

EVOLUTIONARY AND APPLIED APPROACHES FOR THE MANAGEMENT OF
INVASIVE SWALLOW-WORT VINES (*VINCETOXICUM SPP.*): BIOLOGICAL CONTROL,
ECOSYSTEM RESTORATION AND CONSEQUENCES FOR MONARCH BUTTERFLIES
(*DANAUS PLEXIPPUS*)

By

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ABSTRACT

Classical weed biological control uses natural enemies that have co-evolved with their host plants in their native range and provides a sustainable and effective strategy for the long-term management of invasive species. However, its successful implementation encounters several challenges that must be addressed to maximize efficacy. Biological control agents, often reared extensively in quarantine, face issues like adaptation to culture, reduced genetic diversity and inbreeding, which can affect their establishment and efficacy. Upon implementation of biological control, site restoration may be limited without the proper integration of supplemental restoration techniques. Restoration techniques combined with biological control are not extensively studied, limiting their widespread adoption and application. Furthermore, the impact of invasive species to native species of concern is important to optimize management plans. This dissertation investigates the integration of applied and evolutionary approaches in the control of invasive swallow-wort vines (*Vincetoxicum spp.*), emphasizing the importance of genetic background when employing the biocontrol agent *Hypena opulenta*, the restoration of invaded forests, and the implications for the monarch butterfly (*Danaus plexippus*). In Chapter 1, I research the importance of genetic background by creating an outbred population by crossing a long-term laboratory-reared population with individuals from an-established population of *H. opulenta* in the field. These populations are used to understand the role of intraspecific outcrossing in enhancing the fitness and neutral genetic diversity of *Hypena opulenta* with laboratory and field experiments alongside molecular analysis. The results show that increases in neutral genetic variation can translate to tangible fitness benefits in the outbred population measured as increases in production of pupae in the laboratory, and larval feeding rates and second-generation adult production under field conditions. In Chapter 2, I research how biological control, manual removal of swallow-wort, and native seeding, alone and combined, can impact restoration success. The results show that when native seeding is combined with biological control, swallow-wort cover is reduced, and species richness is enhanced. In Chapter 3, I investigate the effects of genetic background and demography in the establishment success of *Hypena*

opulenta by conducting experimental field releases. However, I was not able to understand this relationship because of establishment failure. In repeat releases two years later, where genetic background was the sole focus, the results mirrored patterns seen in the field cage experiments from Chapter 1 with the outbred population eating more plant tissue and producing more adults compared to lower genetic diversity laboratory reared and field collected populations. Finally, in Chapter 4, I explore the oviposition choices of the monarch butterfly in Michigan. I determine the frequency with which monarch butterflies mistakenly lay eggs on swallow-worts under natural field conditions and use laboratory experiments to uncover the underlying mechanisms of their oviposition decisions, aiming to clarify the factors influencing monarch egg-laying behavior. Field results showed that monarchs can lay up to 17% of their eggs on swallow-wort locally, and about 8% overall. Despite oviposition failure in the lab experiment, monarch larvae exhibited 16% mortality in the lab directly attributed to feeding on swallow-wort. This dissertation contributes to the discourse on ecological restoration, biological control, and provides insights for future conservation and management endeavors in ecosystems threatened by invasive species.

*This dissertation is dedicated to my family, pets, and friends.
Thank you for always being there for me.*

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CHAPTER 1:

Intraspecific hybridization improves performance of the biological control agent *Hypena opulenta*

Abstract

Evolutionary processes during species introduction are crucial for understanding population establishment and persistence. Intraspecific hybridization can lead to varied fitness outcomes depending on the genetic distance between parental populations. The link between neutral genetic diversity and fitness in the literature is still uncertain, making it difficult to predict the outcomes of intraspecific hybridization. We investigated the role of intraspecific outcrossing, in enhancing the fitness and genetic diversity of *Hypena opulenta*, a biological control agent used against invasive swallow-wort vines in North America. We created an outbred population by crossing a long-term laboratory-reared population with a field-established population that was derived from the same source as the laboratory population 12 generations prior. The fitness of parental and outbred populations was tested in the laboratory by comparing the number of pupae produced by individual females. In the field the levels of defoliation and second-generation adult emergence by groups of five females was compared. Whole Genome Sequencing (WGS) was conducted on 15-20 individuals from the parental and hybrid populations. Outbreeding increased fitness both under laboratory and field conditions with the outbred females producing the most pupae in the laboratory, the highest defoliation rates of the plants, and the most second-generation adults in the field. Outbreeding increased genome-wide heterozygosity, and despite how recently the parental populations were isolated from each other there was clear population genetic structure between the parental and hybrid populations. These findings show that increases in neutral genetic variation can translate to tangible fitness benefits both under laboratory and field conditions. The results highlight the critical role genetic diversity and

intentional hybridization may play to improve the performance of biological control agents prior to field releases in a novel environment.

1. Introduction

Evolutionary processes during species introduction are key to understanding population establishment and persistence, particularly for organisms released into novel environments (Sakai et al., 2001; Szucs et al., 2014). Such processes, like intraspecific hybridization, or the crossing of individuals within the same species from different populations, can produce diverse fitness outcomes influenced by the genetic distance between the populations involved effects (Colautti et al., 2017; Frankham et al., 2011; Whiteley et al., 2015). While hybridization among genetically similar populations may have negligible effects, increasing genetic distance can lead to outbreeding depression due to the disruption of co-adapted gene complexes (Colautti et al., 2017; Frankham et al., 2011; Templeton et al., 1986; Whiteley et al., 2015). This phenomenon underscores the need for detailed genetic analysis to predict hybridization outcomes, especially when historical data is scarce (Dlugosch et al., 2015). Furthermore, understanding intraspecific hybridization requires examining genetic variation in neutral parts of the genome and clarifying its link to fitness. In the context of biological control, understanding these dynamics is crucial as control agents often face genetic bottlenecks and inbreeding during mass rearing (Bertin et al., 2017; Szűcs et al., 2019). We seek to investigate the fitness effects of intentional hybridization in the biological control agent *Hypena opulenta* Christoph (Lepidoptera: Erebidae), recently introduced to combat invasive swallow-wort vines (*Vincetoxicum spp.*) in North America. By comparing hybridized laboratory and field populations, we aim to elucidate the impact of genetic diversity on fitness, ultimately contributing to the optimization of biological control strategies.

During intraspecific hybridization the genetic distance between the hybridizing populations can mediate the outcome from neutral, to beneficial or negative fitness effects (Colautti et al., 2017; Frankham et al., 2011; Whiteley et al., 2015). Hybridization between populations that are genetically similar may not have any discernable effects on fitness, however, as the genetic distance between hybridizing populations grow, the negative effects of outbreeding depressions can occur (Colautti et al., 2017; Frankham et al., 2011; Templeton et al., 1986; Whiteley et al., 2015). Outbreeding depression may arise as a result of disrupting co-adapted gene complexes upon interbreeding that can vary even within the same environment (Frankham et al., 2011). The degree of divergence between hybridized individuals can make the difference between heterosis and outbreeding depression (Edmands, 1999; Wei & Zhang, 2018). The risk of outbreeding depression is considered to be small if populations have the same karyotype, inhabit similar environments, and have not experienced genetic isolation for more than 500 years (Frankham et al., 2011). Yet it may not always be easy to discern if hybridization will have a positive, neutral, or negative effect when historical data for populations is scarce, therefore genetic analysis to understand population history and divergence is recommended (Dlugosch et al., 2015). In an example by Audet et al. (2017), intraspecific crosses of salmon from two different geographic locations resulted in neither heterosis nor outbreeding depression. In this case, assumptions on population divergence were made based on population history and no molecular analysis was done to determine the degree of population divergence and the genetic diversity of the resulting crosses (Audet et al., 2017). In another example, research investigating the impact of population divergence and geographical distance on the intertidal copepod *Tigriopus californicus* found that F1 individuals exhibited similar or greater performance than their parental lines. However, all but one divergent line – a hybrid line with less than 5% sequence divergence – saw F2 hybrid

breakdown indicating the importance of measuring the degree of divergence (Edmands, 1999). Indeed, past studies have struggled to identify the optimal mating distance that might provide the beneficial effects of heterosis and avoid the negative effects of outbreeding (Wei & Zhang, 2018). A meta-analysis by Wei and Zhang (2018), showed a hump shaped relationship between intraspecific hybrid performance and mating distance across environments, whereas performance boost is minimal (or nearly non-existent) at the shortest mating distances, improves with greater mating distances up to a peak genetic distance, and then declines as mating distance continues to increase. With advanced and increasingly affordable genetic techniques the risk of outbreeding can be reduced, however, it is still unclear if hybridization between recently isolated and genetically similar populations can bring any tangible benefits.

To enhance our understanding of intraspecific hybridization, it is essential to explore the underappreciated significance of genetic variation in the neutral parts of the genome. Kimura's neutral theory of molecular evolution (1968) posits that the genetic diversity observed within and between species is primarily a product of non-selective forces like genetic drift, gene flow, and mutation, rather than natural selection (Kimura, 1968; Neale & Wheeler, 2019). Numerous studies have established a link between heterozygosity and fitness, particularly in highly inbred populations, though these often rely on indirect methods such as breeding pedigrees (Arct et al., 2017; Chapman et al., 2009; Hansson and Westerberg, 2002). Whether this relationship is due to changes at neutral markers, linkage disequilibrium at neutral markers and fitness-related genes, or if heterozygous markers signify overall genomic heterozygosity is unknown (Arct et al., 2017; Hansson and Westerberg, 2002; Miller & Coltman, 2014). Despite this, the correlation between heterozygosity and fitness is not always straightforward, and the role of neutral genetic variation in this context is increasingly seen as potentially overemphasized (Chapman et al., 2009; Sudkya

et al., 2017; Teixeira & Huber, 2021). The effectiveness of using neutral genetic diversity as a marker for conservation efforts is complicated by factors like the intricate relationship between neutral genome-wide diversity and a population's survival or extinction risks, particularly when considering outbreeding and other evolutionary processes like gene-environment interactions and epistasis (Teixeira & Huber, 2021). The variable results from studies on heterozygosity-fitness correlations, which depend on environmental conditions, highlight the complexities of this relationship (Chapman et al., 2009). Presently, research on heterozygosity-fitness correlations is primarily concentrated on populations that have naturally hybridized, using historical data and indicators to decipher the influence of both neutral and non-neutral processes (Chapman et al., 2009; Hansson and Westerberg, 2002).

Classical biological control, which offers a sustainable solution for managing invasive weeds by introducing co-evolved natural enemies from the invader's native region, can improve our understanding of the evolutionary processes that govern intentionally introduced populations and provide insight into their success rates. Globally, the success rate of established, intentionally introduced weed biological control agents stands at 63.2%, and only 23.9% of these agents cause significant damage to the target weed (Schwarzländer et al., 2018). These rates indicate that there are fundamental factors affecting the success of classical biological control, potentially hindering its broader implementation (Brodeur et al., 2018; Cock et al., 2016). Insects used in biological control are often sourced from small native populations and mass-reared in quarantine for several years before release because of the lengthy host-specificity testing and approval processes. This practice subjects laboratory populations to phenomena such as population bottlenecks, founder effects, cultural adaptation, and inbreeding (Bertin et al., 2017; Szűcs et al., 2019). Organisms that are mass-reared often experience fluctuating and low population sizes that can lead to

genetic drift, reduced genetic diversity, the accumulation of recessive deleterious alleles and inbreeding depression in sexually reproducing species (Bertin et al., 2017; Szűcs et al., 2019). Releasing laboratory populations into new environments can impose stressors that exacerbate the negative impacts of inbreeding (Fauvergue et al., 2012; Hufbauer et al., 2013; Szűcs et al., 2017; Szűcs et al., 2019). Introducing new genetic material to founding populations can alleviate inbreeding, enhance adaptive potential, and produce novel genotypes that may be more suited to new environments (Barker et al., 2019; Sinclair et al., 2019; Stewart et al., 2017; Szűcs et al., 2017). This approach is supported by laboratory studies, which have consistently shown that admixed populations exhibit improved establishment success, higher population growth, and greater long-term survival (reviewed in Szűcs et al., 2014). For instance, admixture in pheasant populations has led to the 'catapult effect,' a temporary surge in fitness in initial generations due to heterosis, or 'hybrid vigor', enhancing the population's persistence (Drake, 2006). Yet the long-term effects of intentional intraspecific hybridization after release into the wild are still unclear.

