonia*, were found there (Morin 2015, unpublished); however, that study did not include sentinel winter moth and thus showed correlation but not direct causation.

We were surprised that *P. aequalis* detected in this study seemed to be the only major species that parasitized winter moth pupae. The lack of additional parasitoids and seemingly slow recruitment of *Pimpla* may help explain why winter moth has been such a high-density pest in its introduced region. We looked for parasitism by *P. contemplator* and *P. turionellae*, which are known parasitoids of winter moth in Europe (Wylie 1960), and for *P. turionellae*, which was introduced to southeastern Canada in an attempt to control winter moth (Graham 1958). Further, *P. disparis*, a parasitoid introduced in this region to control gypsy moth, was also a potential candidate since *P. disparis* is highly polyphagous, attacking lepidopterans of at least 14 families (Schaefer et al. 1989). However, these *Pimpla* species were not detected. Besides the two species of *Pimpla* reported here, we only had a few cases of parasitism by other wasps—diapriid and *Cratichneumon* wasps. *Cratichneumon culex* (Muell.) has been recorded as an important parasitoid of winter moth pupae in Europe (East 1974; Frank 1967; Hassell 1969; Varley

91

and Gradwell 1963; Varley et al. 1973a; Varley et al. 1973b; Wylie 1960), and an undescribed *Cratichneumon* species was reared from winter moth in British Columbia (Humble 1985). As far as we know, *Cratichneumon* sp. was not introduced as a classical biological control agent (Embree 1966; Graham 1958).

Our findings suggest an important role of *Pimpla spp.* as understudied parasitoids in the population dynamics of winter moth, an invasive forest pest. Overall, this study shows that biotic resistance is acting on the winter moth invasion. We urge future entomology population studies and biological control practitioners to consider not only the effect of any introduced biocontrol agent but also the effect of native parasitoids and the potential interactions between introduced biocontrol agents and native natural enemies.

CHAPTER 4

THE ROLE OF NATIVE NATURAL ENEMIES OF PUPAE AND THE BIOLOGICAL CONTROL AGENT *CYZENIS ALBICANS* IN THE POPULATION REGULATION OF AN INVASIVE GEOMETRID, WINTER MOTH (*OPEROPHTERA BRUMATA*)

4.1 Abstract

Multiple mortality factors can act on the same life stage of an organism. When these factors interact, they can have a synergistic, antagonistic, or no interacting effect on the host population. To fully understand the role of natural enemies on a population, it is important to evaluate potential interactions among such contemporaneous mortality factors. Here we use the model study organism winter moth (*Operophtera brumata*) in its invasive range in the northeast United States to study potential interactions between the introduced parasitoid *Cyzenis albicans* and generalist ground predators, all of which act on the pupal stage. We also consider additional mortality caused by a native parasitoid *Pimpla*. Prior long-term population studies showed that pupal mortality acting on two earlier invasive populations of winter moth—located in Nova Scotia and British Columbia—increased after *C. albicans* establishment, suggesting that predation on pupae was somehow synergized by the presence of this parasitoid. Roland (1990) suggested several hypotheses to explain this observation. He suggested that synergy between the two factors may arise from one or more of three mechanisms: (1) parasitoids suppress the winter moth population to a density that can be maintained by generalist predators, (2) unparasitized pupae are preferred by predators and thus experience higher mortality rates,

or (3) *C. albicans*, which are present in the soil over winter, sustain predator populations throughout the year more effectively than winter moth alone, which emerges in the fall. We tested these hypotheses by deploying winter moth pupae over six seasons spanning 2005 to 2017 and by modeling pupal predation rates as a function of winter moth density and *C. albicans* establishment. We also compared predation rates on winter moth pupae and *C. albicans* puparia. We found support for the first hypothesis. We detected both temporal and spatial density dependence, but only in the latter years of the study when winter moth densities were lower, presumably due to the added mortality by *C. albicans.* With low-density populations, pupal predators appear to no longer be saturated by the abundance of prey. We found no evidence for the latter two hypotheses. Our findings lend support to the hypothesis that pupal predators are currently having a regulatory effect on winter moth populations, but only now that the population has been reduced, presumably by the introduction of the host-specific larval-pupal parasitoid *C. albicans*.

4.2 Introduction

Evaluating the impact of natural enemies on population dynamics requires quantification of all mortality factors in the study system and must account for interactions among contemporaneous sources of mortality. Natural enemies that act on the host population during the same life stage can act synergistically or antagonistically. These dynamics between contemporaneous mortality factors have important implications for our understanding of population dynamics across taxa (Morris 1965, Caughley 1966, Varley et al. 1973, Botkin and Miller 1974, Barlow and Boveng 1991), for population conservation efforts (Heppell 1998, Jørgensen and Holt 2013), and for biological control

programs of pest species (Elkinton et al. 1996, Liebhold et al. 2000, Myers and Cory

2013, Duan et al. 2014, Van Driesche and Reardon 2014, Murphy et al. 2018). In

particular, in-depth multiyear studies based on life-table analyses and comparative studies

relative to other related populations are necessary to build understanding of insect

population outbreaks and declines, the potential causes of which are still highly debated

and of great interest to theoretical and empirical ecologists (Liebhold and Kamata 2000,

Berryman 2002, Myers and Cory 2013, Myers 2018).

Winter moth (Geometridae, *Operophtera brumata* L.) is a famous model study

organism for understanding insect population dynamics (Varley et al. 1973, Hassell 1980,

Murdoch et al. 1985, Myers and Cory 2013). It provides unique examples of long-term

and detailed life-table analyses, carried out in the pest's native range in Europe (Varley

and Gradwell 1970, Varley et al. 1973) and in its introduced ranges (Embree 1965,

Roland and Embree 1995, Elkinton et al. 2015). Winter moth was accidentally

introduced from Europe into Nova Scotia, Canada, in the 1930s (Hawboldt and Cuming

1950), to Oregon, U.S.A., in the 1950s (Kimberling et al. 1986), and to British Columbia,

Canada, in the 1970s (Gillespie et al. 1978). A fourth, recent introduction of winter moth

to North America has taken place in the northeastern United States (Elkinton et al. 2014,

Elkinton et al. 2015). Comparative analyses among these different populations provide

valuable insights into the variable population dynamics across the moth's range. Winter

moth also provides famous examples of successful biological control (Embree 1966,

Hassell 1980, Caltagirone 1981, Murdoch et al. 1985, Roland and Embree 1995, Kenis et

al. 2017), specifically the suppression of winter moth by a tachinid parasitoid (*Cyzenis*

95

albicans Fallén) and an ichneumonid parasitoid, (*Agrypon flaveolatum* Gravenhorst), both released in Nova Scotia and British Columbia, Canada.

The long-term population dynamics of winter moth have been extensively studied in these invaded areas and compared to the moth's dynamics in its native range. However, even after extensive study, the mechanism underlying winter moth population dynamics in its invasive range remains uncertain. In Nova Scotia, following the introduction of parasitoids and ensuing winter moth population decline, the introduced parasitoids were assumed to be responsible (Embree 1966, 1971, Hassell 1980, Murdoch et al. 1985). However, subsequent studies in Canada, in both Nova Scotia and British Columbia, suggested that predation on winter moth pupae along with parasitism was necessary to regulate winter moth densities (Roland 1990, Pearsall and Walde 1994, Roland 1994, Roland and Embree 1995, Horgan et al. 1999, Raymond et al. 2002, Heisswolf et al. 2009). This latter school of thinking recognizes that mortality in the pupal stage appears to be particularly important in winter moth. First, winter moth has a long pupal period (6–7 months during the summer, representing most of its life) and it pupates in the top layer of soil or leaf litter. This renders the pupae particularly vulnerable to mortality from predation and parasitism. Winter moth caterpillars hatch in synchrony with bud-break of their host plants and feed on the foliage in early spring before dropping to the soil in mid-late May to pupate; they emerge as adults in early winter, from early November through early January (Embree 1965, Elkinton et al. 2015). Second, in the native range of winter moth in Europe, pupal mortality in the soil was the most important regulatory factor due to density-dependence across generations (Varley and Gradwell 1968, Varley et al. 1973). Lastly, prior research shows positive density

dependent predation in low-to-medium density winter moth populations (Varley and Gradwell 1968), and the opposite trend (inverse density dependent predation) when winter moth is in outbreak densities (Raymond et al. 2002).

Much of the research on the population dynamics of winter moth focuses on pupal stage interactions with introduced biocontrol agents. In particular, Roland (1990) showed that pupal mortality in both Nova Scotia and British Columbia increased after parasitoid establishment, suggesting that predation on pupae was somehow synergized by the presence of *C. albicans*. He proposed a number of hypotheses of how *C. albicans* parasitism and predation on the winter moth pupae might interact. He suggested that synergy between the two factors might arise from one or more of three mechanisms: (1) parasitoids suppress the winter moth population to a density that can be maintained via pupal predation by native generalist predators, (2) unparasitized pupae are preferred by predators and thus experience higher mortality rates, or (3) *C. albicans*, which are present in the soil for 10 months of the year (June – March), sustain predator populations throughout the year more effectively than winter moth alone, which are present in the soil for only 6 months of the year (June – November)(Figure 14).

The hypotheses outlined by Roland (1990) have some support from research in Nova Scotia and British Columbia (Roland and Embree 1995, Horgan et al. 1999), but have neither been empirically tested, nor evaluated in the most recent and fourth accidental introduction of winter moth in North America, in the northeastern U.S.A. (Elkinton et al. 2010, Elkinton et al. 2014, Elkinton et al. 2015). Furthermore, while both *C. albicans* and *A. flaveolatum* were introduced in an effort to control previous invasive populations of winter moth, only *C. albicans* was introduced to this fourth invasive

population of winter moth (Elkinton et al. 2015). This provides a unique opportunity to study the role of *C. albicans* alone on winter moth population dynamics, and its interaction with generalist predation rates, without potential interference from an additional introduced parasitoid.

Cyzenis albicans was introduced across the areas infested by winter moth in the northeastern United States, beginning in 2005. Populations of *C. albicans* have now started to establish across much of this new winter moth range (Elkinton et al. 2015). In several of the release sites, a large change in winter moth densities (more than a 90% reduction) has been documented, with only a relatively small percent parasitism (5-15%, Elkinton et al., 2018). This suggests, as Roland (1990) described in Canada, that parasitism alone is not responsible for declines in winter moth densities and instead acts with predation to regulate the population.

To test the three hypotheses proposed by Roland (1990), we (1) analyzed pupal mortality before and after establishment of *C. albicans* and as a function of winter moth pupae density, (2) compared mortality rates between parasitized and unparasitized pupae and (3) analyzed mortality on pupae and tested for a difference in the predator community with and without *C. albicans*.

4.3 Methods

4.3.1 Pupa Deployment

Pupae were deployed in 2005 and from 2013 to 2017 to estimate mortality from predation. They were deployed in eastern Massachusetts at eight study sites used for long-term assessment of winter moth population dynamics (Whited 2007, Elkinton et al. 2014, Elkinton et al. 2015. The deployments included winter moth pupae and *C. albicans* puparia (Table 16), reared from spring larval collections conducted across eastern Massachusetts (Elkinton et al. 2015). The larvae were reared in batches of 500 or fewer individuals in ventilated 20 L (5 gallon) buckets and fed with foliage from the tree species on which they were found. Mortality from viruses, fungus, other diseases, and larval parasitism in these collections were minimal (Broadley et al. 2017). Prepupae were given sifted peat moss for pupation (see details in Broadley et al. 2018) and all resulting pupae were non-destructively checked under a dissecting microscope (Wild Heerbrugg stereo) for parasitism by *C. albicans* or other larval parasitoids. Pupae were stored until use in an incubator (Percival) at 12 °C, in batches of up to 50 individuals in sterile 100 mm x 15 mm polystyrene petri dishes (Fisherbrand) with a mesh lid for ventilation. The pupae were sprayed monthly with water treated with sodium propionate (5 g/L) to prevent mold growth.

Pupae were deployed in either three or five consecutive rounds per year, from mid-June until end of October. Five deployments (one every three weeks) were completed in 2005, 2013, and 2014; three deployments (one every six weeks) were completed in 2015, 2016, and 2017. In 2005, sets of 40 winter moth pupae were

deployed, while in all the subsequent years (2013 – 2017) sets of 100 winter moth pupae and 50 *C. albicans* puparia were deployed. For each deployment, the cocoons were attached to small burlap squares using beeswax, as was done in a study of predation on gypsy moth pupae (Elkinton et al. 1996) and in a study on winter moth pupae parasitized by *C. albicans* (Broadley et al. 2018). Pupae on burlap were placed haphazardly in the soil under the drip line of a red oak (*Quercus rubra*) at the study sites (Table 16). The pupae, attached to the burlap squares, were buried 2.5 cm below the soil surface to mimic natural pupa depths, which are within the upper 5 cm of soil (Embree 1965, Frank 1967, East 1974, Holliday 1977).

The study sites were chosen to coincide with existing winter moth long-term study sites and to reflect a range of winter moth and *C. albicans* establishment histories (Elkinton et al. 2014, Elkinton et al. 2015). The study sites were all located in mixed hardwood forests dominated by red oak (*Q. rubra*) and red maple (*Acer rubrum*). Winter moth pupae were deployed at all sites, but only some sites also received deployments of *C. albicans* puparia due to limited supply.

To test for an effect of our deployment method, we compared mortality of pupae attached to wire tags as compared to pupae attached to burlap squares. In 2005, we buried 30 wired pupae and 30 burlap-attached pupae at two sites and retrieved them two weeks later for evaluation. Both techniques— deployment of pupae on burlap squares (Elkinton et al. 1996, Whited 2007, Broadley et al. 2018) or marking them using wire or string tags (Buckner 1959, 1969, East 1974, Horgan et al. 1999, Raymond et al. 2002, Horgan and Myers 2004)—have been used for past studies of pupal predation. Other methods included placing the pupae over screening or within screening (Roland 1988,

Pearsall and Walde 1994, Heisswolf et al. 2009), coloring the cocoons (Heisswolf et al. 2010), or simply marking the location of the pupae (Tanhuanpaa et al. 1999, Heisswolf et al. 2010).

4.3.2 Estimating site pupal densities and parasitism by *C. albicans*

To estimate winter moth pupal density and percent *C. albicans* parasitism at each site, 16 buckets (16 cm wide x 28 cm long x 10 cm high) were filled 3 cm deep with sifted peat moss and placed under each study tree in late May, before pre-pupal winter moth caterpillars began to spin down from the tree canopies. Each bucket was placed at a randomly selected distance between the tree stem and the edge of the tree canopy, along one of eight evenly-spaced directions radiating from the tree stem, as described in Varley et al. (1973) and Whited (2007). Parasitism rates on winter moth by *C. albicans* were estimated both from the proportion of *C. albicans*-parasitized pupae from these pupal bucket collections and from collections of 100 to 500 late-instar larvae collected from a range of host trees at each site. From these values, we calculated the corresponding *C. albicans* density (puparia/m^2) for each plot.

4.3.3 Pupal mortality estimates

After each deployment, pupae were retrieved and stored in an incubator (Percival) at 12°C in constant darkness until analysis. We characterized the fate of the pupae as intact, predated, parasitized, or diseased. Predation was assumed for pupae that had been

101

removed from the burlap square, pupae with only the crushed cuticle remaining, pupae with holes chewed in them, and ones with evidence of teeth or claw marks in the wax, as shown in Broadley et al. (2018). Parasitism by *Pimpla* wasps was assumed for pupae with wasp emergence holes and pupae that yielded wasp adults or their larvae. *Pimpla* is an important pupal parasitoid (Broadley et al. 2018 and Broadley, unpublished). Disease was inferred for moldy or desiccated pupae. However, for the purposes of this study we excluded the diseased pupae in our mortality estimates since the desiccation or mold likely occurred as a result of rearing conditions. These pupae accounted for a small proportion of mortality (< 6%). To allow for pupal parasitoid development, intact pupae were stored in an incubator (Percival) over the winter, also as outlined in Broadley et al. (2018), and were re-examined for parasitoid emergence or development in the spring.

Once pupal fate and pupal mortality due to *C. albicans* parasitism were determined, predation and *Pimpla* parasitism were calculated for each deployment and year. Predation was calculated as the proportion of retrieved pupae classified as predated. Mortality due to pupal parasitism from *Pimpla* was calculated as the total number of pupae parasitized by *Pimpla* wasps divided by the number of pupae that remained after predation.

Many studies have used marginal attack rates to calculate mortality when contemporaneous sources of morality act in a system (Varley and Gradwell 1968, Royama 1981, Van Driesche 1983, Buonaccorsi and Elkinton 1990, Elkinton et al. 1992, Pearsall and Walde 1994, Broadley et al. 2018, Murphy et al. 2018). This method accommodates the observation that pupal parasitism rates can be obscured by predation, which typically occurs on the pupae whether or not they were parasitized.

To calculate the annual cumulative (life stage-long) mortality rate, we converted the mortality rates for each deployment and each mortality factor to survival rates (S_i) by subtracting the proportion of pupae that did not survive (P_i) from one ($S_i = 1 - P_i$). To account for slight discrepancies in when pupae were first deployed, we estimated pre-experimental mortality of the pupae by taking the nth root of the survivorship of the first deployment, where n is the number of days the first deployment was out. We then raised this to the estimated number of days that elapsed during the pupal period of winter moth before to the onset of the study. Based on prior research (Elkinton et al. 2014, Elkinton et al. 2015), we estimated the start of the pupal period to be 1 June. Next, cumulative survivorship values were calculated as the product of successive survivorships of each deployment (e.g., $S_c = S_1 \times S_2 \times S_3$) and the cumulative mortality values (P_c) were calculated by subtracting the cumulative survival from 1 ($P_c = 1 - S_c$).

To evaluate the rate of mortality of *Cyzenis* puparia over the winter, we deployed an additional set of 100 puparia at each of two sites (study sites B and D in Table 1) from 26 October to 3 April of 2013. To analyze mortality rates of deployments across years and months, even when the exact number of days deployed varied (from 19 to 45), we standardized mortality proportions to the mean deployment duration of 31 days. We subtracted the proportion of pupae that died for each mortality source for each deployment from 1 for the survivorship estimate ($S = 1 - M$) then raised this to the nth root, where n is the true number of days the pupae were deployed (which ranged from 19 to 45 days with a mean of 31 days). The survivorship (S) was raised to an exponent of 31 to yield the expected survivorship over a standardized 31 days ($S_{31} = [(S_i)^{1/n}]^{31}$).

