ORIGINAL ARTICLE

Assessment of pupal mortality in *Hypena opulenta*: An obstacle for establishment of a classical biological control agent against invasive swallow-worts

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[Correction added on 12 MAR 2022, after the first online publication: Copyright line has been updated.]

Abstract

1. Common obstacles for establishment of newly-introduced biological control agents include climate and the activity of native antagonists. In temperate climates, these obstacles can disproportionately affect overwintering life-stages because they are exposed to low winter temperatures, and may rely on passive defence from predators.

2. We conducted a series of field exposure experiments with predator-exclusion treatments, in Ontario, Canada, to identify mortality factors for the pupae of *Hypena opulenta*, a biological control agent for invasive swallow-worts in North America.

3. During two winters, predation rates in containers with large holes, that enabled predation, were relatively low (mean: 23.75%) but non-predation mortality in closed containers was high (mean: 66.25%), particularly during the colder of the two winters (87.5% vs. 52.5%).

4. During the summer, non-predation mortality in closed containers was low (mean: 7.5%) but predation rates in containers with large holes were higher than during the winter (mean: 53.33%), increasing as the summer progressed. Predation in containers with large holes was 70% during late summer, compared with 25% during the spring.

5. Across all seasons, pupal predation was dominated by large non-arthropod predators.

6. *Hypena opulenta* can complete 2 generations per year. Photoperiods that induce diapause occur earlier in the introduced range than in the native range, however, and *H. opulenta* individuals in parts of the introduced range are likely to enter diapause early after a single generation. Our results highlight additional vulnerabilities encountered by such individuals, and can contribute to models predicting population dynamics of *H. opulenta* across its introduced range.

KEYWORDS
biological control, cold-tolerance, *Hypena opulenta*, predation, pupae, swallow-wort, *Vincetoxicum rossicum*

INTRODUCTION

Insects introduced as classical biological control agents experience a host of factors that can influence their chances of success. The most common obstacles for establishment are climatic mismatches, followed by the activity of native antagonists (Stiling, 1993). Such obstacles can disproportionately affect overwintering life-stages in temperate regions, both because they are exposed to low winter

temperatures, and because the period of immobility makes them vulnerable to predation and parasitism. We conducted a series of field exposure experiments with predator exclusion treatments, in Ontario, Canada, to identify the major mortality factors for the pupae of *Hypena opulenta* (Christoph) (Lepidoptera: Erebidae), a newly-established biological control agent for invasive swallow-worts in North America (Bourchier et al., 2019).

Understanding the effects of winter temperatures on the survival of introduced biological control agents can help predict their population dynamics, as well as inform the release program to maximise their chances of success (Byrne et al., 2002; Guo et al., 2012; Uyi et al., 2017). For example, Duan et al. (2020) studied cold-induced mortality of *Spathius galinae* (Hymenoptera: Braconidae), a parasitoid biological control agent for emerald ash borer in North America. Mortality of overwintering *S. galinae* larvae varied from <2% in Connecticut, to between 18%–50% in Michigan. Although the population of *S. galinae* released in North America was collected from a similar latitude to Michigan (Duan et al., 2012), the collection site (Vladivostok, Russia) has a coastal climate, similar to Connecticut. The continental climate of Michigan, including lower winter temperatures, has clearly affected the winter survival of *S. galinae* in the region. Winter temperatures in southern Ontario, Canada, are slightly colder than in Donjetsk, Ukraine, where *H. opulenta* was originally collected. Between 2014 and 2020, following the first release and establishment of *H. opulenta* in Ottawa Canada (Bourchier et al., 2019), minimum winter air temperatures in Ottawa were annually between 5 and 16°C colder than in Donetsk, Ukraine (BioSIM - Régnière et al., 2017). We exposed *H. opulenta* to two Ontario winters in order to determine whether the severity of winter temperatures affected pupal survival.

The biotic resistance hypothesis suggests that introduced species often fail to invade communities because their establishment and spread is hindered by interactions with native species (Elton, 1958). Indeed, biotic resistance has been demonstrated as a major factor that can limit the success of biological control agents (Dray Jr et al., 2001; Wiebe & Obrycki, 2004), accounting for approximately 20.3% of cases in which agents fail to establish (Goeden & Louda, 1976). In Australia and New Zealand, for example, the cinnabar moth, *Tyria jacobaeae* L. (Lepidoptera: Erebidae), has been introduced several times for the control of tansy ragwort, *Senecio jacobaea* L. (Asteraceae). Early releases in Australia were hampered by predation by the native mecopteran, *Harpobittacus nigriceps* L. (Mecoptera: Bittacidae) (Currie & Fyfe, 1938), as well as extensive predation by native ants (Schmidl, 1972). In New Zealand, releases of *T. jacobaeae* were decimated by birds (Dempster, 1971). Despite the effects of wide-ranging antagonists on the success of biological control agents, relatively few programs seek to identify the natural enemies experimentally (Crider, 2011; Goeden & Louda, 1976; Stiling, 1993). Such experiments can be particularly difficult to conduct in cases where the agent is not well established. Although there are established populations of *H. opulenta* in Canada, population densities appear low, and rapid dispersal behaviour makes larvae difficult to find (Bourchier et al., 2019). By focusing on an immobile life-stage, we sought to identify at least some of the important mortality factors that may be contributing to

