EXPLORING THE POTENTIAL OF THE BIOLOGICAL CONTROL AGENT *HYPENA OPULENTA* (CHRISTOPH) (LEPIDOPTERA: EREBIDAE) AT CONTROLLING INVASIVE SWALLOW-WORT VINES IN MICHIGAN AND ITS INDIRECT EFFECTS ON MONARCH BUTTERFLIES

By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Entomology—Master of Science

2021

ABSTRACT

EXPLORING THE POTENTIAL OF THE BIOLOGICAL CONTROL AGENT *HYPENA OPULENTA* (CHRISTOPH) (LEPIDOPTERA: EREBIDAE) AT CONTROLLING INVASIVE SWALLOW-WORT VINES IN MICHIGAN AND ITS INDIRECT EFFECTS ON MONARCH BUTTERFLIES

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This thesis focuses on swallow-wort, an invasive vine related to milkweeds, and the potential of *Hypena opulenta* for providing biological control in Michigan, as well as the indirect effects that the introduction of *H. opulenta* may have on the monarch butterfly. The first chapter covers the background of swallow-wort in its invasive range and outlines previous research done with *H. opulenta* in the United States and Canada.

In the second chapter, the impact and the phenology of *H. opulenta* on black swallow-worts in Michigan is examined in common garden experiments. Swallow-wort survival and reproductive output are measured and compared when different adult densities are released in one study. The second study assessed the phenology of *H. opulenta* when releases took place at different dates either in sunny or shaded sites. In southern Michigan, *H. opulenta* is found to produce two generations per season, though overwintering could not be confirmed. Damage caused by larval feeding was higher in the shade but was not enough to reduce plant fitness regardless of release size or light availability.

Swallow-worts can serve as oviposition sinks for monarchs, however, the presence of *H. opulenta* larvae may serve as an oviposition deterrent. Examination of the indirect effects of *H. opulenta* on monarch butterflies in the third chapter found no evidence that *H. opulenta* deters monarch oviposition. In addition, the utilization of swallow-wort by wild monarch butterflies as an oviposition host in Michigan was confirmed.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Marianna Szucs, for her insight and for her patience as a mentor throughout my time here. I have learned a lot as a student in her lab and have grown as a scientist with her guidance. I would also like to thank the members of my committee, Dr. Douglas Landis and Dr. Ruth Hufbauer, for their expertise and for their support with my research.

Thank you to those who assisted with project design and statistical analyses, Nathan Haan, and Leo Pham. Their help with my project was invaluable.

I am thankful to the members of the Biological Control Lab for their assistance with my research and for their support: Christian Millan-Hernandez, Benjamin Jarrett, Olivia Simaz, Anupreksha Jain, Shelley Linder, Brianna Foster, and Andrew Yoshimoto. Thank you to the undergraduate research assistants who helped out with experimental set up and data collection: Nadja Tabara, Jacqueline Okuniewicz, Jennifer Lee, and Mark Hamlyn.

I would also like to thank my Grandma Jill who fostered my love for biology from a young age and who I have always looked up to. My dad who has always pushed me to do my best and whose strong work ethic has been an example for me. Jeffrey Norris who has provided endless emotional support, proofreads all my emails, and is always around when I need help. Ellie Darling, whose Zoom calls and socially distant lunches kept me from going crazy this year.

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Chapter 1: Invasive status and biological control of swallow-worts in North America

Three swallow-wort species (*Vincetoxicum* spp.) are present in North America that were introduced from Europe as ornamental plants in the 1800's (Monachino, 1957). Two species, black swallow-wort (*V. nigrum*) and pale swallow-wort (*V. rossicum*) have become invasive in the last 30-40 years in eastern Canada and the eastern and midwestern United States (Fig. 1.1). Swallow-worts have likely arrived in Michigan prior to 2008 with populations of black swallow-wort reported in 9 counties, and pale swallow-wort in 27 counties as of 2021 (Midwest Invasive Species Information Network, 2018). Mechanical and chemical approaches have proven largely unsuccessful to control either species. For example, swallow-wort control in Oakland county, Michigan alone cost over $245,000 between 2010 and 2018 for the treatment of 450 acres with herbicides (pers. comm. E. DuThinh). Despite these efforts, swallow-worts have continued to spread and have remained a problem in the county and across the state.

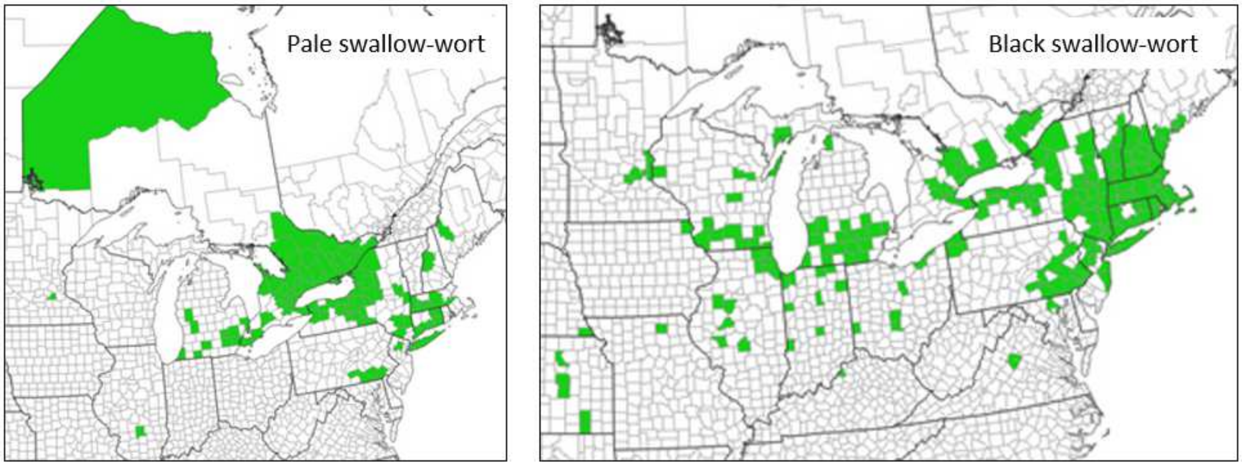

Figure 1.1. Distribution of pale (left) and black swallow-wort (right) in the eastern and midwestern United States and Canada. Source: www.eddmaps.org accessed on March 1, 2021.

To control invasive weeds, such as swallow-worts, biological control - the use of specialist insect herbivores - can provide a long-term and sustainable method. To this end, a defoliating moth, *Hypena opulenta* was imported from the native range of swallow-worts and approved for field release in Canada in 2013 and in the U.S. in 2017.

In this chapter, the biology, ecological impacts, and control methods of swallow-worts are reviewed. In addition, our current knowledge of the biology and potential impact of the biological control agent, *H. opulenta* is summarized.

Swallow-wort biology

Swallow-worts (*Vincetoxicum* spp.), commonly referred to as dog-strangling vines, are long-lived perennials in the Asclepiadaceae family. They can grow in a wide range of environments such as wooded edges, forest understories, open fields, and along roadsides (DiTommaso et al. 2005, Weed et al. 2011). Swallow-worts have a woody rootstock which produces multiple shoots during the spring that grow up to three meters in length (DiTommaso et al. 2005). They flower from spring to late August and can be self- or insect-pollinated. Swallow-worts produce an average of 250 anemochorous seeds per stem which disperse up to 18 meters when seeds are released close to the ground and up to 80 meters when seeds are released by stems that have climbed up a tree (DiTommaso et al. 2018, Cappuccino et al. 2002). Seed dispersal is negatively correlated with seed weight, with lighter seeds dispersing further than heavier seeds, but with heavier seeds being more likely to emerge and survive (Ladd and Cappuccino 2005, Cappuccino et al. 2002). Seeds are viable in the soil for two to three years and have 50-70% germination rate (Cappuccino et al. 2002). More than half of the seeds produced by swallow-wort are polyembryonic meaning that they produce two, three or rarely four seedlings. Polyembryonic seeds are more likely than single-embryo seeds to have at least one seedling survive to the next season (Cappuccino et al. 2002). The combined biomass of polyembryonic seeds is nearly 50% greater than that of single seedlings, however the advantage of polyembryony on seedling biomass dissipates when seedlings are in competition with grasses (Cappuccino et al. 2002). Methods to control swallow-wort aboveground biomass and prevent swallow-wort from climbing would reduce their ability to spread far distances (DiTommaso et al. 2018). Controlling the production of seeds would also be effective in reducing the spread of swallow-wort, though through vegetative growth and spread of root stocks, swallow-worts would likely survive (DiTommaso et al. 2017, Averill et al. 2011).

Swallow-wort ecological impacts

Swallow-worts create dense monocultures which outcompete native vegetation (Ernst and Cappuccino 2005, Weed and Casagrande 2010). Areas composed of mainly pale swallow-wort have been found to have low arthropod diversity compared to neighboring areas with native vegetation. Ernst and Cappuccino (2005) found that, when compared to sites with the native common milkweed or Canada goldenrod (*Solidago altissima),* sites dominated by pale swallow-wort supported fewer arthropod species. Swallow-wort stands had significantly fewer pollen/nectar feeders and seed/sap feeders, and stem borers were absent. By smothering native vegetation, swallow-wort reduces the quality of habitats which can negatively affect grassland birds (DiTommaso et al. 2005). There is some evidence that swallow-worts may be allelopathic. In a laboratory study, the exudates from pale swallow-wort and black swallow-wort reduced the root elongation of butterfly milkweed by 35% and of common milkweed by 16% respectively. In lettuce, root exudates from black swallow-wort stimulated root growth, but reduced germination by 25% (Douglass et al. 2011). However when the actual concentrations of -(-) antofine, the phytochemical thought to be responsible for allelopathy in swallow-wort, were assessed in the soil, the levels detected were far lower than what was used in experiments with lettuce (Gibson et al. 2015). Thus, it is unclear whether realistic levels of phytochemicals extracted from swallow-worts would have allelopathic effects (Gibson et al. 2015).

Swallow-worts are closely related to common milkweed (*Asclepias syriaca*), which are the primary hosts of monarch butterflies (*Danaus plexippus*) (Wassenaar and Hobson 1998). As swallow-worts have spread in the eastern United States, monarchs were found to lay up to 15-25% of their eggs on swallow-worts, but their larvae were unable to develop (Casagrande and Dacey 2007). This research suggests that swallow-worts may serve as an oviposition sink for monarchs posing a threat to monarch populations. However, the findings of these laboratory and field cage experiments from Rhode Island (Casagrande and Dacey 2007) had not been corroborated in other parts of North America. In New York monarchs were found to lay no eggs on swallow-wort and in Ontario, Canada only 0.4% of their eggs were laid on swallow-worts (Mattila and Otis, 2003; DiTommaso and Losey 2003).

Swallow-wort control

Swallow-worts are difficult to control by mechanical or chemical means. Mechanical control by itself is not enough to control swallow-wort populations. While cutting and mowing can reduce seed output, reductions in biomass require repeated tissue removal and the plants often regrow and produce flowers making mechanical methods such as pulling and mowing ineffective and laborious (Averill et al. 2008; DiTommaso et al. 2013). One study found that cutting plants twice a season over two seasons increased swallow-wort cover by 301% and stem density by 73% (DiTommaso et al. 2013). Repeated mowing consistently across many years would likely be necessary to see any reductions in swallow-wort. For example, one study which examined the effects of repeated mowing over 7 years found that mowing 3 to 6 times per season was needed for at least 3 years in order to reduce swallow-wort populations (Milbrath et al. 2016). Using herbicide treatments on swallow-wort have produced mixed results. While herbicides can be effective at reducing biomass and density, different light environments can alter the efficacy of the treatment (DiTommaso et al. 2013). Herbicide treatment in addition to mechanical control methods have the potential to reduce over 80% of stem density and cover, but the same treatments may yield different results in a different site (DiTommaso et al. 2013). This makes it difficult to determine an efficient way to control swallow-wort populations in different environments. In addition, mechanical treatments of swallow-worts are limited to easily accessible areas such as open fields. Swallow-worts found in forest understories may be more difficult to control and require other control techniques.