4.3.4 Predator exclusion and community experiments

In 2013 and 2014, we used a combination of predator exclusion studies and pitfall traps to identify predators in the community and to evaluate their relative contribution to pupal mortality. We ran six rounds of a predator exclusion experiment to quantify the relative role of the different potential pupal predators. Using the same two sites for each round, we ran one round in 2013 and two rounds in 2014. For each round 100 pupae were deployed in a 100 m by 100 m grid with one pupa placed every 1 m^2 in the array. The pupae were attached to burlap squares and these squares were secured to the bottom of one of three cage treatments—cages with 3.2 mm (1/8"), 6.4 mm (1/4"), or 12.7 mm (1/2") square openings or a control (just the wire mesh bottom of one of the other three cages). The cages were placed 2 to 3 cm into the ground and covered with a soil and leaf litter. For both years we used the same sites in Wellesley, MA and Hanson, MA. In 2013 we deployed the array of pupae from 12 August to 20 October, and in 2014 we ran deployments from 26 June to 11 August and again from 18 August to 30 October. At the end of each deployment the pupae were retrieved and evaluated as described above in section 2.2. In 2013, two pitfall traps were placed at each study site, and all pitfall traps were checked once a week. The pitfall traps were each made of a 24 oz plastic cup, buried flush to the surface, covered with a lid elevated five centimeters above with a wire stand, and partially filled with 70% ethyl alcohol solution. All beetle larvae, adult staphylinid and carabid beetles, and any small mammal by-catch were counted.

4.3.5 Statistical analyses

All analyses were run in R 3.4.4 (RCoreTeam 2013) using RStudio, version 1.1.442 (RstudioTeam 2015). For each analysis, the full model was always run initially (including site, year, and deployment effects). The model was evaluated for evidence of skew in the residuals or outliers, and any insignificant predictors ($p > 0.05$) were dropped sequentially starting with the largest p-value until the best fit model was selected using AIC comparisons. We checked for overdispersion and when evidence of overdispersion was noted we applied a quasibinomial or quasipoisson distribution (Crawley 2005, Zuur et al. 2013). For the logistic regression and ANOVA analysis, proportions were weighted by the total number of pupae evaluated. A pseudo-R^2 was calculated by comparing the residual deviance of the fit model against the null model (deviance null model – deviance fit model / deviance null model). All graphical data were displayed using ggplot2 (Wickham 2009).

To analyze the overall magnitude of total pupal mortality, predation on pupae, and pupal parasitism across the years and study sites, we used a logistic generalized linear model with the cumulative mortality by each mortality factor as the response with year and site as fixed effects. To test for a significant difference among sets of the mortality factors (total mortality, predation, and parasitism), we used a multiple comparisons test of the means (Tukey Contrasts), including year and site as factors. The package multcomp (Hothorn et al. 2008) was used for these pairwise comparisons. We looked for seasonality in the predation rate within a year by analyzing monthly mortality rates (as

calculated using methods explained in Section 2.2) over the pupal period (June –

October, as Julian days) with year and site effects.

To test for direct density-dependent effects on predation to the pupae, we

analyzed the cumulative mortality on the winter moth pupae by predation at the long-

term study plots against the underlying density of pupae at these sites. We again

considered year and site effects (temporal and spatial density dependence, respectively).

To test for delayed density dependence, we analyze the mortality from predation against

the prior year's densities. As Roland and Myers (1987) did in their evaluation of a

delayed density effect on pupal weight, we plotted the residuals of the fit model of the

mortality from predation against the same year's density estimates plotted against the

prior year's density.

To test the hypothesis that *C. albicans* puparia may experience mortality from

predation at a rate lower than that of unparasitized winter moth pupae, we used a logistic

ANOVA to compare the annual cumulative mortality experienced by the two different

pupae types over the winter moth pupal season (June – October). We also compared the

mortality of winter moth pupae to *Cyzenis* puparia that were left for the duration of their

own pupal period, which extends through the winter. Subsequently, to test the related

hypothesis that the presence of *Cyzenis* puparia provides an overwintering food source

for resident predators and thus allows them better survival and reproduction rates, we

compared predation rates of winter moth pupae in sites with and without *C. albicans*

establishment and across a *C. albicans* parasitism gradient.

Lastly, to compare predation rates on winter moth pupae by different members of

the predator community, we again used a logistic ANOVA to compare the relative

mortality of the predator exclusion treatments (deployment methods in Section 2.4) with

deployment and treatment as fixed effects. Using logistic regression, we tested for a

correlation between predation rates and the abundance of carabid beetles, staphylinid

beetles, beetle larvae, and small mammals at each site.

4.4 Results

4.4.1 Winter moth pupal mortality

In total, 14,500 winter moth pupae were deployed, and 13,525 burlap squares

were retrieved after their deployment period and evaluated (Table 11). For *C. albicans*

puparia, 3,700 were deployed and 3,507 retrieved. Overall, predation accounted for the

vast majority of pupal mortality (Figure 15). There was no consistent increase or

decrease in total mortality or predation on pupae from 2005 to the end of the study in

2017; however, the rate of predation on pupae varied significantly across years (pseudoR^2

= 0.54, df = 38, p = 0.011); rates in 2013 and 2017 were significantly lower than 2005 (p

= 0.011 and p = 0.031, respectively). Site effects were not significant (p > 0.29).

Parasitism by pupal parasitoids accounted for a minority of pupal mortality and was

significantly lower than predation (p = 0.0001) but ranged from approximately 20 to 40%

of mortality on pupae left behind after predation.

When considering rates of predation on the pupae across the pupal period of

winter moth within a year (June – October, regressing against Julian day the pupae were

retrieved), there was a significant effect of seasonality (pseudoR^2 = 0.37, df = 128, p <

0.0001), with year and site effects (p < 0.0001 and p = 0.001, respectively). Peak

mortality from predation occurred in the third week of August with an average predation rate of 50% of pupae every 31 days.

There was no effect of deployment method (deploying the pupae on burlap squares as compared to deploying with a tag/wire attached to the cocoon) on pupal mortality. For the side-by-side comparison of the two treatments, an equal number of pupae were attacked (58% of 60 pupae from each deployment treatment).

4.4.2 Density dependence of winter moth pupal mortality

There was a significant effect of winter moth pupa density on the resulting mortality from predation of the sentinel pupae (pseudoR^2 = 0.50, df = 24, p < 0.018) with a significant effect of year (Figure 16A; p = 0.0040) and site (Figure 16B; p = 0.026). There was no evidence of delayed density dependence when considering either the current year's rate of predation as compared to the prior year's winter moth density (p = 0.63) or the correlation between the residuals of the model constructed with the current year's predation correlated with the density of pupae in the same year against the prior year's winter moth density (p = 0.81 and p = 0.10 for the model with year and site effects, respectively).

4.4.3 Comparative mortality on winter moth pupae vs *C. albicans* puparia

The predation rates on *C. albicans* puparia were not significantly different than that on winter moth pupae when compared over the same interval (winter moth's pupal stage, June – October) or when factoring in the additional mortality that may act upon *C.*

albicans puparia over the winter before they emerge as adults (June – April, Figure 17; pseudoR^2 = 0.67, df = 19, p = 0.32).   There was a trend toward lower mortality on *C. albicans* puparia than winter moth pupae.

4.4.4 Effect of *C. albicans* presence on winter moth pupal mortality

Cyzenis albicans was successfully established in different years across the sites included in this analysis (e.g., as early as 2010 for Wellesley, MA or as late as 2014 for Hanson, MA). We compared predation rates on winter moth pupae as a function of winter moth density and *C. albicans*-establishment status (with or without *C. albicans*). The effect of established *C. albicans* was significant, with lower rates of predation after *C. albicans* establishment (Figure 18; p = 0.012). However, pupal predation was also only significantly related to density after *C. albicans* establishment (Figure 19; pseudoR^2 = 0.38, df = 33, p = 0.0027). No correlation was detected between predation rates and percent *C. albicans* parasitism (pseudoR^2 = 0.29; df = 36; p = 0.90 and pseudoR^2 = 0.32; df = 31; p = 0.25 for estimates using pupae and larvae collections, respectively).

4.4.5 Predator exclusion and community experiments

In the predator community, we focused on the relative role of small mammals, carabid beetles (adults and larvae), and staphylinid beetles. Using predator exclusion cages, there was a significant effect of treatment (mesh size; Figure 20; pseudoR^2 = 0.48; df = 41; p < 0.0001), and to a lesser extent, deployment number (p = 0.0017). The four size classes of predators contributed equally to pupal mortality; there was an even decline

109

in predation rates on the pupae with each smaller mesh size exclusion cage. No

significant effect was found when regressing predation rates against the abundance of

each of the four taxonomic groups of predators (p = 0.35, p = 0.41, p = 0.40 for small

mammals, carabid beetle adults, staphylinid beetle adults, and total predator abundance

including beetle larvae).

Table 11: Study sites used for deployments of winter moth pupae and *Cyzenis albicans* puparia across the years of the study. The numbers given in the top of each cell (ranging from 0.59 to 1.00) are cumulative predation rates. The numbers in parentheses are the total sample sizes. Data are given only for sites and years that had deployments of sentinel pupae.

| Study Site | GPS Location | Winter moth | | | | | | Cyzenis albicans | | | |
| | | pupa deployments | | | | | | puparium deployments | | | |
		2005	2013	2014	2015	2016	2017	2013	2014	2015	2016
A. Co-op Extension, Hanson, MA	42.048889, -70.873806	1.00 (200)	0.88 (500)	0.96 (500)	0.99 (300)	1.00 (300)	0.72 (300)			0.98 (200)	1.00 (300)
B. Maquan St., Hanson, MA	42.060694, -70.844167		0.80 (750)	0.99 (700)	0.98 (300)	0.81 (300)	0.77 (300)	0.83 (350)	1.00 (200)	0.98 (200)	0.85 (300)
C. Pondview Dr., Falmouth, MA	41.626417, -70.580417		0.81 (500)	0.77 (500)		0.97 (300)	0.79 (300)				0.92 (300)
D. Centennial Park, Wellesley, MA	42.308444, -71.266778		0.78 (750)	0.94 (700)	0.84 (300)	0.93 (300)	0.87 (300)	0.96 (350)	0.87 (200)	0.80 (200)	0.98 (300)
E. Wompatuck SP, Hingham, MA	42.208333, -70.853056	1.00 (200)	0.89 (500)	0.92 (500)	0.97 (300)	0.78 (300)	0.79 (300)			0.59 (200)	0.59 (300)
F. Route 6, Yarmouth, MA	41.686167, -70.287722		0.91 (500)	0.81 (500)		1.00 (300)	1.00 (300)				1.00 (300)
G. Center St, W. Bridgewater, MA	42.020806, -70.982306	0.93 (200)	0.86 (500)	0.88 (500)							
H. Bare Cove Park, Hingham, MA	42.238222, -70.913389	1.00 (200)	0.83 (500)	0.99 (500)							
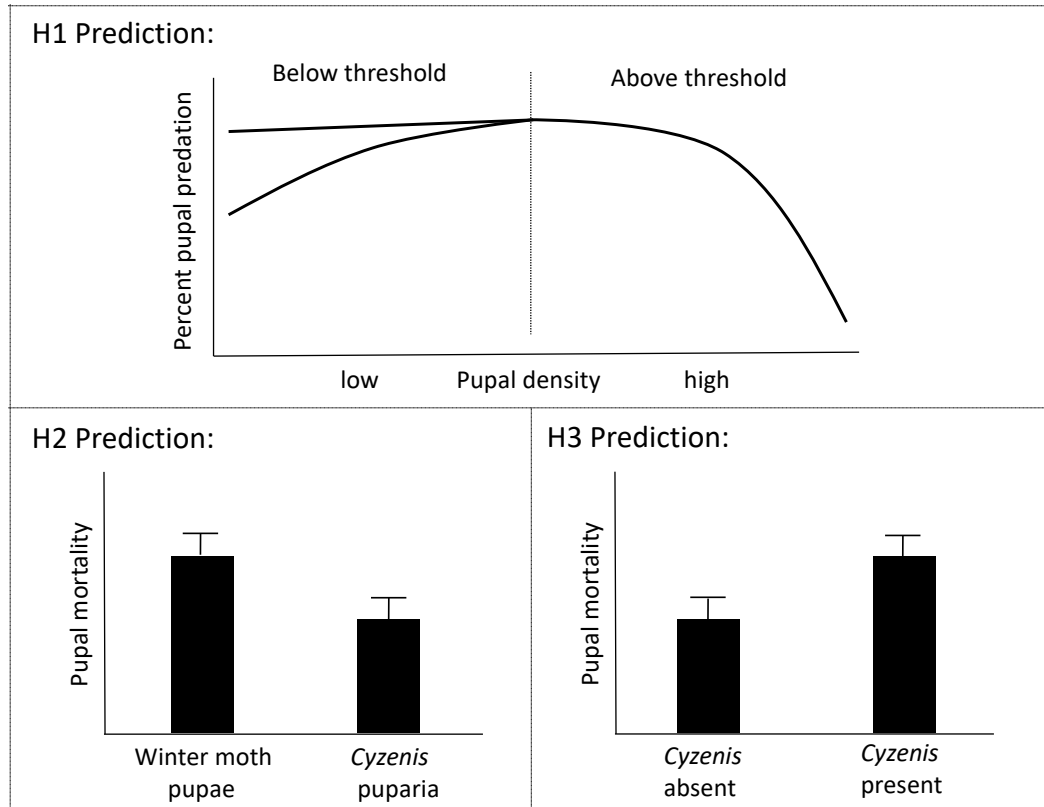
Figure 14: Three hypotheses for how the introduced parastioid *C. albicans* may positively affect predation rates of winter moth pupal predation rates by generalist ground predators in winter moth's introduced range. After Roland (1990).
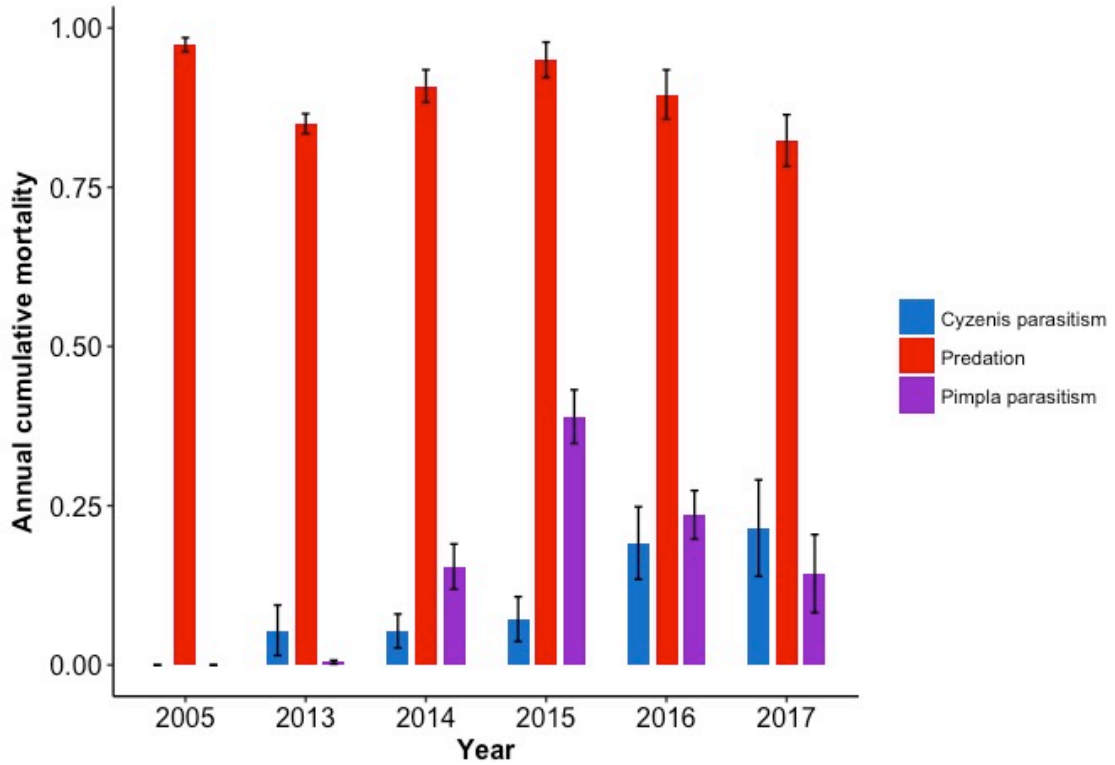
Figure 15: Mortality from *C. albicans* parasitism (blue), mortality from predation (red), and mortality from *Pimpla* parasitism (purple) on winter moth pupae across the six years of study for long-term study sites (first eight sites listed in Table 1). Each mortality source is calculated from what was left behind after the prior mortality source acted on the system. Error bars show standard errors. Data was not collected on *Pimpla* parasitism in 2005 and 2013.
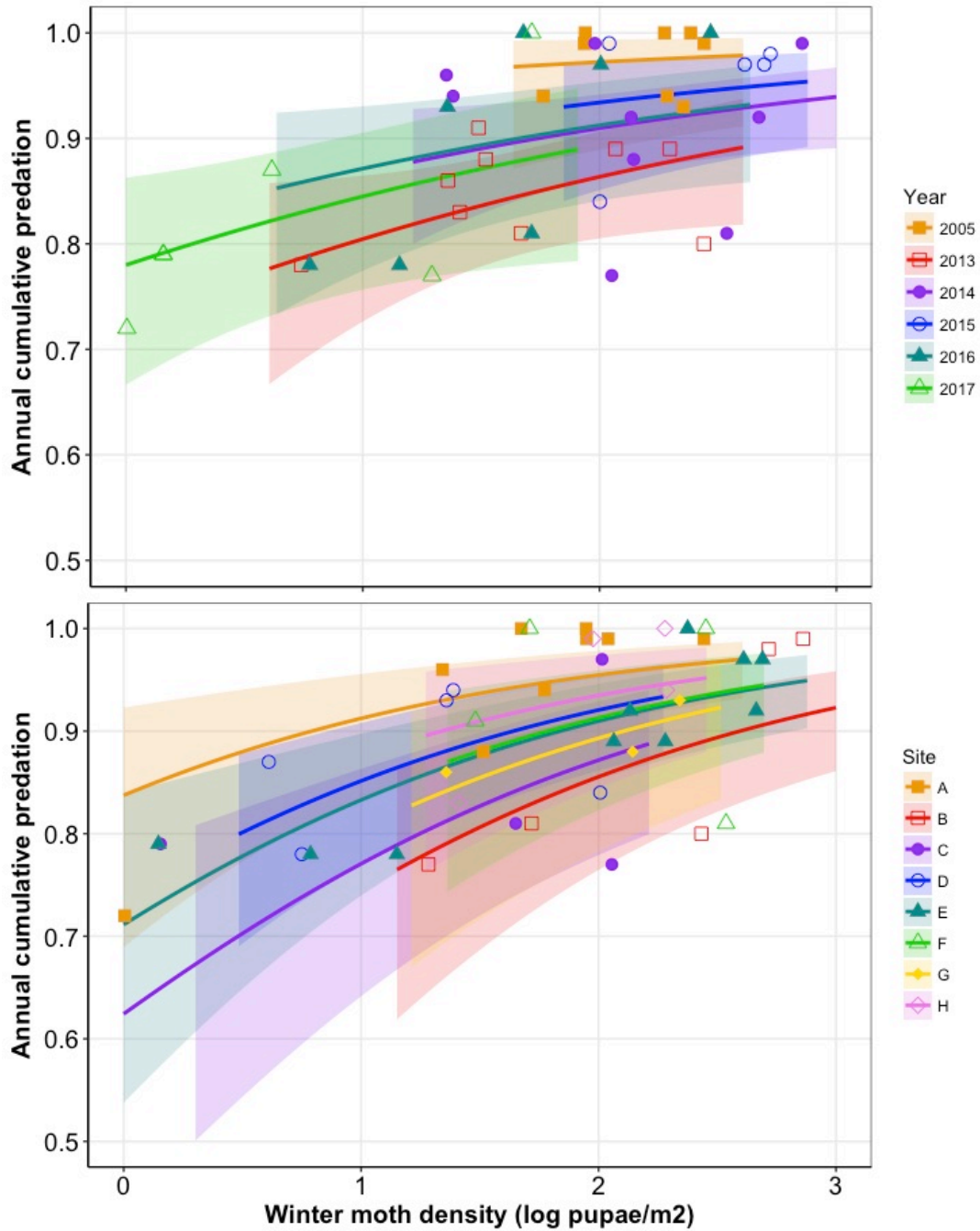
Figure 16: Cumulative mortality from predation by the density of winter moth pupae at the site by (A) year and (B) site. Pupal density is given as the base-10 logarithm of (pupae/m^2) calculated from the oak trees at each site. Different colors indicate different years (A) or sites (B).
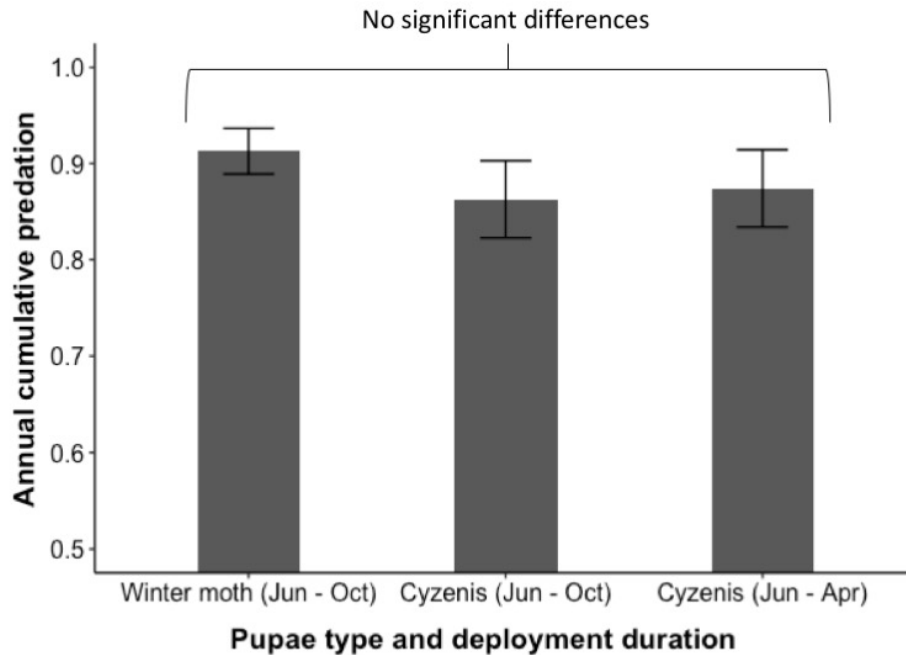
Figure 17: Annual cumulative predation on *Cyzenis albicans* puparia and winter moth pupae across all the sites and years in which both were deployed. There was no significant difference between the two types of pupae, even when additional spring mortality of *C. albicans* is included (right most bar). Error bars show standard errors.
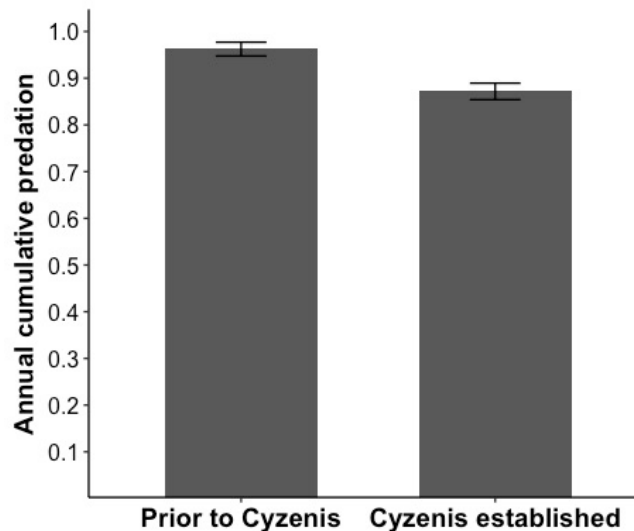
Figure 18: Mortality from predation of winter moth pupae in sites and years prior to *Cyzenis albicans* establishment as compared to sites and years with *C. albicans* establishment. Error bars are constructed using one standard error from the mean.
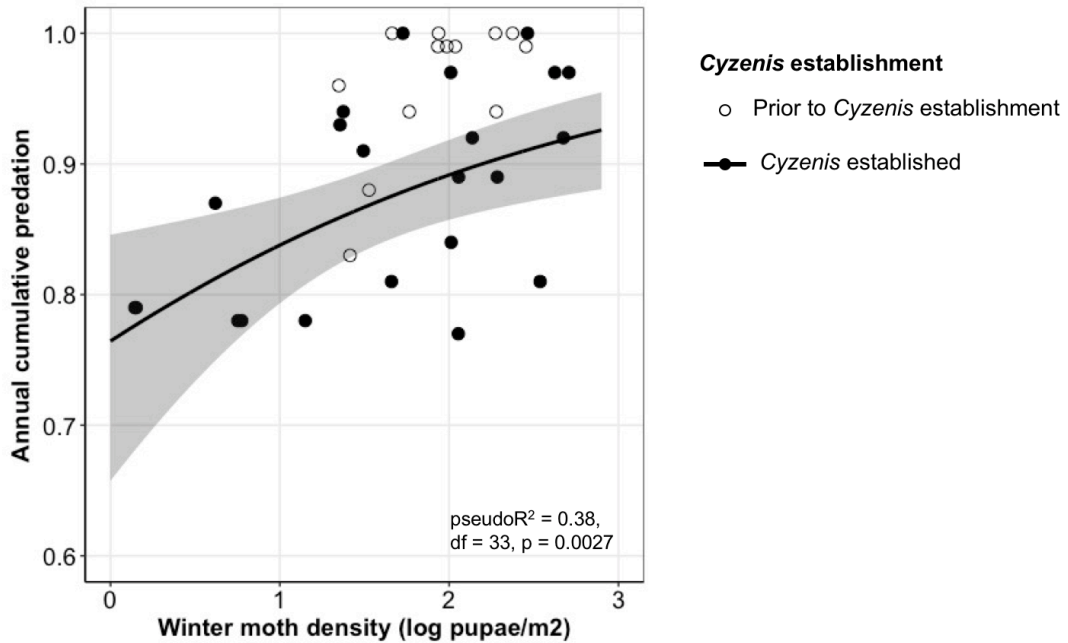
Figure 19: Mortality from predation of winter moth pupae in sites and years prior to *Cyzenis albicans* establishment as compared to sites and years with *C. albicans* establishment against the corresponding pupal density. Pupal density had a significant effect on the resulting predation with *Cyzenis* establishment (black trendline) but was not significant prior to *Cyzenis* establishment.
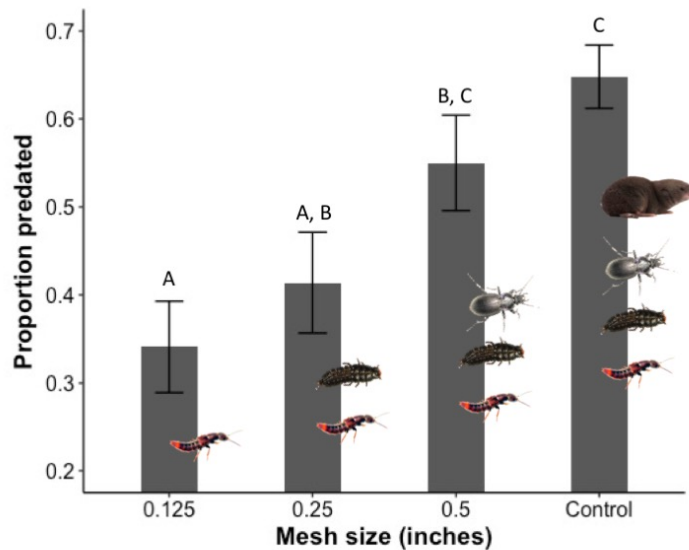
Figure 20: Proportion of pupae predated upon across the predator exclusion cage treatments. The images show the likely predators (staphylinid, beetle larvae, carabid, and small mammal) able to access the pupae for each mesh size. Error bars show standard errors.