the relatively slow population growth and spread of *H. opulenta* in Canada.

Hypena opulenta was initially collected from Ukraine (Weed & Casagrande, 2010), and its native range extends east to Afghanistan and south into Iran and Syria (Fibiger et al., 2010). Releases of the agent began in Canada during 2013 (Young & Weed, 2014) and in the U.S. during 2017 (Tewksbury, unpublished data). Adult *H. opulenta* emerge in the spring and females lay around 400 eggs, predominantly on the underside of leaves (Weed & Casagrande, 2010). Larvae develop through four or five instars before pupating in the soil, or occasionally attached to leaves. In Canada, the moths undergo one or two generations per year (Hazlehurst et al., 2012; Jones et al., 2020). The final summer generation then overwinter as pupae in the soil. Knowledge of the moth's life-cycle allowed us to experimentally expose pupae to predators and abiotic conditions at relevant times of the year. In North America, *H. opulenta* has been observed entering diapause early in the season, after only a single generation. Photoperiod is the main factor that drives diapause in *H. opulenta* (Weed & Casagrande, 2010) and the threshold day-length that stimulates diapause (between 15 and 16 h) occurs earlier in the season across the introduced range than across the moth's native range in Donetsk (Jones et al., 2020). Indeed, it has been predicted that *H. opulenta* is unlikely to undergo a second summer generation at the southern extreme of its introduced range (Harrisburg, Pennsylvania, USA) (Jones et al., 2020). The limitation of *H. opulenta* populations to a single summer generation is likely a major factor slowing the establishment of the agent in parts of North America (Jones et al., 2020). Not only does univoltinism reduce the scope for population growth in *H. opulenta*, but the associated occurrence of early diapause exposes pupae to different and enhanced mortality factors. We aimed to explore the mortality factors, such as increased predation risk, associated with early and extended diapause in *H. opulenta*.

We conducted two winter pupal exposures ending in the spring of 2019 and 2020. In addition, we carried out three pupal exposures at different times during the summer of 2019. In each case, a set of exclusion treatments were used to broadly identify the type of predation most affecting *H. opulenta* pupae. We aimed to quantify winter mortality in *H. opulenta* and determine how sensitive this might be to variable winter temperatures. We also set out to understand when *H. opulenta* pupae were at most risk from predation and relate this vulnerability to populations that undergo one or two generations per year.

METHODS

Insect production

All *H. opulenta* used in this study were from laboratory-reared colonies maintained at the University of Toronto, ON, and Agriculture and Agri-Food Canada in Lethbridge, AB. The populations were descendants of insects originally collected in 2006 and 2012 from Donetsk, Ukraine by CABI-Switzerland (Weed & Casagrande, 2010). In the

University of Toronto colony, larvae were reared individually in clear plastic containers in order to standardise the rearing conditions for each pupa used in the experiments. Larvae were held at 20–25°C, 40%–60% relative humidity (RH), and a 16:8 h (L:D) diel period. Larvae were fed on individual *Vincetoxicum rossicum* leaves collected from the same field site in Uxbridge, Ontario, where the exposure experiments were carried out. Leaves were replaced every 48 h or more frequently if necessary. For diapause induction, eggs were moved to a growth chamber with the same conditions as described above but with a 12:12 h (L:D) diel period in which they were reared as above until pupation. Approximately 3 weeks after pupation, pupae were moved to plastic containers that had a bed of moist cotton or vermiculite (0–3 mm grain), and they were gradually cooled down over 2 weeks before they were stored in a continuously dark growth chamber at 2–5°C for 3–5 months.