Biological control agents provide a unique opportunity to use intentional hybridization to understand how neutral genetic variation affects fitness related traits including population performance, establishment, and persistence. Here we explore the fitness effects of intraspecific hybridization for a recently approved biological control agent *Hypena opulenta* that is being released to control invasive swallow-wort vines (*Vincetoxicum spp.*) in North America. *Hypena opulenta* had been reared in quarantine for over a decade prior to approval for field release in the U.S. and our laboratory population was founded with few individuals (< 20 females). We hybridized this likely inbred laboratory population with a population established in the field and compared the fitness of the two parental (laboratory population vs. field population) and the

outbred populations in the field and in the laboratory. We also assessed the molecular genetic diversity of each population and evaluate if they are genetically distinct. We expected relatively low yet significant genetic divergence between the laboratory and field populations and that the outbred population would exhibit higher heterozygosity and increased fitness compared to the laboratory population. We hypothesized that the outbred and field populations may have similar performance in the field but that the effects of outbreeding may be more apparent in laboratory assays reflecting gene-environment interactions. Ultimately, we expected that intraspecific hybridization would increase neutral genetic variation and be correlated with increased fitness under different environmental conditions.

2. Methods

2.1 Study System

Invasive swallow-wort vines were introduced to North America in the mid to late 1800s (Cappuccino, 2002; Weed & Casagrande, 2010). Two species were introduced: pale swallow-wort (*Vincetoxicum rossicum* Kelopow) and black swallow-wort (*Vincetoxicum nigrum* L.) as ornamentals from Ukraine and Mediterranean countries, respectively (Ditommaso et al., 2005). Both have spread in southeastern Canada and the northeast United States. These herbaceous, perennial vines invade a variety of habitat types including forests, crop fields, pastures, road and trail edges, and home gardens (Ditommaso et al., 2005; Weed & Casagrande, 2010). Swallow-wort vines outcompete native vegetation and form dense monocultures because of their aggressive growth habits and lack of natural enemies (Ditommaso et al., 2005; DiTommaso et al., 2018). Swallow-worts have proven difficult to control by chemical and cultural methods, they have adverse effects on arthropod diversity (Ernst & Cappuccino, 2005), reduce grassland

habitat for birds (Ditomaso et al., 2005), and are an oviposition trap for monarch butterflies (Alred et al., 2022; Casagrande & Dacey, 2007).

The biological control agent *Hypena opulenta* was approved for release in the United States in 2017 (Stewart, 2017). It feeds on the leaves of swallow-wort in all five of its larval stages before it enters the pupal stage to undergo metamorphosis, then emerging asynchronously as adults (Weed & Casagrande, 2010). *H. opulenta* is a bivoltine species in Michigan, meaning it can complete two generations per year (Alred et al., 2022). However, like many other insects, this agent enters diapause in the winter, a state of halted development that allows it to survive extreme conditions (Tauber et al., 1986, Weed & Casagrande, 2010). However, diapause in *H. opulenta* is facultative (occurring in the pupal stage) and is mainly regulated by the photoperiod (Jones et al., 2020; Weed & Casagrande, 2010). The point at which 50% of *H. opulenta* enter diapause is known as the critical photoperiod, which happens when daylight lasts 15h and 35 min (Jones et al., 2020; Weed & Casagrande, 2010). In Michigan, release success can be maximized by releasing shortly before the summer solstice to increase exposure to Michigan's maximum photoperiod conditions (15 h 20 min; East Lansing, MI) (Alred et al., 2022; Jones et al., 2020). In another main release location – Canada – *H. opulenta* was approved for release in 2013 and field releases started in September of 2013. Establishment of *H. opulenta* was confirmed in 2015 in Ontario (Bourchier et al., 2019). Overwintering success in Michigan wasn't confirmed until the summer of 2023 and monitoring for field establishment is still ongoing. There are several theories to explain the delay in establishment of *H. opulenta* for Canada and the United States. Firstly, mismatched photoperiod between the collection site in Ukraine and release latitudes in North America could be causing early diapause (Jones et al., 2020). Secondly, *H. opulenta* appear to be very vulnerable to predation in the field in Canada (Jones et al., 2022). Early

diapause can cause *H. opulenta* to spend longer periods of time in its pupal stage increasing its risk of predation. Thirdly, we suspect that reduced genetic variation or inbreeding could be causing performance issues in field populations causing delayed establishment and stagnant or reduced population growth (Szűcs et al., 2019).

2.2 Moth populations and rearing

Population background

Before shipment to Michigan, the first lab population of *Hypena opulenta* was created from wild caught individuals in Donetsk, Ukraine in 2006 from four pupae and 32 larvae (Weed et al., 2011). Population maintenance started at CABI EU-CH (Delémont, Switzerland) before shipment to the University of Rhode Island (URI) which then started quarantine and population rearing in 2008 (Hazlehurst et al., 2012). URI provided a larval shipment to Michigan State University (MSU; East Lansing, MI) in June of 2018 which founded the MSU population with 18 females and 22 males.

Additional *H. opulenta* individuals were shipped to MSU in 2020 that originated from a field population in Canada (Bourchier et al., 2019). This Canadian population was founded from the same base population as the MSU population from URI but six years (or 12 generations) earlier, in 2012. Field releases in Canada started in 2013 and resulted in overwintering success and establishment in Ontario by May 2015 (Bourchier et al., 2019). Individuals collected in the field in Canada in 2019 were reared for one generation in quarantine at URI and then were shipped as pupae to MSU in May of 2020. This population was founded with 15 females and 13 males.

Reciprocal crossings between the MSU laboratory and Canadian field population were conducted in summer 2020. The original MSU population from 2018 will be referred to as the laboratory adapted population, while the population shipped from Canada will be called the field

population, and the cross between the two will be called the outbred population. The outbred population was created through reciprocal crosses of 5 males and 3 females from the field population (shipped May 2020) and 5 males and 8 females of the lab population. Three separate cages were set up for reciprocal crosses. The first cage allowed five females from the lab population to mate freely with three males from the field population. The second cage allowed three females from the field population to freely mate with five males from the lab population. The third cage allowed three females from the lab population to freely mate with the two males from the field population.

Rearing and population maintenance

Both species of swallow-wort were used for rearing as *H. opulenta* does not discriminate between species for oviposition or feeding (Hazlehurst et al., 2012). However, by 2021 most moths were reared on *V. rossicum* due to its availability. Field collected plants of both *Vincetoxicum* spp. from infestations across Michigan including Grand Rapids and several towns in Oakland County were transplanted into 1.14 L square plastic pots using SUREMIX® all-purpose perlite mix (Michigan Grower Products Inc., United States). Potted swallow-wort was grown in the greenhouse at $26.6\pm 4.9^{\circ}\text{C}$ from March through September. In the fall the potted plants were placed outside at the MSU Entomology Farm until the following spring. In March/April the overwintering plants were brought back in the greenhouse and repotted prior to use in rearing in subsequent seasons. Rearing was conducted in the laboratory in mesh cages (40 x 40 x 60 cm, Restcloud). In each cage 6 potted swallow-wort plants were placed on a tray with a 0.6-1.3 cm layer potting soil. Rearing cages were kept in a laboratory at $22\text{-}25^{\circ}\text{C}$ with ambient humidity under 16:8h L:D photoperiod provided by supplemental lighting (4ft, 80 Watt; Barrina®; Zhongshan, China). Adult moths were fed with a 1:5 ratio of honey to water in 2 oz

cups with a cotton wick. Honey water was changed for freshness when molding was detected, and cages were misted with distilled water daily. Potted swallow-wort was watered as needed.

Between March and August of each year, pairs of adults were released into the mesh cage setup with no more than 5 pairs per cage. Adults oviposited onto the potted swallow-wort plants until death. Larvae were allowed to feed on the potted swallow-wort and were supplemented with cut stems of swallow-wort in water picks (9 x 2.5 cm) in each cage. Cut-stem swallow-wort was sourced from potted swallow-wort early in the season when swallow-wort was not yet available in the field but was cut from field infestations starting in late May. Larvae fed in the mesh cages until 3rd-5th instar when they were removed to a 2.4 L round plastic mixing container (HDX[®], Home Depot; United States) with a cut hole in the lid covered with mesh for ventilation. Containers were supplemented with swallow-wort in water picks and provided paper towels for pupation. Containers were placed in a laboratory under 16:8h L:D lights and checked daily to every other day for pupae. Under high volume conditions, some larvae were allowed to pupate in mesh cages.

Pupae were collected from plastic containers and mesh cages. They were then sexed and placed, in groups of 8 or less into 0.35 L transparent round plastic containers with vermiculite (Vigoro[®]; United States) moistened with distilled water. During the continuous rearing season, these containers of pupae were placed on the benchtop under 16:8h L:D lights until emergence. In August, pupae containers were placed in incubators under 12:12h L:D at 20°C to induce diapause. The temperature was lowered by 5°C each month until 5°C was reached and maintained. The containers with pupae were remoistened once a month until removal and placement under 16:8h L:D and 22-25°C in early spring. This rearing protocol has been used for this laboratory population since 2018 and was used in subsequent population crosses.

2.3 Laboratory Experiment

To compare the performance of the three *H. opulenta* populations (laboratory, field and outbred), we tested how many pupae are produced by individual females as a proxy for performance. Counts from the pupal stage were chosen as an indicator of performance because survival up to this point would incur the most impact to swallow-wort. Individual females were allowed to mate and lay eggs for 72 hours in rearing cages. One to two males were used for mating based on availability, but the numbers were kept consistent in each block. We exclusively used *V. rossicum* for egg laying which was transplanted from field sites in Oakland County, MI to pots in the greenhouse in the spring of 2021 and 2022. The larvae were removed from the rearing cages at the 4th and 5th instar stage and transferred to rearing containers where they pupated (see rearing methods above). For logistical reasons these fitness experiments were conducted at two locations (laboratory and greenhouse) in staggered time frames (temporal blocks) over a two-year period (2021 and 2022). Overall, the number of replications (individual females tested) for each of the three treatments (laboratory, field and outbred populations) ranged between 68-87. Two temporal blocks were setup both in 2021 and in 2022. In 2021, the first block was setup in the greenhouse and the second block in the laboratory. In 2022, both temporal blocks were tested in the laboratory. In the greenhouse experiment the temperature was $25.6 \pm 4.8^{\circ}\text{C}$ and beyond natural daylight hours artificial lights were used to ensure 16:8 L:D photoperiod. In the laboratory the cages were kept under ambient temperature and humidity with artificial lighting set to 16:8h L:D. The number of replications for each year and temporal block and the location of assays are summarized in Table 1.1.

Table 1.1 Summary of the years, blocks, and replicates for the laboratory experiment.

Year	Block	Population	Number of Replicates	Location
2021	1	Field	4	Lab
2021	1	Lab	4	Lab
2021	1	Outbred	4	Lab
2021	2	Field	4	Lab
2021	2	Lab	4	Lab
2021	2	Outbred	4	Lab
2022	1	Field	39	Greenhouse
2022	1	Lab	33	Greenhouse
2022	1	Outbred	37	Greenhouse
2022	2	Field	15	Lab
2022	2	Lab	20	Lab
2022	2	Outbred	23	Lab
2022	3	Field	6	Lab
2022	3	Lab	13	Lab
2022	3	Outbred	19	Lab

2.4 Field Experiment

To compare the performance of the three populations of *H. opulenta* under field conditions, adults were released in cages setup in a natural infestation of pale swallow-wort at a field site in Oakland County, MI (42.7087042, -83.5732006). Cages were square frames made of polyvinyl

chloride and covered with a fine white mesh that were staked to the ground over naturally growing swallow-wort (60 x 60 x 60 cm; Gardeners Advantage). Cages were setup within ~500 square meters of infestation in early June of 2023. In each cage three types of annual flower plugs were planted (one each of marigold, begonia, and impatiens) to provide nectar for adult moths. There were 14 replicates for each population treatment (field, lab, and outbred) totaling 42 cages. A total of five female and five male adult moths were released in each cage on June 14th, 2023. Four of the pairs of *H. opulenta* adults were allowed to mate for 48 hours prior to release under ambient laboratory conditions in small mesh cages (12 x 12 x 12, Restcloud) and were given honey water. As more adults became available, another pair was added to each replicate 24 hours prior to release. Adults ranged from 1 day old to 5 days old at the time of release. Adults were allowed to lay eggs until death (1-3 weeks). Swallow-wort stem counts within each cage were taken before release. Percent defoliation was assessed for five randomly chosen stems in each cage once on July 10th, 2023, when fifth instar larvae were present, and pupation had started. Percent defoliation is used as a proxy for fitness for each population treatment. The number of 2nd generation adults emerged in each cage was checked weekly after the start of pupation until the end of August. Adults were released from the cage upon each monitoring date to avoid double counts later.