115

4.5 Discussion

We used a multifaceted approach to evaluate the role and interaction of two important, contemporaneous mortality factors acting on the pupal stage of winter moth— predation from generalist ground predators and parasitism by *Cyzenis albicans*— and their effect on population dynamics of this model study organism. Our findings lend support to the hypothesis that pupal predators only have a regulatory effect on winter moth populations after the moth's population has been reduced (presumably by the introduction of the host-specific larval-pupal parasitoid *C. albicans*). This is one of three hypotheses proposed by Roland (1990) to explain the trend noted in all introduced populations of winter moth in North America, where establishment of *Cyzenis* led to a larger decline in winter moth population densities than can be explained by the additional mortality caused by *C. albicans*.

We detected both spatial and temporal direct density dependent effects. Temporal density dependent effects were more evident in the most recent year of study, when the population of winter moth was at its lowest for the study years and the lowest recorded since long-term life-table studies in Massachusetts commenced in 2004 (Elkinton et al. 2015). Our data suggests that since winter moth densities have decreased, presumably due to mortality by *C. albicans*, pupal predators are no longer saturated by the abundance of prey. These generalist predators can now regulate winter moth density. A similar trend was detected in the long-term population studies conducted on winter moth in Nova Scotia (Embree 1965); mortality in the pupal stage did not govern the survivorship between generations until after *C. albicans* was established. This density dependent effect was detected in our data even with relatively low parasitism by *C. albicans* (ca

116

13% in 2016 and 18% in 2017). Additionally, Massachusetts's population densities have now been reduced to levels comparable to those historically found in winter moth's endemic range in Europe (Varley and Gradwell 1968, East 1974, Kowalski 1977). However, in 2005 and 2013 – 2015, populations in Massachusetts were at levels that matched other invasive, outbreaking populations of winter moth at or above a predator saturation threshold (Raymond et al. 2002, Horgan and Myers 2004, Heisswolf et al. 2009). Together this suggests that the invasive population of winter moth in the northeast United States is now behaving much like the native population in Europe and no longer represents an outbreak population.

Predation was by far the main source of mortality on winter moth pupae, resulting in more than 75% loss of the pupae deployed. The rates of pupal mortality from predators were similar to those found in prior studies in British Columbia (Roland 1988, Horgan et al. 1999, Horgan and Myers 2004), Nova Scotia (Macphee et al. 1988, Pearsall and Walde 1994), and Europe (Varley et al. 1973, East 1974, Hansen et al. 2009, Heisswolf et al. 2010). However, our estimate of pupal mortality was higher than some other estimates of pupal mortality in Europe (East 1974, Klemola et al. 2009, Klemola et al. 2014, Pepi et al. 2017). This discrepancy was particularly evident when comparing data from studies of predation rates in winter moth outbreak years, or at high elevation sites, likely because the predator saturation point had been surpassed (Tanhuanpaa et al. 1999, Raymond et al. 2002, Heisswolf et al. 2009, Klemola et al. 2014). As in other parts of in winter moth's introduced range (Roland 1990), after predation, we found mortality by native pupal parasitoids to be the next highest mortality factor for winter moth pupae. In the current study, this was parasitism on the pupae by a *Pimpla* ichneumonid wasp,

while in Roland (1990) the wasp species was not specified. In another similarity to previous studies conducted in winter moth's introduced range (Neilson 1965, Cunningham et al. 1981, Roland 1990, Pearsall and Walde 1994, Horgan et al. 1999, Burand et al. 2011, Broadley et al. 2017), fungal, viral, or other unknown reasons that the pupation failed accounted for a small portion of mortality in the pupae in our study.

Combined with evidence from previous winter moth literature, our findings lend support to the hypothesis that different suites of mortality factors operate in low-density versus outbreak populations of winter moth. Parasitism by *C. albicans* caused little to no detectable mortality in endemic low-density winter moth populations (Varley et al. 1973). However, when introduced to outbreak populations in Canada, *C. albicans* reached high levels of parasitism and with three to six years of establishment brought about a decline in winter moth population densities (Macphee et al. 1988, Pearsall and Walde 1994, Roland 1994, Horgan et al. 1999). In both Nova Scotia and British Columbia, the population of winter moth crashed four to five years after the first release of *C. albicans* (Roland 1990). More specifically, Embree (1965) found that defoliation by winter moth was reduced to negligible levels three years after *C. albicans* reached 10% parasitism. Indeed, while parasitism by *C. albicans* was non-existent or negligible at the start of our study, *C. albicans* parasitism rates steadily increased each year and shifted winter moth from a consistently high-density population showing no regulation by predators to a population that is now regulated by predators.

We did not find significant differences between predation on unparasitized pupae compared to parasitized pupae. We found no difference when comparing the two pupal types for the duration of winter moths' pupal period (June – October) nor when we

included the additional mortality *C. albicans* experiences overwinter as a pupa. Pupae parasitized by *C. albicans* are smaller than unparasitized pupae and have a thicker and tougher cuticle. Some studies found that *Cyzenis* puparia suffered lower mortality than winter moth pupae (Roland 1990, Horgan and Myers 2004). However, other studies (Hassell 1969, Horgan and Myers 2004) did not find lower rates of mortality on *C. albicans* puparia and, in fact, Horgan and Myers (2004) found that *C. albicans* puparia suffered from overall higher rates of mortality. Winter moth pupae and *C. albicans* puparia vary in size from year to year (Horgan and Myers 2004); thus, it may be size rather than parasitism status that influences mortality rate differences between these two pupa types. We also found very little additional mortality to *C. albicans* puparia during the winter. This is in contrast to the predictions made by Hassell (1969) but aligns with predictions made by Horgan and Myers (2004). In the northeast U.S., as long as snow cover is present, temperatures in the leaf litter and upper soil layer are close to freezing between November and March in Massachusetts and can dip much colder without snow (Elkinton et al, unpublished). As was found in British Columbia (Horgan and Myers 2004), cold winter temperatures limits predation by invertebrates on *C. albicans* puparia over the winter.

While we did detect positive density-dependent effects on the pupae from generalist ground predators and found this effect to be strongest in the recent, lower-density years, we found no direct evidence that the presence of *C. albicans* causes an increase in predation rates on the winter moth pupae. As in Nova Scotia and British Columbia (Embree 1965, Roland 1988, 1990, Roland and Embree 1995), we found that the presence of *C. albicans* acts on winter moth population to reduce density below the

saturation threshold such that generalist predators can then respond spatially and temporally to the densities. However, unlike these previous studies we did not find that the presence of *C. albicans* increased predation rates. Instead, the presence of *C. albicans* appeared to decrease overall levels of predation. We suggest that this is because *C. albicans* pupae are a less-preferred prey item than winter moth pupae (Roland 1990, Horgan and Myers 2004). Thus, when predators encounter a high proportion of cocoons that are *C. albicans* rather than winter moth, they are likely to switch to other food sources and leave in search of better feeding grounds (Holling 1959, Murdoch 1969). Such behavior has been found in other studies where predator preference for prey items depends on their parasitism status (Lafferty 1992, Al-Zyoud and Sengonca 2004, Gehman and Byers 2016, Murphy et al. 2018). In the current study, this interaction results in a density dependent response of pupae, but overall lower predation rates. While there is convincing evidence from Nova Scotia and British that *C. albicans* parasitism synergizes with predation (Embree 1965, Roland 1988, 1990, 1995, Roland and Embree 1995), complementary research did not detect a relationship between parasitism by *C. albicans* and subsequent declines in in winter moth densities (Kimberling et al. 1986, Bonsall and Hassell 1995, Horgan et al. 1999). We also did not detect a synergistic relationship between *C. albicans* parastism and predation, but our data suggests that the two mortality factors are additive.

There has been much debate in the literature about which predators are most important in terms of applying top-down pressure on the winter moth population. We found that small and large predators contributed equally to the mortality experienced by the pupae. Various studies have come to different conclusions as to whether beetles

(primarily carabid and staphylinid) or small mammals were more important predators on winter moth pupae, with results depending on the region, season, duration, and method of study. A number of studies determined that small mammals were the most important predators of winter moth pupae (Embree 1965, Buckner 1969), while others concluded that carabid or staphylinid beetles were most important (East 1974, Kowalski 1977, Pearsall and Walde 1994, Roland and Embree 1995, Horgan and Myers 2004). In addition to our results, other studies on winter moth pupal predation have also concluded that all three categories contribute equally (Frank 1967, Horgan et al. 1999, Heisswolf et al. 2010). Seasonally, most mortality occurred between the end of July and the end of August; however, mortality on the pupae extended through October. It is likely that both components of the predator community (mammalian and invertebrate) contribute to the top-down regulation of winter moth densities. In some sites and years one predator may provide more control while in other sites or years control may result from a different species. The constant fluctuation but consistent pressure from all components of the predatory community obscure any obvious association between densities of particular taxa and predation rates.