Pupal field exposures

Partial life-table experiments were conducted at a field site in Uxbridge, Ontario, Canada (44.088681, −79.106804) using a complete-block design. The experiment aimed to determine the major mortality factors affecting overwintering *H. opulenta* pupae, as well as for pupae occurring at various times between April to October. Each experimental block consisted of three treatment cages constructed using a PVC pipe, 17 cm in length and 15 cm in diameter. For treatment one, two 3 cm diameter holes were drilled in opposite sides of the cylinder and a fine (<1 mm gauge) plastic mesh glued over the holes to allow for air flow, but ensuring all predators and parasitoids were excluded from the treatment. For treatment two, 31 holes (8 mm in diameter) were drilled around the circumference of the cylinder in three parallel lines, the lowest of which was 4 cm from the base. These holes were left open to allow access to the majority of insect predators and parasitoids. For treatment three, two square holes (4 cm by 4 cm) were cut in opposite sides of the cylinder, 4 cm from the base. These holes were designed to allow access to larger predators including rodents and small birds.

Three cylindrical cages for each experimental block were placed in an equilateral triangle formation ∼25 cm apart from each other. Cylinders were hammered into the soil using a rubber mallet to a depth of 4 cm, and the cages were completed by placing a solid plastic lid over the cylinders. Ten experimental blocks were constructed for a total of 30 cages. An additional experimental block was constructed with a LogTag temperature sensor (LogTag USA, Union, New Jersey, USA) in each cage to determine whether and how each cage type affected temperature. A fourth LogTag sensor was buried 1 cm below the soil surface outside of the experimental cages to give a baseline soil temperature and to confirm that the cages themselves did not greatly alter conditions for the pupae. All LogTag sensors were programmed to take temperature readings every hour throughout the study. The 10 experimental blocks were all placed in semi-shaded locations a minimum of 3 m apart from each other, and the same 10 block-sites were used for every exposure. Four *H. opulenta* pupae

were placed in each cylinder for a total of 120 pupae, and a thin layer of soil and dried leaves was placed over the pupae to mimic natural pupation conditions.

For the two winter experiments, pupae were placed in the field site after the first frost on 26 November and 28 October for years one and two respectively. Experimental start dates were chosen to minimise the shock of moving the pupae from storage conditions (5°C) into the field, while minimising exposure to warm autumn conditions. Remaining pupae were then recovered from cages after snow cover had receded on 9 April and 12 April for years one and two respectively. Pupae were returned to the laboratory and placed individually in small plastic pots with a thin layer of damp cotton wool in the base. Pupae were stored at 25°C and monitored for adult emergence. For each experimental cage, we recorded the number of pupae removed by predators, the number of pupae that emerged as adults, and the number of pupae that avoided predation, but did not emerge as adults. Pupae that failed to emerge were dissected to give some indication of cause of death. Pupae with visible fungus, or with liquefied contents, were presumed to have succumbed to some form of pathogenic disease; that is, a fungus or microorganism. Pupae containing partially developed adults were recorded as having died from freezing or non-freezing cold stress.

For the three experiments between April and October, a metal cage frame (183 × 183 × 183 cm) was constructed over each experimental block (BioQuip Products, Rancho Dominguez, CA, USA). The bottom 1 m of each cage was left open to allow access to predators, but the top and upper sides of the cage were lined with a thick amber coloured mesh (Lumite®) to provide shade and prevent experimental cages from overheating. The initial trial covered the spring period to assess predation rates experienced by *H. opulenta* pupae following overwintering and prior to emergence, in mid-summer to assess mortality experienced by *H. opulenta* pupating between summer generations, and in late-summer to assess predation risk associated with early diapause that has been observed in many North American populations. The three experiments were set up on 26 April, 17 July, and 23 August respectively. In each case, 120 pupae were used, as in the winter experiments, and pupae were monitored twice per week. Predation was assumed to have occurred when pupae were absent, or if visible pupal remains were present. Adult emergence was recorded when adult moths were present and/or when empty pupal cases were found. Field monitoring continued in each experiment until adult emergence within the cylinders had stopped for a period of 1 week. The monitoring period lasted for a period of 84, 36, and 72 days for the spring, mid-summer, and late-summer experiments respectively.

Statistical analyses

For the winter exposures, predation, adult emergence, and non-predation mortality were compared between years using generalised linear models. In the case of predation, only the open (large-hole) containers were included in the analysis, as these were the containers in which all predators had access to pupae. In the case of adult

emergence, all treatment containers were included in the analysis. In the case of non-predation mortality, only the closed (control) containers were included in the analysis, as these were the only pupae guaranteed to be unaffected by predators. The effects of the exclusion treatments on predation, adult emergence, and non-predation mortality were analysed for each year separately using Kruskal-Wallis tests. Post hoc testing was conducted using Dunn-Bonferroni comparisons.