Classical biological control involves importing and releasing natural enemies from a pest species' native range into its invasive range. Due to the severity of swallow-wort infestations and the difficulty in controlling swallow-worts by chemical and mechanical means, a biological control program was initiated in 2001 in North America (Tewksbury et al. 2002, Weed et al. 2011). Surveys conducted in Ukraine and in southern Europe, where black and pale swallow-worts are native, identified five potential biological control agents (Weed and Casagrande 2010). These insects included a defoliator beetle *Chrysolina aurichalcea asclepiadis*, a root feeding flea beetle *Liprus punctatostriatus*, a seed eating fly *Euphranta connexa*, and two defoliator moths *Abrostola asclepiadis* and *Hypena opulenta*. The leaf feeding moth *H. opulenta* was found to have the highest potential and thus were tested for host-specificity on 82 plant species that are either native to North America or are of economic importance (Hazlehurst et al. 2012). The plant test list included several species in the family Asclepiadaceae such as common milkweed, swamp milkweed (*Asclepias incarnata*), and butterfly weed (*Asclepias tuberosa)*, which are important hosts for monarchs. *Hypena opulenta* was only able to survive to adulthood on pale swallow-wort, black swallow-wort, and white swallow-wort (*Vincetoxicum hirundinaria)*. While there was reported feeding on *Gonolobus stephanotrichus* and on *Urtica dioica,* neither supported larval development (Hazlehurst et al. 2012). One larva developed on *Boehmeria cyclindrica* but did not pupate. These findings led to the conclusion that *H. opulenta* would not threaten any native or economically important plants in North America (Hazlehurst et al. 2012). As a result, in 2013, *H. opulenta* was approved for release in Canada, and in 2017 in the U.S.

Hypena opulenta

It is important to understand the biology of *Hypena opulenta* Christoph (Lepidoptera: Erebidae) if it is going to be reared and released as a biological control agent. *Hypena opulenta* was discovered feeding on black swallow-wort in low-light forest understories in Ukraine. It feeds on black and pale swallow-wort and has similar egg production, larval performance, and adult longevity regardless of which host it is reared on (Weed et al. 2011, Hazlehurst et al. 2012). The adult is a small dark brown moth with pale yellow hind wings (Fig. 1.2) and females are capable of laying up to 600 eggs each, which are laid singly, often on the underside of swallow-wort leaves (Weed and Casagrande 2010). The larvae feed on swallow-wort leaves or seedpods as they develop through five instar stages before pupating in the soil. They are capable of producing at least two generations per year depending on photoperiod and host plant quality (Weed and Casagrande 2010).

Experiments to determine *H. opulenta* impact on swallow-wort have been conducted in laboratory and greenhouse studies. In laboratory experiments, as few as two *H. opulenta* larvae have been shown to cause significant reduction in aboveground biomass on a single pale swallow-wort plant although the same number of *H. opulenta* did not cause the same amount of damage on black swallow-wort (Weed and Casagrande 2010). Even though larvae feeding on foliage caused impressive reductions in biomass and was capable of reducing seed productions, the single generation of larval feeding was not enough to kill the plants (Weed and Casagrande 2010). Another study found that 3-6 larvae are sufficient to significantly reduce biomass for black swallow-wort as well as pale swallow-wort (Milbrath and Biazzo 2016). Light-conditions may also impact *H. opulenta* performance on swallow-wort. In a greenhouse study, larvae were capable of killing black swallow-wort seedlings with repeated defoliation under low-light conditions, but the same result was not found with swallow-worts under high-light conditions (Weed and Casagrande 2010).

Figure 1.2. Adult *H. opulenta*. (Photo: B. Alred).

Field releases of *H. opulenta* larvae in Ontario, Canada began in 2013 and 2014. From these releases, overwintering success was confirmed in 2015 and 2016. In 2017 and 2018, larvae and larval damage were found up to 2.0 km away from the original release sites confirming establishment and spread (Bourchier et al. 2019). Field releases of *H. opulenta* have begun in 2018-2020 in Rhode Island, Massachusetts, Connecticut and Michigan in the U.S., but as of spring 2021 establishment has not been confirmed.

Chapter 2: Impact and phenology of the biological control agent, *Hypena opulenta* on *Vincetoxicum nigrum* in the field in Michigan

Introduction

Classical biological control involves importing and releasing natural enemies from a pest species' native range into its invasive range and can be one of the only long-term and sustainable solution to invasive weed problems (McFadyen 1998, Clewley et al. 2012, Schwarzlander et al. 2018, Heimpel and Mills 2017). While selection of biological control agents has historically focused on the assessment of safety and climate matching with the planned release area, recent testing increasingly involves pre-release assessments to predict the potential impact of the agents on the target weed (Barratt et al. 2010, McClay and Balciunas 2005). Impact experiments may be carried out in the native range of invasive plants, however, field populations of the targeted invasive can be sparse and a suite of natural enemies besides the potential biocontrol agent may be present. In the field, cage studies may be conducted where various densities of the biological control agent are released (McClay and Balciunas 2005, Briese 1996, 2004, Briese et al. 2002, 2003). The per-capita impact of agents can also be assessed under laboratory or greenhouse conditions either in the native range or in quarantine in the introduced range using potted plants (McClay and Balciunas 2005, Gerber et al. 2008, Kloppel et al. 2003, Reddy and Mehelis 2015). Alternatively, in cases when the agent itself is not available for experimentation simulated herbivory tests can be conducted (Raghu and Dhileepan 2005, Raghu et al. 2006). These pre-release assessments can be valuable at predicting what densities the agent would need to achieve in the field for measurable impact on the target weed (Sheppard 2003, McClay and Balciunas 2005, Morin et al. 2009). However, the ultimate tests of effectiveness can only be carried out in the field in the introduced range where the invasive plants grow and where the biocontrol agent is subject to local environmental conditions.

Black swallow-wort (*Vincetoxicum nigrum* (L.) Moench) and pale swallow-wort (*Vincetoxicum rosicum* (Kleopow) Barbar.) are perennial vines from Europe that have become invasive in the last 30-40 years in eastern Canada and the eastern and midwestern United States (DiTommaso et al. 2005, Milbrath 2010). Swallow-worts can grow in a wide range of environments including pastures, old fields, understories of woodlands, ruderal areas, roadsides, flood plains and in no-till agricultural fields (DiTommaso et al. 2005, Weed et al. 2011). They often form monocultures replacing or smothering native vegetation and have cascading ecological effects on native ecosystems by reducing arthropod diversity and habitat for native birds (Ernst and Cappuccino 2005, DiTommaso et al. 2005). Swallow-worts are also related to milkweeds (Asclepiadaceae), the primary hosts of monarch butterflies (*Danaus plexippus*) and can serve as oviposition sinks for monarchs (Casagrande and Dacey 2007). Monarchs were shown to lay 15-25% of their eggs on swallow-worts, but these invasive plants are not suitable for larvae development and all eggs laid on swallow-worts eventually die (Matilla and Otis 2003, Casagrande and Dacey 2003).

Established populations of swallow-worts are difficult to control by mechanical or chemical means. Swallow-worts can spread quickly as they produce polyembrionic seeds, which means that a single seed can produce 1-4 seedlings (DiTommaso et al. 2017). Pulling, cutting or mowing plants are ineffective, laborious and often impossible in hard to access areas. Cutting and mowing may reduce seed output, however, any reduction in biomass requires repeated tissue removal and plants often grow back and produce flowers after mechanical control given their ability to reproduce vegetatively (Averill et al. 2008, DiTomasso et al. 2013, Milbrath and Biazzo 2016). Chemical control can be effective at reducing biomass and density of swallow-worts, however, there is no single method that works in each habitat. Different herbicides can yield differential success in low-light or high-light environments, such as in forest understories versus in open pastures (DiTommaso et al. 2013).

Given the severity of infestations and the difficulty of controlling swallow-worts by mechanical and chemical approaches a biological control program was initiated in 2001 in North America (Tewksbury et al. 2002, Weed et al. 2011). Surveys conducted in southern Europe, the native range of pale swallow-wort, and in Ukraine, the native range of black-swallow-wort led to the discovery of a defoliating moth, *Hypena opulenta* Christoph (Lepidoptera: Erebidae) (Weed and Casagrande 2010). *Hypena opulenta* can attack both black and pale swallow-worts and is highly host-specific to the genus

Vincetoxicum and thus was approved for field release in Canada in 2013 and in the United States in 2017 (Hazlehurst et al. 2012, Casagrande et al. 2011).

Moths in the family Noctuidae, the former classification of *H. opulenta*, have around 36% overall success rate as weed biological control agents using combined metrics for establishment and impact (Heimpel and Mills 2017). To predict the impact of *H. opulenta,* numerous pre-release efficacy studies were carried out. Early assessments using artificial defoliation showed that simulated herbivory can reduce seed production with a one-time 90% defoliation event resulting in 80% lower seed set in recently established field populations of pale swallow-wort (Doubleday and Cappuccino 2011). Biomass and seed production of potted black and pale swallow-wort plants also decreased following 100% artificial defoliation with greater losses and high mortality in shaded compared to high-light environments (Milbrath 2008). Experiments with *H. opulenta* showed that feeding by only 2 larvae per plant can cause significant reductions in above ground biomass of pale swallow-wort, but not of black swallow-wort (Weed and Casagrande 2010). In this study, larval feeding was found to reduce seed production of both pale and black swallow-wort but was not able to kill plants in a single generation (Weed and Casagrande 2010). A greenhouse study using potted plants found that 3-6 larvae per stem could significantly reduce biomass of both pale and black swallow-wort and that two rounds of defoliation of black swallow-wort under low light conditions killed plants (Milbrath and Biazzo 2016). Impact of *H. opulenta* in the field was assessed at one location in Ontario where over 700 larvae were released in field cages covering naturally growing pale swallow-wort either in sunny or shaded habitats. The high larval densities of 5 and 10 larvae per stem in the sun and shade respectively resulted in 75 -100% defoliation, but instead of reducing plant fitness it increased seed production in the shaded plots (Livingstone et al. 2019).

While laboratory and greenhouse studies have provided insight into how *H. opulenta* feeding may impact swallow-wort in different light environments, open field releases and cage experiments have investigated *H. opulenta* phenology in North America. Field releases, field cage and laboratory experiments have shown that *H. opulenta* can complete two generations a year in Ontario but only when released at dates with >15h daylight (Jones et al. 2020, Bourchier et al. 2019). Field releases of *H. opulenta* started five years later in the United States than in Canada, and as of 2020 there were no established populations. Thus, it is still unknown what the phenology of *H. opulenta* may be in more southern locations where the window of >15 h daylight is narrower than in Canada. In addition, all the impact experiments to date used larval densities that inflicted 75-100% defoliation of swallow-wort plants but monitoring of established field populations showed that larval damage five year post-release is usually under 1% and may reach 20-28% only locally in Canada (Bourchier et al. 2019). More studies are needed that use more realistic larval infestation and defoliation levels to predict the effectiveness of *H. opulenta* in the field.