Mortality from parasitism by the native wasp *Pimpla*, while much lower than that from predation, was still notable. With the increase in *Pimpla* parasitism on winter moth pupae from 2013 to 2015, it appeared that *Pimpla* was being recruited to the system as was seen by European parasitoids on winter moth in northern Norway by Vindstad et al. (Vindstad et al. 2013). If the increase in parasitism by *Pimpla* had continued, recruitment of parasitoids could explain the apparent synergistic relationship between *Cyzenis* and predation. However, with the subsequent years' data, it seems that *Pimpla* varies from

year to year. Further, we detected high levels of parasitism by *Pimpla* in central Massachusetts 50 km west of the nearest winter moth population (Broadley et al., unpublished). This shows that attacks on winter moth pupae by *Pimpla* are not linked to the presence of winter moth populations because unlike *C. albicans* (Embree and Sisojevic 1965, Elkinton et al. 2015), *Pimpla* is a generalist (Fitton et al. 1988, Bennett 2008). We recorded no data on *Pimpla* in 2005 and 2013 because we had not yet learned to identify the emergence holes it makes in winter moth pupae nor learned to hold the pupae to allow for wasp development. The recent two years of data suggest that while *Pimpla* parasitism levels fluctuate from year to year, *Cyzenis* parasitism levels are steadily increasing.

Lastly, considering our evaluation of pupa deployment methods, we found no difference in the predation rate when we deployed our pupae tethered to a wire as compared to those attached to burlap squares. Thus, we can compare across studies that have deployed pupae using these different methods. However, we do not claim that our experimental predation rates are equal to the rates on naturally occurring pupae. Instead, these rates are an experimental test of predation rate that are comparable across treatments, years, sites, and studies.

Overall, we explain the role of contemporaneous mortality factors acting on the pupal stage and their interaction on the resulting population dynamics of our study organism. Extensive research has been conducted using winter moth as a model organism to understand the interactions between parasitoids and predators and the role of pupal predation as a density-dependent regulatory factor. Much of this research has been conducted with invasive populations of winter moth and has focused on the interaction

between introduced host-specific parasitoids (*Cyzenis albicans*, a classical biological

control agent) and native generalist predators (biotic resistance). Our study lends support

to the current hypothesis that the introduction of *C. albicans* results in significant indirect

mortality on winter moth; reducing the population of winter moth to densities low enough

to be regulated by pupal predators, as occurs in winter moth's native range in Europe.

However, our data suggest that the two mortality factors are additive, but not synergistic.

These findings are important for both a better understanding of population dynamics,

particularly in terms of what regulates insect outbreaks, and in understanding the current

biological control program on winter moth in the northeast United States.

COMPARATIVE POPULATION ECOLOGY OF AN INTRODUCED GEOMETRID (*OPEROPHTERA BRUMATA*.) AND ITS NATIVE CONGENER (*OPEROPHTERA BRUCEATA*)

5.1 Abstract

Winter moth (*Operophtera brumata* L.) (Lepidoptera: Geometridae) is a newly invasive species from Europe to the northeastern United States, which causes widespread defoliation of a wide variety of deciduous trees. In this region, it co-occurs, and sometimes hybridizes with, a native congener, Bruce spanworm (*O. bruceata* Hulst) (Lepidoptera: Geometridae), a species with a nearly identical life cycle and an equally wide host range. Bruce spanworm is ubiquitous in the northern United States and southern Canada, but populations almost always remain at low density. Outbreaks of the species are rare and short-lived. Here we explore why the two species have such different population dynamics. We discovered a microsporidian in Bruce spanworm that causes high mortality among larvae and pupae, especially in high-density populations, which are rare. We recovered no microsporidia in winter moth in this region. We also report high levels of parasitism by an ichneumonid wasp, *Agrypon* sp., in low-density populations of Bruce spanworm in Massachusetts. This wasp appears to be closely related to *Agrypon flaveolatum* Gravenhorst, which was introduced from Europe to control invasive winter moth populations in Nova Scotia in the 1950s and to Vancouver Island in the 1970s. However, the *Agrypon* recovered from Bruce spanworm's CO1 sequences are distinct from those recovered from winter moths from Norway, Nova

Scotia, and Vancouver Island, all of which have nearly identical sequences. Also, the sequences of *Agrypon* sp. from Bruce spanworm are a close match to sequences from *A. flaveolatum* reared from the autumnal moth (*Epirrita autumnata* Borkhausen) in Finland, where it apparently does not attack winter moth. A third clade of *Agrypon* was found attacking winter moth in the northeastern United States, but at very low levels. Bruce spanworm populations are subject to top-down mortality from a pathogen and parasitoid that do not affect winter moth, which may explain the differences in their population dynamics.

5.2 Introduction

The causes of insect population outbreaks and regulation are of interest to theoretical and empirical ecologists (Myers and Cory 2013, Myers 2018). One approach to determining the underlying causes of outbreaks and regulation is to use comparative studies of a species that exhibits variable population dynamics across its range over time (Elkinton and Liebhold 1990, Tanhuanpaa et al. 1999, Raymond et al. 2002); another approach is to study two related species that exhibit contrasting dynamics but share a common range (Roland and Embree 1995, Watt and Woiwod 1999, Hansen et al. 2009, Heisswolf et al. 2009, Heisswolf et al. 2010, Vindstad et al. 2013). In this study, we use the latter approach to better understand the population dynamics of winter moth (*Operophtera brumata* L.) (Lepidoptera: Geometridae) in its introduced range in the northeastern United States. We compared populations of winter moth in the northeast United States (hereafter, simply 'the northeast') with sympatric populations of its native congener, Bruce spanworm (*Operophtera bruceata* Hulst) (Lepidoptera: Geometridae).

125

Winter moth is a famous model study organism for understanding insect population dynamics (Varley et al. 1973, Hassell 1980, Myers and Cory 2013, Myers 2018) and, in North America, is a destructive, invasive forest pest (Cuming 1961, Embree 1967, Simmons et al. 2014, Elkinton et al. 2015). The population dynamics of winter moth in its native range (Europe) have been extensively studied and were used to develop foundational theories in insect population ecology (Varley and Gradwell 1970, Varley et al. 1973, Hassell 1980). Winter moth was accidentally introduced into Nova Scotia, Canada in the 1930s (Hawboldt and Cuming 1950), to Oregon, U.S.A. in the 1950s (Kimberling et al. 1986), and to British Columbia, Canada, in the 1970s (Gillespie et al. 1978). A fourth introduction of winter moth to North America has recently occurred in the northeastern United States (Elkinton et al. 2010, Elkinton et al. 2015). However, the suppression of winter moth by a tachinid parasitoid (*Cyzenis albicans* Fallén) and an ichneumonid wasp (*Agrypon flaveolatum* Gravenhorst) in Nova Scotia and British Columbia is a famous example of successfull biological control (Embree 1966, Hassell 1980, Caltagirone 1981, Murdoch et al. 1985, Roland and Embree 1995, Kenis et al. 2017). Biological control work is being implemented against winter moth in the northeast and shows promising results (Elkinton et al. 2018). However, the success of this biological control program likely depends in part on mortality caused by native natural enemies, including pathogens, parasitoids, and predators (Roland 1990, Horgan et al. 1999, Broadley et al. 2017, Broadley et al. 2018, Donahue et al. in press).

The introduced range of winter moth in the northeast overlaps with that of Bruce spanworm, which is a native congener of winter moth. Bruce spanworm has a similar life history to that of winter moth, shares the same pheromone, and can hybridize with winter

moth (Pivnick et al. 1988, Gwiazdowski et al. 2013, Havill et al. 2017). Bruce spanworm is widespread in northern North America and southern Canada (Elkinton et al. 2010, Gwiazdowski et al. 2013). However, in contrast to winter moth in the northeast United States, Bruce spanworm is remains at low densities and only occasionally undergoes local, short-lived outbreaks (Brown 1962, Rose and Lindquist 1997). This difference in density is occurs even where the two species are sympatric (Broadley et al. 2017, Donahue et al. in press). We propose that this difference in population dynamics exists because winter moth, in its introduced range, has been released from its natural enemies, while Bruce spanworm is regulated by indigenous natural enemies. Based on research on gypsy moth (*Lymantria dispar* L), we expect host-specific pathogens play an important role in the population dynamics when Bruce spanworm densities are high but expect generalist predators or parasitoids to play an important role when densities are low (Campbell 1975, Campbell and Sloan 1977, Elkinton and Liebhold 1990, Elkinton et al. 1996).

Bruce spanworm is an ideal model species to investigate the role of native, natural enemies affecting *Operophtera* in the northeast. Here we aim to quantify the population dynamics of winter moth as compared to Bruce spanworm in the northeast and measure the prevalence of larval pathogens (nucleopolyhedrovirus and microsporidia) and parasites (*Agrypon* spp.) in the two species. Comparison between the two species will help us understand why winter moth is an outbreak species, whereas Bruce spanworm outbreaks are rare.

5.3 Methods

5.3.1 Sample collection

Bruce spanworm and winter moth larvae were collected from the field as fourth or fifth instars in late May of 2012 – 2016 (Table 12). In 2012 and 2013, Bruce spanworm larvae were collected from a localized outbreak near Millinocket, Maine (Township 2, Region 8 NWP). This is 150 km inland from the area where winter moth has been detected in Maine (Elkinton et al. 2010, Elkinton et al. 2015). In 2014, Bruce spanworm was undetectable at this site, and collections of Bruce spanworm in 2014 and 2015 were made from the Mohawk Trail State Forest in Charlemont, Massachusetts and from Savoy Mountain State Forest in Savoy, Massachusetts. These collection sites are 60 km from the westernmost location of y winter moth in Massachusetts (Elkinton et al. 2010, Elkinton et al. 2014). In all four years, winter moth larvae were collected from long-term study sites in eastern Massachusetts where winter moth was abundant and Bruce spanworm was rare or nonexistent in the study years (Elkinton et al. 2014, Elkinton et al. 2015, Havill et al. 2017). The long-term study sites were in Wellesley, MA (42.308444, -71.266778), Hanson, MA (two sites: 42.048889, -70.873806 and 42.060694, -70.844167), Hingham, MA (2 sites: 42.208333, -70.853056 and 42.238222, -70.913389), Yarmouth, MA (41.686167, -70.287722), and Falmouth, MA (41.626417, -70.580417) (Elkinton et al. 2018). For all collections, collection rate (caterpillars/minute) was recorded as a proxy for larval density. Collection rate was calculated from the number of caterpillars collected for each site divided by the number of collectors and the number of minutes spent collecting at that site.

5.3.2 Rearing for estimate larval and pupal mortality

Larvae were reared in batches of 500 or fewer in ventilated 20 L (5 gallon) buckets provisioned with the foliage from the tree species from which the caterpillars were collected. Winter moth larvae were reared separately from Bruce spanworm larvae, and larvae of the same species that were collected from different sites were reared separately. The foliage was insertedinto wet Oasis Floral Foam bricks (Smithers-Oasis North America) to keep the foliage fresh and upright. Foliage was replaced every other day and any cadavers were removed. Larvae pupated in a week or less, which is not enough time for NPV or microsporidia infections to spread among larvae (Wigley 1976). All dead larvae found during rearing were placed individually in sterile 1.5 mL microcentrifuge tubes (Fisherbrand) and immediately frozen at −20 °C. When the remaining larvae started to show signs of pupating (thickening body shape and rolling a leaf edge over themselves), sifted peat moss was added to the bottom of the buckets as a pupation site. Later, pupae were sifted from this peat moss and counted.

Healthy pupae were stored in an incubator (Percival) until the following spring to allow further development of moths or parasitoids. Pupae were stored at 12 °C until the beginning of December, when the temperature was lowered to 9.5 °C. At the end of December, the temperature was adjusted to 4 °C. The pupae were kept in dark with no day/night cycle, and once a month they were sprayed with a sodium propionate solution (5 g sodium propionate/L of water) to prevent mold. Starting in late March, the temperatures were gradually increased in increments of 4 °C per week until April, when

pupae were taken out of storage and held at room temperature for emergence. Any parasitoids that emerged were identified to family using Goulet and Huber (1993) and Triplehorn and Johnson (2005) and stored in 95% ethanol at -20 °C for molecular or morphological identification.

5.3.3 Microscopy for prevalence of visible infections

Larval cadavers were homogenized in molecular-grade water and viewed under a light microscope (Carl Zeiss Standard 14) at 400x. All cadavers collected in 2013 and 2014 were scanned, and any visible infections of NPV or microsporidia were noted. Only cadavers whose tissues were predominately filled with virus particles or microsporidia spores were counted as being infected by that agent. A subsample of the microscopy evaluations determining infection by NPV and microsporidia were confirmed with molecular analyses (Broadley et al. 2017, Donahue et al. in press). Disease incidences were compared only in 2013-2015, as cadavers were not saved from the 2012 Bruce spanworm or winter moth collections. Also, it should be noted that from the 2015 collection of Bruce spanworm we only recovered three cadavers.

5.3.4 Molecular analysis of *Agrypon* wasps

A subset of the emerged *Agrypon* sp. wasps was used for molecular analysis. In addition, *Agrypon* wasps from winter moth collection in British Columbia, Nova Scotia, and Norway, as well as *Agrypon* from autumnal moth (*Epirrita autumnata* Borkhausen) and from grey mountain carpet moth (*Entephria caesiata* Denis and Schiffermüller) from

Finland (Table 13), sent to us by Tero Klemola, Department of Biology, University of Turku, Turku, Finland were included in the molecular analysis.

DNA was extracted using the QIAGEN DNeasy Blood and Tissue Kits, following the company protocol with the following modifications: for adults, DNA was extracted from a single leg removed from the specimen, and DNA was eluted twice in 100 μl Buffer AE instead of once with 200 μL. All DNA extractions were stored at -20°C for subsequent analysis. A portion of the mitochondrial locus cytochrome c oxidase subunit I (CO1) was amplified using standard PCR techniques. A master mix was prepared using the following ratios of reagents per sample: 17.3 μl nuclease free water, 0.5 μl dNTPs, 5 μl 5X GoTaq Buffer (Promega), 0.2 μl GoTaq (Promega), 0.5 μl of both the forward and reverse primer (10 μM each), and 1 μl of eluted DNA. To amplify a 710 bp fragment of CO1, the primer set LCO (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994) were used with the thermocycler profile outlined by Hebert *et al.* (2003).

PCR reactions were run on a BioRad T100 thermocycler, and the resulting PCR products were visualized on an 1.5% agarose gel stained with SYBERsafe (Invitrogen, Carlsbad, CA) to verify amplification. Samples that produced bands of the expected fragment size for each locus were then cleaned prior to sequencing using Exonuclease 1 (ThermoScientific) and Thermolabile Recombinant Shrimp Alkaline Phosphatase (New England BioLabs). The resulting products were submitted to The Yale University DNA Analysis Facility on Science Hill for Sanger sequencing in both sense and anti-sense orientations.

The resulting sequences were then visualized, the forward and reverse sequences were aligned, and sequences were edited using Geneious R8.1.8 and R9 (Biomatters Ltd.). The ends of the aligned sequences were trimmed by hand to remove primer sequences and so that all sequences had a high-quality score (>90% HQ nucleotide reads). The presence of heterozygous sites was determined by Geneious and encoded using the appropriate IUPAC-IUB ambiguity codes. All ambiguous regions were subsequently inspected by eye. Additionally, for our CO1 fragment sequences, we looked for evidence of nuclear mitochondrial DNAs (NUMTs) or pseudogenes by examining for the presence of stop codons based on translation with Invertebrate Mitochondrial DNA genetic code.

The sequences were trimmed to the shortest sequence. We used the Geneious alignment algorithm in Geneious v. 8.1.8 (Kearse et al. 2012) to generate a sequence alignment. A sequence from *Agrypon flexorium* downloaded from the Barcode of Life Database (accession number BBHYE400-10) was used as the outgroup. A neighbor-joining analysis was run in Geneious using 1,000 bootstrap replications and a majority rule (50%) consensus threshold. The resulting gene tree was then visualized using FigTree Version 1.4.2 (Rambaut 2014).

5.3.5 Statistical analysis

To calculate the standard error of the mortality of the larval collections and of the pupal collections, the standard error of a proportion was calculated.

$$\sqrt{\frac{p(1-p)}{n}}$$

The symbol p is proportion dying and n is the sample size. Logistic regression

analyses were performed in R 3.4.4 (RCoreTeam 2013) using RStudio, version 1.1.442

(RstudioTeam 2015). We analyzed the effect of year and species on (1) larval mortality

and pupal mortality, (2) proportion of pupae that died from virus and microsporidia, and

(3) proportion of pupae with *Agrypon* sp. parasitism. For each analysis, the model was

evaluated for evidence of skew in the residuals or of outliers. We checked for

overdispersion, and when evidence of overdispersion was noted, we applied a

quasibinomial distribution. Quasibinomial analyses do not generate AIC values; thus, to

select the best fit model, we compared the residual deviance of the fit model to that of the

null model. A pseudo-R^2 was calculated by comparing the residual deviance of the fit

model against the null model (deviance null model – deviance fit model / deviance null

model).

5.4 Results

5.4.1 Mortality of larvae and pupae

Across collection years (2012 -2016), Bruce spanworm consistently experienced

higher mortality in both larvae (p(species) = 0.030; p(year) = 0.32; df = 3; pseudoR^2 =

0.89; Figure 21A) and pupae (p(species) = 0.016; p(year) =0.025; df= 3; pseudoR^2 = 0.94;

Figure 21B) than winter moth. This was especially true in the first two years (2012 and

2013) when the Bruce spanworm larvae were collected from outbreak populations. In

these two years, the Bruce spanworm caterpillars were just as easy to collect as winter

moth in outbreak areas and could be collected at the same rate (Figure 22). However, in

2014 no Bruce spanworm caterpillars were found at the Maine site, and Bruce spanworm

larvae were collected from low-density sites in western Massachusetts (Table 12).

Collection of even a very few Bruce spanworm caterpillars in 2014 and 2015 took many

hours, with a collection rate of only 0.03 and 0.02 caterpillars/minute or 1.6 and 1.2

caterpillars/hour. In other words, it took 3 individuals 12 hours to collect the 170 Bruce

spanworm caterpillars in 2014 and 2 individuals 12 hours to collect the 87 caterpillars in

2015. This is in contrast to 3.5 to 4 caterpillars/minute for the other collections of winter

moth and Bruce spanworm.

5.4.2 Larval cadavers with visible infections

Occlusion bodies with the characteristic of NPV were found in both winter moth

and Bruce spanworm cadavers. For both species, 10 to 30% of the cadavers showed

heavy loads of NPV (Figure 23) and there was no significant difference in virus load

between winter moth and Bruce spanworm or by year (p(species) = 0.12, p(year) = 0.44,

df = 2, pseudoR^2 = 0.93). No microsporidia were detected in the winter moth collections

and there was no significant difference in microsporidia prevalence across years (p =

0.33, df = 3, pseudoR^2 = 0.56).