For the three summer period exposures (spring, mid-summer, and late-summer), predation, adult emergence, and non-predation mortality were compared among exposures using Kruskal-Wallis tests, with Dunn-Bonferroni tests for pairwise comparisons. Only the relevant treatment containers were included in these analyses as described above for the winter exposures. In the case of predation, the three summer period exposures were analysed by comparing the number of individuals that had been removed by predators at the 5-week point of each exposure. This allowed us to control for the different exposure lengths of each experiment.

wThe effects of exclusion treatments on pupal predation, adult emergence, and non-predation mortality were then compared for each exposure separately using Kruskal-Wallis tests. Post hoc testing was conducted using Dunn-Bonferroni comparisons as above. All statistical analyses were conducted using SPSS version 27 (IBM, Armonk, USA), and an alpha value of 0.05 was used across all analyses.

RESULTS

Winter exposures

The treatment containers did not have any significant effect on the temperature. For example, during year one, average temperatures ranged from $-0.849°C$ in the containers with small-holes to $-1.082°C$ in the containers with large-holes while the average temperature measured in the soil outside of the experimental containers was $-0.952°C$. There were, however, differences in temperature between years one and two, with the average temperature in the soil being higher in year two ($1.84°C$) than in year one ($-0.95°C$). The minimum temperature experienced throughout the winter, in the soil, was also higher in year two ($-4.1°C$) than in year one ($-7.7°C$) (Figure 1).

Between year comparisons: Predation from open (large-hole) containers was higher in year two ($40\% \pm 14.04\%$) (mean $\pm$ SE) than in year one ($7.5\% \pm 3.81\%$) (N = 20, χ^2 = 5.54, df = 1, p = 0.019) (Figure 2a). Successful adult emergence, across all three treatments combined, was higher in year two (38.33 ± 5.18) than in year one ($10\% \pm 3.30\%$) (N = 60, χ^2 = 21.94, df = 1, p < 0.001) (Figure 2b). Death from non-predation causes, in closed (control) containers was higher in year one ($87.5\% \pm 6.71\%$) than in year two ($52.5\% \pm 6.92\%$) (N = 20, χ^2 = 14.62, df = 1, p < 0.001) (Figure 2c).

Treatment comparisons year 1: During the first winter exposure, the treatment containers had no effect on predation rate (Figure 2a),
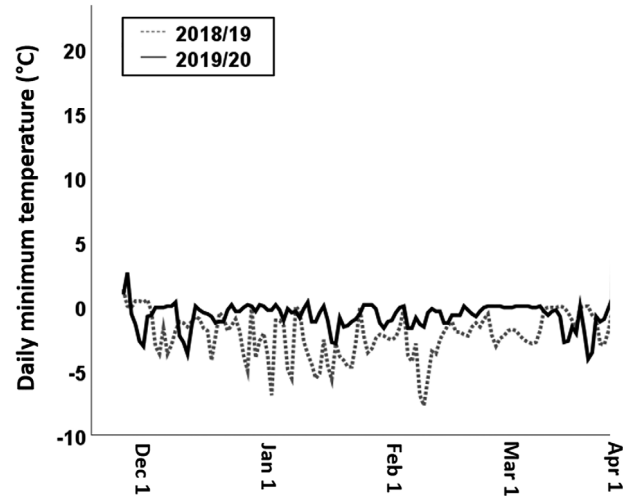

FIGURE 1 Daily minimum temperatures recorded throughout the winters of 2018/19 (winter 1) and 2019/20 (winter 2) in southern Ontario. Temperatures were recorded using a LogTag sensor buried 1 cm below the soil surface. Temperature readings were taken every hour throughout the experiments

successful adult emergence (Figure 2b), or death from non-predation causes (Figure 2c) (Table 1).

Treatment comparisons year 2: During the second winter exposure, predation was significantly higher in open (large-holes) containers ($40\% \pm 14.04\%$) compared with closed (control) containers (0 ± 0) (Figure 2a). Successful adult emergence (Figure 2b), and death from non-predation causes (Figure 2c) did not differ among treatments during the second winter exposure (Table 1).

Exploring non-predation winter mortality: In the first winter exposure, a total of 103 pupae died from non-predation causes, of which four (3.88%) were visibly infested with a white fungus. Dissections of the remaining pupae revealed that 69 (66.99%) contained liquefied remains, while the remaining 30 (29.12%) contained partially or apparently fully developed adults that had failed to eclose. In the second winter exposure, a total of 56 pupae died from non-predation causes, of which 19 (33.92%) were visibly infested with a white fungus. Dissections of the remaining pupae revealed that 32 (57.14%) contained liquefied remains, while the remaining 5 (8.92%) contained partially or apparently fully developed adults that had failed to eclose.