We conducted common garden experiments to assess the impact and phenology of *H. opulenta* in southern Michigan on black swallow-worts in 2019 and 2020. In one experiment, we released different densities of *H. opulenta* (1, 2, or 5 pairs) in field cages to achieve different levels of defoliation. In a separate experiment we released 4-5 pairs of *H. opulenta* in field cages at different dates, either in a sunny or shaded site to assess its phenology and impact under various light conditions. For all experiments we monitored larval damage, moth phenology, swallow-wort stem density and seed production. In addition, we assessed overwintering success in the impact experiment. We predicted that larval damage in plots with the highest adult releases (5-pair) would result in complete defoliation and measurable fitness effects on swallow-worts. We also assumed that when *H. opulenta* is released around the summer solstice it would show a bivoltine life cycle, in accordance with observations from Canada.

Materials and Methods

Study species

Hypena opulenta Christoph is a small (1.1 ± 0.1 cm in length) dark brown moth with pale yellow hind wings in the family Erebidae (Weed and Casagrande 2010). Adults emerge in late spring and females begin laying eggs within two days. Individual females can produce up to 600 eggs with an average of 410 eggs and live for an average of 17 days. Eggs are laid singly, often on the underside of swallow-wort leaves or on the petioles and take about 3 to 4 days to hatch in the laboratory at 20° C. Larvae develop through five instars. Early instar larvae skeletonize leaves, leaving window-like feeding damage, while late instars feed on the entire leaf with the potential to completely defoliate the plant. Larvae develop in approximately 19 days then pupate in the soil. Total development from egg to adult takes 37 days on average under laboratory conditions (Fig. 2.1) (Weed and Casagrande 2010). This short development time allows *H. opulenta* to complete multiple generations per season in its native range (Weed and Casagrande 2010, Jones et al. 2020).

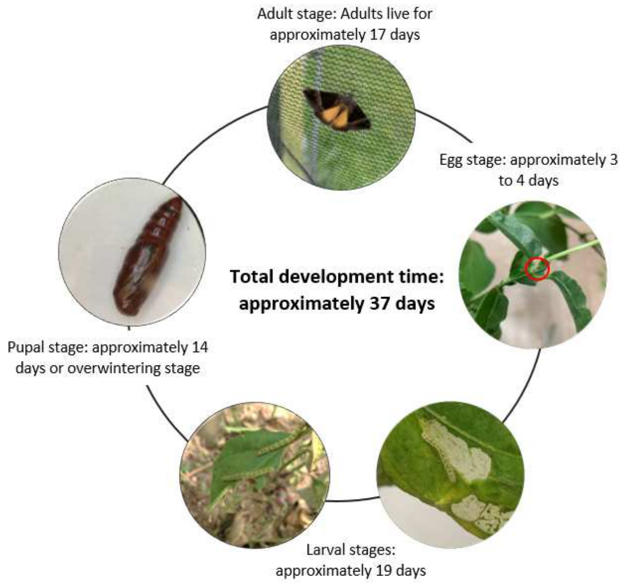

Figure 2.1. The development of *H. opulenta* at 20°C laboratory conditions as reported by Weed and Casagrande (2010). (Images by B. Alred).

Insect rearing

Hypena opulenta used in all experiments originated from a laboratory rearing at Michigan State University (MSU). The laboratory colony was established with 18 females and 22 males shipped as larvae from the University of Rhode Island on 3 May and 8 June 2018. Adults were paired in 30 x 30 x 30 cm mesh cages (Bioquip®) that were placed early in the season (May – June) under lights at 16:8h L:D photoperiod and later in the season on bench tops under natural daylight conditions at 23-25˚C. Black swallow-wort plants that were transplanted from an infestation on MSU campus into 1.14 L square plastic pots using SUREMIX perlite potting media were provided for oviposition. Adults were provisioned with honey water provided via a cotton wick inserted into a 60 mL cup. Three to five pairs of adults were released in each cage that contained 4-6 potted plants and a thin layer of soil at the bottom on a plastic tray that was kept moist at all times. Adults were removed from the cages after one week of oviposition and provided with fresh plants in new cages until their death weekly. This rotation served to limit the number of larvae in each cage. Larvae were kept in the mesh cages until the fourth to fifth instar stages and supplemented with stems of both black and pale swallow-worts inserted into 7.5 cm floral water tubes. Pale swallow-wort stems used for supplementation were cut at an infestation near Holly, MI (N 42°84’62 – W -83°41’28) throughout the season. Late instar larvae were removed from the cages and placed in groups of 20-30 in 2.4 L transparent round plastic containers (HDX, Home Depot with a mesh top for ventilation. In each container swallow-wort stems were provided in floral tubes. Pupation took place in these containers in paper towels. Pupae were sexed and males and females were placed separately in moistened vermiculite (Vigoro®) in 0.35 L clear round plastic containers in groups of eight. Pupae were placed on bench tops and within one to two weeks a new generation of adults emerged. Late season pupae (August-September) were prepared for diapause by placing them in an incubator at 12:12h L:D and gradually lowering the temperature by 5˚C each month from 20˚C in September to 4˚C by December.

Impact experiment

A two-year common garden experiment was conducted at the MSU Entomology Research Farm to test the impact of different densities (1, 2, 5 pairs or no release control) of *H. opulenta* on swallow-worts. One year prior to the start of the experiment 18 research plots ($2m^2$ each) were established in spring 2018 by transplanting 40 black swallow-wort plants in each. In spring 2019, 60 additional plants were added to each plot. The plants were collected from Lowell, MI as rootstocks. The plots were situated about one meter apart in an east to west direction along a fence line and were surrounded by grassland on the north side, a 10 m strip of grass and rows of grapes on the south side. In May 2019, 16 of the 18 plots were covered with a 4m x 4.6m x 2m mesh tent (Coleman® Instant Screenhouse), which was left on until late September. In 2020, home-made, 2m x 2m x 2m grey mesh (Gardzen) cages with a zipper running along the middle of one side were used instead of the screenhouses that held up better against the elements during the 5 months of deployment. The two uncovered plots served to test for cage effect on plant growth.

The different *H. opulenta* release treatments and the cage effect treatment were assigned randomly among the 18 plots. Each different release size had four replications. *Hypena opulenta* adults that emerged prior to the summer solstice (21 June in 2019) were kept for 7-10 days in the laboratory in cages with one potted plant in pairs corresponding to the release size treatments to ensure that they laid eggs. On 28 June 2019 when all pairs were confirmed to lay eggs, adults were released in the field cages. The single potted swallow-wort plant on which they laid eggs prior to release and the cup with honey solution was also placed in the cages to include the full complement of eggs in the experiment and for provisioning the adults.

To monitor the impact of the different treatments the following measurements were taken in 2019 and 2020: swallow-wort stem density, the number of seedpods produced, *H. opulenta* larval feeding damage, the number of larvae, number of second-generation adults in year one and the number of emerging adults in year two. The number of swallow-wort stems were counted in early June and at the end of the season in September in both years. The number of seedpods were counted in late September in both years. Larval feeding damage was measured weekly in 2019 and every three weeks in 2020 by choosing 10 swallow-wort stems randomly in each plot, counting the number of leaves on each stem and estimating the percent larval damage on each leaf. Mean damage was calculated by dividing the cumulative percent damage with the total number of leaves. On each of the 10 randomly chosen stems the number of larvae were also recorded upon each monitoring. Second generation adults in 2019 were monitored by searching the cages for five minutes weekly. In 2020, cages were placed back on the plots on 22 May and emergence of overwintering adults was monitored.

A fungus was observed attacking the swallow-wort plants in the experimental plots in late June 2019, which was identified as Cercospora leaf spot by the MSU Diagnostic Services in July. This fungus created a black soot-like layer on the underside of leaves and spread quickly covering 80-90% of leaves by the end of July. The fungicide Flint Extra® (Bayer) was applied to all 18 experimental plots on 26 July 2019 when *H. opulenta* was in the pupal stage to avoid exposing larvae to the chemical. In 2020, the fungus was present again, and covered 90-100% of swallow-wort leaves by the end of the season.

Phenology experiment

Hypena opulenta was released at different dates in common garden plots at a sunny and a shaded site to assess its seasonal phenology and its performance under different light conditions. The sunny site was located at the MSU Entomology Research Farm and was established by transplanting 20 black swallow-wort rootstocks in each of 16 plots (1m^2 each) one year prior to the start of the experiment. In 2020 spring, stems were counted, and additional plants were added if needed to standardize plant densities at 20 plants/plot. The plots were positioned 0.5 m apart in a row running north to south and were surrounded by grassland on each side with a wood edge about 6 m to the west and a row of blueberry bushes about 4 m to the north. The shaded site was located about 4 km to the north in a forested area on

MSU campus, where black swallow-wort plants grew naturally under pine trees. At both sites 1m x 1m x 1m mesh cages were placed over the plots (sunny sites) or the naturally growing swallow-wort plants (shaded site).

Hypena opulenta adults were released at two dates, on 25 June and 17 July 2020 at both sites. There were four replications for each release date and four control plots where no insects were released. Treatments were randomly assigned among the plots. Five females and four males were released per cage except for the 17 July release at the sunny site where four females were released. To monitor *H. opulenta* development and damage in relation to the timing of releases during 2020, the same data were collected in these plots as described for the previous experiment including stem density, seed pod numbers, larval damage, presence of larvae and adults.

Statistical analyses

All data were analyzed in R version 3.6.1 (R Core Team, 2019) using the package 'glmmTMB' (Brooks et al. 2017) and 'emmeans' version 1.5.2-1 to get model generated means (Lenth, 2020). With the package 'glmmTMB' general linear models or generalized linear mixed models (GLMM) can be fitted to count data that is either zero-inflated or not using maximum likelihood estimation (Brooks et al. 2017). Since the data collected for our common garden experiments consist of repeated observations taken over time in the same fixed plots, they are correlated. To account for this correlation, we included treatment nested within replication as a random effect and used GLMMs either with or without zero-inflation for all analyses. In cases when the data were overdispersed (the variance was greater than the mean) a negative binomial distribution was used. The same data were collected both for the impact, and the phenology experiments that included the larval damage over time, the number of swallow-wort stems and seedpods, and thus they were analyzed similarly for the different experiments. Larval damage and seedpod count data were zero-inflated and thus zero-inflation models were used. In addition, seedpod counts were overdispersed and thus were modeled with a negative binomial distribution. For all analyses full models with interactions and null models with main effects only were compared using likelihood ratio tests to select the model with the best fit.

Damage to swallow-wort leaves over time as a function of release numbers was compared by including treatment (number of insects released), date (monitoring dates over the season), and the interaction of treatment and date in the model. To assess the effect of releasing different numbers of *H. opulenta* on swallow-wort stem numbers, treatment, season (early versus late season counts), and year (2019 and 2020) were included as fixed effects in the model. A similar model was used for seedpod numbers by excluding ‘season’ as an effect since those were counted only at the end of the season each year.

For the phenology experiment the sunny and shaded sites were analyzed separately. Seedpod counts at the shaded site were not analyzed as there was only a single seedpod across all treatments and replications. Larval damage as a function of release dates was compared including treatment, monitoring dates, and the interaction of treatment and monitoring dates in the model. The influence of release date of *H. opulenta* on swallow-wort stem numbers was assessed with a model that included treatment and season as fixed effects. Seedpod numbers were compared with treatment as a fixed effect.