5.4.3 Percent parasitism by and phylogenetic analysis of *Agrypon* wasps

Both winter moth and Bruce spanworm experienced parasitism by *Agrypon* sp.wasps; however, Bruce spanworm had significantly higher parasitism rates than winter moth in our study sites in eastern Massachusetts and Maine (p <0.025, df = 4, pseudoR^2 = 0.71; Figure 24). While parasitism rates were not significantly different across years (p = 0.15), there was a trend toward higher parasitism detected in the lower density collections of Bruce spanworm than in higher density collections of Bruce spanworm. From molecular analyses using the CO1 loci of *Agrypon* sp. collected from winter moth and Bruce spanworm in these study sites as well as from the Canada release sites and from Europe, we found that the *Agrypon* specimens reared from Bruce spanworm were distinct from those that we reared from winter moth. These Bruce spanworm *Agrypon* collections' COI genes differed by 4% or 22-23 bp for the *Agrypon* specimens reared from winter moth collected in Massachusetts and by 2% or 9-12 bp from the *Agrypon flaveolatum* specimens collected from winter moth from British Columbia and Nova Scotia. We never found any cross-parasitism by *Agyrypon* species between their two host species (Figure 25). We also detected a different *Agrypon* species from winter moth in Massachusetts. This parasitoid clade was 4-5% (25 or 26 bp) different from that of the *A. flaveolatum* that we reared from winter moth from Nova Scotia or British Columbia, and it was 4% (22 or 23 bp) different from the *Agrypon* specimens we reared from Bruce spanworm.

Table 12: Bruce spanworm and winter moth larvae collections made each year in May, the resulting pupae at beginning of the pupal period, of those pupae the number that were intact (i.e. not diseased, desiccated, or parasitized), and the number of larval cadavers recovered from the rearing and subsequently used for comparisons of pathogen prevalence. The number of collections sites for each year and species is included in parentheses after the collection site name. No cadavers were collected from the 2012 collections.

Year	Species	Collection Site	Larvae Collected	Total pupae	Pupae intact	Larval cadavers
2012	Bruce spanworm	Millinocket, Maine (1, T2R8 TWP)	1055	300	193	NA
2012	Winter moth	Eastern MA study sites (7)	5479	5273	4246	NA
2013	Bruce spanworm	Millinocket, Maine (1, T2R8 TWP)	433	159	57	173
2013	Winter moth	Eastern MA study sites (7)	6547	5559	4810	37
2014	Bruce spanworm	Charlemont and Savoy, MA (2)	170	137	95	25
2014	Winter moth	Eastern MA study sites (7)	6956	6071	5413	186
2015	Bruce spanworm	Charlemont and Savoy, MA (2)	87	57	50	3
2015	Winter moth	Eastern MA study sites (7)	4103	4063	3841	51

Table 13: *Agrypon* sp. wasp sample collection locations.

Sample number	Site	Host	Year
395	Millinocket, ME	Bruce spanworm	2013
396	Millinocket, ME	Bruce spanworm	2013
151	Charlemont, MA	Bruce spanworm	2014
152	Charlemont, MA	Bruce spanworm	2014
153	Charlemont, MA	Bruce spanworm	2014
154	Charlemont, MA	Bruce spanworm	2014
155	Charlemont, MA	Bruce spanworm	2014
156	Charlemont, MA	Bruce spanworm	2014
114	British Columbia	Winter moth	2013
115	British Columbia	Winter moth	2013
116	British Columbia	Winter moth	2013
117	British Columbia	Winter moth	2013
118	British Columbia	Winter moth	2013
119	British Columbia	Winter moth	2013
120	British Columbia	Winter moth	2013
121	British Columbia	Winter moth	2013
122	British Columbia	Winter moth	2013
125	British Columbia	Winter moth	2013
126	British Columbia	Winter moth	2013
242	Nova Scotia	Winter moth	2015
243	Nova Scotia	Winter moth	2015
244	Nova Scotia	Winter moth	2015
32	Norway	Autumnal moth	2005
43	Norway	Autumnal moth	2005
44	Norway	Autumnal moth	2005
49	Norway	Autumnal moth	2004
34	Finland	Autumnal moth	2009
35	Finland	Autumnal moth	2009
36	Finland	Autumnal moth	2009
38	Finland	Autumnal moth	2005
39	Finland	Autumnal moth	2005
41	Finland	Autumnal moth	2005
206	Finland	Autumnal moth	2006
207	Finland	Autumnal moth	2006
208	Finland	Autumnal moth	2006
220	Finland	Autumnal moth	2005
221	Finland	Autumnal moth	2005
222	Finland	Autumnal moth	2005
226	Finland	Grey mountain carpet moth	2010
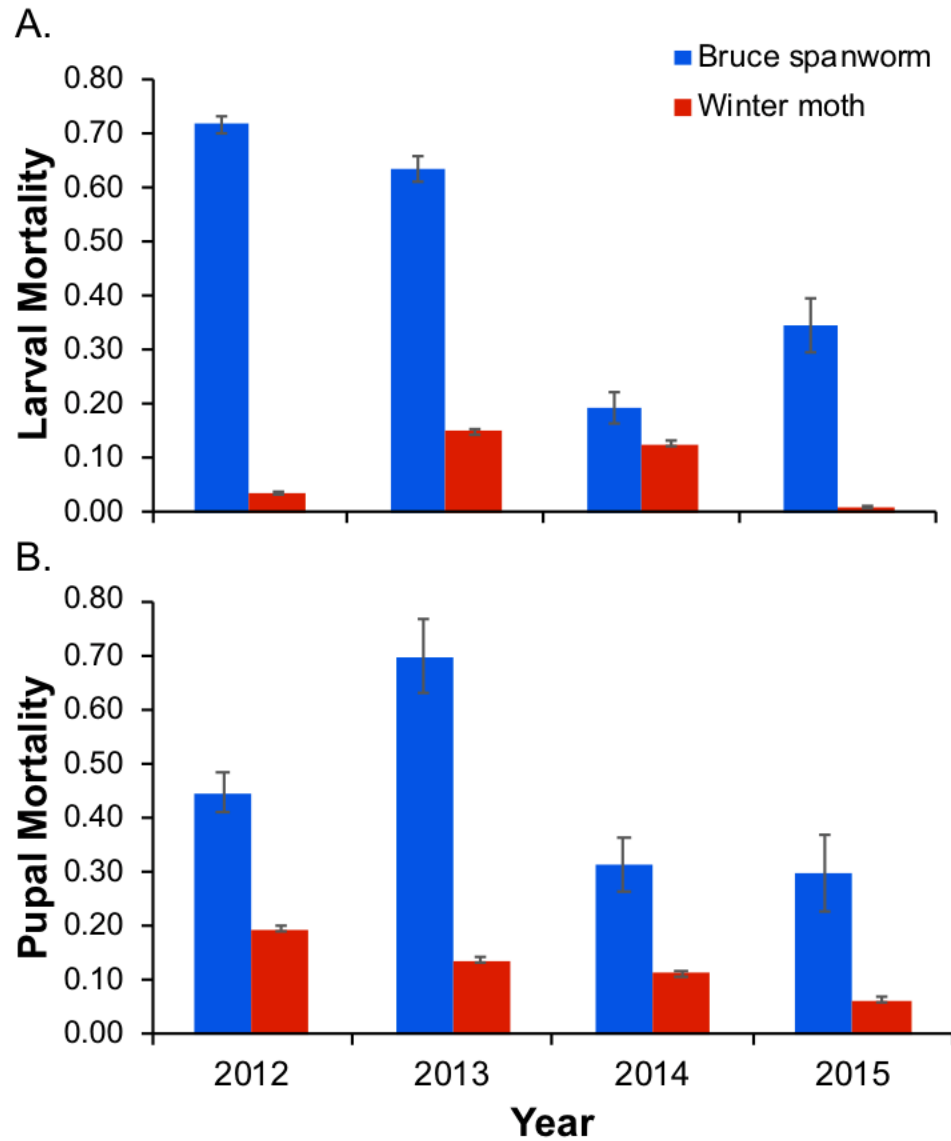
Figure 21: Mortality of larvae (A) and pupae (B) by year of Bruce spanworm (blue) and winter moth (red). The first two years of Bruce spanworm collections (2012 and 2013) were from high-density populations while the second two years (2014 and 2015) were from low density populations. Error bars show standard errors.
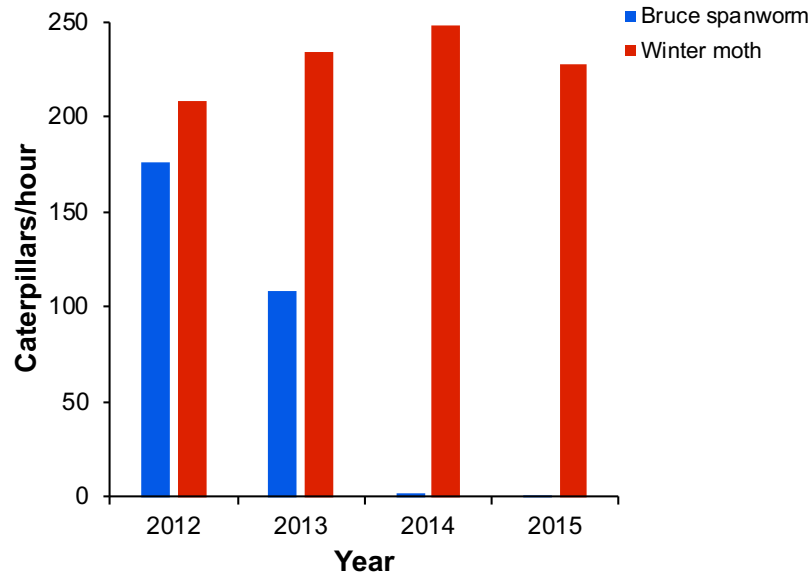
Figure 22: Caterpillars collected per minute for each year and species.
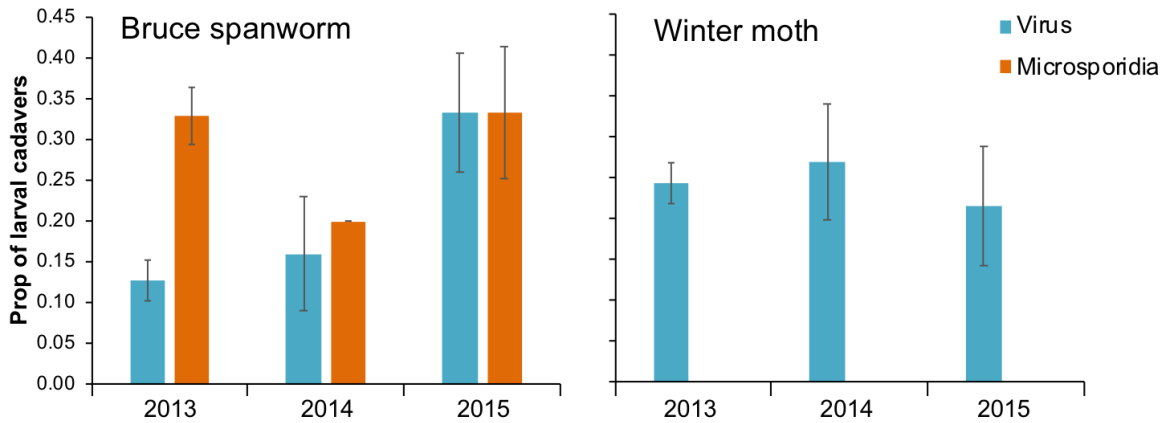
Figure 23: Proportion of larval cadavers with visible infections by nucleopolyhedrovirus and microsporidia. Error bars show standard errors.
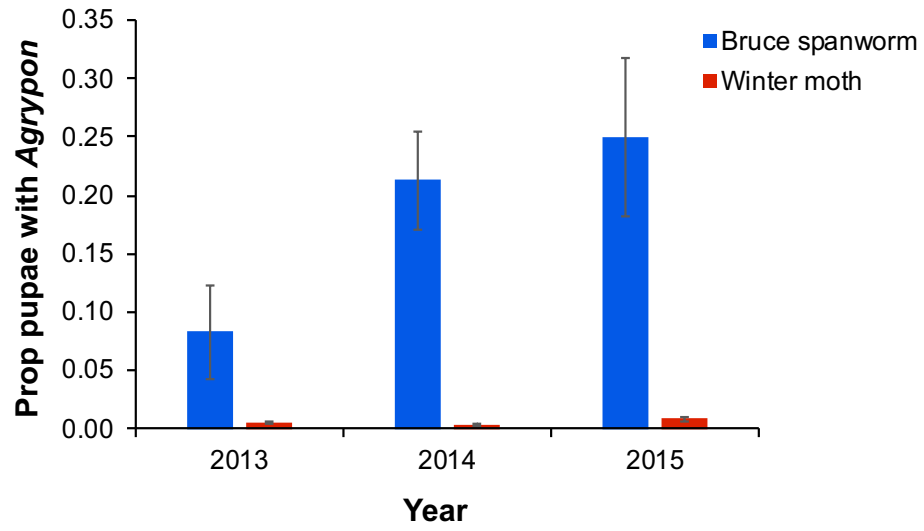
Figure 24: Percent parasitism by *Agrypon* wasps on Bruce spanworm as compared to winter moth. Error bars show standard errors.
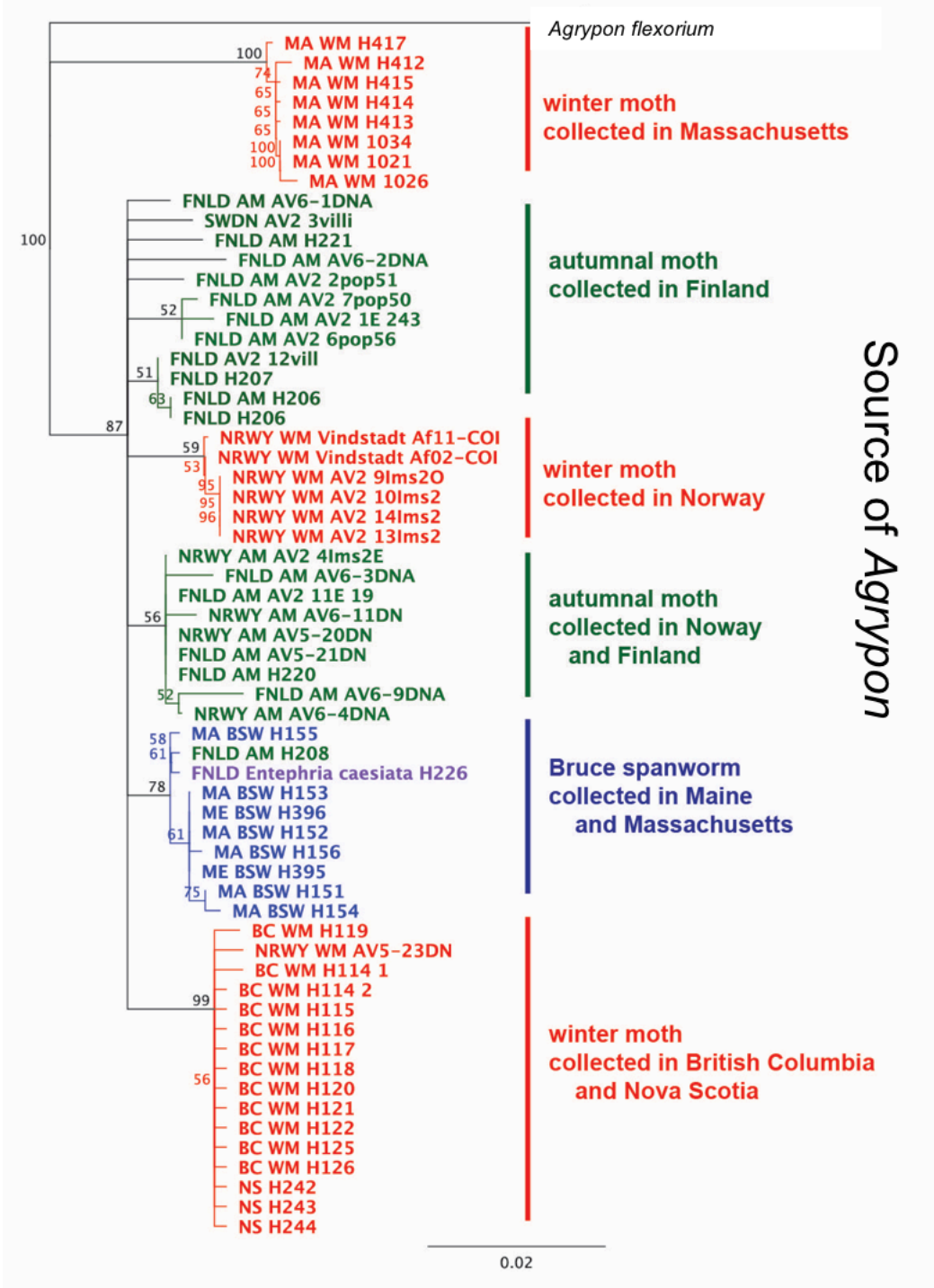
Figure 25: Neighbor joining tree of a 570 bp region of the CO1 loci with the sequences from our *Agrypon* species samples. The color of the text indicates the host: Red indicates winter moth, green indicates autumnal moth, blue indicates Bruce spanworm, and purple indicates gray mountain carpet moth. The number to the left each node represents the bootstrap support value for the branch.

141

5.5 Discussion

To better understand the population dynamics of winter moth in its introduced range in the northeastern United States, we compared winter moth larval and pupal mortality to that of Bruce spanworm, its native congener. In the northeastern United States, winter moth is a high-density, invasive species (Elkinton et al. 2015) and Bruce spanworm is a native species, which is typically at low densities (Brown 1962, Rose and Lindquist 1997). We found that winter moth experienced lower larval and pupal mortality than did Bruce spanworm. While the populations of winter moth have recently been decreasing in Massachusetts, presumably due to classical biological control efforts (Elkinton et al. 2015, Elkinton et al. 2018), during the period of the present study, winter moth caterpillars were still consistently of higher density and easier to collect than Bruce spanworm caterpillars from low-density populations.

The findings from this study have important implications for our understanding of why, winter moth has been in high-density populations in this region while Bruce spanworm only showed local, short-duration outbreaks or was at low densities. Bruce spanworm was affected by several natural enemies in its larval and pupal stages, including viruses, fungal infections, and parasitism Bruce spanworm likely also experiences additional mortality from generalist predators and parasitoids of the larval and pupal stages, but these are likely similar to those that affect winter moth (Elkinton et al. 2015, Pepi et al. 2016, 2017, Broadley et al. 2018) and likely cause comparable rates of mortality. The mortality factors acting on Bruce spanworm appear to maintain its populations at low density. This is in contrast to winter moth, which, during the study period, showed consistently high densities and low rates for larval and pupal mortality.

Together, these findings suggest that winter moth is at such high densities in its invasive range because it has been released from the top-down regulation from natural enemies that have co-evolved with it in its native range. This is referred to as e enemy release, where a non-indigenous species gains an advantage because it arrives in a new habitat without its specialized natural enemies.