Summer exposures

Between season comparisons: Predation from open (large-hole) containers differed significantly among spring/summer seasons after 5 weeks of exposure (N = 30, t = 9.889, df = 2, p = 0.007). Predation was significantly higher during the mid-summer ($65\% \pm 9.27\%$) (N = 20, t = −12.80, df = 1, p = 0.003) and late-summer exposures ($67.5\% \pm 9.89\%$) (N = 20, t = −13.30, df = 1, p = 0.002) compared with the spring exposure ($12.5\% \pm 5.59\%$). There was no difference in predation rate between the mid-summer and late-summer exposures (N = 20, t = −0.50, df = 1, p = 1.000) (Figure 2d).
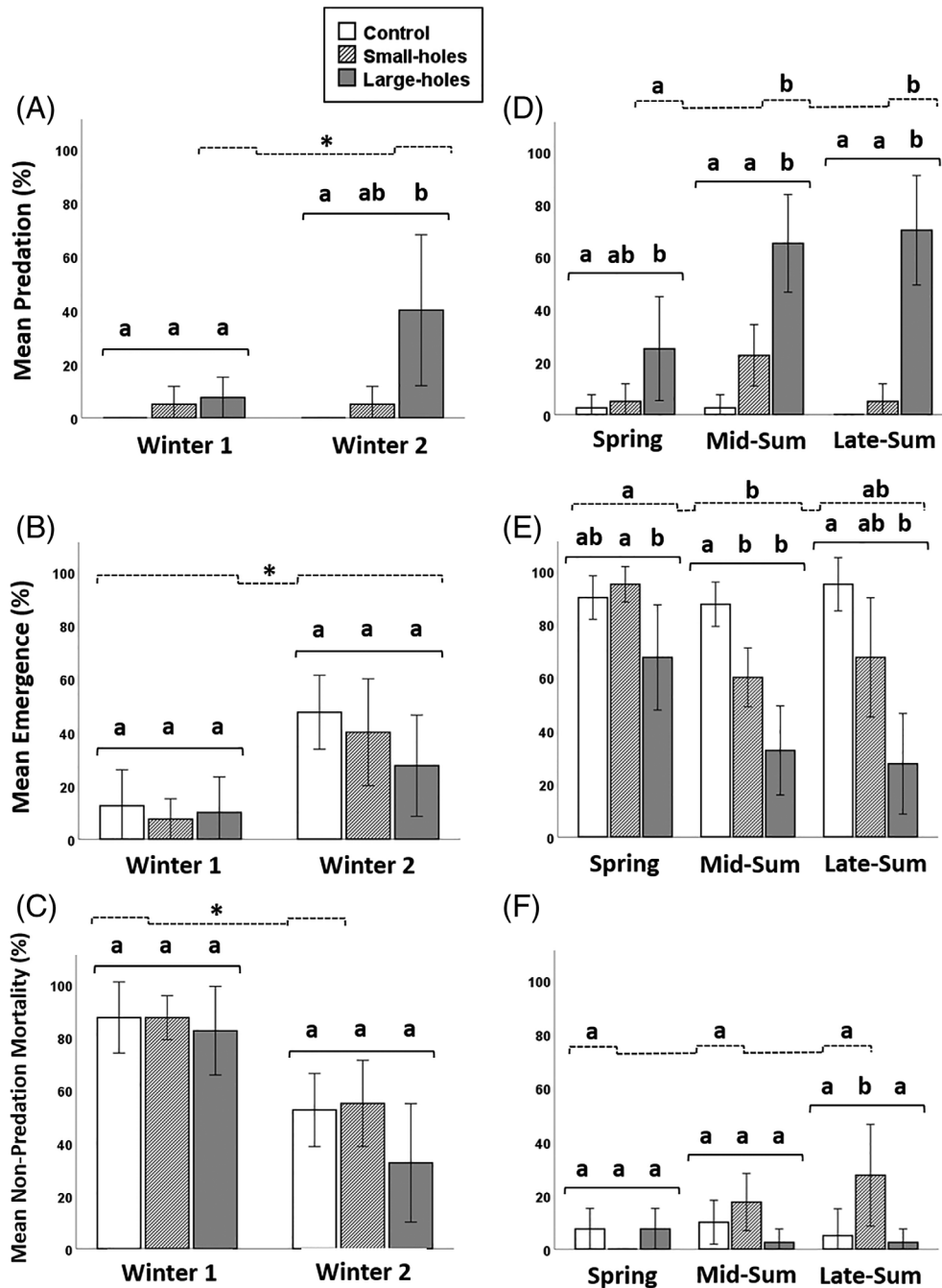
FIGURE 2 Mean predation/adult emergence/non-predation mortality across exclusion treatments during winter exposures (a, b, and c) and summer season exposures (d, e, and f) in southern Ontario. Mid-sum and late-sum refer to mid and late summer respectively. Error bars represent standard error. Lower brackets represent Kruskal-Wallis comparisons among exclusion treatments. Higher brackets represent generalised linear models (a, b, and c) or Kruskal-Wallis (d, e, and f) comparisons between years/seasons. Lower case letters indicate significant differences among exclusion treatments and among summer season exposures. Asterisks represent significant differences between winter years ($\alpha = 0.05$)