Results

Impact experiment

Larval damage by *H. opulenta* to swallow-wort leaves did not differ significantly across the different release sizes overall (treatment: $F_{4,1397} = 0.76$, $p = 0.554$); however, the significant interaction between treatment and monitoring dates (treatment*date: $F_{28,1397} = 4.63$, $p < 0.0001$) revealed that over time plant damage increased with the number of *H. opulenta* released. Larval damage increased as the season progressed (monitoring date: $F_{7,1397} = 14.43$, $p < 0.0001$). Early season damage was similar across the different release size treatments but by week five plots where 5-pairs of *H. opulenta* had been released had significantly higher damage than the control plots (pairwise contrast: p = 0.016). After seven weeks, the 5-pair treatment was significantly different from the 1-pair treatment (pairwise contrast: p = 0.035), and at the end of the season, 17 September 2019, the damage in the 5-pair treatment was significantly higher than each of the other treatments (pairwise contrast 1 vs. 5-pairs: p = 0.031, 2 vs. 5-pairs p = 0.048) (Fig. 2.2).

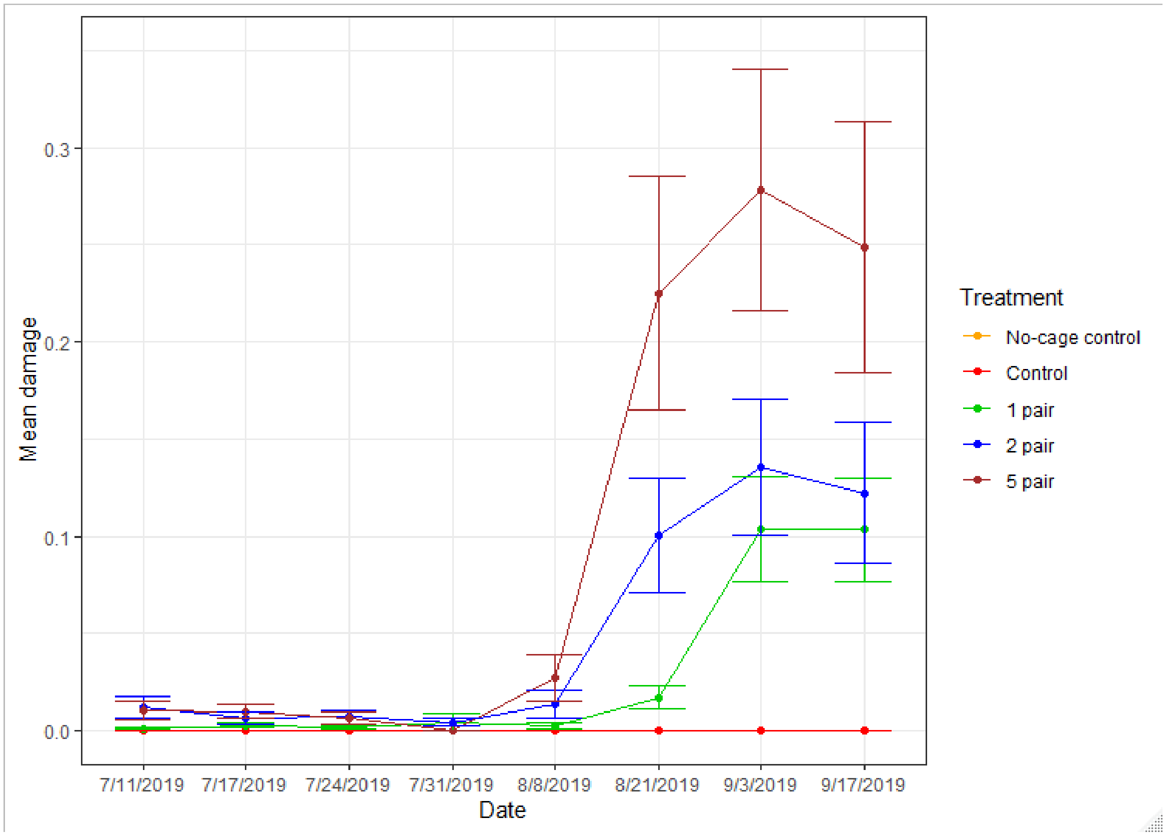

Figure 2.2: Proportion of black swallow-wort plants defoliated (non-model means ± SE) in common garden plots in southern Michigan between July and September where 1, 2, or 5 pairs of *H. opulenta* adults were released or no releases took place (no-cage control and control plots).

First generation adult moths were detected in three plots during the 3rd and 4th weeks of July 2019, in two of the 5-pair and one of the 2-pair treatments. However, adults likely emerged in more plots given that increasing larval damage was observed in eight plots during August and September. Few larvae were observed during the first generation but in the second generation larvae numbers were higher and there were significantly more larvae in the 5-pair treatment than in the 2 pair treatment (pairwise comparison: p = 0.018) but there were not significantly more larvae in the 5-pair treatment than the 1-pair treatment (pairwise comparison: p = 0.186). In 2020, no adult emergence was found and no larval damage.

The number of swallow-wort stems was not affected by the number of *H. opulenta* released in the plots (treatment: $F_{4,63} = 0.92$, p = 0.460) and there were no differences in stem numbers between the years 2019 and 2020 (year: $F_{1,63} = 1.45$, p = 0.234). Stem numbers declined from the beginning to the end of the season regardless of *H. opulenta* release size (season: $F_{1,63} = 12.73$, p = 0.0007) (Fig. 2.3). In 2019, at the start of the experiment mean swallow-wort stem numbers were 44.9 (± 3.1 SE), which declined to 40.2 (± 3.3) per plot by the end of 2019 (Fig. 2.3). In 2020, mean stem numbers early in the season were 43.6 (± 3.1) and declined to 38.1 (± 2.7) by the end of the season (Fig. 2.3). Seedpod numbers were similar across the treatments ($F_{4,351} = 0.01$, p = 0.983). Plants produced overall more seeds in 2020 (2.87 ± 0.2) than in 2019 (1.89 ± 0.16) (year: $F_{1,351} = 0.01$, p = 0.983), but it was not correlated with the amount of larval feeding in the previous year (the model without interaction was more parsimonious).

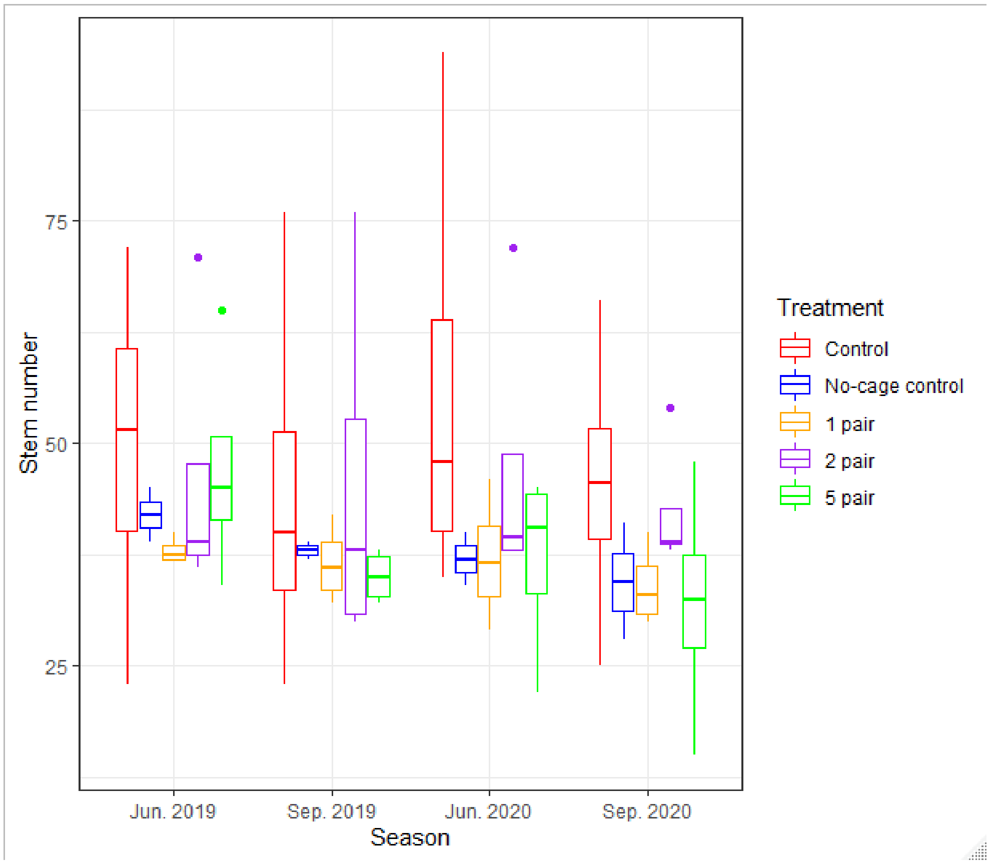

Figure 2.3: Mean black swallow-wort stem numbers recorded at the beginning and end of the season in 2019 and 2020 in common garden plots where 1, 2, or 5 pairs of *H. opulenta* adults were released or no releases took place (no-cage control and control plots). Dots indicate outlier observations, the horizontal line indicates the median with the box representing the interquartile range and vertical lines are 1.5 times the interquartile range.

Phenology experiment

At the shaded site larval damage differed between the different release dates (treatment: $F_{2,698} = 32.84$, $p < 0.0001$) and it increased over time (monitoring date: $F_{5,698} = 22.14$, $p < 0.0001$). As the season progressed there was higher damage in plots where adult *H. opulenta* was released on 25 June than on 17

July (treatment*monitoring date interaction: $F_{10,698} = 41.41$, $p < 0.0001$) (Fig. 2.4a). Stem numbers were not affected by the release date of *H. opulenta* (treatment: $F_{2,24} = 1.78$, $p = 0.190$), and remained the same during the season (65.9 ± 3.66) (season: $F_{1,24} = 0.004$, $p = 0.975$). Very few plants produced seeds at the shaded site in general, and only one within the experimental plots.

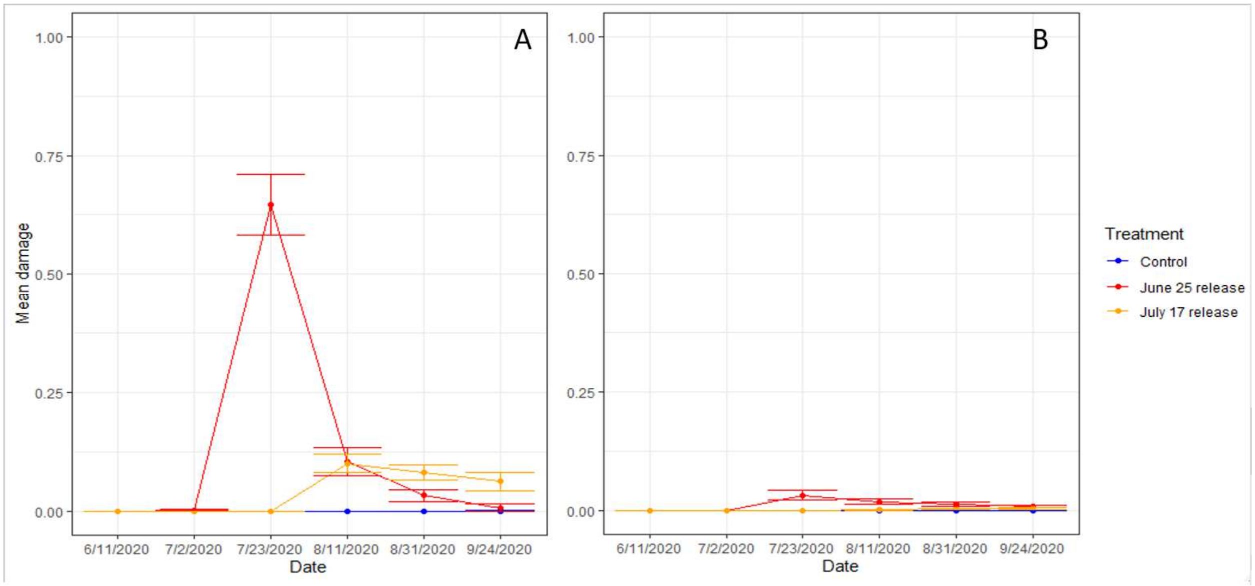

Figure 2.4. Proportion of black swallow-wort plants defoliated (non-model means ± SE) at a shaded (A) and at a sunny (B) site by *H. opulenta* adults released either on 25 June or 17 July 2019.