Microsporidia are important fungal pathogens of insects that typically show low virulence, causing chronic infections that reduce fecundity and population growth of the host (Hajek et al. 2007, Solter et al. 2012). Our finding, that microsporidia were present in Bruce spanworm but not winter moth in the northeastern United States, is consistent Donahue et al. (in press), who showed that these infections were caused by two species of *Nosema/Vairimorpha* microsporidia in Bruce spanworm. We also detected NPV infections in both winter moth and Bruce spanworm cadavers and found that the proportion of cadavers with visible viral infections was similar between the two species. This supports an earlier study that showed that in the introduced range, both winter moth and Bruce spanworm experience low incidences of NPV, but that each host species is infected by its own virus species, which do not cross infect the other congener moth (Broadley et al. 2017).

The pathogens present in each of the two species' populations appear to act differently. The incidence of microsporidia in Bruce spanworm was higher in larvae collected from a high-density population (2013) than from a low-density population (2014). Furthermore, the Bruce spanworm collection site used in 2013 was checked the following year, and Bruce spanworm densities were found to have declined precipitously. Together, this suggests that when Bruce spanworm populations are in a high-density

phase, microsporidia may cause an epizootic able to reduce the host's population density. On the other hand, winter moth larvae consistently experienced lower mortality than Bruce spanworm, even though for the past decade winter moth in the northeastern USA has existed at consistently high density (Elkinton et al. 2015, Elkinton et al. 2018), at levels where one would expect to see a disease epizootic (Hajek 2004). This difference may help to explain why, in contrast to winter moth within its introduced range, Bruce spanworm is typically present in low-density populations, and why when Bruce spanworm does outbreak it does so on a local scale, and the high-density populations disappear quickly (Brown 1962, Rose and Lindquist 1997), as did our population in central Maine.

Bruce spanworm not only experienced more larval mortality from pathogens, but also from parasitism, compared to winter moth. We found a much higher percent parasitism by *Agrypon* wasps on the Bruce spanworm than on the winter moth, and *Agrypon* sp. parasitism was higher when Bruce spanworm densities were lower than when Bruce spanworm densities were higher. We also compared the CO1 barcoding loci of *Agrypon* wasps collected from (1) Bruce spanworm, (2) winter moth collected in the northeast, (3) the *Agrypon* speciesintroduction sites in Canada, and (4) Norway and Finland. Surprisingly, we found that the *Agrypon* sp. collected from Bruce spanworm was most closely related to the *Agrypon* recovered from autumnal moth (*E. autumnata*) collected in Finland. In Finland, *Agrypon* sp. can be reared from the autumnal moth, but it does not attack winter moth (Klemola, unpublished). The *Agrypon* specimens collected from winter moth from British Columbia and from Nova Scotia all were nearly identical and matched published sequences for *A. flaveolatum*. *Agrypon flaveolatum* has not been

introduced to Massachusetts due lack of information on its host preference and taxonomy (Elkinton et al. 2015). However, we detected another *Agrypon* wasp in our Massachusetts winter moth collections, which was never detected in our Bruce spanworm collections.

Agrypon sp. wasps are known to play an important role in the population dynamics of winter moth and were studied in the biological control project against winter moth in Canada. In contrast, the influence of *Agrypon* sp. parasitism on Bruce spanworm is largely unstudied. One year after introduction to Nova Scotia in 1956, *A. flaveolatum* was recovered in large numbers (Graham 1958). Subsequently, winter moth populations in Nova Scotia declined dramatically in 1962, following high levels of both *C. albicans* and *A. flaveolatum* parasitism (40-60%) and remained at low density in subsequent years (Embree 1965, 1966, Macphee et al. 1988, Roland and Embree 1995). Studies of the role of these two parastioids showed that parastism by *C. albicans* exceeded that caused by *A. flaveolatum* at high winter moth densities but the reverse was true at lower densities (Embree 1966, Macphee et al. 1988, Roland and Embree 1995). This reflects what we found with the *Agrypon* sp. present in Bruce spanworm; parasitism was higher in the collections from Bruce spanworm that were from lower density populations than from the high-density population.

Following the research conducted in Nova Scotia, both *C. albicans* and *A. flaveolatum* were also introduced to Victoria, British Columbia to control a new invasion of winter moth in the 1970s (Gillespie et al. 1978). These introductions resulted in the subsequent decline of winter moth densities (Embree and Otvos 1984, Roland 1988, 1994, Roland and Embree 1995). Parasitism by *C. albicans* varied between 40-80% during this period, but, in contrast to Nova Scotia, parasitism by *A. flaveolatum* never

145

exceeded 2%. Similar levels of parasitism by both species prevailed in collections of winter moth larvae from Victoria, British Columbia between 2007 and 2013 (G. Boettner, unpublished data). This suggests that *A. flaveolatum* may play an important role in some locations where winter moth has invaded, but not in others. Since the host range of *A. flaveolatum* is not known, *A. flaveolatum* will not be introduced to Massachusetts, but if we consider the population dynamics of Bruce spanworm as a proxy for that of winter moth, the data suggest that *A. flaveolatum* could have played an important role.

The current study used light microscopy to identify the primary pathogens in the cadavers recovered. However, due to the life cycle of microsporidia, the spore stage is the only easily recognizable life stage (Ptaszyńska 2014), and light phase microscopy is known to result in false negative diagnoses (Sokolova et al. 2004). Similarly, NPV can also be hard to detect using light microscopy. However, we confirmed our visual diagnoses with molecular approaches in other work (Broadley et al. 2017, Donahue et al. in press). These studies showed that what we identified as microsporidia and NPV by visual scan were indeed microsporidia and NPV. However, from both of these studies we also found that our PCR technique was not always sensitive enough or broad enough to pick up the signal.

Our results support the hypothesis that winter moth in the Northeast exhibits outbreak populations because, in contrast to Bruce spanworm, it lacks several natural enemies. By studying two related species that exhibit variable dynamics but share their range, we highlighted differences that suggest what may be allowing the invasive winter moth to maintain high population densities, while its native congener, Bruce spanworm, has consistently low population densities.

BIBLIOGRAPHY

Al-Zyoud, F., and C. Sengonca. 2004. Preference by prey consumption of Serangium parcesetosum Sicard (Col., Coccinellidae) by feeding on different prey stages, species and parasitized prey. Journal of Pest Science 77:197-204.

Andersen, J. C., M. E. Gruwell, G. E. Morse, and B. B. Normark. 2010. Cryptic Diversity in the Aspidiotus nerii Complex in Australia. Annals of the Entomological Society of America 103(6):844-854. doi: 10.1603/an10060

Andersen, J.C., Wagner, D.L., 2016. Systematics and biological control, in: Van Driesche, R.G., Simberloff, D., Blossey, B., Causton, C., Hoddle, M.S., Wagner, D.L., Warner, K.D. (Eds.), Integrating Biological Control into Conservation Practice. John Wiley & Sons, Ltd., Chichester, UK, pp. 105–129.

Avise, J. C., and K. Wollenberg. 1997. Phylogenetics and the origin of species. P Natl Acad Sci USA 94(15):7748-7755. doi: 10.1073/pnas.94.15.7748

Ball, S.L., Hebert, P.D.N., Burian, S.K., Webb, J.M., 2005. Biological identifications of mayflies (Ephemeroptera) using DNA barcodes. J. N. Am. Benthol. Soc. 24(3), 508–524. https://doi.org/10.1899/04-142.1

Barlow, J., and P. Boveng. 1991. Modeling age-specific mortality for marine mammal populations. Marine Mammal Science 7:50-65.

Baum, D. A., and K. L. Shaw. 1995. Genealogical Perspectives on the Species Problem. In: P. C. Hoch and A. G. Stephenson, editors, Molecular and experimental approaches to plant biosystematics. Missouri Botanical Garden, St. Louis, MO. p. 289-303.

Bell, R. A., C. D. Owens, M. Shapiro, and J. R. Tardif. 1981. Development of mass rearing technology. Pages 599–633 in D. C. C. and M. M. L., editors. The gypsy moth: Research toward integrated pest management. Technical Bulletin 1584. U.S. Department of Agriculture, Forest Service, Washington, DC.

Bennett, A. M. R. 2008. Review and identification keys to the ichneumonid parasitoids (Hymenoptera : Ichneumonidae) of Nearctic Choristoneura species (Lepidoptera : Tortricidae). Canadian Entomologist 140(1):1-47.

Berryman, A. A. e. 2002. Population cycles: the case for trophic interactions. Oxford University Press, Oxford, UK.

Bonsall, M. B., and M. P. Hassell. 1995. Identifying density-dependent processes - a comment on the regulation of winter moth. Journal of Animal Ecology 64:781-784.

Botkin, D. B., and R. S. Miller. 1974. Mortality Rates and Survival of Birds. The American Naturalist 108:181-192.

Broadley, H. J., M. Boucher, J. P. Burand, and J. S. Elkinton. 2017. The phylogenetic relationship and cross-infection of nucleopolyhedroviruses between the invasive winter moth (Operophtera brumata) and its native congener, Bruce spanworm (O. bruceata). Journal of Invertebrate Pathology 143:61-68. doi: 10.1016/j.jip.2016.11.016

Broadley, H. J., E. A. Kelly, J. S. Elkinton, R. R. Kula, and G. H. Boettner. 2018. Identification and impact of hyperparasitoids and predators affecting Cyzenis albicans (Tachinidae), a recently introduced biological control agent of winter moth (Operophtera brumata L.) in the northeastern U.S.A. Biological Control 121:99-108.

Brodeur, J. 2000. Host specificity and trophic relationships of hyperparasitoids. In: M. E. Hochberg and A. R. Ives, editors, Parasitoid Populaiton Ecology. Princeton University Press, Princeton, New Jersey.

Brown, C. E. 1962. The Life History of the Bruce Spanworm, Operophtera bruceata. The Canadian Entomologist.

Buckner, C. H. 1959. The assessment of larch sawfly cocoon predation by small mammals. Canadian Entomologist 91:275-282.

Buckner, C. H. 1969. Common shrew (Sorex araneus) as a predator of winter moth (Operophtera brumata) near Oxford England. Canadian Entomologist 101:370-&.

Buonaccorsi, J. P., and J. S. Elkinton. 1990. Estimation of contemporaneous mortality factors. Res Popul Ecol 32:151-171.

Burand, J. P., W. Kim, A. Welch, and J. S. Elkinton. 2011. Identification of a nucleopolyhedrovirus in winter moth populations from Massachusetts. J Invertebr Pathol 108:217-219.

Callaway, R. M., D. Montesinos, K. Williams, and J. L. Maron. 2013. Native congeners provide biotic resistance to invasive Potentilla through soil biota. Ecology 94(6):1223-1229. doi: 10.1890/12-1875.1

Caltagirone, L. E. 1981. Landmark examples in classical biological-control. Annual Review of Entomology 26:213-232.

Campbell, R. W. 1975. The gypsy moth and its natural enemies. U.S. Dept. Agric. Info. Buill. 381.

Campbell, R. W., and R. J. Sloan. 1977. Natural regulation of innocuous gypsy moth populations. Environmental Entomology 6:315-322.

Caughley, G. 1966. Mortality Patterns in Mammals. Ecology 47:906-918.

Carlson, R. W. 2009. Superfamily Ichneumoidea. http://www.discoverlife.org/proceedings/0000/6

Carpenter, J. M., and W. C. Wheeler. 1999. Towards simultaneous analysis of morphological and molecular data in Hymenoptera. Zoologica Scripta 28(1-2):251-260. doi: 10.1046/j.1463-6409.1999.00009.x

Carrillo-Gavilan, A., X. Moreira, R. Zas, M. Vila, and L. Sampedro. 2012. Early resistance of alien and native pines against two native generalist insect herbivores: no support for the natural enemy hypothesis. Funct Ecol 26(1):283-293. doi: 10.1111/j.1365-2435.2011.01931.x

Cooper, D., J. S. Cory, D. A. Theilmann, and J. H. Myers. 2003. Nucleopolyhedroviruses of forest and western tent caterpillars: cross-infectivity and evidence for activation of latent virus in high-density field populations. Ecological Entomology 28:41-50.

Cory, J. S. 2010. The Ecology of Baculoviruses. Pages 411-428 in S. Asgari and K. Johnson, editors. Insect Virology. Caister Academic Press, Norfolk, UK.

Cory, J. S. 2015. Insect virus transmission: different routes to persistence. Current Opinion in Insect Science 8:130-135.

Cory, J. S., and J. H. Myers. 2003. The ecology and evolution of insect baculoviruses. Annual Review of Ecology Evolution and Systematics 34:239-272.

Caughley, G. 1966. Mortality Patterns in Mammals. Ecology 47:906-918.

Cuming, F. G. 1961. The distribution, life history, and economic importance of the winter moth Operophtera brumata (L.) (Lepidoptera, Geometridae) in Nova Scotia. The Canadian Entomologist 93:135-142.

Cunningham, J. C., N. V. Tonks, and W. J. Kaupp. 1981. Viruses to control winter moth, Operophtera brumata, (Lepidoptera: Geometridae) Journal of the Entomological Society of British Columbia 78:17-24.

Dearborn, K. W., S. B. Heard, J. Sweeney, and D. S. Pureswaran. 2016. Displacement of Tetropium cinnamopterum (Coleoptera: Cerambycidae) by Its Invasive Congener Tetropium fuscum. Environ Entomol 45(4):848-854. doi: 10.1093/ee/nvw045

deRivera, C. E., G. M. Ruiz, A. H. Hines, and P. Jivoff. 2005. Biotic resistance to invasion: Native predator limits abundance and distribution of an introduced crab. Ecology 86(12):3364-3376. doi: 10.1890/05-0479

Dettman, J. R., D. J. Jacobson, and J. W. Taylor. 2003. A multilocus genealogical approach to phylogenetic species recognition in the model eukaryote Neurospora. Evolution 57(12):2703-2720.

Dettman, J. R., D. J. Jacobson, and J. W. Taylor. 2006. Multilocus sequence data reveal extensive phylogenetic species diversity within the Neurospora discreta complex. Mycologia 98(3):436-446. doi: 10.3852/mycologia.98.3.436

Diez, J. M., J. J. Sullivan, P. E. Hulme, G. Edwards, and R. P. Duncan. 2008. Darwin's naturalization conundrum: dissecting taxonomic patterns of species invasions. Ecology Letters 11(7):674-681. doi: 10.1111/j.1461-0248.2008.01178.x

Donahue, K. L., H. J. Broadley, J. S. Elkinton, J. P. Burand, W. F. Huang, and J. C. Andersen. in press. Using the SSU, ITS, and Ribosomal DNA Operon Arrangement to Characterize Two Microsporidia Infecting Bruce spanworm, Operophtera bruceata (Lepidoptera: Geometridae). Journal of Eukaryotic Microbiology.

Duan, J. J., K. J. Abell, L. S. Bauer, J. Gould, and R. Van Driesche. 2014. Natural enemies implicated in the regulation of an invasive pest: a life table analysis of the population dynamics of the emerald ash borer. Agricultural and Forest Entomology 16(4):406-416. doi: 10.1111/afe.12070

Duan, J. J., P. B. Taylor, R. W. Fuester, R. R. Kula, and P. M. Marsh. 2013. Hymenopteran parasitoids attacking the invasive emerald ash boror (Coleoptera: Buprestidae) in western and central Pennsylvania. Florida Entomologist 96(1):166-172. doi: 10.1653/024.096.0122

East, R., 1974. Predation on soil-dwelling stages of winter moth at Wytham Woods Berkshire. J. Anim. Ecol. 43(3), 611–626. http://www.jstor.org/stable/3526

Ehler, I.E., 1979. Utility of facultative secondary parasites in biological control. Environ. Entomol. 8(5), 829–832. https://doi.org/10.1093/ee/8.5.829

Elkinton, J. S., G. H. Boettner, A. Liebhold, and R. Gwiazdowski. 2015. Biology, Spread, and Biological Control of Winter Moth in the Eastern United States. USDA Forest Service Publication, Morgantown, West Virginia.

Elkinton, J. S., G. H. Boettner, H. J. Broadley, R. Reardon, and R. D. Weeks Jr. 2018. Biological control of the winter moth in the northeastern North America. USDA Forest Service Forest Health Assessment and Applied Science Team 2018-03:0-9.

150

Elkinton, J. S., G. H. Boettner, M. Sermac, R. Gwiazdowski, R. R. Hunkins, J. Callahan, S. B. Scheufele, C. P. Donahue, A. H. Porter, A. Khrimian, B. M. Whited, and N. K. Campbell. 2010. Survey for Winter Moth (Lepidoptera: Geometridae) in Northeastern North America With Pheromone-Baited Traps and Hybridization With the Native Bruce Spanworm (Lepidoptera: Geometridae). Annals of the Entomological Society of America 103:135-145.

Elkinton, J.S., Buonaccorsi, J.P., Bellows, T.S., Van Driesche, R.G., 1992. Marginal attack rate, k-values and density dependence in the analysis of contemporaneous mortality factors. Res. Popul. Ecol. 34, 29–44. https://doi.org/10.1007/BF02513520

Elkinton, J.S., Healy, W.M., Buonaccorsi, J.P., Boettner, G.H., Hazzard, A.M., Smith, H.R., 1996. Interactions among gypsy moths, white-footed mice, and acorns. Ecology 77(8), 2332–2342. http://www.jstor.org/stable/2265735

Elkinton, J. S., and A. M. Liebhold. 1990. Population dynamics of gypsy moth in North America. Annual Review of Entomology 35:571-596.

Elkinton, J. S., A. Liebhold, G. H. Boettner, and M. Sremac. 2014. Invasion spread of Operophtera brumata in northeastern United States and hybridization with O.bruceata. Biological Invasions 16:2263-2272.

Elton, C. S. 1958. The Ecology of Invasions by Animals and Plants. Methuen, London, England.

Embree, D. G. 1965. The population dynamics of the winter moth in Nova Scotia, 1954–1962. Memoirs of the Entomological Society of Canada 97:5-57.

Embree, D.G., Sisojevic, P., 1965. The bionomics and population density of Cyzenis albicans (Fall.) (Tachinidae: Diptera) in Nova Scotia. Can. Entomol. 97(6), 631–639. https://doi.org/10.4039/Ent97631-6

Embree, D. G. 1966. The role of introduced parasites in the control of the winter moth in Nova Scotia. Canadian Entomologist 98:1159-1168.

Embree, D. G. 1967. Effects of winter moth on growth and mortality of red oak in Nova Scotia. Forest Science 13:295-299.

Embree, D. G., and I. S. Otvos. 1984. Operophtera brumata (L.), winter moth (Lepidoptera: Geometridae). Pages 353-357 Biological Control Programmes Against Insects and Weeds in Canada, 1969-1980.