Successful adult emergence, across all three treatments combined, differed significantly among the three spring/summer season exposures (N = 90, t = 10.762, df = 2, p = 0.005). Adult emergence was higher in spring (84.16% ± 4.23%) than in mid-summer (60% ± 5.44%) (N = 60, t = 3.213, df = 2, p = 0.004). There was no significant difference in adult emergence between spring and late summer (63.33% ± 7.16%) (N = 60, t = 2.180, df = 2, p = 0.088), or between

mid-summer and late-summer (N = 60, t = −1.33, df = 2, p = 0.905) (Figure 2e).

Death from non-predation causes in closed (control) containers did not differ among spring/summer season exposures (N = 30, t = 1.736, df = 2, p = 0.420) (Figure 2f).

Treatment comparisons spring: During the spring, predation was significantly higher in open (large-hole) containers (25% ± 9.86%)

TABLE 1   Between treatment comparisons for predation, adult emergence, and non-predation mortality for both winter exposures

	Winter 1			Winter 2		
Predation						
	N	Test (df)	p	N	Test (df)	p
Treatment	30	3.360 (2)	0.186	30	9.545 (2)	0.008*
Control*Large-holes	20	N/A	N/A	20	8.571 (1)	0.010*
Control*Small-holes	20	N/A	N/A	20	2.222 (1)	0.408
Small-holes*Large-holes	20	N/A	N/A	20	3.333 (1)	0.204
Adult emergence						
	N	test (df)	p	N	test (df)	p
Treatment	30	0.341 (2)	0.843	30	2.235 (2)	0.327
Control*Large-holes	20	N/A	N/A	20	N/A	N/A
Control*Small-holes	20	N/A	N/A	20	N/A	N/A
Small-holes*Large-holes	20	N/A	N/A	20	N/A	N/A
Non-predation mortality						
	N	test (df)	p	N	test (df)	p
Treatment	30	0.253 (2)	0.881	30	3.529 (1)	0.171
Control*Large-holes	20	N/A	N/A	20	N/A	N/A
Control*Small-holes	20	N/A	N/A	20	N/A	N/A
Small-holes*Large-holes	20	N/A	N/A	20	N/A	N/A

Note: Treatments were compared using Kruskal-Wallis tests, and Dunn's-Bonferroni tests were conducted for pairwise comparisons. Test statistics represent Kruskal-Wallis H values. Asterisks highlight significant difference ($\alpha = 0.05$).

compared with closed (control) containers (2.5% ± 2.5%) (Figure 1d). Adult emergence was significantly lower in the open (large-hole) containers (67.5 ± 9.89) than in small-hole containers (95% ± 3.33%) (Figure 2e). Death from non-predation causes did not differ among treatments during the spring exposure (Figure 2f) (Table 2).

Treatment comparisons mid-summer: During the mid-summer exposure, predation was significantly higher in open (large-hole) containers (65% ± 9.27%) compared with closed (control) containers (2.5% ± 2.5%) and small-hole containers (22.5% ± 5.83%) (Figure 2d). Successful adult emergence was significantly lower in the open (large-hole) containers (32.5% ± 8.73%) than in closed (control) containers (87.5% ± 4.16%) (N = 20, t = 4.276, df = 1, p < 0.001). Adult emergence was also lower in small-hole containers (60% ± 5.52%) than in closed (control) containers (Figure 2e). Death from non-predation causes did not differ among treatments in mid-summer (Figure 2f) (Table 2).

Treatment comparisons late-summer: In late-summer, predation was significantly higher in open (large-hole) containers (70% ± 10.41%) compared with closed (control) containers (0% ± 0%) and small-hole containers (5% ± 3.33%) (Figure 2d). Adult emergence was significantly lower in the open (large-hole) containers (27.5% ± 9.46%) than in closed (control) containers (95% ± 5%) (Figure 2e). Finally, Non-predation mortality in late summer was significantly higher in the small-hole containers (27.5% ± 9.46%) than in the closed

(control) containers (5% ± 5%) and the open (large-hole) containers (2.5% ± 2.5%) (Figure 2f) (Table 2).

DISCUSSION

Over the two winters of our study, predation rates were low whereas non-predation mortality was high, particularly during the colder of the two winters. During the summer months, non-predation mortality was low, but predation rates were higher, increasing as the summer progressed. Across all seasons, pupal predation appeared to be dominated by larger predators, most likely rodents or small birds.

