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.

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**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 (arthropods and small mammals) and hyperparasitism by three ichneumonid genera: *Phygeadeum*, *Pimpla*, and *Gelis*. Retrievial of sentinel puparia revealed high mortality due to generalist ground predators and hyperparasitism by three ichneumonid genera: *Phygeadeum*, *Pimpla*, and *Gelis*. These predators and hyperparasitoids are native generalist species and while their presence is high, they seem to have little influence on the biological control efforts of winter moth (*Operophtera brumata*) in the northeast U.S.

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1. Introduction

High mortality from disease, predation, and hyperparasitism has the potential to interfere with introduced parasitoids in biological control programs 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 tachnid 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 microscopic 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 Sisiojevic, 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 Sisiojevic, 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. Hyperparasitism 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. Materials and methods

2.1. 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. Pupae were pre-sifted through a screen (with 3 mm × 3 mm openings). This allows for later removing pupae 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 paralysis. 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 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 mixed-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 × 28 cm length × 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 1) 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 August to 22 September 2016.
September until 1 November 2016.

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 0.5 m 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 (Per- cival) in constant dark until analysis.

2.2. 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, 100 mm × 15 mm poly styrene 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. 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 (Fig. S1A), and evidence of teeth or claw marks left in the wax (Fig. S1B). Parasitism was inferred for any puparia with distinct wasp emergence holes (Fig. S1C) 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 and parasitism. For the purposes of this study, we excluded mortality due to unknown causes, including potentially diseased, mouldy, 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 take into account 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_t) by subtracting the proportion dying from one (1 − M). 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_t)^{1/42}]^{42}). This conversion enabled us to compare mortalities across years and months in the face of small differences in the 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} × 2nd deployment, S_{42} × 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_{W} = 1 − S_{W}).

2.4. 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.

| Table 1 | Locations and coordinates for study sites where sentinel puparia of Czernik albicans were deployed in 2015 and 2016. An X 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, -70.841617 | X | X |
| Wompack 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 |
2.5. 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 daumenorum 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 contemptor (Müller), Pimpla dispersis (Viereck), Pimpla turionellae (L.), and Pimpla hesperus (Townes) in the USNM were also examined. Pimpla contemptor, P. dispersis, 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 Campsilura concinnata (Meigen) (Sharov and Izhevskiy, 1987). Pimpla hesperus has been reported as a parasite of winter moth and its native congener Brue spanworm Hulst (Operophthera bruceata) in British Columbia (Humble, 1985). Lastly, four species of Gelis (i.e., Gelis areator Panzer, Gelis acarorum L., Gelis dissectens Förster, and Gelis rufogaster Thunberg) have been reported as associated with winter moth in Europe (Secher, 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.

2.6. 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.

2.6.1. DNA extraction, amplification, and sequencing

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 100μl Buffer AE instead of 200μl (Step 7). The DNA extractions were stored at ~20°C. A master mix was prepared using the following amounts per sample: 17.3μl nuclease free water, 0.5μl dNTPs, 5μl GoTaq Buffer, 0.2μl GoTaq, and 0.5μl 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.

2.6.2. Phylogenetic analysis

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 Hubble (1985) reported Phygadeuon sp. from undetermined Cyzenis (i.e., either C. albicans or Cyzenis pululla, Townsend), Mastrus sp. from ichneumonid primary parasites of winter moth, and Buathra dorsicarina (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 loci. 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 (Kearey 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).

3. Results

3.1. Pupal mortality

Cyzenis albicans experienced consistently high mortality throughout the study (Fig. 1). Cumulative mortality was not significantly different between years (df = 21, χ² = 0.001, p = 0.98), but there was a significant difference (df = 21, χ² = 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 (χ² = 0.90, p = 0.34). Mortality due to hyperparasitism was significantly lower than predation (χ² = 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 = 12, χ² = 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 (Fig. S2). Sentinel puparia deployed earlier in the season (July-August) had the highest percent hyperparasitism, but there was no significant difference (df = 21, χ² = 2.5, p = 0.29) in hyperparasitism among deployment periods (Fig. 2). Neither winter moth pupae nor C. albicans pupal densities were significant predictors of cumulative total mortality (df = 1,8, χ² = 0.0001, p = 0.99 and df = 1,9, χ² = 0.018, p = 0.89, respectively) or hyperparasitism (Fig. 3, df = 1,8, χ² = 0.62, p = 0.43 and df = 1,9,
3.2. Hyperparasitoid identification by morphology

Three ichneumonid wasp genera were identified using morphological features: Phygadeuon (Cryptinae), Pimpla (Pimplinae), and Gelis (Cryptinae) (Fig. 5A–C). 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 Colombia (Humble, 1985). However, other species of *Phygadeuon* 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 *Coccogygimimus hesperus*) was reported by Humble (1985) as a parasitoid of winter moth and Bruce spanworm in British Colombia 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. reator* 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. disceden*, 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.

3.3. 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

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**Fig. 1.** 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, \( \alpha = 0.05 \)).

**Fig. 2.** 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, \( \alpha > 0.05 \)).
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 S1, Fig. 4). 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 (Fig. 4). These were all matches of between 98.8% and 100% identity to the 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 Pimpla wasps (the ﬁve individuals that most closely matched sequences in GenBank and BOLD were labeled Pimplinae sp./Pimpla sp. versus the three sequences that most closely matched sequences labeled P. aequalis) 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 pinpmine wasps in the winter moth system.

3.4. 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 2). 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.

4. 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

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**Fig. 4.** *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 *Phygadeuon* sp. sequences generated from this study are bolded and fit with representative sequences from NCBI GenBank and BOLD.
rearing and biological study is necessary to elucidate the role of *Gelis* species in the winter moth food web in the northeastern U.S. Unidifferentiated 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.

Cumulative 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; Embree and Roland, 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* is unrelated to either winter moth or primary parasitoid densities (Fig. 3). The hyperparasitoids discovered – *Phygadeuon* sp., *Pimpla* sp. 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; pipilne 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 (Fig. 4) 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 S1) 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; Ivies 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.

### Table 2

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

<table>
<thead>
<tr>
<th>ID</th>
<th>2015 Adult (n = 33)</th>
<th>2015 Larvae (n = 68)</th>
<th>2016 Adult (n = 19)</th>
<th>2016 Larvae (n = 61)</th>
<th>Total (n = 101)</th>
<th>Total (n = 80)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Phygadeuon</em></td>
<td>0.70</td>
<td>0.63</td>
<td>0.65</td>
<td>0.84</td>
<td>0.66</td>
<td>0.70</td>
</tr>
<tr>
<td><em>Pimpla</em></td>
<td>0.12</td>
<td>0.10</td>
<td>0.11</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td><em>Gelis</em></td>
<td>0.09</td>
<td>0.00</td>
<td>0.00</td>
<td>0.03</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Unknown</td>
<td>0.09</td>
<td>0.27</td>
<td>0.21</td>
<td>0.16</td>
<td>0.34</td>
<td>0.30</td>
</tr>
</tbody>
</table>

5. Conclusions

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 north-eastern 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.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocontrol.2018.01.011.

References