Faillace, C. A., N. S. Lorusso, and S. Duffy. 2017. Overlooking the smallest matter: viruses impact biological invasions. Ecology Letters 20(4):524-538. doi: 10.1111/ele.12742

Feeny, P. 1970. Seasonal changes in oak leaf tannins and nutrients as a cause of spring feeding by winter moth caterpillars. Ecology 51:565-581.

Fitton, M.G., Shaw, M.R., Gauld, I.D., 1988. Pimpline Ichneumon-flies: Hymenoptera, Ichneumonidae (Pimplinae). Royal Entomological Society of London, London, U.K.

Flanders, S.E., 1963. Hyperparasitism, a mutualistic phenomonon. Can. Entomol. 95(7), 716–720. https://doi.org/10.4039/Ent95716-7

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R., 1994. DNA primers for the amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. 3, 294–299.

Frank, J.H., 1967. Effect of pupal predators on a population of winter moth Operophtera brumata (L) (Hydriomenidae). Journal of Animal Ecology 36:661-621.

Frank, J. H. 1967. Insect predators of pupal stage of winter moth Operophtera brumata (L) (Lepidoptera - Hydriomenidea). Journal of Animal Ecology 36:375-&.

Frost, S. W. 1964. Insects Taken in Light Traps at the Archbold Biological Station, Highlands County, Florida. The Florida Entomologist 47(2):129-161. doi: 10.2307/3493289

Garnas, J. R., M. A. Auger-Rozenberg, A. Roques, C. Bertelsmeier, M. J. Wingfield, D. L. Saccaggi, H. E. Roy, and B. Slippers. 2016. Complex patterns of global spread in invasive insects: eco-evolutionary and management consequences. Biological Invasions 18(4):935-952. doi: 10.1007/s10530-016-1082-9

Gauld, I. D. 1991. The Ichneumonidae of Costa Rica,1. Introduction, keys to subfamilies, and keys to the species of the lower pimpliform subfamilies Rhyssinae, Poemeniinae, Acaenitinae and Cylloceriinae. Memoirs of the American Entomological Institute 47:1-589.

Gillespie, D. R., T. Finlayson, N. V. Tonks, and D. A. Ross. 1978. Occurrence of Winter Moth, Operophtera brumata (Lepidoptera Geometridae), on Southern Vancouver-Island, British-Columbia. Canadian Entomologist 110:223-224.

Gehman, A. L. M., and J. E. Byers. 2016. Non-native parasite enhances susceptibility of host to native predators. Oecologia 183:919-926.

Goldson, S.L., Wratten, S.D., Ferguson, C.M., Gerard, P.J., Barratt, B.I.P., Hardwick, S., McNeill, M.R., Phillips, C.B., Popay, A.J., Tylianakis, J.M., Tomasetto, F. 2014. If and when successful classical biological control fails. Biological Control. 72, 76-79, https://doi.org/10.1016/j.biocontrol.2014.02.012.

Goulet, H., and J. T. Huber. 1993. Hymenoptera of the world: An identification guide to families. Centre for Land and Biological Resources Research, Ottawa, Ontario.

Grabenweger, G., P. Kehrli, I. Zweimuller, S. Augustin, N. Avtzis, S. Bacher, J. Freise, S. Girardoz, S. Guichard, W. Heitland, C. Lethmayer, M. Stolz, R. Tomov, L. Volter, and M. Kenis. 2010. Temporal and spatial variations in the parasitoid complex of the horse chestnut leafminer during its invasion of Europe. Biological Invasions 12(8):2797-2813. doi: 10.1007/s10530-009-9685-z

Graham, A. R. 1958. Recoveries of introduced species of parasites of the winter moth, Operophtera brumata (L.) (Lepidoptera: Geometridae), in Nova Scotia. The Canadian Entomologist 90:595-596.

Graham, R. I., W. I. Tyne, R. D. Possee, S. M. Sait, and R. S. Hails. 2004. Genetically variable nucleopolyhedroviruses isolated from spatially separate populations of the winter moth Operophtera brumata (Lepidoptera: Geometridae) in Orkney. Journal of Invertebrate Pathology 87:29-38.

Groeneveld, L. F., D. W. Weisrock, R. M. Rasoloarison, A. D. Yoder, and P. M. Kappeler. 2009. Species delimitation in lemurs: multiple genetic loci reveal low levels of species diversity in the genus Cheirogaleus. Bmc Evolutionary Biology 9doi: 10.1186/1471-2148-9-30

Guindon, S., J. F. Dufayard, V. Lefort, M. Anisimova, W. Hordijk, and O. Gascuel. 2010. New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. Systematic Biology 59:307-321.

Gwiazdowski, R. A., J. S. Elkinton, J. R. Dewaard, and M. Sremac. 2013. Phylogeographic Diversity of the Winter Moths Operophtera brumata and O. bruceata (Lepidoptera: Geometridae) in Europe and North America. Annals of the Entomological Society of America 106(2):143-151. doi: 10.1603/an12033

Haas-Stapleton, J. O. Washburn, and L. E. Volkman. 2004. P74 mediates specific binding of Autographa californica M nucleopolyhedrovirus occlusion-derived virus to primary celluar target in the midgut epithelia of Heliothis virescens larvae. Journal of Virology 78:6786-6791.

Hajek, A.E., 2004. Natural Enemies: An Introduction to Biological Control. Cambridge University Press. New York, New York.

Hajek, A. E., M. L. McManus, and I. D. Junior. 2007. A review of introductions of pathogens and nematodes for classical biological control of insects and mites. Biological Control 41:1-13.

Hansen, N. M., R. A. Ims, and S. B. Hagen. 2009. No Impact of Pupal Predation on the Altitudinal Distribution of Autumnal Moth and Winter Moth (Lepidoptera: Geometridae) in Sub-Arctic Birch Forest. Environmental Entomology 38:627-632.

Harvey, J.A., 2008. Comparing and contrasting development and reproductive strategies in the pupal hyperparasitoids Lysibia nana and Gelis agilis (Hymenoptera: Ichneumonidae). Evol. Ecol. 22(2), 153–166. https://doi.org/10.1007/s10682-007-9164-x

Hassell, M.P., 1969. A study of the mortality factors acting upon Cyzenis albicans (Fall.), a tachinid parasite of the winter moth (Operophtera brumata (L.)). J. Anim. Ecol. 38(2), 329–339. https://doi.org/10.2307/2774

Hassell, M.P., 1980. Foraging strategies, population models and biological control: a case study. J. Anim. Ecol. 49(2), 603–628. http://www.jstor.org/stable/4267

Hassell, M. P. 2000. Host-parasitoid population dynamics. Journal of Animal Ecology 69(4):543-566. doi: 10.1046/j.1365-2656.2000.00445.x

Hassell, M. P., and D. J. Rogers. 1972. Insect parasite responses in the development of population models. Journal of Animal Ecology 41(3):661-676.

Havill, N. P., Elkinton, J.S., Andersen, J. C., Hagen, S. B., Broadley, H. J., Boettner, G. J., Caccone, A. 2017. Asymmetric hybridization between non-native winter moth, Operophtera brumata (Lepidoptera: Geometridae), and native Bruce spanworm, Operophtera bruceata, in the Northeastern United States, assessed with novel microsatellites and SNPs. Bull Entomol Res. 107:241-250.

Hawboldt, L. S., and F. G. Cuming. 1950. Cankerworms and winter moth in Novia Scotia. Forest Insect Investigations; bi-monthly progress report 6:1-2.

Hebert, P.D.N., Cywinska, A., Ball, S.L., deWaard, J.R., 2003. Biological identifications through DNA barcodes. Proc. R. Soc. Lond. [Biol.] 270(1512), 313–321. https://doi.org/10.1098/rspb.2002.2218

Hebert, P. D. N., S. Ratnasingham, E. V. Zakharov, A. C. Telfer, V. Levesque-Beaudin, M. A. Milton, S. Pedersen, P. Jannetta, and J. R. deWaard. 2016. Counting animal species with DNA barcodes: Canadian insects. Philos T R Soc B 371(1702)doi: 10.1098/rstb.2015.0333

Heisswolf, A., M. Kaar, T. Klemola, and K. Ruohomaki. 2010. Local outbreaks of Operophtera brumata and Operophtera fagata cannot be explained by low vulnerability to pupal predation. Agricultural and Forest Entomology 12:81-87.

Heisswolf, A., N. Klemola, T. Ammunet, and T. Klemola. 2009. Responses of generalist invertebrate predators to pupal densities of autumnal and winter moths under field conditions. Ecological Entomology 34:709-717.

Heppell, S. S. 1998. Application of Life-History Theory and Population Model Analysis to Turtle Conservation. Copeia 1998:367-375.

Heraty, J., F. Ronquist, J. M. Carpenter, D. Hawks, S. Schulmeister, A. P. Dowling, D. Murray, J. Munro, W. C. Wheeler, N. Schiff, and M. Sharkey. 2011. Evolution of the hymenopteran megaradiation. Molecular Phylogenetics and Evolution 60(1):73-88. doi: 10.1016/j.ympev.2011.04.003

Hobbs, R. J., E. Higgs, and J. A. Harris. 2009. Novel ecosystems: implications for conservation and restoration. Trends Ecol Evol 24(11):599-605. doi: 10.1016/j.tree.2009.05.012

Holliday, N.J., 1977. Population ecology of winter moth (Operophtera brumata) on apple in relation to larval dispersal and time of bud burst. J. Appl. Ecol. 14(3), 803–813. http://www.jstor.org/stable/2402812

Holliday, N.J., 1985. Maintenance of the Phenology of the Winter Moth (Lepidoptera, Geometridae). Biological Journal of the Linnean Society 25:221-234.

Holling, C. S. 1959. The components of predation as revealed by a study of small mammal predation of the European pine sawfly. Canadian Entomologist 91:293-320.

Holling, C. S. 1973. Resilience and Stability of Ecological Systems. Annual Review of Ecology and Systematics 4(1):1-23. doi: 10.1146/annurev.es.04.110173.000245

Horgan, F.G., Myers, J.H., 2004. Interactions between predatory ground beetles, the winter moth and an introduced parasitoid on the Lower Mainland of British Columbia. Pedobiologia 48(1), 23–35. https://doi.org/10.1016/j.pedobi.2003.07.002

Horgan, F. G., J. H. Myers, and R. Van Meel. 1999. Cyzenis albicans (Diptera : Tachinidae) does not prevent the outbreak of winter moth (Lepidoptera : Geometridae) in birch stands and blueberry plots on the lower mainland of British Columbia. Environmental Entomology 28:96-107.

Hostetter, D. L., and B. Puttler. 1991. A new broad host spectrum nuclear polyhedrosis virus isolated from a celery looper, Anagrapha flacifera (Kriby), (Lepidoptera, Noctuidae) Environmental Entomology 20:1480-1488.

Hothorn, T., F. Bretz, and P. Westfall. 2008. Simultaneous Inference in General Parametric Models. Biometrical Journal 50:346 - 363.

Huelsenbeck, J. P., and F. Ronquist. 2001. MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17:754-755.

Hughes, D. S., R. D. Possee, and L. A. King. 1993. Activation and detection of latent baculovirus resembling Mamestra brassicae nuclear polyhedrosis virus in M. brassicae insects. Virology 194:608-615.

Humble, L., 1985. Final-instar larvae of native pupal parasites and hyperparasites of Operophtera spp. (Lepidoptera: Geometridae) on Southern Vancouver Island. Can. Entomol. 117(5), 525–534. https://doi.org/10.4039/Ent117525-5

Ives, W. G. H., and J. C. Cunningham. 1980. Application of nuclear polyhedrosis virus to control Bruce spanworm (Lepidoptera: Geometridae). The Canadian Entomologist 112:741-744.

Jeschke, J., L. Gómez Aparicio, S. Haider, T. Heger, C. Lortie, P. Pyšek, and D. Strayer. 2012. Support for major hypotheses in invasion biology is uneven and declining. NeoBiota 14doi: 10.3897/neobiota.14.3435

Jørgensen, C., and R. E. Holt. 2013. Natural mortality: Its ecology, how it shapes fish life histories, and why it may be increased by fishing. Journal of Sea Research 75:8-18.

Jurkovicova, M. 1979. Activation of latent virus infections in larvae of Adoxophyes orana (Lepidoptera: Tortricidae) and Barathra brassicae (Lepidoptera: Noctuidae) by foreign polyhedra. Journal of Invertebrate Pathology 34:213-223.

Keane, R. M., and M. J. Crawley. 2002. Exotic plant invasions and the enemy release hypothesis. Trends Ecol Evol 17(4):164-170. doi: 10.1016/s0169-5347(02)02499-0

Kearse, M., R. Moir, A. Wilson, S. Stones-Havas, M. Cheung, S. Sturrock, S. Buxton, A. Cooper, S. Markowitz, C. Duran, T. Thierer, B. Ashton, P. Mentjies, and A. Drummond. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647-1649.

Kellogg, S.K., Fink, L.S., Brower, L.P., 2003. Parasitism of native luna moths, Actias luna (L.) (Lepidoptera: Saturniidae) by the introduced Compsilura concinnata (Meigen) (Diptera: Tachinidae) in central Virginia, and their hyperparasitism by trigonalid wasps (Hymenoptera: Trigonalidae). Environ. Entomol. 32(5), 1019–1027. https://doi.org/10.1603/0046-225X-32.5.1019

Kenis, M., B. P. Hurley, A. E. Hajek, and M. J. W. Cock. 2017. Classical biological control of insect pests of trees: facts and figures. Biological Invasions 19:3401-3417.

Kenis, M., H. E. Roy, R. Zindel, and M. E. N. Majerus. 2008. Current and potential management strategies against Harmonia axyridis. Biocontrol 53(1):235-252. doi: 10.1007/s10526-007-9136-7

Kim, W., J. S. Elkinton, G. Boettner, and J. P. Burand. 2011. Possible origin of nucleopolyhedrovirus in winter moth populations in Massachusetts. Society of Invertebrate Pathology, Halifax, Nova Scotia, Canada.

Kimberling, D. N., J. C. Miller, and R. L. Penrose. 1986. Distribution and Parasitism of Winter Moth, Operophtera-Brumata (Lepidoptera, Geometridae), in Western Oregon. Environmental Entomology 15:1042-1046.

Klemola, N., A. Heisswolf, T. Ammunet, K. Ruohomaki, and T. Klemola. 2009. Reversed impacts by specialist parasitoids and generalist predators may explain a phase lag in moth cycles: a novel hypothesis and preliminary field tests. Annales Zoologici Fennici 46:380-393.

Klemola, T., T. Andersson, and K. Ruohomaki. 2014. Delayed density-dependent parasitism of eggs and pupae as a contributor to the cyclic population dynamics of the autumnal moth. Oecologia 175:1211-1225.

Klemola, T., T. Andersson, and K. Ruohomaki. 2008. Fecundity of the autumnal moth depends on pooled geometrid abundance without a time lag: implications for cyclic population dynamics. Journal of Animal Ecology 77(3):597-604. doi: DOI 10.1111/j.1365-2656.2008.01369.x

Klopfstein, S., and F. Ronquist. 2013. Convergent intron gains in hymenopteran elongation factor-1 alpha. Molecular Phylogenetics and Evolution 67(1):266-276. doi: 10.1016/j.ympev.2013.01.015

Kowalski, R. 1977. Further elaboration of winter moth population models. Journal of Animal Ecology 46:471-482.

Krombein, K. V., P. D. Hurd, D. R. Smith, and B. D. Burks. 1979. Symphyta and Apocrita (Parasitica), Catalog of Hymenoptera in America North of Mexico No. 1. Smithsonian Institute Press, Washington, DC.

Lafferty, K. D. 1992. Foraging on Prey that are Modified by Parasites. American Society of Naturalists 140:854-867.

Lange, M., H. Wang, H. Zhihong, and J. A. Jehle. 2004. Towards a molecular identification and classification system of lepidopteran-specific baculoviruses. Virology 325:36-47.

Leius, K. 1960. Attractiveness of Different Foods and Flowers to the Adults of some Hymenopterous Parasites. The Canadian Entomologist 92(5):369-376. doi: 10.4039/Ent92369-5

Levine, J. M., P. B. Adler, and S. G. Yelenik. 2004. A meta-analysis of biotic resistance to exotic plant invasions. Ecology Letters 7(10):975-989. doi: 10.1111/j.1461-0248.2004.00657.x

MacPhee, A., Newton, A., McRae, K.B., 1988. Population studies on the winter moth Operophtera brumata (L.) (Lepidoptera: Geometridae) in apple orchards in Nova Scotia. Can. Entomol. 120(1), 73–83. https://doi.org/10.4039/Ent12073-1

Maron, J. L., S. Harrison, and M. Greaves. 2001. Origin of an insect outbreak: escape in space or time from natural enemies? Oecologia 126(4):595-602.

Maron, J. L., and M. Vila. 2001. When do herbivores affect plant invasion? Evidence for the natural enemies and biotic resistance hypotheses. Oikos 95(3):361-373. doi: 10.1034/j.1600-0706.2001.950301.x

Matosevic, D., and G. Melika. 2013. Recruitment of native parasitoids to a new invasive host: first results of Diyocosmus kuriphilus parasitoid assemblage in Croatia. Bulletin of Insectology 66(2):231-238.

McDonald, R.C., Kok, L.T., 1991. Hyperparasites attacking Cotesia glomerata (L.) and Cotesia rubecula (Marshall) (Hymenoptera: Braconidae) in southwestern Virginia. Biol. Control 1(2), 170–175. https://doi.org/10.1016/1049-9644(91)90116-H

McKinley, D. J., D. A. Brown, C. C. Payne, and K. A. Harrap. 1981. Cross-infectivity and activation studies with four baculoviruses. Entomophaga 26:79-90.

McNeil, J.N., Rabb, R.L., 1973. Life histories and seasonal biology of four hyperparasites of the tobacco hornworm, Manduca sexta, (Lepidoptera: Sphingidae). Can. Entomol. 105(8), 1041–1052. https://doi.org/10.4039/Ent1051041-8

Menkis, A., E. Bastiaans, D. J. Jacobson, and H. Johannesson. 2009. Phylogenetic and biological species diversity within the Neurospora tetrasperma complex. Journal of Evolutionary Biology 22(9):1923-1936. doi: 10.1111/j.1420-9101.2009.01801.x

Miller, M. A., W. Pfeiffer, and T. Schwartz. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. Proceedings of the Gateway Computing Environments Workshop (GCE), New Orleans, LA

Mills, N.J., Gutierrez, A.P., 1996. Prospective modelling in biological control: an analysis of the dynamics of heteronomous hyperparasitism in a contton-whitefly-parasitoid system. J. Appl. Ecol. 33(6), 1379–1394. http://www.jstor.org/stable/2404778

Morin, H. 2015. Winter Moth (Operophtera brumata L.) Natural Enemy Diversity and Abundance in Infested Areas in Midcoast Maine, The University of Maine.