2.5 Molecular Genetic Diversity and Structure

Samples

Adult *H. opulenta* from the laboratory adapted population were saved in 2021 (n = 15), from the field population in 2020 (n = 8) and in 2021 (n = 7) and from the outbred population in 2020 (n = 10) and 2021 (n = 10). For the outbred population the 2020 collections represent two generations post-crossing, and the 2021 collections are third and fourth generation post-crossing.

The samples were stored at -80°C until shipment to the Novogene Corporation Inc. (Durham, NC, USA).

DNA Extraction, Sequencing, and Bioinformatics

DNA extraction, library preparation, whole genome sequencing and sequence alignment were performed by Novogene. Whole genome sequencing libraries were prepared using the Illumina Standard 350bp DNA library. Samples were not mixed for library construction and were checked with Qubit and real-time PCR for size detection and distribution. The libraries were then pooled and sequenced by Novogene on NovaSeq 6000 (Paired-End 150bp).

Novogene completed a standard bioinformatic analysis including data quality control, alignment with a reference genome and statistics of sequencing depth and coverage. The sequencing error rate for all samples was 0.03% with the percent of Phred-scaled quality scores greater than 30 all above 91%. Reads were aligned to the *Hypena proboscidalis* reference genome available through NCBI (iIHypProb1.1; GCA_905147285.1; Boyes, et al., 2023). The mapping rate ranged from 28.55% - 30.43% and average depth ranged from 16.10X – 21.46X.

Genome-wide Diversity

ANGSD (v.0.935) was used to calculate the global, genome-wide nuclear heterozygosity of individual samples. This software uses the entire genome and incorporates genotype uncertainty by using genotype likelihoods (GLs). This method is particularly useful for non-model organisms with low to medium coverage (Sand Korneliussen et al., 2014).

For each sample a reference genome is provided as the ancestral state. We first took the site allele frequency likelihood, which is based on individual genotype likelihoods assuming Hardy-Weinberg equilibrium, by using the -doSaf 1 option. Filters were used since the input files are raw alignments (BAM and FASTA files). The filter -C 50 was used to adjust mapping quality for

excessive mismatches. Then reads were removed if mapping quality fell below 30 (-minMapQ 30), minimum base quality score was below 20 (-minQ 20), they were flagged if above 255 (-remove_bads), if location was not unique during mapping (-uniqueOnly), and filter for pairs of reads with both mates mapped correctly (-only_proper_pairs). The regions used were filtered (-rf) to include all 30 autosomes and exclude the sex chromosome. Genotype likelihoods were calculated using -GL 1 (SAMtools method). The result was then used to obtain the maximum likelihood estimate of the SFS by using the subprogram realSFS. Heterozygosity was then calculated by using the data in the SFS by taking the number of heterozygous sites and dividing it by the total number. This method was adapted from de Jager et al., 2021.

Population Structure

PCAngsd (v.1.21) was used to calculate individual allele frequencies and their covariance for structured populations using principal component analysis (PCA) using genotype likelihoods (Meisner and Albrechtsen, 2018). PCAngsd requires files to be input in a beagle format. BAM files were converted to beagle files using ANGSD and the -doGlf 2, -GL 1 and -SNP_pval 1e-6 to work with sites with this p-value less than 1e-6. During conversion, the regions were again filtered to include autosomes 1-30 for consistency. Individual allele frequencies were calculated by simply running PCAngsd by inputting the beagle file.

2.6 Statistical Methods

All analyses were performed in R 4.3.0 (R Core Team, 2023). We used the *DHARMA* package to calculate the residuals to assess best model fit for all non-parametric models. This package assesses the model in several ways including QQ plot, the Kolmogorov-Smirnov test (K.S. test), dispersion testing, and zero-inflation testing. Chi-squared values were provided for mixed models using the Anova function from the *car* package (Zeileis & Hothorn, 2002). All

post-hoc pairwise comparisons were performed using the *emmeans* package and were adapted for parametric tests by specifying for the Tukey adjustment (Lenth et al., 2021).

Laboratory Experiment

In this experiment, we measured if a female succeeded or failed in producing any pupae and then the number of pupae produced by each female. Statistically, we compared the production of pupae by population in two ways. First, we evaluated whether any pupae were produced (hereafter “production success”) with a generalized linear mixed model with a binomial distribution using the *lme4* package (Bates et al., 2015). If no pupae were produced, then the replicate was assigned a “0”. If one or more pupae were produced, then the replicate was assigned a “1”. The model contained population (lab, field, outbred) as a fixed effect and replicates nested within block nested within year as the random effect.

Second, we evaluated the number of pupae produced per female using a generalized linear mixed model via the *glmmTMB* package with a Poisson distribution (Brooks et al., 2017). The best model contained population (lab, field, outbred) as a fixed effect and replicates nested within block nested within year as the random effect. The *glmmTMB* package allows for a zero-inflation parameter to be applied to a model with a Poisson distribution. The zero-inflation parameter was set to vary by population such that the probability that observations had structural zeros depended on population.

Field Experiment

In this experiment, we measured if a female succeeded or failed in producing any larvae by noting evidence of larval feeding and then measured the rate of defoliation averaged over each stem. We also measured if any pupae succeeded or failed to produce a second generation and how many adults were produced in the second generation. To compare performance between the

three treatments, we used evidence of larval feeding. If a cage exhibited no feeding, then the replicate was assigned a “0”. If a cage exhibited any feeding, then the replicate was assigned a “1”. A generalized linear mixed model was used with a binomial distribution with the *glmmTMB* package in R (Brooks et al., 2017). We used population (field, lab, and outbred) as the fixed effect and swallow-wort stem density as the random effect.

In addition, we compared total defoliation per stem as a proportion (between 0 and 1) using a generalized linear mixed model with a beta distribution in the *glmmTMB* package in R (Brooks et al., 2017). This package allows a zero-inflation parameter that permits zeros in the data because beta distributions are bound between greater than zero and less than one yet is often zero or one inflated. This bypass allowed us to set the zero-inflation parameter to vary by population. We used population (field, lab, and outbred) as the fixed effect and swallow-wort stem density as the random effect. One replicate in the field population was missed during data collection and therefore $n = 13$ for this treatment while $n = 14$ for the other populations.

Adult emergence success by treatment was evaluated by assigning cages with no emergence a “0” and one or more emergence a “1”. A generalized linear model was used, from the *lme4* package, with a binomial distribution with population and emergence date as fixed effects (Bates et al., 2015). The number of adults that emerged by each treatment with a generalized linear model with a Poisson distribution, from the *lme4* package (Bates et al., 2015). We used two fixed effects, population and emergence date. Emergence was only recorded on two dates with only one cage with one adult emergence at the later date. In a separate analysis, only population was used as fixed effect and the number of emerging adults were summed for the two dates.

Genome-wide Diversity

Heterozygosity values were assessed for normality using a Shapiro-Wilk test then modeled with a one-way ANOVA using the *stats* package (R Core Team, 2023). Population (lab, field, outbred) was used as the fixed effect. A Q-Q plot and Levene's test was used to assess model assumptions from the *car* package (Fox and Weisberg, 2019).

3. Results

3.1 Laboratory Fitness Experiment

The probability that at least one pupa would be produced was not affected by population ($\chi^2 = 3.55$, $df = 2$, $p = 0.17$). However, there was a trend of higher probability for the outbred population producing at least one pupa (52%), compared to 40% probability for the field, and 36% probability for the lab population.

The number of pupae produced was affected by population ($\chi^2 = 20.81$, $df = 2$, $p < 0.001$) (Fig. 1.1). The outbred population produced the most pupae (mean [95% confidence interval], n = number of replicates, N = number of individual pupae; 34.01 [23.94, 48.31], $n = 87$, $N = 2352$) compared to the field population (29.74 [22.85, 38.69], $n = 68$, $N = 1160$) and the lab population (28.76 [20.03, 41.29], $n = 74$, $N = 1369$) (Fig. 1). Effect sizes show a 14% increase in pupae production by the outbred population when compared to the field population (pairwise comparison: $p < 0.01$) and an 18% increase when compared to the lab population (pairwise comparison: $p = 0.0001$). Whereas the lab and field population are not significantly different from each other. This is reflected in the nearly doubling in pupal production in the outbred population, despite slightly lower replication in the field and lab populations (Table 1).

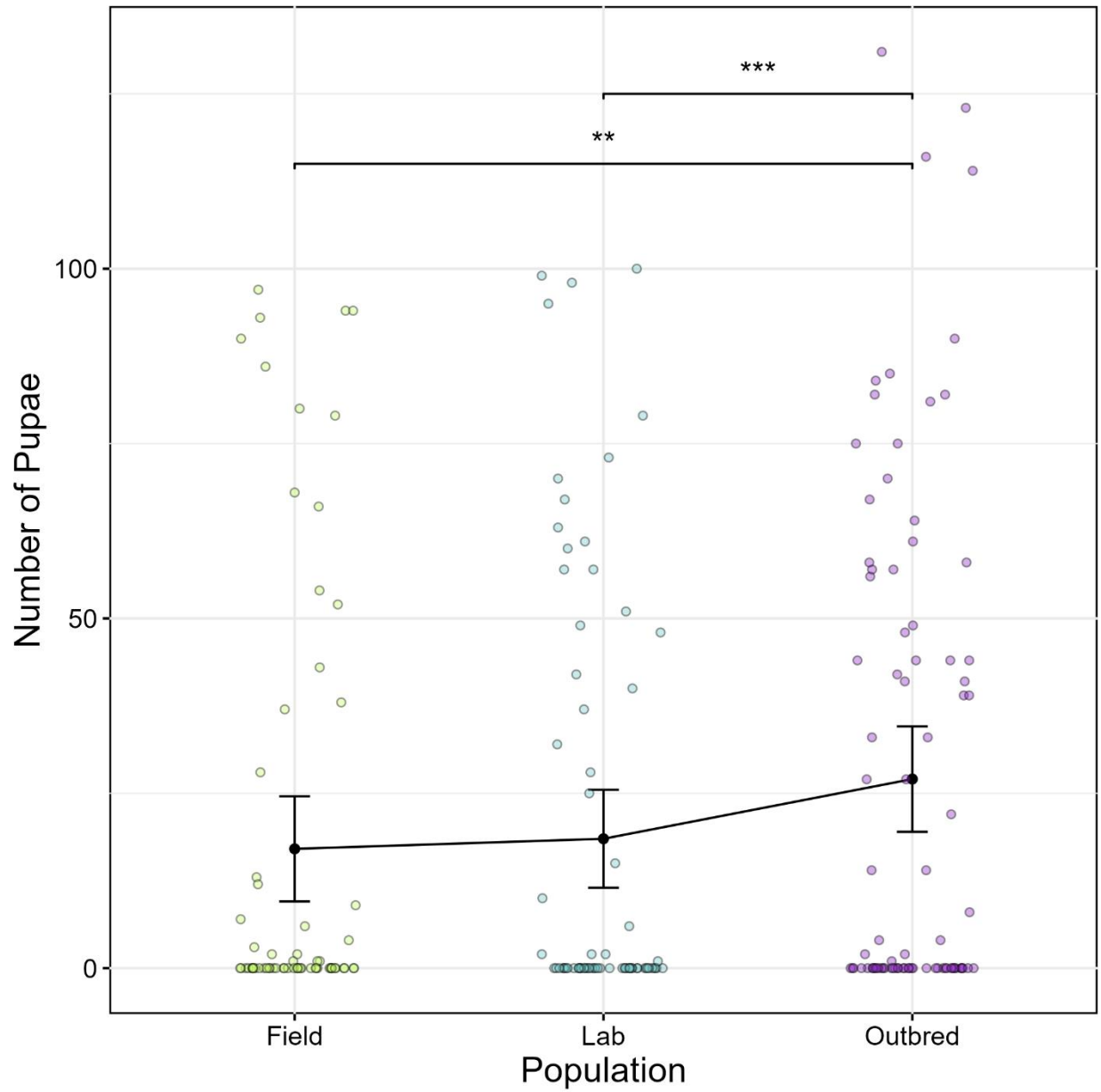


Figure 1.1 Means and 95% confidence intervals of the number of pupae produced per female by population (field, lab, outbred). Observations have been jittered to show individual points.