Predation over the winter months was uncommon and occurred mostly from the containers with large holes. These results suggest that predation in the winter can mostly be attributed to larger animals such as small birds and rodents. Birds are voracious predators of many insect pests (Eilers & Klein, 2009; Kirk et al., 1996; Mols & Visser, 2002). Indeed, two species of tits (Parus spp.) have been shown to provide effective biological control of the horse chestnut leaf miner (HCLM), Cameraria ohridella Deschka and Dimic (Lepidoptera, Gracillariidae), in Europe (Mösch et al., 2018). Rodents such as mice remain active predators throughout the winter. Watmough and Kfir (1995) studied pupal predation of Helicoverpa armigera Hbn. (Lepidoptera, Noctuidae) in grain crop fields, and found that mice accounted for approximately 100% of winter predation.

TABLE 2 Between treatment comparisons for predation, adult emergence, and non-predation mortality in all three summer season exposures

	Spring			Mid-summer			Late-summer		
Predation									
	N	Test (df)	p	N	Test (df)	p	N	Test (df)	p
Treatment	30	7.110 (2)	0.029*	30	19.830 (2)	<0.001*	30	24.329 (2)	<0.001*
Control*Large-holes	20	−2.499 (1)	0.037*	20	−4.441 (1)	<0.001*	20	−4.553 (1)	<0.001*
Control*Small-holes	20	−0.433 (1)	1.000	20	−1.941 (1)	0.157	20	−0.634 (1)	0.100
Small-holes*Large-holes	20	−2.056 (1)	0.199	20	2,5 (1)	0.037*	20	−3.919 (1)	<0.001*
Adult emergence									
	N	test (df)	p	N	test (df)	P	N	test (df)	p
Treatment	30	7.169 (2)	0.028*	30	18.416 (2)	<0.001*	30	16.125 (2)	<0.001*
Control*Large-holes	20	1.856 (1)	0.190	20	4.276 (1)	<0.001*	20	4.007 (1)	<0.001*
Control*Small-holes	20	−0.743 (1)	1.000	20	2.455 (1)	0.042*	20	1.783 (1)	0.224
Small-holes*Large-holes	20	2.599 (1)	0.028*	20	1.821 (1)	0.206	20	2.225 (1)	0.078
Non-predation mortality									
	N	test (df)	p	N	test (df)	P	N	test (df)	p
Treatment	30	3.625 (2)	0.163	30	5.555 (2)	0.062	30	8.354 (2)	0.015*
Control*Large-holes	20	N/A	N/A	20	N/A	N/A	20	0.098 (1)	1.000
Control*Small-holes	20	N/A	N/A	20	N/A	N/A	20	−2.453 (1)	0.043*
Small-holes*Large-holes	20	N/A	N/A	20	N/A	N/A	20	2.551 (1)	0.032*

Note: Treatments were compared using Kruskal-Wallis tests, and Dunn's-Bonferroni tests were conducted for pairwise comparisons. Test statistics represent Kruskal-Wallis H values. Asterisks highlight significant difference (α = 0.05).

Helicoverpa armigera pupae are comparable to *H. opulenta* in that they overwinter 2–3 cm under the soil surface (Watmough & Kfir, 1995).

We found winter mortality of *H. opulenta* from non-predation causes to be quite high, averaging 66.24% over the two winter seasons. These results are comparable to those of Johansen (1997), who conducted a similar study with cabbage moth, *Mamestra brassicae* (Lepidoptera: Noctuidae), and observed overall pupal mortality averaging 90% over three winter seasons. Preliminary experiments to understand cold tolerance in *H. opulenta* have revealed the species to be somewhat freeze-tolerant, with a supercooling point around −14°C (Ian Jones. Unpublished data). Given that the lowest temperatures experienced during the colder of two winters in this experiment was −7.7°C, death through freezing should have been low. Supercooling point is, however, not always a reliable predictor of overwintering mortality due to cold. It has been shown that other climatic variables can affect both freezing temperature, and non-freezing mortality related to cold (Tsutsui et al., 1988; Turnock & Bodnaryk, 1991). Extended exposures to cold temperatures above the supercooling point can also affect insect mortality (Chandler et al., 2020). Tsutsui et al. (1988), for example, found that the supercooling point for *Mamestra brassicae* pupae is around −20°C, however, in wet conditions freezing can occur at temperatures as high as −4°C. In addition to changes in the supercooling point, it is also possible that pupae may have died from cold stress unrelated to freezing. Turnock and Bodnaryk (1991) found that winter mortality of *Mamestra configurata*

Walker (Lepidoptera: Noctuidae) was primarily a result of non-freezing cold stress, and that this cause of death was often characterised by incomplete pupal development or failed ecdysis. In the present study, we dissected pupae that failed to emerge in spring. Of those pupae, the number that appeared disease-free, but that had suffered incomplete adult development or failed ecdysis, was higher for year one (30; 29.12%), the colder of the two winters, compared to year two (5; 8.92%). Although we cannot rule out disease as a cause of mortality based on the lack of visible symptoms, our results suggest that overwinter mortality of *H. opulenta* resulting from abiotic factors may vary greatly depending on the winter conditions.