Morris, R. F. 1965. Contemporaneous Mortality Factors in Population Dynamics. The Canadian Entomologist 97:1173-1184.

Morse, H. E., and B. B. Normark. 2006. A molecular phylogenetic study of armored scale insects (Hemiptera: Diaspididae). Systematic Entomology 31:338-349.

Murdoch, W. W. 1969. Switching in General Predators: Experiments on Predator Specificity and Stability of Prey Populations. Ecological Monographs 39:335-339.

Murdoch, W. W., J. Chesson, and P. L. Chesson. 1985. Biological-Control in Theory and Practice. American Naturalist 125:344-366.

Murillo, R., M. S. Hussey, and R. D. Possee. 2011. Evidence for covert baculovirus infections in a Spodoptera exigua laboratory culture. Journal of General Virology 92:1061-1070.

Murphy, T. C., J. R. Gould, R. G. Van Driesche, and J. S. Elkinton. 2018. Interactions between woodpecker attack and parasitism by introduced parasitoids of the emerald ash borer. Biological Control 122:109-117.

Myers, J. H. 2018. Population cycles: generalities, exceptions and remaining mysteries. P R Soc B 285(1875)doi: 10.1098/rspb.2017.2841

Myers, J. H., and J. S. Cory. 2013. Population Cycles in Forest Lepidoptera Revisited. Pages 565-592 in D. J. Futuyma, editor. Annual Review of Ecology, Evolution, and Systematics, Vol 44.

Necols, J. R., W. C. Kauffman, and P. W. Schaefer. 1992. Significane of host-specificity in classical biological control. Selection criteria and ecological consequences of importing natural enemies:41-52.

Neilson, M. M. 1965. Effects of a cytoplasmic polyhedrosis on adult Lepidoptera. Journal of Invertebrate Pathology 7:306-314.

Nofemela, R.S., 2013. The effect of obligate hyperparasitoids on biological control: Differential vulnerability of primary parasitoids to hyperparasitism can mitigate trophic cascades. Biological Control. 65(2): 218-224. https://doi.org/10.1016/j.biocontrol.2013.02.003

O'Donnell, K. M. 2015. The relationship between the winter moth (Operophtera brumata) and its host plant in coastal Maine. Masters Thesis, The University of Maine.

Oaten, A., and W. W. Murdoch. 1975. Switching, Functional Response, and Stability in Predator-Prey Systems. The American Naturalist 109(967):299-318. doi: 10.1086/282999

Pearsall, I.A., Walde, S.J., 1994. Parasitism and predation as agents of mortality of winter moth populations in neglected apple orchards in Nova Scotia. Ecol. Entomol. 19(2),190–198. http://dx.doi.org/10.1111/j.1365-2311.1994.tb00409.x

Pearson, D. E., Y. K. Ortega, O. Eren, and J. L. Hierro. 2018. Community Assembly Theory as a Framework for Biological Invasions. Trends Ecol Evol 33(5):313-325. doi: 10.1016/j.tree.2018.03.002

Pepi, A. A., H. J. Broadley, and J. S. Elkinton. 2016. Density-dependent effects of larval dispersal mediated by host plant quality on populations of an invasive insect. Oecologia 182:499-509.

Pepi, A. A., H. J. Broadley, and J. S. Elkinton. 2017. Erratum to: Density-dependent effects of larval dispersal mediated by host plant quality on populations of an invasive insect (vol 182, pg 499, 2016). Oecologia 185:533-535.

Pepi, A. A., O. P. L. Vindstad, M. Ek, and J. U. Jepsen. 2017. Elevationally biased avian predation as a contributor to the spatial distribution of geometrid moth outbreaks in sub-arctic mountain birch forest. Ecological Entomology 42:430-438.

Pivnick, K. A., D. L. Barton, J. G. Millar, and E. W. Underhill. 1988. Improved Pheromone Trap Exclusion of the Bruce Spanworm Operophtera bruceata (Hulst) (Lepidoptera, Geometridae) When Monitoring Winter Moth Operophtera brumata (L) Populations. Canadian Entomologist 120:389-396.

Ptaszyńska, A. A. 2014. The validation of microscopic techniques for identification and differentiation of Microsporidia. European Journal of Medical Technologies 1:1-5.

Quicke, D.L.J. 2015. The Braconid and Ichneumonid Parastoids Wasps: Biology, Systematics, Evolution, and Ecology. John Wilely and Sons, Ltd. West Sussex, U.K.

Quicke DLJ, Laurenne NM, Fitton MG, Broad GR (2009) A thousand and one wasps: a 28S rDNA and morphological phylogeny of the Ichneumonidae (Insecta: Hymenoptera) with an investigation into alignment parameter space and elision Journal of Natural History 43:1305-1421 doi:10.1080/00222930902807783

Rambaut, A. 2014. FigTree Tree Figure Drawing Tool.

Raymond, B., A. Vanbergen, A. Watt, S. E. Hartley, J. S. Cory, and R. S. Hails. 2002. Escape from pupal predation as a potential cause of outbreaks of the winter moth, Operophtera brumata. Oikos 98:219-228.

Raymond, B., and R. S. Hails. 2007. Variation in plant resource quality and the transmission and fitness of the winter moth, Operophtera brumata, nucleopolyhedrovirus. Biological Control 41:237-245.

Raymond, B., S. Hartley, J. S. Cory, and R. S. Hails. 2005. The role of food plant and pathogeninduced behaviour in the persistence of a nucleopolyhedrovirus. Journal of Invertebrate Pathology 88:47-57.

RCoreTeam. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Richardson, D. M., and P. Pysek. 2006. Plant invasions: merging the concepts of species invasiveness and community invasibility. Progress in Physical Geography 30(3):409-431. doi: 10.1191/0309133306pp490pr

Rodriguez V.A., Belaich M.N., Gomez D.L.M., Sciocco-Cap A., Ghiringhelli P.D., Identification of nucleopolyhedrovirus that infect Nymphalid butterflies Agraulis vanilla and Dione juno, J. Invertebr. Pathol. 106, 2011, 255-262.

Rodriguez, V. A., M. N. Belaich, D. L. M. Gomez, A. Sciocco-Cap, and P. D. Ghiringhelli. 2011. Identification of nucleopolyhedrovirus that infect Nymphalid butterflies Agraulis vanillae and Dione juno. Journal of Invertebrate Pathology 106:255-262.

Roelofs, W. L., A. S. Hill, C. E. Linn, J. Meinwald, S. C. Jain, H. J. Herbert, and R. F. Smith. 1982. Sex-pheromone of the winter moth, a geometrid with unusually low-temperature pre-copulatory responses. Science 217:657-659.

Roland, J. 1986. Parasitism of winter moth in British Columbia during buildup of its parasitoid Cyzenis albicans - Attack rate on oak vs. apple. Journal of Animal Ecology 55:215-234.

Roland, J., 1988. Decline in winter moth populations in North America: direct versus indirect effect of introduced parasites. J. Anim. Ecol. 57(2), 523–531. http://www.jstor.org/stable/4922

Roland, J. 1990. Interaction of parasitism and predation in the decline of winter moth in Canada. Pages 289-301 in A. Watt, S. R. Leather, and A. F. Hunter, editors. Population Dynamics of Forest Insects. Intercept Ltd, Andover, Hampshire, UK.

Roland, J. 1994. After the decline: What maintains low winter moth density after successful biological-control. Journal of Animal Ecology 63:392-398.

Roland, J., and D. G. Embree. 1995. Biological-control of the winter moth. Annual Review of Entomology 40:475-492.

Roland, J., and J. H. Myers. 1987. Improved Insect Performance from Host-Plant Defoliation - Winter Moth on Oak and Apple. Ecological Entomology 12:409-414.

Rose, A. H., and O. H. Lindquist. 1997. Insects of eastern hardwood trees; Revised 1997. Department of the Environment, Canadian Forestry Service, Minister of the Environment, Government of Canada, Ottawa, Ontario, Canada.

Royama T., 1981. Evaluation of mortality factors in insect life table analysis. Ecol. Monogr. 51(4), 495–505. http://www.jstor.org/stable/2937326

RstudioTeam. 2015. RStudio: Integrated Development Environment for R. RStudio, Inc., Boston, MA.

Sakai, A. K., F. W. Allendorf, J. S. Holt, D. M. Lodge, J. Molofsky, K. A. With, S. Baughman, R. J. Cabin, J. E. Cohen, N. C. Ellstrand, D. E. McCauley, P. O'Neil, I. M. Parker, J. N. Thompson, and S. G. Weller. 2001. The population biology of invasive species. Annual Review of Ecology and Systematics 32:305-332. doi: 10.1146/annurev.ecolsys.32.081501.114037

Schauber, E. M., M. J. Connors, B. J. Goodwin, C. G. Jones, and R. S. Ostfeld. 2009. Quantifying a dynamic risk landscape: heterogeneous predator activity and implications for prey persistence. Ecology 90:240-251.

Schaefer, P. W., R. W. Fuester, R. J. Chianese, L. D. Rhoads, and R. B. Tichenor. 1989. Introduction and North-Amherican establishment of Coccygomimus disparis (Hymenoptera, Ichneumonidae), a polyphagus pupal parasite of Lepidoptera, including gypsy moth. Environ Entomol 18(6):1117-1125.

Schenk, D., and S. Bacher. 2002. Functional response of a generalist insect predator to one of its prey species in the field. Journal of Animal Ecology 71(3):524-531. doi: 10.1046/j.1365-2656.2002.00620.x

Schonrogge, K., G. N. Stone, and M. J. Crawley. 1995. Spatial and temporal variation in guild structure - parasitoids and inquilines of Andricus quercuscalicis (Hymenoptera, Cynipidae) in its native and alien ranges. Oikos 72(1):51-60. doi: 10.2307/3546037

Schooler, S.S., De Barro, P., Ives, A.R., 2011. The potential for hyperparasitism to compromise biological control: why don't hyperparasitoids drive their primary parasitoid hosts extinct? Biol. Control 58(3), 167–173. https://doi.org/10.1016/j.biocontrol.2011.05.018

Sechser, B., 1970. Der Parasitenkomplex des Kleinen Frostspanners (Operophtera brumata L.) (Lep., Geometridae) unter besonderer Berücksichtigung der Kokonparasiten. I. Teil. Z. Angew. Entomol. 66, 1–35.

Shea, K., and P. Chesson. 2002. Community ecology theory as a framework for biological invasions. Trends Ecol Evol 17(4):170-176. doi: 10.1016/s0169-5347(02)02495-3

Simmons, M. J., T. D. Lee, M. J. Ducey, J. S. Elkinton, G. H. Boettner, and K. J. Dodds. 2014. Effects of Invasive Winter Moth Defoliation on Tree Radial Growth in Eastern Massachusetts, USA. Insects 5:301-318.

Smirnoff, W. A. 1964. Nucleopolyhedrosis of Operophtera bruceata (Hulst) (Lepidoptera: Geometridae). Journal of Insect Pathology 6:384-386.

Sokolova, Y. Y., I. M. Sokolv, and J. R. Fuxa. 2004. Identification of Microsporidia infections in nature: light microscopy or PCR? Protistology 3:273-281.

Solter, L. F., J. J. Becnel, and D. H. Oi. 2012. Microsporidian Entomopathogens. Pages 221-263 Insect Pathology. Academic Press, San Diego, CA.

Starrett, J., and M. Hedin. 2007. Multilocus genealogies reveal multiple cryptic species and biogeographical complexity in the California turret spider Antrodiaetus riversi (Mygalomorphae, Antrodiaetidae). Mol Ecol 16:583-604.

Strauss, A., A. White, and M. Boots. 2012. Invading with biological weapons: the importance of disease-mediated invasions. Functional Ecology 26:1249-1261.

Sullivan, D. J. 1987. Insect Hyperparasitism. Annual Review of Entomology 32:49-70.

Sullivan, D.J., Völkl, W., 1999. Hyperparasitism: multitrophic ecology and behavior. Annu. Rev. Entomol. 44(1), 291–315. https://doi.org/10.1146/annurev.ento.44.1.291

Swofford, D. L. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Tanhuanpaa, M., K. Ruohomaki, P. Kaitaniemi, and T. Klemola. 1999. Different impact of pupal predation on populations of Epirrita autumnata (Lepidoptera; Geometridae) within and outside the outbreak range. Journal of Animal Ecology 68:562-570.

Tenow, O., A. C. Nilssen, H. Bylund, R. Pettersson, A. Battisti, U. Bohn, F. Caroulle, C. Ciornei, G. Csoka, H. Delb, W. De Prins, M. Glavendekic, Y. I. Gninenko, B. Hrasovec, D. Matosevic, V. Meshkova, L. Moraal, C. Netoiu, J. Pajares, V. Rubtsov, R. Tomescu, and I. Utkina. 2013. Geometrid outbreak waves travel across Europe. Journal of Animal Ecology 82(1):84-95. doi: 10.1111/j.1365-2656.2012.02023.x

Townes, H., 1969. The genera of Ichneumonidae, Part 1. Mem. Am. Entomol. Inst. 11, i–ii+1–300.

Townes, H., 1970. The genera of Ichneumonidae, Part 2. Mem. Am. Entomol. Inst. 12, i–iv+1–537.

Townes, H. K. 1940. A revision of the Pimplini of Eastern North America (Hymenoptera, Ichneumonidae). Annals of the Entomological Society of America 33(2):283-323.

Townes, H.K., Townes, M., Walley, G.S., Townes, G., 1960. Ichneumon-flies of America north of Mexico: 2 Subfamily Ephialtinae, Xoridinae, Acaenitinae. US Natl. Mus. Bull. 216, 1–676.

Triplehorn, C. A., and N. F. Johnson. 2005. Borror and DeLong's Introduction tot he Study of Insects. 7th ed. Brooks/Cole, a division of Thomson Learning, Inc. , Belmont, CA.

Troubridge, J. T., and S. M. Fitzpatrick. 1993. A revision of the North American Operophtera (Lepidoptera, Geometridae). Canadian Entomologist 125:379-397.

Van Driesche, R. G. 1983. Meaning of "Percent Parasitism" in Studies of Insect Parasitoids. Environ Entomol 12(6):1611–1622.

Van Driesche, R., Hoddle, M., Center, T., 2008. Control of Pests and Weeds by Natural Enemies: An Introduction to Biological Control. Blackwell Publishing, Malden, MA.

Van Driesche, R. G., and R. Reardon. 2014. The use of classical biological control to preserve forests in North America. United States Department of Agriculture, Forest Service, Morgantown, West Virginia.

van Oers, M. M., E. A. Herniou, M. Usmany, G. J. Messelink, and J. M. Vlak. 2004. Identification and characterization of a DNA photolyase-containing baculovirus from Chrysodeixis chalcites. Virology 330:460-470.

Varley, G. C., and G. R. Gradwell. 1963. Predatory insects as density dependent mortality factors, Proceedings of the 16th International Congress of Zoology, Washington, 20-27 August 1963 No. 1, Washington, D.C. p. 240.

Varley, G.C., Gradwell, G.R., 1968. Population Models for the Winter Moth. In: T. R. E. Southwood Ed., Insect Abundance, Symposium, Royal Entomological Society, London.

Varley, G.C., and Gradwell, G.R., 1970. Recent advances in insect population dynamics. Annual Review of Entomology 15:1-24.

Varley, G. C., G. R. Gradwell, and M. P. Hassell. 1973. Insect population ecology: an analytical approach. University of California Press.

Vindstad, O. P. L., S. B. Hagen, J. U. Jepsen, L. Kapari, T. Schott, and R. A. Ims. 2011. Phenological diversity in the interactions between winter moth (Operophtera brumata) larvae and parasitoid wasps in sub-arctic mountain birch forest. Bulletin of Entomological Research 101(6):705-714. doi: Doi 10.1017/S0007485311000277

Vindstad, O. P. L., T. Schott, S. B. Hagen, J. U. Jepsen, L. Kapari, and R. A. Ims. 2013. How rapidly do invasive birch forest geometrids recruit larval parasitoids? Insights from comparison with a sympatric native geometrid. Biological Invasions 15(7):1573-1589. doi: 10.1007/s10530-012-0393-8

Waage, J., and D. Greathead. 1985. Insect Parasitoids. Academic Press Limited, San Diego, CA.

Watt, A. D., and I. P. Woiwod. 1999. The effect of phenological asynchrony on population dynamics: analysis of fluctuations of British macrolepidoptera. Oikos 87:411-416.

Whited, B. M., 2007. The population ecology of winter moth (Operophtera brumata) in eastern Massachusetts. M.S. Thesis in Organismic and Evolutionary Biology. University of Massachusetts, Amherst.

Whiting, M. F., J. C. Carpenter, Q. D. Wheeler, and W. C. Wheeler. 1997. The strepsiptera problem: Phylogeny of the holometabolous insect orders inferred from 18S and 28S ribosomal DNA sequences and morphology. Systematic Biology 46(1):1-68.

Wickham, H. 2009. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.

Wigley, P. J. 1976. The epizootics of a nuclear polyhedrosis virus disease of winter moth, Operophtera brumata, L., at Wistman's Wood, Dartmoor. Doctoral Dissertation, Oxford University.

Wylie, H. G. 1960. Insect parasites of the winter moth, Operophtera brumata (L.) (Lepidoptera: Geometridae) in western Europe. Entomophaga 5:111-129.

World Ichneumonoidea 2011. Taxonomy, Biology, Morphology and Distribution (2012) Home of Ichneumonoidea. http://www.taxapad.com Accessed accessed 17 August 2017

Yu, D. S. K., C. van Achterberg, and K. Horstmann. 2016. World Ichneumonoidea 2015 Taxapad 2016. Home of Ichneumonoidea, Nepean, Ontario, Canada.

Yu, D. S. K., C. van Achterberg, and K. Horstmann. 2012. World Ichneumonoidea 2011. Taxonomy, Biology, Morphology and Distribution Taxapad. Home of Ichneumonoidea, Nepean, Ontario, Canada.

Zappala, L., U. Bernardo, A. Biondi, A. Cocco, S. Deliperi, G. Delrio, M. Giorgini, P. Pedata, C. Rapisarda, G. T. Garzia, and G. Siscaro. 2012. Recruitment of native parasitoids by the exotic pest Tuta absoluta in Southern Italy. Bulletin of Insectology 65(1):51-61.

Zuur, A. F., J. M. Hilbe, and E. N. Ieno. 2013. A Beginner's Guide to GLM and GLMM with R. Highland Statistics Ltd., Newburgh, United Kingdom.