Asterisks indicate significant differences between treatments at the 0.05(*), 0.01(**), 0.001 (***) levels.

3.2 Field Experiment

The probability that a cage would produce at least one larva (i.e., visible feeding damage) was not significant by population ($\chi^2 = 2.81$, $df = 2$, $p = 0.25$) (field: $p = 0.458$; lab: $p = 0.522$; outbred: $p = 0.096$). However, the outbred population had the highest probability of success (96%), the lab population the second highest success probability (77%) and the field population the lowest success probability (63%) in terms of replicated field cages showing any feeding damage. Post-hoc pairwise comparisons were not significant.

Percent defoliation was affected by population ($\chi^2 = 18.24$, $df = 2$, $p < 0.001$) (Fig. 1.2). The outbred population had the highest mean defoliation (0.17 [0.13, 0.21], mean [95% confidence interval], $n = 14$) and the field population the second lowest mean (0.13 [0.08, 0.18], $n = 13$), lacking significance from each other. This is likely due to the number of cages that initially produced larvae (see results above), which was lower in the field population when compared to the outbred. Ultimately, the field adapted population produced similar rates of defoliation to the outbred population in cages that showed any larval feeding. The outbred population produced significantly more defoliation than the lab population (0.04 [0.02, 0.06], $n = 14$) (pairwise comparison: $p < 0.001$). And the field population had higher rates of defoliation when compared to the lab population (pairwise comparison: $p < 0.001$). Ultimately, the number of cages that produce larvae for each population and then the distribution of the pupal number influences the significance of this non-linear model.

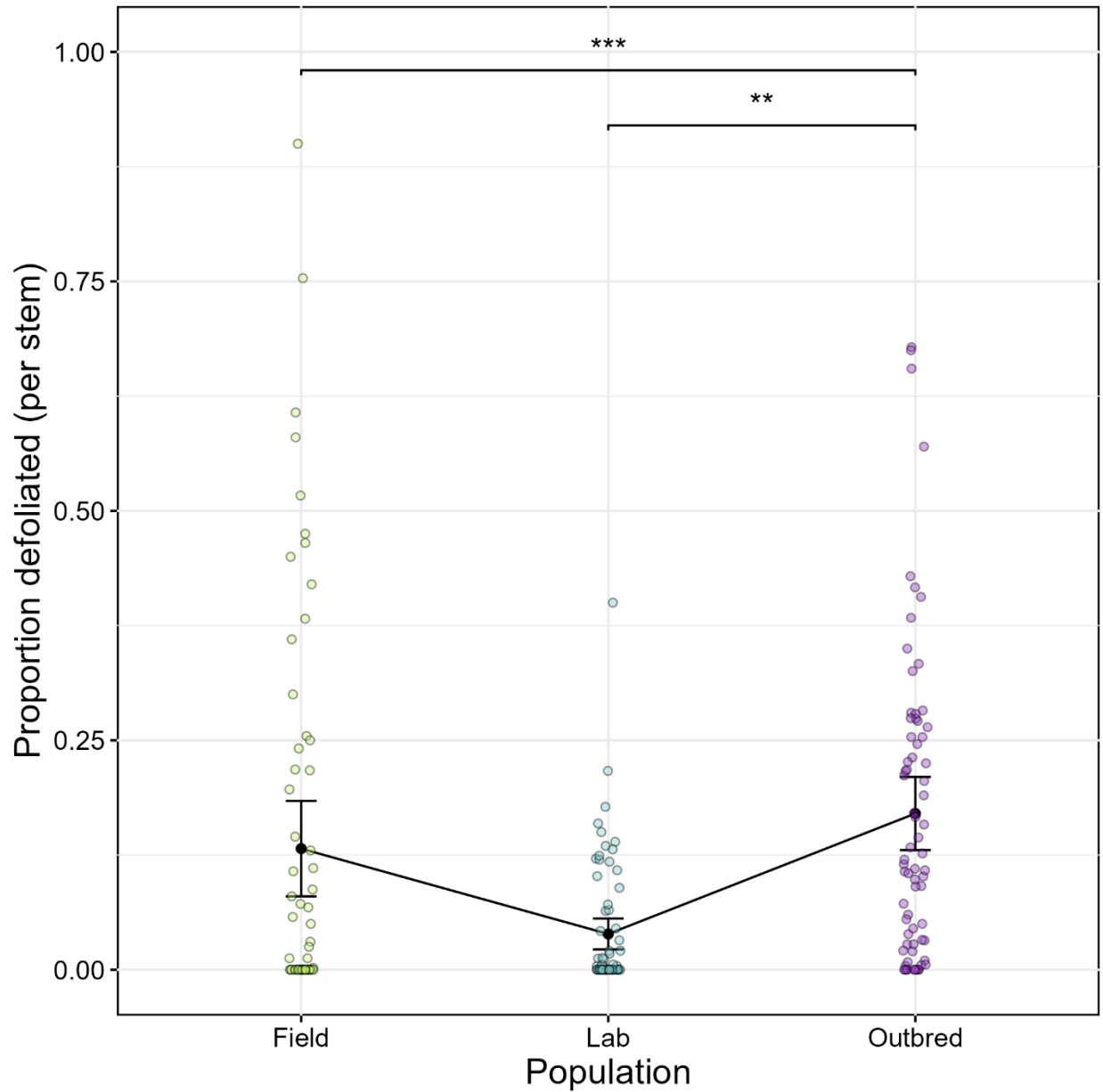


Figure 1.2. Mean and 95% confidence intervals of the proportion of leaf matter defoliated per stem by population (field, lab, outbred). Observations have been jittered to show individual points. Asterisks indicate significant differences between treatments at the 0.05(*), 0.01(**), 0.001 (***) levels.

The number of adults that emerged was affected by population ($\chi^2 = 7.97$, $df = 2$, $p < 0.05$) (field: 0.012 [0.0006, 0.069]; mean [95% confidence interval], $n = 13$, $N = 3$) (lab: 1.24 [0.27,

6.29], $n = 14$, $N = 4$) (outbred: 4.02 [1.30, 17.55], $n = 14$, $N = 13$). The number of adults that emerged depended on date ($\chi^2 = 19.79$, $df = 1$, $p < 0.001$). The outbred population produced 3.3 times more adults than the field population (pairwise comparison: $p = 0.076$) and 2.3 times more adults than the lab population (pairwise comparison: $p = 0.098$) thus neither pairwise comparisons were significant. The two dates that produced adults were significantly different as 97% of the adults emerged by August 8th, 2023, compared to August 16 (pairwise comparison: $p < 0.01$). When dates were combined to assess the number of emerged adults the results for population differences were similar, and the pairwise comparison between the outbred population and the lab population became marginally significant (pairwise comparison: $p = 0.057$).

The probability that at least one adult would emerge was not affected by population ($\chi^2 = 4.56$, $df = 2$, $p > 0.05$). The probability of emergence depended on date ($z = \chi^2 = 15.10$, $df = 1$, $p < 0.001$). The probability that a second generation of adults would emerge was 1% for the field and the lab populations, and 5% for the outbred population (pairwise comparisons were not significant). Adults were 22% more likely to emerge by August 8th than August 16th (pairwise comparison: $p < 0.01$).

3.3 Molecular Genetic Diversity

Genome-wide Diversity

The mean global heterozygosity of the outbred population was significantly higher than the field and the lab populations' ($F = 16.59$, $df = 2$, $p < 0.0001$) (Fig. 1.3). The heterozygosity of the outbred population was 12% higher than the field (pairwise comparison: $p = 0.0001$) and 13% higher than the lab populations (pairwise comparison: $p < 0.0001$). Heterozygosity of the field, and the lab population did not differ ($p = 0.968$).

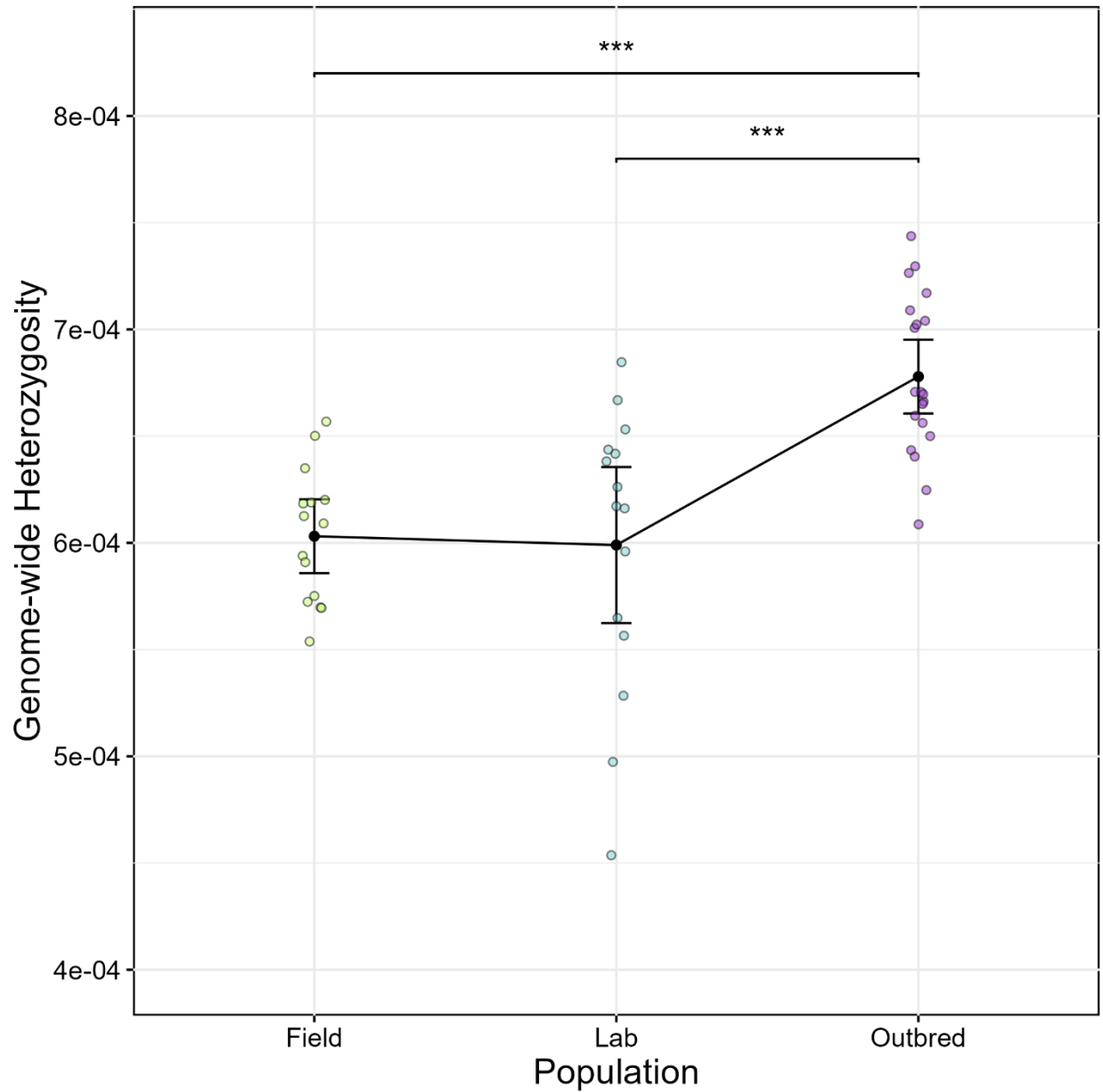


Figure 1.3. Mean and 95% confidence interval of genome-wide heterozygosity by population (field, lab, outbred). Observations have been jittered to show individual points. Asterisks indicate significant differences between treatments at the 0.05(*), 0.01(**), 0.001 (***) levels.