At the sunny site there was very little larval feeding damage on the plants regardless of when *H. opulenta* was released (treatment: $F_{2,699} = 0.34$, $p = 0.710$). Damage increased somewhat over time (monitoring date: $F_{5,698} = 22.14$, $p < 0.0001$) with larvae from the 25 June releases feeding more than larvae from the 17 July release (treatment*monitoring date interaction: $F_{10,699} = 10.74$, $p < 0.0001$) (Fig. 2.4b). Stem numbers did not differ between the treatments (treatment: $F_{2,26} = 0.07$, $p = 0.935$) or change over the season (season: $F_{1,26} = 0.45$, $p = 0.509$). Treatment did not affect seedpod numbers (treatment: F2,114 = 0.47, p = 0.629) and overall few seedpods were produced (2.4 ± 0.1).

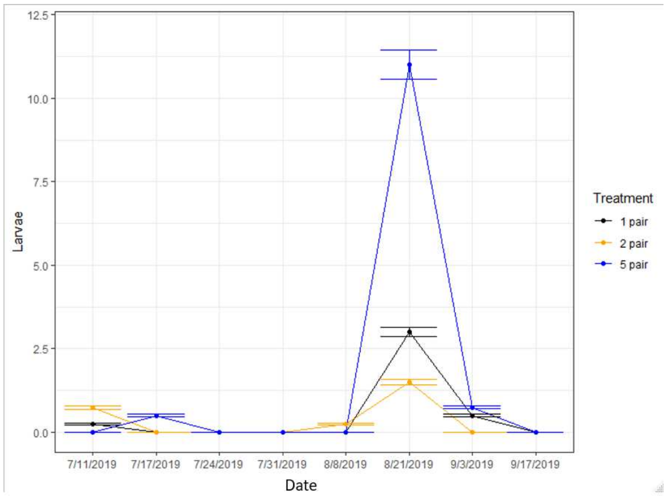

Figure 2.5. Mean larval densities (non-model mean ± SE) of *H. opulenta* on black swallow-wort in common garden plots in southern Michigan between July and September 2019 from releases of 1, 2, or 5 pairs of *H. opulenta* adults on 28 June 2019.

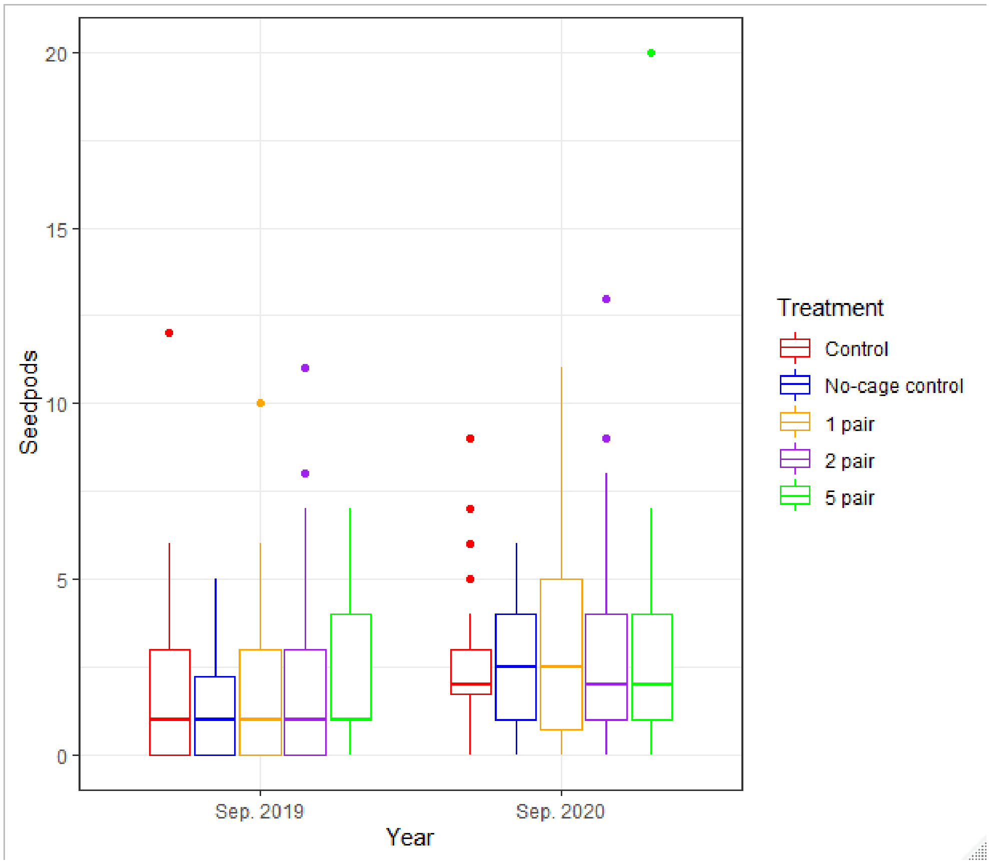

Figure 2.6. Mean number of seedpods recorded for the impact experiment at the end of the season in 2019 and 2020. Dots indicate outlier observations, the horizontal line indicates the median with the box representing the interquartile range and vertical lines are 1.5 times the interquartile range.

Discussion

This study examined the impact that the biological control agent, *H. opulenta,* may have on black swallow-wort, and its phenology in southern Michigan. Through manipulation of the release size and the date of releases we aimed to determine the best ways to release *H. opulenta* to ensure establishment and to maximize their impact on swallow-wort populations. We found that *H. opulenta* can be bivoltine in southern Michigan since in two separate common garden experiments first generation adults emerged in July and second generation larvae were present during August. However, overwintering success could not be confirmed during the course of this 2-year study. Damage caused by one season of larval feeding did not affect swallow-wort fitness at any larval densities.

To test how establishment success may be impacted by release size and to achieve different levels of larval densities in our experimental plots we released 1, 2, or 5 pairs of *H. opulenta* adults. Given that a single female can lay on average 410 eggs (Weed and Casagrande 2010), and that there were on average 45 stems in each of our plots we expected to achieve larval densities ranging from 9 - 45 larvae per stem. In contrast, larvae densities in our plots in 2019 remained low and ranged from 0-12 for the single pair releases to 0-39 for the 5-pair releases (Fig. 2.5). Similarly, in our phenology experiment in 2020 larval damage rates at the sunny site remained below 13% in any individual plot indicating low larval densities, which were largely undetectable despite releasing 4-5 pre-mated females per cage (Fig. 2.4b). These findings indicate that fecundity measured in the laboratory may not be a good predictor of oviposition rates in the field. Alternatively, there may be significant mortality of early stages of *H. opulenta* caused by abiotic and/or biotic factors. This could mean that *H. opulenta* population growth may be slower in the field than would be predicted based on the high fecundity of individual females. In Ontario, 4-5 years following the release of about 2000 larvae in 2013 and 2014, and over 200 adults in 2014 and 2015, larval damage rates were still under 1% overall, indicating relatively slow population growth despite completing two generations each year (Bourchier et al. 2019). Our plots to test the effect of release size were located in the sun, however, the screenhouse used to cover the plots provided some shade since it had black netting on all four sides and a dark polyester top. It is possible that the shading provided by this cage still allowed more sunlight through than a site would get that is in partial shade or under a tree canopy. *Hypena opulenta* was collected from a forested site in Ukraine and it has been hypothesized that they may perform better in shaded environments (Weed and Gassman 2006, Weed and Casagrande 2010). In agreement with this, we found up to 100% larval feeding damage in one plot at the shaded site in the phenology experiment and an average of up to 64.6% larval feeding damage across all replications within a treatment, which is the highest compared to all other experiments that were located in the sun. However, larval numbers at the shaded site were still magnitudes lower than expected based on the fecundity of females. In Ontario, the highest concentration of larvae was also found at the most shaded location providing further support to the hypothesis that *H. opulenta* prefer shade (Bourchier et al. 2019).

As expected, larval damage increased with higher release size but only later in the season (Fig. 2.2). Few larvae were seen, and damage was very low early in the season during the first generation with larvae and their feeding becoming visible mostly in the second generation in August. These findings align with results from the Ontario field release where first generation larvae were not detected until two years following *H. opulenta* releases, while second generation larvae were observed within the same year of releases (Bourchier et al. 2019). The detection of second generation larvae in our plots confirm expectations that *H. opulenta* should be bivoltine at most planned release sites in North America (Weed and Casagrande 2010, Bourchier et al. 2019, Jones et al. 2020). However, it is unclear if the second generation of larvae was able to complete their development in Michigan since we did not detect adult emergence from any of the plots in the following spring, and thus cannot confirm overwintering success. Even though the question of whether higher release size increase establishment success cannot be answered with this experiment, the higher larval densities and damage in plots with higher release sizes align with results from previous studies that show that population growth positively correlates with release size (Grevstad 1999, Memmott et al. 2005). There are also examples of a single female *Galerucella* beetle (Grevstad 1999) and two, four, or ten psyllids (*Arytainilla spartiophila*) founding populations that persisted for multiple years (Memmott et al. 2005). Our results indicate that at least five, but ideally more than five female *H. opulenta* should be used for individual releases to increase its establishment success.

The infestation by *Cercospora* leaf spot could have contributed to the lack of establishment in our impact experiment. *Cercospora* species are plant pathogenic fungi that infect a wide variety of plants and can cause yield loss of crops, notably of sugar beet and celery (Weiland and Koch 2004, Crous and Braun 2003, Raid 2004). An isolate of *Cercospora* has even been considered for use as a mycoinsecticide against weedy morning glory species in sugarcane fields (Nechet and Halfeld-Vieria 2019). The presence of this fungus, which causes stunting of the leaves and defoliation, likely decreased the quality of swallow-wort leaves and could have impacted *H. opulenta* development. *Cercospora* infestation of swallow-worts was also observed in Rhode Island and its potential to provide biocontrol of swallow-worts is being explored (L. Tewksbury pers. comm.).