Future work should further clarify the minimum temperatures and durations of cold that *H. opulenta* can survive. Other climatic factors such as snow-cover and soil moisture content should also be considered. The insulating properties of snow, for example, have been shown to reduce mortality in the overwintering pupae of cotton bollworm, *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) (Huang, 2017). Conversely, high levels of soil moisture can lead to increased pupal mortality due to lack of oxygen (Zheng et al., 2013). Experiments similar to those that we have conducted here should consider employing an additional control group, in which pupae are retained in controlled laboratory conditions. This would provide a baseline for pupal survival that would eliminate pupal viability as a source of variation in field survival. Although our experiments lacked this control, rearing conditions were the same across all experiments

and we weighed all pupae prior to each exposure to insure relative uniformity of size (0.086 ± 0.0007 g) (mean ± SE).

Predation during the summer months was significantly higher in containers with large holes than in those with small holes or control containers. Similar to the winter months, this suggests that the majority of predation in the summer can be attributed to larger predators such as rodents and small ground-dwelling birds. These results are similar to those of Hastings et al. (2002) and Gschwantner et al. (2002), who determined that rodents were the main summer predators of gypsy moth pupae, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae). Relatively few studies have addressed rates of pupal predation in Lepidoptera, much less identified the predators involved. Farias et al. (2020), however, conducted multiple horizontal life tables for the diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae), and found that ants were the most important predators. Although we observed one species of ant gaining access to multiple experimental cages, *Formica subsericea* (Say) (Hymenoptera: Formicidae), we recorded only a few cases of suspected ant predation.

The predation rate of summer pupae was higher in the mid- and late-summer than in the spring suggesting that key predators are more active and abundant later in the season. These results highlight the importance of multivoltinism for the success of *H. opulenta* as a biological control agent (Jones et al., 2020). Not only will populations of *H. opulenta* undergoing only one generation be lower in number, but the early onset of diapause will expose pupae to the highest rates of predation for an extended period. For example, based on our observations of overall pupal survival, if a population of 1000 *H. opulenta* entered diapause in mid-summer after a single generation, only 89.38 would survive until the onset of winter (1000*proportion surviving in open containers in mid-summer*proportion surviving in open containers in late summer), and only 21.53 would be alive the following spring (1000*proportion surviving in open containers in mid-summer*proportion surviving in open containers in late summer*Mean proportion surviving winter). Previous studies have indicated that the survival rates of diapause pupae in the soil can be low. Bonsignore et al. (2015) collected pupae of the pine processionary moth, *Thaumetopoea pityocampa* (Denis & Schiffermüller) (Lepidoptera: Notodontidae), in the spring of 2012. Of the 5440 pupae collected only 646 were undamaged, and the overall parasitism rate was 77.6%. These results further highlight the potential impacts of an extended diapause on noctuid moth populations.

The number of pupae dying from non-predation causes in the summer months was low (10%) and did not differ among exposure treatments, suggesting that predation was the main source of mortality among summer pupae. We observed very little incidence of disease among summer pupae, and no evidence of parasitism was found throughout the study.

Overall, our results highlight two important sources of mortality for *H. opulenta* pupae, late-season predation that may disproportionately affect populations undergoing only a single generation, and winter mortality due to abiotic factors. Late-season predation is likely to affect the establishment and spread of *H. opulenta* in the lower latitudes of the North American swallow-wort invasion, as short summer photoperiods dictate that only one summer generation is likely (Jones et al., 2020). Winter mortality is also likely to affect *H. opulenta* differentially along a latitudinal gradient. Priority must be to further elucidate the abiotic factors that contribute to winter mortality in *H. opulenta* in order to determine whether a northern threshold exists for their establishment. Our results can contribute to models predicting population dynamics of *H. opulenta* across its introduced range. In order to facilitate such predictions, future work must focus of mortality rates of larvae and adults, as well as adult fecundity in the field.

AUTHORS CONTRIBUTION

Ian Jones contributed to project design, data collection, analysis, and paper writing. Sandy Smith contributed to funding acquisition, project design, and paper writing. Rob Bourchier contributed to funding acquisition, project design, and paper writing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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PUPAL MORTALITY IN HYPENA OPULENTA

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535

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