Population Structure

The population structure of *H. opulenta* based on the principal component analysis of allele frequencies displays three distinct populations (Fig. 1.4). Two individuals from the field

population deviate from the group by clustering more closely with the lab population. The outbred population is inversely correlated with the field population. The lab population is more closely correlated to the outbred population yet is still distinct as its first principal component is positive. Populations seem to be primarily divided by the first principal component, however some individuals in each population are more similar, falling closer to zero.

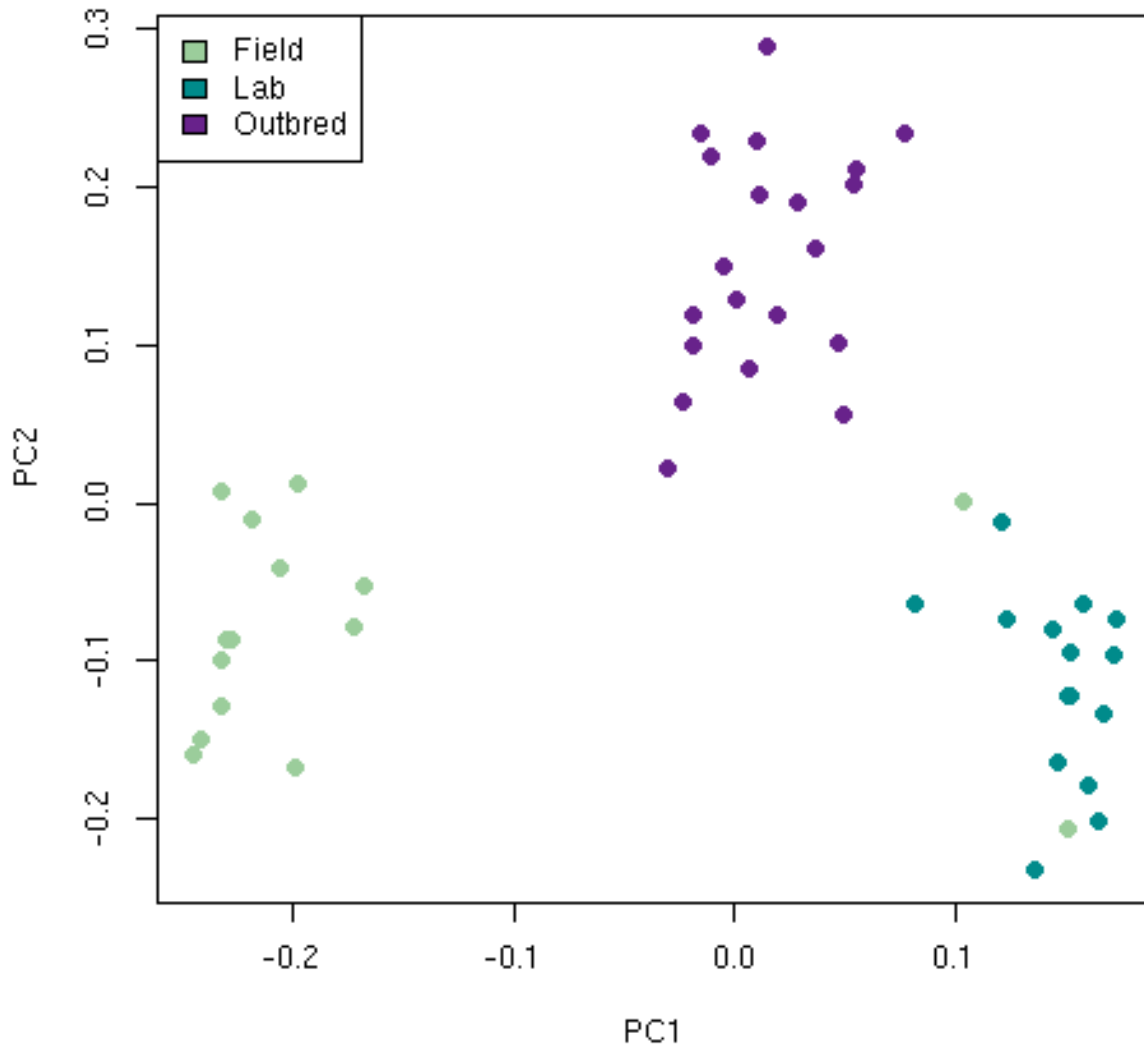


Figure 1.4. Population structure using individual allele frequencies of *Hypena opulenta* from the field, the lab, and outbred. The principal component analysis (PCA) plot is the percentage of variation explained by each principal component.

4. Discussion

This study shows that genetic structure can develop among recently isolated populations of the same species and that outcrossing between populations separated by 5 years in the field, or approximately 10 generations, can lead to fitness benefits in the outbred population. Notably, intraspecific hybridization between the lab and field populations led to increased heterozygosity and enhanced multiple fitness metrics in the hybrid offspring. Our findings indicate that neutral genetic diversity is a reliable predictor of performance across various environments.

In our laboratory experiments, we observed that outbred females consistently produced more pupae, indicating enhanced fecundity and/or better survival of larvae to the pupal stage. This higher fecundity or larval survival in outbred populations could have been due to hybridization increasing heterozygosity and the masking of deleterious alleles compared to the laboratory population. The field population, despite its successful establishment in natural settings, exhibited lower fitness than the laboratory adapted population when tested in the laboratory. Considering heterozygosity alone these results may be unexpected. However, the rearing and release history of the two populations can explain the observed better performance of the lab population. The field population has likely undergone similar pre-release challenges as our lab population, including adaptation to culture and heterozygosity loss during mass-rearing. Moreover, this population faced novel biotic and abiotic conditions upon field release and novel selection pressures. Adaptation to the field conditions could have led to further reductions in heterozygosity and low population sizes if there was a hard selection event that resulted in high mortality of suboptimal or unsuitable genotypes in the novel environment. In contrast, the lab population has been reared continuously in the lab for over 45 generations and likely adapted to the rearing conditions, experiencing genetic drift and inbreeding. Thus, it is not entirely

surprising that under laboratory conditions the lab population would outperform the field population. In the lab experiment, we tested F3 to F7 hybrids and in the field experiment we tested F8 to F9 hybrids. Despite testing later generation hybrids where the effects of heterosis are less pronounced, we still saw clear fitness benefits of hybridization. This indicates that the positive effects of hybridization can be lasting in this system, as seen in others (Szűcs et al., 2014; Wagner et al., 2017). Over time, continuous lab conditions are likely to erode genetic variation in the outbred population. This raises the question for biocontrol practitioners of what the ideal timing for field releases of laboratory created hybrids may be.

In a field environment, the field population outperformed the lab population which is in line with expectations and shows how testing long-term laboratory reared populations in the lab environment could lead to artifacts that do not hold up under natural conditions. Overall, the outbred population had the best performance in the field when looking at the metrics of successful egg laying with subsequent larval feeding and the production of second-generation adults. However, defoliation rates were similar between the outbred and field populations despite lower heterozygosity of the latter suggesting that adaptation to field conditions can yield similar benefits as hybridization in some metrics. These observations imply a greater potential for sustained biocontrol effectiveness of the outbred population in field conditions. Limited literature exists on field performance of hybrids that is coupled with genetic data (Abbott et al., 2016). Most studies focus on naturally hybridizing populations, as seen in the case of the weed biological control agent *Longitarsus jacobaeae* (Szűcs et al., 2019). This study reported increased densities and impact of hybridized ragwort flea beetle populations on tansy ragwort compared to parental populations. Conversely, much of the research in this area has concentrated on invasive species, such as the naturally admixed populations of the Caribbean lizard *Anolis*

sagrei, where increased body size of the hybrids correlated with colonization success (Kolbe et al., 2007). Similar patterns of genetic admixture contributing to the success of several invasive plant species, including a salt marsh grass (*Spartina alterniflora*), a monkeyflower (*Mimulus guttatus*), and the yellow star-thistle (*Centaurea solstitialis*), have been observed (Qiao et al., 2019; Li et al., 2018; Barker et al., 2017). To our knowledge, no published studies have moved beyond correlative experiments to intentionally perform intraspecific hybridization and assess hybrid performance in the field. Our study fills this gap by experimentally releasing controlled hybrid populations to discern performance differences, thus eliminating confounding factors like founding size variability and number of introduction events (Baker et al., 2003; Dlugosch et al., 2015; Szűcs et al., 2017). Genomic analysis further enhances our understanding of these experiments.

Our heterozygosity analysis was instrumental in interpreting the laboratory and field experiment results, demonstrating the benefits of increased heterozygosity in hybrids whose parental generations were recently isolated. The extent of divergence between populations plays a critical role in determining hybridization outcomes—either positive (heterosis) or negative (outbreeding depression). In *Drosophila littoralis* studies, for instance, hybrids with low divergence (seven generations) exhibited heterosis, which dissipated after another seven generations of rearing. In contrast, outbreeding between more divergent lines (fifteen generations) showed no advantage over inbred lines and even displayed lower fecundity than control lines, likely indicating the presence of outbreeding depression (Pekkala et al., 2012). In our case, however, we did not find such results suggesting that the level of divergence between *H. opulenta* populations was not large enough to cause outbreeding depression, yet substantial enough to induce higher performance. Variability in genetic variation within laboratory

populations also showed it can lead to differences in population structure. For example, *Aedes aegypti* strains, isolated by being reared in different labs, showed significant genetic differentiation, suggesting that minor environmental differences can prompt genetic divergence (Gloria-Soria et al., 2019). In another example, hybridization of two inbred *Drosophila melanogaster* lines resulted in breeding success improvements (Heber et al., 2012). Even though the parental populations of our hybrid population experienced different environmental conditions, they both had reduced heterozygosity and produced hybrids with increased performance like in the previous example. Our findings contribute to the understanding that hybridization of recently isolated populations can create distinct genetic structures and heterozygosity changes, correlating with fitness differences. This has implications for managing captive or inbred populations, which might be overlooked for intentional hybridization. Our study also demonstrates that, despite similar genetic variation rates, recently isolated populations can differ significantly. We found that neutral genetic variation effectively predicts performance, underscoring the value of neutral genetic diversity. However, the predictive power of genome-wide diversity for endangered species on the IUCN's Red List is limited, possibly due to complexities like gene-environment interactions and epistasis, suggesting that functional genetic diversity might be more critical (Teixeira & Huber, 2021). DeWoody et al. (2021) emphasized the longstanding association between neutral genetic diversity and fitness, noting reduced genetic diversity in endangered species compared to non-threatened ones. They argue that neutral diversity is unquestionably linked to functional diversity and improvements can be made by integrating complex factors like demographic history and ecological relationships into these considerations. Our study supports the notion that neutral genetic diversity significantly predicts fitness, especially when gene-environment interactions are considered. Future studies should

further explore this by directly manipulating genetic variation in different organisms with varied histories, contributing to the body of evidence supporting the role of neutral genetic diversity.

In the realms of conservation and restoration, the critical role of genetic diversity has been well-established (DeWoody et al., 2021; Chapman et al., 2009; Hufford et al., 2003). However, it is a relatively unexplored avenue in biological control. The success of biocontrol programs is hindered by low establishment and limited control success that may stem from genetic problems that biocontrol agents experience prior to field releases, as detailed above. While we demonstrate here that crossing of recently isolated populations can give a fitness boost that also translates to better field performance, the use of intentional hybridization is still rare in the practice of biocontrol (Szucs et al. 2019). Previously, incidences of unintentional hybridization have led to fitness boosts in biocontrol populations. For example, it is postulated post-release that population admixture in the predatory ladybird *Cryptolaemus montrouzieri* increased establishment based on later laboratory experiments (Li et al., 2018). This aversion to genetic manipulation of biocontrol agents partly stems from fears that it might lead to changes in host specificity of agents, compromising the safety of biocontrol programs (Hoffman et al., 2002; Mathenge et al., 2010; Szűcs et al., 2019). However, there is no evidence that intraspecific hybridization would result in either host shifts or host range expansion and less than 1% of species released for weed biocontrol exhibit non-target effects (Clark et al., 2023; Dieckhoff et al., 2017; Hinz et al., 2019; Szűcs et al., 2019). On the other hand, it can increase establishment success and persistence of populations in the field, as shown here. These insights have significant implications not only for biological control programs aimed at boosting performance of biocontrol agents but also for other fields grappling with the challenges of captive breeding and release. Particularly, populations that are endangered, extirpated, or extinct in the wild, often characterized by small

numbers and limited gene flow, could substantially benefit from strategic intraspecific hybridization. Currently, species are encountering an array of increasing challenges, such as biological invasions, habitat degradation, fragmentation, and climate change, all necessitating multifaceted and interdisciplinary approaches for resolution. These challenges are leading to a rise in isolated and diminishing native species populations (Dawson et al., 2011; Urban, 2015). In scenarios with restricted gene flow, deliberate hybridization between populations could be a vital strategy to avert extinction. The rapidly evolving climate conditions are likely to necessitate more frequent species reintroductions and adaptation to new habitats. Therefore, ongoing research into the role of genetic diversity under both laboratory and field conditions remains crucial. Furthermore, these insights could shed light on the factors that underlie the success of invasive species. From biocontrol to conservation and restoration, the continued study of the role of genetic variation is invaluable.