The average feeding damage of 27.6% we saw in the 5-pair release plots in August 2019 is similar to those found in the release quadrats in Ontario, where 21-28% of leaf area was consumed, and in 2018 about 20% of defoliation was observed at a hedgerow locally (Bourchier et al. 2019). In the phenology experiment, at the shaded site the release of five females around the summer solstice resulted in over 64.6% average defoliation (Fig. 2.4). In pre-release studies, evaluating the potential impact of *H. opulenta*, usually higher damage was inflicted on the plants and higher larval densities were used in feeding assays than what is being observed in the field so far (Milbrath and Biazzo 2016, Doubleday and Cappuccino 2011, Milbrath et al. 2016, Weed and Casagrande 2010). For example, Weed and Casagrande (2010) used two to eight larvae per plant, achieving nearly 100% defoliation of pale swallow-wort with four and eight larvae. Milbrath and Biazzo (2016) used three to six larvae per stem resulting in 62-100% defoliation on both black and pale swallow-worts. Artificial defoliation studies removed 50 -100% (Milbrath et al. 2016, Milbrath 2008) and up to 90% of tissue (Doubleday and Cappuccino 2011). These studies often found significant reductions in plant growth (Milbrath and Biazzo 2016), seed production (Doubleday and Cappuccino 2011, Milbrath et al. 2016) or even mortality (Weed and Casagrande 2010) as a result of defoliation. In a field experiment in Ontario, where *H. opulenta* larvae were caged on pale swallow-wort at densities of about 10 larvae per stem in the sun and 5 larvae per stem in shade environments, almost 100% defoliation was seen after three weeks of feeding in the shade and over 75% defoliation in the sun (Livingstone et al. 2020). In this study, the high levels of defoliation resulted in increased seed production in the shade sites and had no effect on seed production in the sun environments (Livingstone et al. 2020). In our common garden field experiments, where damage levels were closer to those seen in open field releases of *H. opulenta*, there was no change in the fitness of black swallow-wort in terms of stem and seedpod numbers within the season or the following year (Fig. 2.6).

Swallow-worts appear to be quite tolerant to defoliation, especially when growing in high-light conditions (Milbrath and Biazzo 2016, Milbrath et al. 2016). To observe any negative fitness effects of folivory usually multiple rounds of defoliation were needed and the plants had to be grown under low light (Milbrath and Biazzo 2016, Milbrath et al. 2016, Milbrath 2008). In shaded environments seed production is low and plant fitness is reduced, which can impede recovery of swallow-worts from defoliation (DiTommaso et al. 2005, Milbrath 2008, Hotchkiss et al. 2008). In accordance with this we found almost no seed production in our plots growing under the forest canopy compared to open fields.

The multiple rounds of defoliation that may result in control could be achieved in Michigan, since both our phenology and impact experiments confirmed that *H. opulenta* can produce two generations within a season when released around the summer solstice (21 June). Jones et al. (2020) predicts that the window within which adults should be released to get two generations a year falls between 29 May and 16 July for East Lansing, Michigan when the daylength is over 15 hours. Jones et al. (2020) also found that 50% of the population enters diapause at 15 h 35 min photoperiod and the proportion of pupae entering diapause increases as daylength decreases (Jones et al. 2020). The daylength was around 15 h 20 min when *H. opulenta* emerged from our lab rearing and was released in the field in both 2019 in the impact experiment and in 2020 as the first treatment of the phenology experiment. Thus, it is likely that more than 50% of first generation pupae produced in the experimental plots went into diapause. This could partly explain the relatively low number of larvae seen during the second generation. Having a large proportion of pupae diapausing relatively early in the season can be maladaptive because they can be exposed to predation and to high summer temperatures for long periods, which increases mortality (Jones et al. 2020). However, diapause induction can evolve relatively quickly and over time *H. opulenta* may adapt to enter diapause later in the season. In *Diorhabda carinulata* beetles released to control invasive *Tamarix* species the critical daylength for diapause induction evolved within five years or 10 generations to synchronize beetle phenology better with the climate during their southward expansion (Bean et al. 2012).

Given the effect of low light on swallow-wort plant fitness, the better performance and higher impact of *H. opulenta* in shaded habitats, and their ability of producing two generations within a season, the emerging consensus is that biological control with *H. opulenta* is more likely to succeed on swallow-wort infestations in forested areas (Milbrath and Biazzo 2016, Milbrath et al. 2016, Weed and Cassagrande 2010). Our results provide support to this prediction as we saw higher defoliation overall in the shaded environment and the production of two generations when releases took place at >15 h daylength. We have not yet detected any reduction in fitness of black swallow-wort plants in our plots, but it is not surprising after just one season of feeding at relatively low larval densities. Since most swallow-wort infestations in Michigan are located within shaded or partially shaded habitats, once *H. opulenta* populations reach higher densities in the field it is more likely that we begin to see the long-term negative impact of repeated defoliation on plant fitness.

Chapter 3: Does the presence of the biological control agent, *Hypena opulenta* on swallow-worts deter monarch oviposition?

Introduction

The arrival of exotic plant species into the habitat of native herbivorous insects can present novel resources (Tong and Shapiro 1989) but may also serve as ecological traps (Casagrande and Dacey 2007). The survival and development of holometabolous insects whose larval stages have limited mobility are dependent on the oviposition decisions of the females since the host plant chosen by the adult will serve as an early food source for the larval stages. Females, however, do not always make optimal oviposition choices and may choose to oviposit on plants which are unsuitable for larval development (Ries and Fagan 2003, Schlaepfer et al. 2005, Casagrande and Dacey 2007, Faldyn et al. 2018). For example, the diamondback moth *(Plutella xylostella)* has been recorded to prefer to lay eggs on *Barbarea vulgaris*, a plant on which its larvae cannot develop (Badenes-Perez et al. 2006), and similar examples are abound in several other Lepidoptera species (Berenbaum 1981, Straatman 1962, Chew 1977). The invasion of North America by garlic mustard *Alliaria petiolata* provides one such example, as the native West Virginia white butterfly (*Pieris virginiensis*) were found to lay more eggs on garlic mustard than on its native mustard host *Cardamine diphylla*, despite it being toxic for their larvae (Davis and Cipollini 2014). These mistaken oviposition choices have likely contributed to the decline of *P. virginiensis* and *P. oleracea* (Davis and Cipollini 2014).

Monarch butterflies may also be threatened by plant species that can act as ecological traps as the density of their native milkweed *(Asclepiadaceae)* host plants decrease due to herbicide use (Thogmartin et al. 2017, Inamine et al. 2016, Pleasants and Oberhauser 2013, Zaya et al. 2017), and as invasive species that are related to milkweeds become more abundant. Two European swallow-wort species, pale and black swallow-wort (*Vincetoxicum rossicum* and *V. nigrum*) that are related to milkweed have become invasive in the midwestern and eastern United States and southeastern Canada (DiTommaso et al. 2005). Also called dog strangling vines, swallow-worts can grow in many of the same places as milkweeds, including in disturbed areas along railway lines, highways and other transportation corridors, in natural areas, as well as in no-till corn and soybean fields and pastures (DiTommaso et al. 2005, Weston et al. 2005, Weed and Casagrande 2010). Therefore, monarchs are often presented with choices between milkweeds and swallow-worts. In Rhode Island, monarchs were found to lay 15-25% of their eggs on swallow-worts (Casagrande and Dacey 2007). However, all monarchs that hatch on swallow-worts will eventually die since the larvae are not adapted to feed on this exotic plant (Casagrande and Dacey 2007, Mattila and Otis 2003). Thus, swallow-worts may act as an oviposition sink to monarchs (Casagrande and Dacey 2007), which might accelerate their decline (Inamine et al. 2016).

The host plant selection of monarchs is influenced by multiple factors besides food quality, such as the experience of females with different plant species and the risk of larval competition or predation (Jones and Agrawal 2019). Jones and Agrawal (2019) found that when monarchs showed an oviposition preference towards a suboptimal milkweed host *Asclepias i. pulchra*, prior oviposition experience on a more suitable milkweed species, *Asclepias i. incarnata* could alter this preference. More importantly, the presence of a monarch larva on the suboptimal host could reverse preference towards that unsuitable plant, likely because monarchs try to avoid cannibalism of their freshly laid eggs and/or larval competition when multiple individuals feed on the same plant (Jones and Agrawal 2019). These results suggest that the presence of another herbivorous larvae on a suboptimal host plant, such as swallow-wort, may act as a deterrent to monarch oviposition.

Given that swallow-worts are not native to North America, few indigenous herbivorous insect species were found to feed on them at very low densities (Milbrath 2010), and thus those are unlikely to deter monarch oviposition. However, a classical biological control agent, a defoliating moth *Hypena opulenta* (Christoph) (Lepidoptera: Eribidae) was approved for field release in 2017 in the U.S. (USDA APHIS, 2017). This moth, originating from the native range of swallow-worts in Ukraine is a specialist on swallow-worts (*Vincetoxicum* spp.) and can feed on both pale and black swallow-wort (Hazelhurst et al 2012). *Hypena opulenta* larvae feed on the foliage of swallow-worts in a similar manner as monarch larvae feed on milkweeds, therefore, their presence may act as a deterrent for monarch oviposition. To test this hypothesis, we conducted a 2-year field study where naturally occurring monarch butterflies in lower Michigan were presented with common milkweed and swallow-wort plants with and without *H. opulenta*. In addition, we evaluated host choice of laboratory reared monarchs between milkweeds and swallow-worts that were either free of or attacked by *H. opulenta*.

Materials and Methods

Field experiments 2019-2020

To assess the preference of monarchs for laying eggs on swallow-wort and to test whether the oviposition choices of females would be influenced by the presence of *H. opulenta* on swallow-wort, open field tests were conducted over two years on the south campus of Michigan State University. In 2019, potted plants representing three treatments: milkweed (control), swallow-wort infested with *H. opulenta* and non-infested swallow-worts were placed at six locations on 8 August 2019 (Fig. 3.1). At each location, the treatments were replicated four times by using four potted plants that were arranged in groups, 2-3 m apart, to mimic small patches of milkweed or swallow-wort.

Figure 3.1: Map of field sites located on the south campus of Michigan State University in East Lansing, Michigan. Sites used in 2019 are shown in red (1-6). In 2020, all sites from 2019 were reused and nine extra sites were added (shown in yellow 7-15).

For the milkweed control, common milkweed (*Asclepias syriaca*) was used that had been grown from seed at the Wildtype Native Plant Nursery in Mason, MI, USA in spring 2019. Plants were transplanted into 11 L plastic pots in June 2019. For the swallow-wort treatments, pale swallow-wort plants were used that had been collected from an infestation in Holly, MI, USA (N 42°84'62 – W 83°41'28) in July 2019 and transplanted to either 7.5- or 19 L plastic pots. Milkweed treatments had one or two stems, and swallow-wort treatments had two to four stems per pot. For the experiments, two of the 7.5 L and two of the 19 L pots were used at each site for each swallow-wort treatment. The biological control agent, *H. opulenta,* was reared in the laboratory on black swallow-wort. For the treatment of swallow-wort with *H. opulenta*, groups of four plants were infested with eight 3rd to 5th instar *H. opulenta* larvae and 12 adults in 61 cm x 61 cm x 142 cm mesh cages (Bioquip®) for 48 hours. After infestation, the adults were removed, and eggs were confirmed to be present on at least one of the plants from each of the cages prior to being set out in the field. Larvae remained on the plants to feed throughout the experiment.

Plants were set out in the field on 9 August and monitored daily between 4-7 pm for monarch eggs until 22 August 2019. The number of eggs found were recorded and removed from the plants so they would not discourage future monarch oviposition. Sites were located on south campus in a range of environments including along forest edges, agricultural fields, experimental orchards, and near open fields. At each field site milkweeds and egg-laying monarchs were present, which was confirmed by checking naturally growing milkweeds for monarch eggs.