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CHAPTER 2:

Restoring swallow-wort (*Vincetoxicum spp.*) invaded woodlands: swallow-wort control and native seeding diversifies plant communities

Abstract

Invasive plant species pose significant threats to ecosystem integrity and biodiversity, particularly in forested regions. This study evaluates the efficacy of restoration strategies in swallow-wort invaded woodlands in Michigan. Three different management strategies were applied to three swallow-wort infested sites: defoliation of swallow-wort to simulate feeding by a biological control agent, manual removal of swallow-wort, native seeding and some combination of these treatments over a 3-year period. Plant cover, species richness, species diversity, and swallow-wort stem densities were monitored for 3 years. While all treatments reduced swallow-wort cover, the combination of manual removal and native seeding most significantly reduced swallow-wort cover and density and enhanced native species richness and diversity, although outcomes varied by site. Our results suggest that integrating defoliation with the addition of native seeds emerges as the second most effective strategy, having an impact on swallow-wort stem density. Given the laborious demands of manual removal, this discovery holds significant relevance for the feasibility of applying biological control with native species reintroduction in practical settings. At certain locations, treatments involving only the addition of native seeds proved effective in curbing swallow-wort stem density. Defoliation by itself, that served to mimic feeding by a moth biological control agent, did not enhance native species presence. For effective swallow-wort suppression and to boost native species, proactive native revegetation is essential. When combined with biological control, this approach offers a less labor-intensive method for managing swallow-wort, particularly for large infestations.

1. Introduction

Invasive plant species present a formidable challenge to the integrity and resilience of ecosystems globally, often outcompeting native flora, altering habitats, and diminishing biodiversity (Huston et al., 1994; Levine et al., 2003; Vitousek et al., 1997). The traditional arsenal against such invasions—mechanical removal, chemical applications, and prescribed burns—while potentially effective, demands substantial labor, incurs high costs, and risks adverse ecological impacts (Pile Knapp et al., 2023, Weidlich et al., 2019). Furthermore, attempts to control invasive plants often result in mixed success (Kettenring & Adams, 2011). Combining multiple management strategies might enhance efficacy and produce synergistic effects while mitigating the negative consequences of individual techniques (reviewed in Lake & Minter, 2018). However, the success of these combinations likely varies, and the role of native revegetation in conjunction with active removal of the invaders remains understudied (Kettenring & Adams, 2011; Schuster et al., 2018).

The majority of literature in invasive species control primarily focuses on a few select methods. According to a systematic global review by Weidlich et al. (2020), non-chemical controls were employed in 57.7% of cases, although countries with higher HDI (Human Development Index) reduced this number in preference for herbicide. The most prevalent methods of non-chemical control globally are prescribed fire and mowing, while hand pulling was done in just 12.3% of cases and cutting in 9.7% of cases. Insects and clipping were employed at less than 2.5% each. This is contrasted with chemical controls, accounting for 42.3% of interventions globally, where glyphosate was the most used herbicide. In the biome of interest for this study – eastern North American forests – herbicides are used in 64% of control

cases emphasizing the need for effective, alternative methods within this biome (Pile Knapp et al., 2023).

The frequent use of non-chemical methods aligns with efforts to mitigate potential negative impacts associated with chemical controls, such as environmental pollution and health risks to humans (Weidlich et al., 2020). Such non-chemical methods include biological control, the intentional introduction of natural enemies to support long term control of exotic pests (Heimpel & Mills, 2017). However, the regulatory capabilities of biological control agents are frequently surpassed due to the robustness of invasive species—a result of their swift growth, prolific reproduction, and ability to drastically alter soil chemistry and hydrology—underscoring the necessity for innovative management strategies (Blossey, 1999; Cuddington, 2011). Currently published research underscores the value of integrating biological control with native plant seeding to bolster native plant richness and diversity, but examples in different systems that span multiple years are still limited (Cutting & Hough-Goldstein, 2013; Lake et al., 2014). According to a review by Lake & Minter (2018). The few studies (n = Only 55) that experiment with biological control combined with other management techniques for invasive weed control mostly use herbicides in combination with biological control (36.4%) and biological control together with plant competition (23.6%). Mechanical tactics were the least studied (3.6%) and approaches that deployed more than two control methods were minimal (16.4%). The efficacy of such integrated interventions varies across systems and treatment. For instance, the integration of biological control with native revegetation shows promise in enhancing native plant species richness and diversity in mile-a-minute (*Persicaria perfoliate*) invaded systems (Cutting & Hough-Goldstein, 2013, Lake et al., 2014). On the contrary, herbicides can alter the dynamics of and be toxic to a biological control agent as was in the case of the biocontrol agent moth

Wheeleria spilodactylus for the invasive weed *Marrubium vulgare* (Ainsworth, 2003).

Understanding these nuances may be particularly important in scenarios where biological control agents alone are insufficient to suppress invasive species significantly and local native propagules are scarce.

Mechanical methods are often selected when herbicide use is inappropriate or undesirable, although these can be labor-intensive and costly. The effectiveness of these treatments can depend on timing, with certain phenological stages of the plant being more susceptible to control measures (Pile Knapp et al., 2023). Manual and mechanical controls, such as stem girdling, clipping, uprooting, and mowing, are frequently utilized due to their direct removal of biomass. However, the absence of complete eradication of below-ground parts may lead to regrowth, indicating a need for follow-up interventions (Pile Knapp et al., 2023). Mechanical treatments targeting susceptible phenological stages could amplify their effectiveness, such as mowing after midsummer to reduce seed production of invasive grasses (Pile Knapp et al., 2023). In pale swallow-wort (*Vincetoxicum rossicum* (Kleopow) Barbar), manual removal showed that this approach significantly reduced the target weed but did not increase native plant diversity or cover, reaffirming the importance of native revegetation (Collings et al., 2023).

Native revegetation efforts play a crucial role in the restoration process, particularly in the context of controlling invasive species. These efforts not only aim to suppress the invasive species but also to reestablish native plant communities that have been displaced or diminished (Kettenring & Adams, 2011; Riebkes et al., 2018). Native revegetation contributes to the restoration of ecosystem functions and biodiversity by providing habitat, food resources for wildlife, and restoring soil health and water regulation (Lake et al., 2014). Moreover, the introduction of native plants can create competitive pressures against invaders, reducing their

dominance and preventing reinvasion (Cutting & Hough-Goldstein, 2013; Pile Knapp et al., 2023). In some cases, native plants selected for revegetation have specific traits that allow them to effectively compete with invasive species, such as deep rooting systems that can access water resources or specific allelopathic properties that inhibit the growth of invaders (Assis et al., 2020). Competitive dynamics within plant communities can be leveraged, as demonstrated by an experiment involving the over sowing of native species, which selectively suppressed the invasive annual *Rapistrum rugosum* (Simmons, 2005). These strategies, when combined with other management techniques like biological control or mechanical removal, might significantly enhance the success of restoration efforts by creating more resilient and diverse ecosystems.

Restoration strategies must account for the temporal dynamics of invasive species control to optimize recovery efforts. The timing of native revegetation appears to be important, with previous literature suggesting that early integration with biological control can occupy the niche that would otherwise be taken by reinvading weeds (Assis et al., 2020; Kettenring & Adams, 2011). In addition to the timing of control, the duration of control and monitoring has significantly influenced the current knowledge in restoration ecology. Initial positive trends in restoration can reverse over time, emphasizing the need for long-term monitoring and adaptive management (Cordell et al., 2016). The timing of active control and monitoring varies, with 15% of cases engaged in active control for a period of 2 to 5 years, and a smaller fraction, 7%, extended beyond 5 years (Kettenring & Adams, 2011). Most studies conducted monitoring for a duration of 1 to 2 years (22%), with monitoring for 2 to 5 years slightly less common (18%) and significantly less frequent for periods exceeding 5 years (6%). The predominant approach to implementing control methods was a single application, noted in 28% of studies (Kettenring & Adams, 2011). This emphasizes the importance of diversifying the duration of control efforts and

enhancing the scope of monitoring to include multiple, adaptive management strategies. In addition to such temporal effects, forests invaded by vines pose unique spatial challenges. Globally, vines rank as the most detrimental invasive plants, given their ability to rapidly colonize and dominate vertical and horizontal spaces that also complicate control efforts (David & Lake, 2023). This often necessitates tailored management approaches such as targeted manual removal and the use of vine-specific biocontrol agents (David & Lake, 2023).

In this research, we combined three different treatment methods, that were applied over a 3-year period to control the invasive vine, pale swallow-wort (*Vincetoxicum rossicum*) in Michigan woodlands. We wanted to incorporate the potential impact of a recently approved but not yet established biocontrol agent and other environmentally friendly approaches. Swallow-wort has shown resilience to mowing and cutting, and herbicide control takes repeat application which is further complicated by the risk to non-target species (Biazzo & Milbrath, 2019; Collings et al., 2023; DiTommaso et al., 2013). As a result of such challenges, we chose to include artificial defoliation of swallow-wort, manual removal of swallow-wort and seeding of native plants. By juxtaposing these methods both individually and collectively, we aimed to evaluate their individual and combined efficacy. We hypothesized that combining treatments will prove more effective than any single strategy, with manual removal and supplementary native seeding anticipated as the most successful approach. To evaluate this hypothesis, we examined various metrics including percent cover, species richness, diversity indices, and the density of swallow-wort for the various treatments.

2. Methods

2.1 Study System

Swallow-wort vines – which primarily invade eastern North American forests – reduce functional diversity, plant richness, and arthropod diversity (Carboni et al., 2021; Collings et al., 2023; Ernst & Cappuccino, 2005; Sodhi et al., 2019). This experiment was conducted at three such invaded sites in Oakland County, Michigan. According to the Michigan Natural Features Inventory, these three sites are categorized as dry-mesic or mesic southern forest (Cohen et al., 2020; Kost et al., 2007). The first site was located in a small recreational area within a residential area in Oxford, Michigan (42° 49' 19.887"N, 83° 17' 14.0058"W). The 4.5 hectares infestation is predominantly pale swallow-wort and the overstory is dominated by American elm (*Ulmus americana*), bitternut hickory (*Carya cordiformis*), and sugar maple (*Acer saccharum*). The size of the swallow-wort infestation at this site allowed us to include all treatments of interest. The second site was located on private property in a rural area (42° 42' 22.1616"N, 83° 36' 28.0038"W). This site was predominantly pale swallow-wort but with patchier and a less dense infestation than the first site. It was dominated by American elm (*Ulmus americana*), sugar maple (*Acer saccharum*) and oak trees (*Quercus spp.*). The third site was located on private property in a rural area near the previous site (42° 42' 18.8418"N, 83° 37' 32.3436"W). This site had a more mixed understory than the previous two but with dense patches of swallow-wort in certain areas. It was dominated heavily by black cherry (*Prunus serotina*) in the overstory. The size of the infestations at these two sites required us to reduce the number of treatments we were able to include. To differentiate the two reduced treatment sites, the former site which has a moderate swallow-wort infestation will be called the reduced moderate site. The latter site which has a patchy but dense infestation will be called the reduced dense site.

2.2 Experimental Design

Due to infestation size and site availability, sites varied in the number of treatments. We conducted all treatments at one site and a reduced treatment design at two sites. At the full treatment site, six treatments were executed in plots of 4m x 4m. At the reduced treatment sites, four different treatments were applied within plots measuring 2m x 2m. Treatments were implemented using a randomized complete block design and were replicated four times per site. All plots had a minimum of 1m buffer between them and each plot contained a 1m x 1m subplot positioned in the center of the plot. The restoration treatments included: (1) Control plots with no intervention, (2) Artificial defoliation of pale swallow-wort, (3) Seeding and/or planting seedlings of native species, (4) Defoliation of pale swallow-wort combined with seeding and/or planting of native species, (5) Manual removal of pale swallow-wort, and (6) Manual removal of pale swallow-wort followed by seeding and planting of native species. At the full treatment site, all the above listed treatments were applied, and the seed addition treatments included the planting of native seedlings, while only treatments 1 – 4 were applied at the reduced treatment sites with addition of seeds but not of native plant seedlings. Planting of native seedling happened at just one site due to availability of native plant plugs. Each site was established in the fall of 2020.

A defoliating biological control agent – the moth *Hyponomeuta opulenta* Christoph (Lepidoptera: Erebidae) – was introduced in the United States as a control method for swallow-wort vines (Stewart, 2017). However, effectively implementing biological control measures can present significant challenges in the field. The swallow-wort biocontrol agent had not yet successfully overwintered in Michigan and had not exhibited sustainable populations at the time of this experiment. To circumvent this issue, we used artificial defoliation to simulate feeding by *H.*

opulenta. Artificial defoliation represents a practical approach to simulating the impact of biocontrol agents, providing insights into the invasive species' resilience to herbivory and the compatibility with other restoration strategies to assess the potential of biocontrol agents before their introduction and establishment. Several studies use artificial defoliation as a method to evaluate the effects of herbivory on a target plant, with one study specifically investigating the differences between simulated and insect herbivory and finding no difference (Kirby et al., 1997; Milbrath, 2008; Milbrath et al., 2016; Watt et al., 2007; Wirf, 2006). This technique, while experimental, complements traditional restoration methods by offering a proxy for the ecological pressures exerted by enemies of invasive species. Therefore, for the defoliation only treatment and defoliation plus seed addition treatment (all sites), feeding by the biocontrol agent was mimicked by hand. Defoliation was conducted by ripping each leaf on a swallow-wort stem in half. This level of defoliation was chosen because laboratory studies show that two larvae per stem can cause up to a 50% reduction in leaf area on pale swallow-wort (Weed & Casagrande, 2010). At the full treatment site, in each year from 2021 to 2023, defoliation took three to four weeks to implement due to the large size of the plots. Defoliation for this site was conducted in the month of July. At the reduced treatment sites, defoliation took a week each and was completed within the first two weeks of August.

The manual removal of swallow-wort was conducted at the full treatment site in treatments five and six. The removal of swallow-wort plants was conducted in October of 2020 by digging all swallow-wort stems out by the root and raking with a hard-tine rake to remove straggling root balls and smooth the surface. Another manual removal was performed in spring of 2021 to account for any roots or plants missed at the first removal.

For treatments three and four (all sites), and treatment six (full treatment site only) that involved the addition of native plant seeds we used a native mesic woodland mix from Native Connections (local Michigan genotypes; Kalamazoo, Michigan) supplemented with woodland species from Michigan Wildflower Farm (Portland, Michigan). Seeding was conducted at the recommended application rate by Native Connections of approximately 10 kg/ha of forbs and 25 kg/ha of grasses (30% forbs to 70% grasses). Core species were seeded yearly while some species varied each year based on yearly availability (see a list of native plant included in the seed mix in Appendix 1). At all sites in these treatments, seeding was conducted from fall 2020 to winter 2023 according to season specific recommendations. Each year, one seeding treatment was conducted once in mid-October to January and one seeding treatment was conducted in mid-April to mid-June for a total of six seed additions. Each fall/winter, seeding was performed by raking the leaf layer from each plot. The soil was raked with a soft tine rake and seeds were hand dispersed. Seeds were tamped into the soil by stomping and leaves were replaced on each plot.

We added native plantings to the full treatment site to take advantage of the increased size of the plots and the additional treatments. Native plant plugs were grown from the same seed mix used for the seed addition treatment by seeding the mix into a 51 x 25 cm plant growing tray with drain holes using SUREMIX® all-purpose perlite mix (Michigan Grower Products Inc., United States). Trays were seeded in December of 2021 and placed outside for overwintering. Trays were brought into the greenhouse in early April of 2021 and placed under 16:8h L:D lamps at 22-25°C. Species that grew in abundance were repotted into 1.14 L square plastic pots after reaching approximately 2cm in height. Species that were abundant enough for planting were *Ageratina altissima* (white snakeroot), *Epilobium coloratum* (cinnamon willowherb), *Anemone virginiana* (tall thimbleweed), *Oenothera biennis* (common evening primrose), and *Monarda*

fistulosa (wild bergamot). Plugs were planted in any plot assigned to the seed addition treatment outside of the 1m x 1m subplot but in the main plot, 70-80cm from the edge of the main plot. Species were planted in a square, with at least one species per side (minimum 4 per plot). All species except *Epilobium coloratum* and *Monarda fistulosa* were planted in replicates of 2 per side (8 per plot). Transplantation occurred in June of 2021 and replacements were planted in July 2021 if transplantation failed. All plugs were watered immediately after planting.

Data Collection

Data collection took place in August to September from 2021 to 2023. Percent cover was taken as a proportion of the subplot in each treatment allowing for overlap between species, therefore allowing the total percent cover of all species to exceed 1. Species richness was taken for the entire plot, including the subplot. For percent cover, each species was categorized into a descriptive status as either ‘swallow-wort’, ‘invasive’, ‘native’, ‘non-native’, and ‘unknown’. The statuses were classified using the University of Michigan Herbarium online flora search (Reznicek & Walters, 2011). The ‘swallow-wort’ status only included the percent cover of swallow-wort, whereas the percent cover of the ‘invasive’ status includes all other invasive species other than swallow-wort. An invasive species is a non-native species introduced to an ecosystem that causes or is likely to cause economic, environmental, or human health harm (Higman et al., 2009). These species often outcompete native plants because they lack natural population controls such as predators, competitors, and diseases from their native environment (University of Michigan Environment, Health & Safety). The status ‘non-native’ included plant species that would not be deemed invasive but did not originate in Michigan (U.S. Department of the Interior). If a plant species could not be identified, often due to damage to the plant and/or difficulty of identification in early life stages, it was placed in the ‘unknown’ status. Statuses

were altered for species richness so that swallow-wort was included in the ‘invasive’ category, as there was only one species of swallow-wort at each site.

Likewise, a species was categorized as either seeded or not seeded, referring to species that were actively seeded as part of the treatment or species that were not actively seeded, or naturally occurring. In years 2022 and 2023, abundance was taken for each species in the subplot for diversity indices calculations and swallow-wort stem density. Each species was identified at minimum to family and at most to species. For consistency, if a plant was identified to the level of genus or the level of species at first identification, it was kept at that level of identification for the rest of the experiment. All forbs were identified to genus and often species. All trees were identified to genus. Grasses, however, were only identified to family for percent cover as it was difficult to disentangle species visually. Grasses in the full treatment site and one of the reduced treatment sites were labeled as ‘native’ as there were no other grasses present at the site and grass was only present where active seeding occurred. Grasses at the last reduced treatment site could not be confidently identified and were left in the ‘unknown’ status category. If grasses produced seed heads, they were identified to species for species richness data collection. Latin names were formatted so that each taxonomic name was in a ‘family *genus species*’ format. If genus or species was unknown, it was labeled as ‘*spp*’. Grasses were excluded from abundance counts.

Native plantings at the full treatment site were checked for survival in 2022 and 2023. The orientation of each plug was tracked by being assigned a side of the planting square which was kept consistent for each plot. Additionally, the replicate per side was noted.

2.2 Statistical Analysis

All analyses were performed in R 4.3.0 (R Core Team, 2023). We constructed different models for each response variable: percent cover, species richness, swallow-wort stem density, and native planting survival. We provide chi-squared values for each model by using the Anova functions from the *lmtest* and the *car* package (Fox & Weisberg, 2019; Zeileis & Hothorn, 2002).

2.2.1 Percent Cover

To assess if there were any differences in the percent cover by treatment, we chose to use a beta family distribution which can be used for analyzing continuous proportions (Douma & Weedon, 2019). Percentages were converted to proportions equal to or between 0 and 1 to use this distribution. We used the *glmmTMB* package which allows us to implement a generalized linear mixed model using the beta family distribution (Brooks et al., 2017). Fixed effects for this model were additive and included treatment (control, defoliation, seed, defoliation + seed, swallow-wort removal, swallow-wort removal + seed), year (2021, 2022, 2023), status ('swallow-wort', 'invasive', 'native', 'non-native', or 'unknown'), and site (full treatment site, first reduced treatment site, second reduced treatment site). To account for repeated annual sampling of each plot, plot replicate was used as a random effect (percent cover ~treatment + year + status + site + (1|replicate)).

Additionally, we assessed if species that were actively seeded differed in their percent cover by treatment (treatments three, four, and six). To do this, we used a nearly identical model as the previous model but added the Latin name as a fixed effect (percent cover of seeded species ~ treatment + Latin name + year + site + (1|replicate)). We also added a zero-inflation parameter, which was set with the same fixed and random effects as the conditional model. This was added

because beta family distributions cannot equal zero or one but a zero-inflation parameter allows the model to include zeros.

To assess both models, we used the *DHARMA* package which provides residuals for generalized linear mixed models (Hartig, 2022). Post-hoc comparisons were performed using the *emmeans* package (Lenth et al., 2021).

2.2.2 Species Richness

To assess overall species richness, we first assessed the normality of species richness using a Shapiro-Wilk's test from the *stats* package (R Core Team, 2023). Once species richness was found to fit the assumptions of normality, a linear mixed effects model was used for analysis from the *lme4* package (Bates et al., 2015). Fixed effects for this model were additive and included treatment (control, defoliation, seed, defoliation + seed, swallow-wort removal, swallow-wort removal + seed), year (2021, 2022, 2023), and site (full treatment site, first reduced treatment site, second reduced treatment site). Plot replicate was used as a random effect (percent cover ~ treatment + year + site + (1|replicate)). Model fit was assessed by plotting residuals and creating a Q-Q plot from the *stats* package (R Core Team, 2023).

Species richness was also analyzed by status ('native', 'invasive', 'non-native', or 'unknown'). To do this, we again assessed the normality of species richness using a Shapiro-Wilk's test from the *stats* package after separating the number of species by status (R Core Team, 2023). After separation, species richness did not follow the assumptions of normality, therefore we implemented a generalized linear mixed effects model with a Poisson distribution, which is often used to model count data. Fixed effects for this model included treatment (control, defoliation, seed, defoliation + seed, swallow-wort removal, swallow-wort removal + seed), year (2021, 2022, 2023), status ('swallow-wort', 'invasive', 'native', 'non-native', or 'unknown'), and

site (full treatment site, first reduced treatment site, second reduced treatment site). We added an interaction between treatment and status, but site and year were additive. Plot replicate was used as a random effect (percent cover \sim treatment * status + year + site + (1|tepligate)). To assess this model, we used the *DHARMA* package which provides residuals for generalized linear mixed models (Hartig, 2022). Post-hoc comparisons for both models were performed using the *emmeans* package (Lenth et al., 2021).

2.2.3 Swallow-wort Stem Density

To assess if swallow-wort stem density varied by treatment, we first assessed the normality of the count of swallow-wort stems using a Shapiro-Wilk's test from the *stats* package (R Core Team, 2023). Fixed effects included treatment (control, defoliation, seed, defoliation + seed, swallow-wort removal, swallow-wort removal + seed), year (2022, 2023), and site (full treatment site, first reduced treatment site, second reduced treatment site). An interaction between treatment and site was added. Plot replicate was used as a random effect (percent cover \sim treatment * site + year + (1|replicate)). Swallow-wort stem density did not meet the assumptions of normality, so a generalized linear mixed effect model was performed using a Poisson distribution. However, the model exhibited significant overdispersion after assessment for overdispersion using the *DHARMA* package (Hartig, 2022). To address this issue, we created a similar model using negative binomial distribution, which is often used to counteract overdispersion. We reassessed dispersion of the negative binomial model and found it to be satisfactory. Additionally, the Poisson model and the negative binomial model were compared using alkaline information criteria (AIC) from the *bbmle* package (Bolker and Team, 2022). The negative binomial model produced the lowest AIC, confirming our model choice.