In 2020, this field experiment was repeated with some changes to the experimental design. The number of field sites was increased from six to fifteen (Fig. 3.1), and at each field site the replication of plants for each treatment was reduced from four to two. These changes were implemented to increase exposure of the different treatments to monarchs, and to potentially increase the power for statistical analyses.

Potted milkweed plants that over-wintered outside were reused in 2020. Half of the swallow-wort plants were reused and new plants were collected in 2020 to replace the other half. For the swallow-wort treatments equal numbers of plants were used from 2019 and 2020. Plants for the swallow-wort with *H. opulenta* treatment were infested with larvae and adults similarly as described for 2019. To increase the apparent feeding damage to the plants, larvae and adults were left on for 15-20 days, until each swallow-wort was at least 50% eaten. Because the availability of larvae was delayed, plants infested with *H. opulenta* were moved to the field later than the milkweed control and non-infested swallow-wort treatments.

Milkweed plants and non-infested swallow-wort plants were set out to the field on 6 August and swallow-wort plants infested with *H. opulenta* were set out on 15 August 2020. The presence of monarch eggs was monitored daily until 29 August 2020. All eggs found were removed.

Laboratory experiment

To test the affinity of monarchs to lay eggs on swallow-wort and to assess how the presence of *H. opulenta* on swallow-wort may influence monarch oviposition, choice and no-choice experiments were conducted in the laboratory in 2019. In the no-choice experiments, 2 females and 1 male monarchs were provided either with one milkweed or with one swallow-wort plant. In the choice experiments, 2 females and 1 male monarchs were caged with one milkweed and one swallow-wort plant or with one milkweed and one swallow-wort infested by *H. opulenta*. Each of the above four treatments were replicated four times with the experimental cages arranged in a completely randomized design.

In the no-choice experiments one pea plant was also placed within each replication. This served to test for potential egg dumping, following the protocols of Casagrande and Dacey (2007), that may happen when monarchs are restricted in their oviposition choices. Plants for the choice tests where swallow-worts had to be infested by *H. opulenta* were prepared by placing 16 3rd to 5th instar larvae and

12 adults on groups of four plants in a 60 cm x 60 cm x 60 cm domed mesh cage (Bioquip®) for 24-hours. There were eggs and at least two *H. opulenta* larvae on all swallow-wort plants used for this treatment. Milkweeds were potted in 11 L plastic pots and swallow-wort plants in 1.3 L plastic pots. Monarch butterflies used for the experiment originated from a colony at MSU that was established using monarch eggs collected in the area in May 2019. Monarchs were reared for two generations on common milkweed prior to the start of the experiment. For the rearing, larvae were fed cut milkweed stems in 42 cm x 33 cm x 17 cm plastic containers that were placed on bench tops under natural daylight conditions in a laboratory that was kept at ambient temperatures. Pupae were moved to mesh mating cages (75 cm x 75 cm x 115 cm) and emerging adults were supplied with Gatorade® via a cotton wick inserted into a 60 ml cup. Adults were approximately 14 days old at the start of experiments.

The experiment was conducted between 16-23 August 2019 by checking all plants daily for monarch eggs. Every day, monarchs that died were removed and replaced with a living monarch of the same sex. Eggs were counted and removed each morning.

Statistical Analyses

All analyses were conducted in R version 3.6.1 (R Core Team, 2019) using the "emmeans" package version 1.5.2-1 (Lenth, 2020). Monarch eggs recorded at each site were summed across treatments, and sites where no monarch eggs were recorded were excluded from analyses (Tab. 2.1). To assess whether plant treatment (milkweed, swallow-wort, or swallow-wort with *H. opulenta*) was a significant predictor of monarch oviposition a generalized linear model with a Poisson distribution was used. The full model containing treatment and site as explanatory variables and a null model with only site as a fixed effect were compared with a likelihood ratio test to assess if plant treatment had a significant effect. The number of monarch eggs laid on the different plant treatments were compared using the method of least squares means with treatment and site as fixed effects in the 'emmeans' package. Data for 2019 and 2020 were analyzed separately. Pairwise comparisons for treatment were examined for 2019. For 2020, no monarch eggs were laid after the inclusion of the swallow-wort with *H. opulenta* treatment, so pairwise comparisons for treatment were not conducted. Data for the laboratory experiment was not analyzed since all monarch eggs were laid on milkweeds only.

Site	Year	Number of eggs
1	2019	11
2	2019	6
3	2019	2
4	2019	7
5	2019	0
6	2019	2
1	2020	2
2	2020	3
3	2020	2
4	2020	5
5	2020	0
6	2020	4
7	2020	0
8	2020	2
9	2020	0
10	2020	2
11	2020	0
12	2020	5
13	2020	11
14	2020	0
15	2020	7

Table 2.1. The sites used in 2019 and 2020 and the sum number of monarch eggs recorded at each. Where 0 eggs were recorded, sites were excluded from analyses.

Results

Field experiments 2019 and 2020

Monarchs laid significantly more eggs on milkweeds than on swallow-worts either with or without *H. opulenta* both in 2019 ($F_2 = 8.403$; p = 0.0002) and in 2020 ($F_1 = 13.645$; p = 0.0002) (Fig. 3.2). There were no differences in the number of eggs laid at the different sites in 2019 or in 2020 (2019: $F_4 = 2.16$, p = 0.071), 2020: $F_9 = 1.69$, p = 0.083). The models that included plant treatment predicted the data better than without treatment in both years (2019: p = 3.588e-05, 2020: p = 1.452e-17). In total, 21 eggs were laid on milkweeds, 4 on swallow-worts without *H. opulenta*, and 3 on swallow-worts with *H. opulenta* across all field sites in 2019 (Fig. 3.3). There was no difference in the number of eggs laid on swallow-worts without and with *H. opulenta* present (pairwise contrast: p = 0.925). The four eggs laid on swallow-wort without *H. opulenta* constitute 14.3%, and the three eggs laid on swallow-wort with *H. opulenta* constitute 10.7% of the total 28 eggs laid on experimental plants in 2019. In 2020, 42 eggs were laid on milkweed, one egg on swallow-wort without *H. opulenta* (2.3% of all eggs) and no eggs were laid on swallow-wort with *H. opulenta* (Figure 3.2). The number of eggs laid on swallow-wort without *H. opulenta* was significantly lower than on milkweed (pairwise contrast: p =0.0002). Considering that all monarch eggs were laid prior to August 15 when the swallow-wort plants infested with *H. opulenta* were placed to the field, differences between swallow-worts with and without *H. opulenta* were not investigated in 2020.

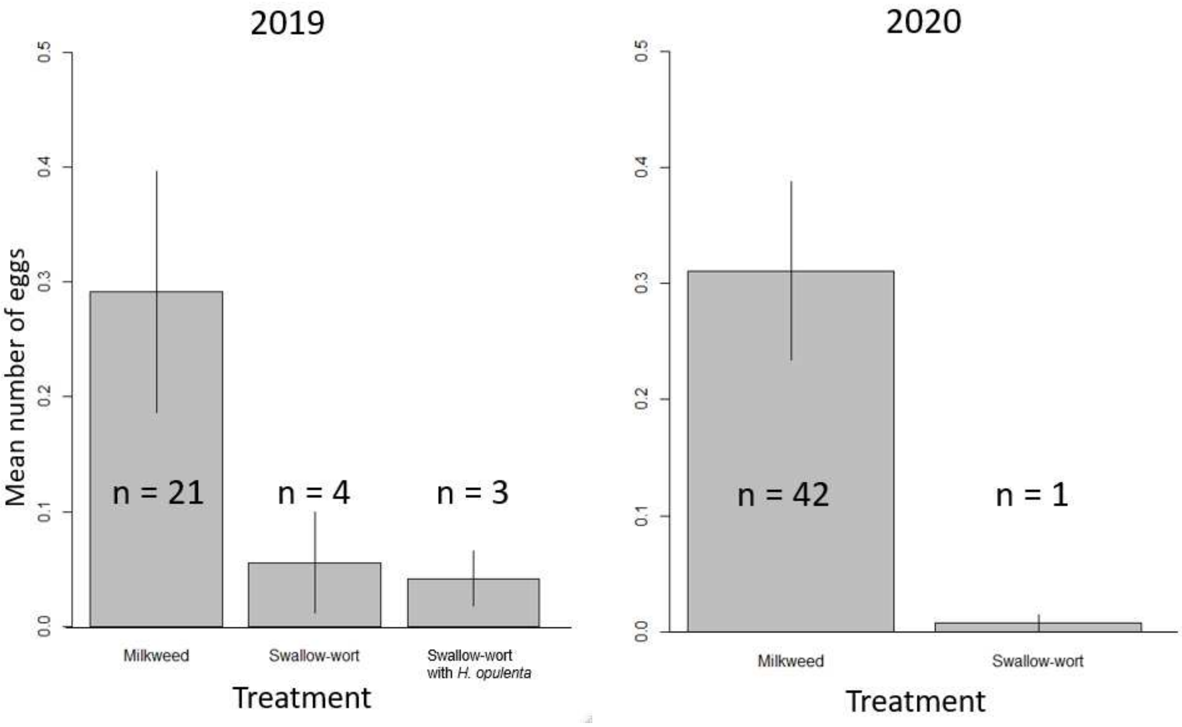

Figure 3.2. Number of monarch butterfly eggs laid (non-model mean ± SE) in 2019 (left) and in 2020 (right) on experimental milkweed plants and on swallow-worts that were either infested or not with *H. opulenta*. 'n' indicates the total number of eggs found on each treatment.

Laboratory experiment

A total of 1315 monarch eggs were laid on milkweeds over the course of this experiment with a daily average of 13.7 per plant. No eggs were laid on any swallow-wort plants regardless of whether *H. opulenta* was present or not. No eggs were laid on the pea control plants either.

Discussion

Our field experiments showed that monarchs strongly prefer milkweeds over swallow-worts, nevertheless they still laid up to 14% of their eggs on swallow-worts. We did not find evidence that the presence of the biological control agent, *H. opulenta* on swallow-worts would deter oviposition by monarch butterflies. Our field experiments utilized monarchs naturally present in the area exhibiting their innate host searching and host acceptance behaviors, and thus provide a realistic assessment of their oviposition choices. In 2019, monarchs laid 14.3% of their eggs on swallow-wort plants that were free of heterospecific herbivores and a similar portion, 10.7% of eggs on swallow-worts with *H. opulenta* feeding and larvae present. These results suggest that the presence of the foreign *H. opulenta* that monarchs have not previously encountered may not elicit the same response as the presence of a conspecific larva, which was found to dissuade monarch oviposition on milkweeds (Jones and Agrawal 2019).

Interspecific competition is known to be important in shaping species interactions among phytophagous insects (Kaplan and Denno 2007, Denno et al. 1995), and the likelihood of competition deterring oviposition has been well documented in a range of insects, particularly in Lepidopterans (Vasconcellos-Neto and Monterio 1993, Honda 1995, Hilker et al. 2008, Schonhoven, Jones and Agrawal 2019). For example, *Pieris* butterflies mark their eggs with pheromones (Schoonhoven 1990) and the confused tigerwing butterfly, *Mechanitis lysimnia* will adjust the number of eggs laid based on plant size and the presence of conspecifics to avoid intraspecific competition (Vasconcellos-Neto and Monterio 1993). The presence of conspecific or heterospecific larvae may also act as a deterrent by leaving feeding marks and frass on the plants (Ditrick et al. 1983, Mitchell and Heath 1985, Renwick and Radke 1980, Hilker and Klein 1989, Nufio and Papaj 2001). In our experiment, the swallow-wort plants were infested by both *H. opulenta* larvae and egg laying adults, and thus had visible feeding marks and both early and later instar larvae ($n < 5$) when they were presented to monarchs in the field. Despite these cues indicating the presence of a possible competitor monarchs still laid similar number of eggs on these plants than on non-attacked swallow-wort plants. In 2020, we aimed to increase the visible feeding damage and the number of larvae on the swallow-wort plants compared to 2019 levels by increasing the feeding and oviposition period of *H. opulenta* from 2 days to 15-20 days. Unfortunately, by the time the higher level of infestation was achieved, monarch oviposition ceased in the field and we were unable to test if it would have had greater deterrence (Fig. 3.3).

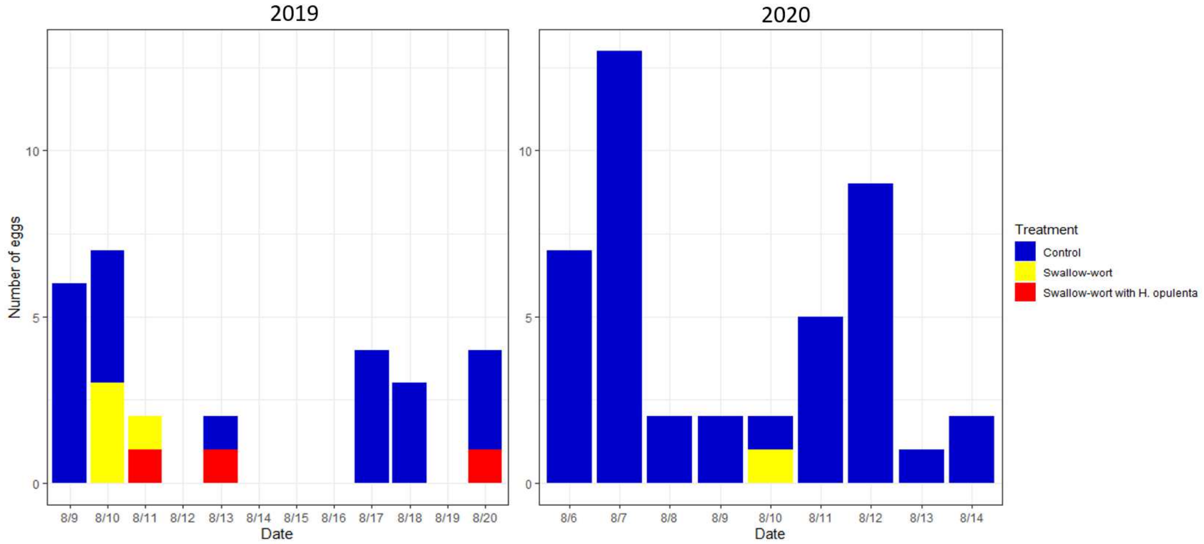

Figure 3.3. Number of eggs laid daily by monarch butterflies on milkweed, swallow-wort and swallow-wort plants infested with the biocontrol agent *H. opulenta* in 2019 (left) and 2020 (right) in an open field experiment.

Our results, with regards to monarch oviposition on swallow-worts under natural conditions, corroborate findings from Rhode Island where 0-15.4% of monarch eggs were found on swallow-worts in field sites where both milkweeds and swallow-worts grew in mixed stands (Casagrande and Dacey 2007). Both in our study in Michigan and in the surveys in Rhode Island, monarchs showed a clear preference for milkweed, yet in some years still laid a non-trivial portion of their eggs on swallow-worts, especially at sites where milkweed densities were relatively low and swallow-wort densities were high (Casagrande and Dacey 2007). At our study sites in East Lansing in Michigan the reverse was true, with swallow-worts being rare and milkweeds being more common in the area where the experiments took place. Despite the higher availability of milkweeds relative to swallow-worts, monarchs still laid up to 14.3% (total 25% between the two swallow-wort treatments) in 2019 and 2.3% of their eggs in 2020 on swallow-worts. Annual variation in monarch egg counts on swallow-worts is expected as there can be natural variation in monarch densities (Casagrande and Dacey 2007).

It is possible that our experiment underestimated the proportion of monarch eggs that may be laid on swallow-worts. The experiment took place late in the season, in August, when naturally growing milkweed plants in the area start to senescence. Our potted milkweeds were younger, and thus likely attracted higher rates of monarch oviposition compared to the naturally growing older milkweeds (Haan and Landis 2019). This could have inflated the number of monarch eggs found on our experimental milkweed plants and skewed the proportion of eggs found on swallow-worts.

Monarchs laid all their eggs on milkweeds and none on swallow-worts in our choice and no-choice laboratory experiments regardless of whether *H. opulenta* was present or not. These results align well with those of DiTommaso and Losey (2003) who also did not find oviposition on swallow-worts in cage choice test. In contrast, monarchs were shown to lay 0.9% of their eggs on swallow-worts in a choice test (Mattila and Otis 2003), and 20.3% in a no-choice and 21.7% in a choice laboratory experiment (Casagrande and Dacey 2007). The reason for this discrepancy likely lies in the source of monarchs used in the different experiments. Casagrande and Dacey (2007) pointed out that experiments that used wild caught monarchs found oviposition and those where monarchs were reared in the laboratory did not find oviposition on swallow-worts. Monarchs can alter their oviposition choices based on experience, and prior exposure to an alternative host can increase the likelihood of accepting this host for oviposition in the future (Jones and Agrawal 2019). The monarchs we used had been reared in the laboratory for multiple generations on common milkweed, without exposure to alternate host or non-host plants, therefore the lack of oviposition on swallow-worts in this experiment is not surprising. It seems clear now from all the above experiments that the source of monarchs can change the outcome of tests and that only field collected individuals should be used in future studies evaluating their oviposition choices.

Our results provide additional evidence that swallow-worts may serve as oviposition sinks for monarchs (Casagrande and Dacey 2007). In addition, we found that the presence of a potential competitor in the form of the foliage feeder biological control agent *H. opulenta* may not be enough to deter monarchs from laying eggs on this non-host plant. However, biological control of swallow-worts is still in the early stages in North America, with *H. opulenta* establishment confirmed only at a few locations in Canada (Bourchier et al. 2019, Livingstone et al. 2020). As *H. opulenta* populations expand geographically and grow in density with increasing feeding damage and exposure to monarchs the interactions between these species may change over time.

SUMMARY

As invasive swallow-worts spread through the eastern and midwestern United States and southern Canada, they create dense monocultures and reduce native biodiversity. One of their most notable ecological impact is that swallow-worts may serve as oviposition sinks for monarch butterflies given their close relatedness to milkweeds, the primary hosts of monarchs. Controlling swallow-worts through mechanical or chemical means is often costly and labor intensive, but using biological control could provide a long-term, cost-effective solution. *Hypena opulenta* is a specialist herbivore which feeds indiscriminately on both black and pale swallow-wort. Laboratory and greenhouse studies examining the potential of *H. opulenta* to provide control to swallow-wort have found *H. opulenta* can cause high levels of damage to swallow-wort (Weed and Casagrande 2010) and can cause swallow-wort mortality under certain conditions, especially with multiple rounds of defoliation and in shaded environments (Milbrath and Biazzo, 2016). However, results found in the laboratory are not always repeatable in the field, thus it is necessary to assess *H. opulenta* performance in field settings to determine the true potential of *H. opulenta* to control swallow-wort. The experiments conducted in this thesis aimed to determine the impact that could be expected from *H. opulenta* in southern Michigan on invasive swallow-worts and how the introduction of this biocontrol agent may influence oviposition choices of the monarch butterfly.

To determine the impact that *H. opulenta* can have on swallow-wort in southern Michigan, different densities of *H. opulenta* adults were released into field cages with transplanted swallow-wort. Larger release sizes correlated with higher levels of feeding damage to the plants, however measurements of swallow-wort fitness were not impacted significantly by the release sizes. This experiment confirmed the ability of *H. opulenta* to be bivoltine in Michigan, as first generation adults were observed in three plots approximately three weeks after the initial release. In other plots, levels of larval damage increased late in the season and larvae were observed late in the season which indicates a second generation of *H. opulenta* was present in more plots than the cages where first generation adults were observed. While *H.*

opulenta was able to complete two generations in the first season, we were unable to confirm *H. opulenta* establishment as adults were not observed the following season.

By changing the date of release of *H. opulenta* we aimed to determine the optimal time for release in Michigan to increase the chances of *H. opulenta* having multiple generations. Releases that took place around the summer solstice (June 20) resulted in a successful second generation of *H. opulenta,* while releases that occurred after the solstice resulted in *H. opulenta* entering diapause. Previous work examining *H. opulenta* diapause induction has found that 50% of the population enter diapause around 15 h 35 min daylight conditions and the percent which enter diapause increases as daylength decreases. Around the summer solstice in southern Michigan daylength is around 15 h 20 min which suggests that many of the *H. opulenta* released may have entered diapause and future releases in southern Michigan should occur earlier in the season.

The indirect effects of introducing *H. opulenta* to control swallow-wort on the monarch butterfly were also examined. Swallow-worts are closely related to milkweeds, and although monarch larvae are unable to develop on swallow-wort, previous research has indicated that they may lay 15-25% of their eggs on swallow-wort (Casagrande and Dacey, 2007). We examined whether the presence of *H. opulenta* larvae feeding on swallow-wort would serve as an oviposition deterrent to monarchs. While there was no evidence from our field experiments that the presence of *H. opulenta* on swallow-wort deterred monarch oviposition, monarchs did choose swallow-wort as a host under field conditions. This supports the findings of Casagrande and Dacey (2007) that swallow-wort may serve as an oviposition sink. No monarch eggs were laid on swallow-wort during the laboratory experiment. While the laboratory experiment did not aid in our understanding of whether *H. opulenta* larvae deters oviposition, it is likely that the source of the monarchs used in the laboratory experiments would alter the results, and future monarch laboratory experiments should consider whether monarchs have been laboratory reared or caught in the wild.

The experiments conducted in this thesis are important as they examine *H. opulenta* in the field through manipulative experiments. Field experiments with *H. opulenta* are necessary to supplement the laboratory and greenhouse studies done to date, and to gain a better understanding of the true potential of *H. opulenta* as a biological control agent as well as how to release *H. opulenta* to maximize its efficiency. Often it takes upwards of 20 years to determine whether biological control agents are successful at providing adequate control to an invasive species (McFayden, 2000). We were able to determine that *H. opulenta* can be bivoltine in southern Michigan if released prior to or around the summer solstice. While we were unable to confirm *H. opulenta* establishment or detect if larval damage had any significant impact on swallow-wort fitness, the findings of this study will be important in guiding future releases in Michigan.

APPENDIX

APPENDIX 1

RECORD OF DEPOSITION OF VOUCHER SPECIMENS

The specimens listed below have been deposited in the named museum as samples of those species or other taxa, which were used in this research. Voucher recognition labels bearing the voucher number have been attached or included in fluid preserved specimens.

Voucher Number: 2021-03

Author and Title of thesis: B. Alred
Exploring the potential of the biological control agent *Hypena opulenta* (Christoph) (Lepidopetera: Erebidae) at controlling invasive swallow-wort vines in Michigan and its indirect effects on monarch butterflies

Museum(s) where deposited:
Albert J. Cook Arthropod Research Collection, Michigan State University (MSU)

Specimens:

Family	Genus-Species	Life Stage	Quantity	Preservation
Erebidae	*Hypena opulenta*	adult	10	pinned

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