2.2.5 Native Planting Survival

At the full treatment site, native plant plugs were individually assessed for survival and were assigned a “0” if they did not survive and a “1” if they did survive. A generalized linear mixed effects model was used with a binomial distribution using the *lme4* package (Bates et al., 2015). Treatment (seed, defoliation + seed, removal + seed), year (2021, 2022), and species planted (*Ageratina altissima*, *Monarda fistulosa*, *Epilobium coloratum*, *Oenothera biennis*, and *Anemone virginiana*) were all included in the fixed effects. Replicate was included as a random effect by nesting the treatment replicate, the orientation (planting square side: 1-4) within the treatment replicate and the replicate within orientation (survival ~ treatment + species planted + year + (1|orientation replicate: orientation: treatment replicate)).

3. Results

3.1 Percent Cover

Percent plant cover varied by treatment ($\chi^2 = 38.17$, $df = 5$, $p < 0.001$), by year ($\chi^2 = 9.28$, $df = 2$, $p < 0.01$), by plant status ($\chi^2 = 684.49$, $df = 4$, $p < 0.001$), and by site ($\chi^2 = 25.15$, $df = 2$, $p < 0.001$) (Fig. 2.1). All treatments were significantly different from the control (pairwise comparison: $p < 0.03$) except the seed addition treatment. The defoliation, seed addition, defoliation plus seed addition treatments all increased percent plant cover when compared to the removal treatment (pairwise comparisons: $p < 0.01$). Overall, the percent cover in 2022 was 1.2 times higher than in 2023 (pairwise comparison: $p < 0.01$) but other comparisons were not significant. The total percent plant cover was dominated by swallow-wort (41.97%) and native species (41.56%). A smaller proportion was covered by other invasive species than swallow-wort (7.70%), non-native species (3.24%), and unknown species (5.56%). Native species were 0.2 times the percent cover of swallow-wort (pairwise comparison: $p < 0.001$) and 1.6 times the

percent cover of non-native species (pairwise comparison: $p < 0.01$). Additionally, comparisons of the percent cover between swallow-wort and invasives, swallow-wort and non-natives, and swallow-wort and unknown species were significant (pairwise comparisons: $p < 0.001$). All other comparisons were not significant. The reduced dense treatment site exhibited significantly higher plant cover from both the reduced moderate site and the full treatment site (pairwise comparisons: $p < 0.001$). However, the reduced moderate site and the full treatment site were not significantly different from each other.

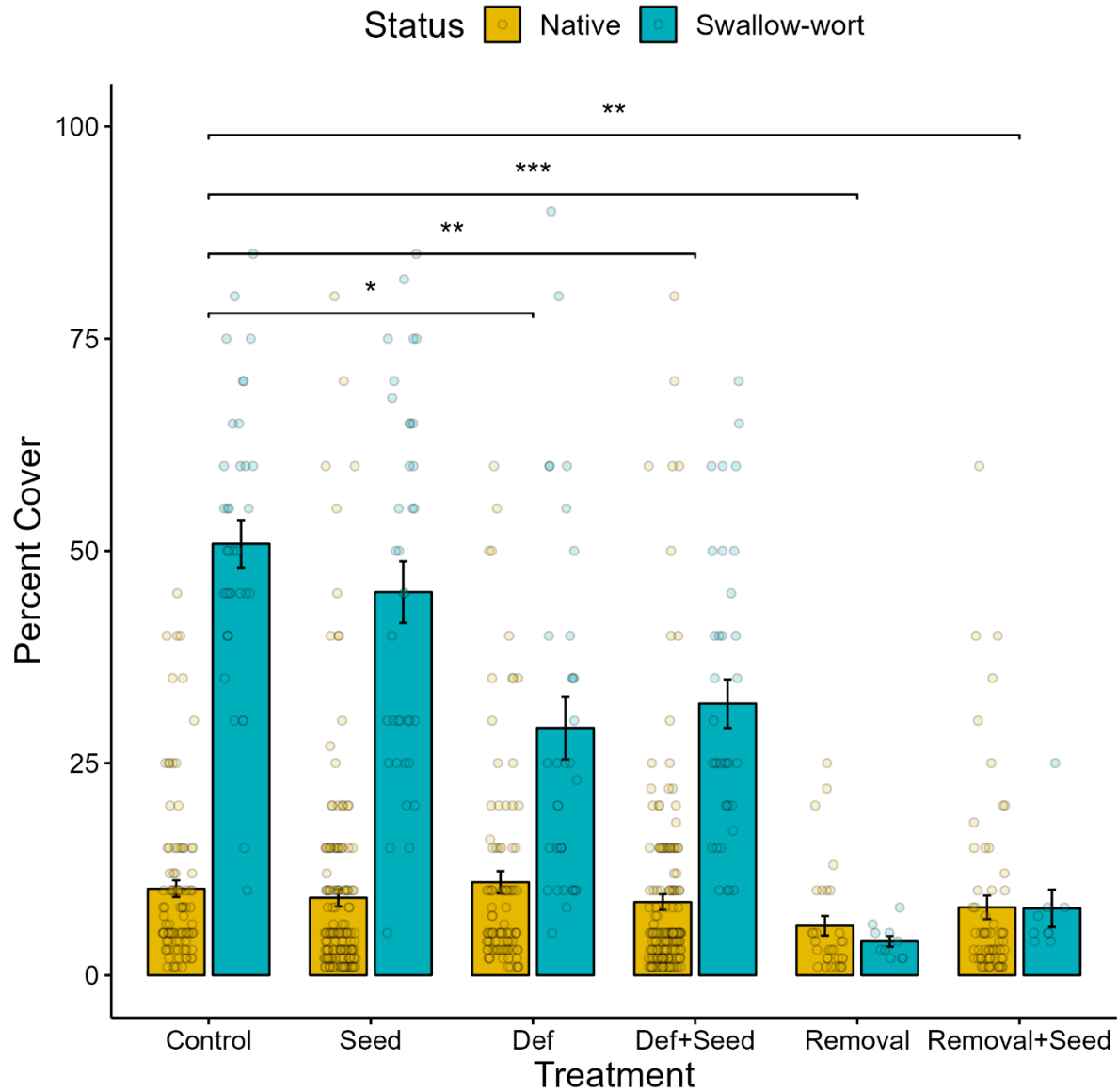


Figure 2.1. Mean and standard error of the percent plant cover by treatment (control, swallow-wort defoliation, swallow-wort removal, native seed addition, swallow-wort defoliation + native seed addition, swallow-wort removal + native seed addition) categorized by status (native or swallow-wort). The categories of invasive, non-native, and unknown for plant invasion status were not included for readability. Observations have been jittered to show individual points. Asterisks indicate significant differences between treatments at the 0.05(*), 0.01(**), 0.001 (***) levels.

Percent plant cover of species that were added with a seed mix varied by treatment ($\chi^2 = 10.12$, $df = 2$, $p < 0.01$), by year ($\chi^2 = 24.14$, $df = 2$, $p < 0.001$), by Latin name ($\chi^2 = 60.61$, $df = 12$, $p < 0.001$), but not by site ($\chi^2 = 2.37$, $df = 2$, $p = 0.31$) (Fig. 2.2). The removal plus seed addition treatment exhibited 1.7 times the seeded plant cover of the seed addition only treatment (pairwise comparison: $p < 0.01$) and 1.5 times the defoliation plus seed treatment (pairwise comparison: $p < 0.05$). However, the seed and the defoliation plus seed treatment were not significant from each other. The year 2023 had higher seeded plant cover than both 2021 and 2022 (pairwise comparisons: $p < 0.01$), doubling the amount of seeded plant cover from 2021 to 2023. The species that dominated the seeded plant cover were native grasses and species in the family Asteraceae, particularly *Ageratina altissima*. The cover of native grasses was significantly higher than *Zizia aurea*, *Rudbeckia* spp., *Monarda fistulosa*, *Oenothera biennis*, and *Thalictrum dioicum* (pairwise comparisons: $p < 0.05$).

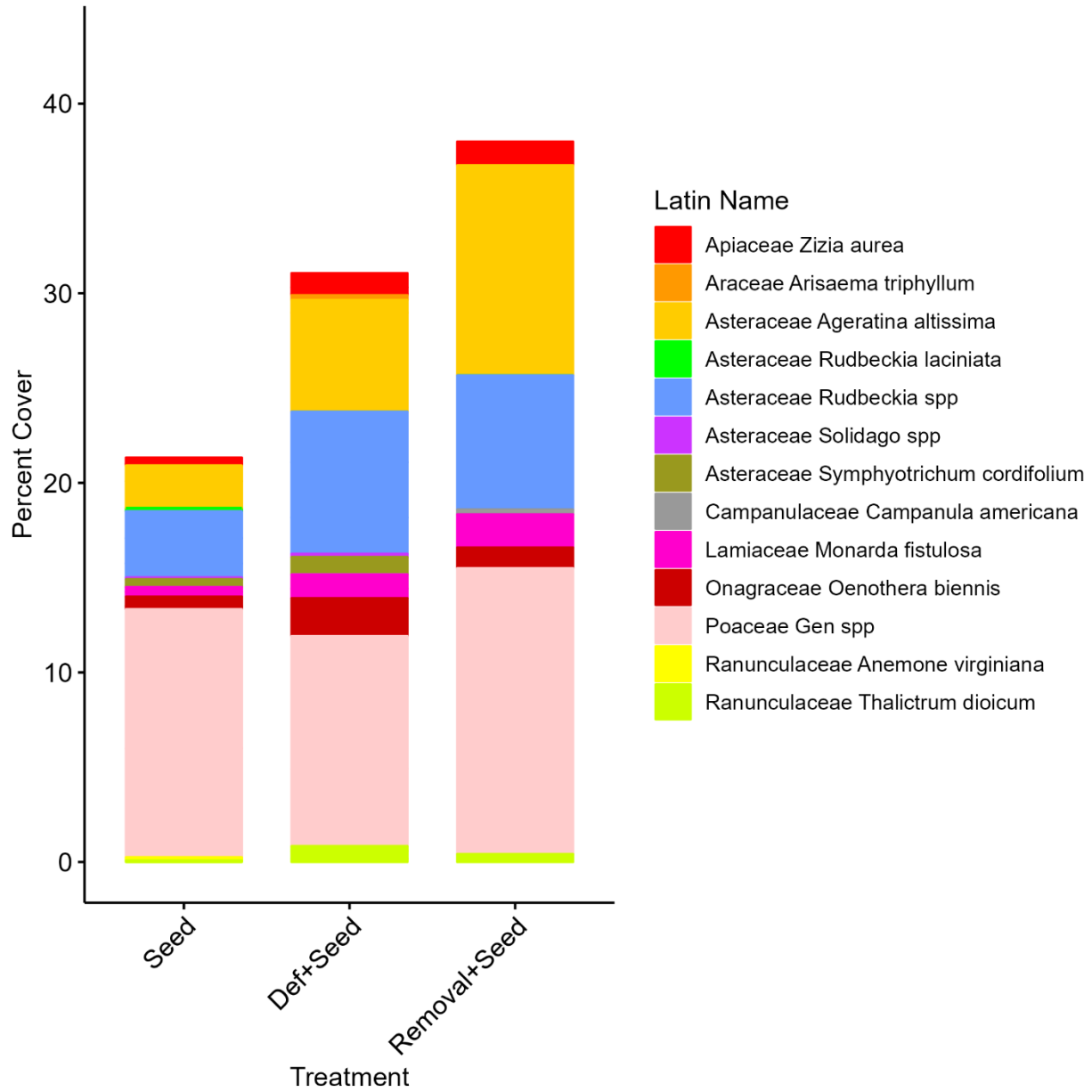


Figure 2.2. Mean percent plant cover of actively seeded species by treatment (seed, def + seed, removal + seed). The species are given by their Latin names in a ‘Family Genus species’ format.

3.2 Species Richness

Species richness varied by treatment ($\chi^2 = 104.83$, $df = 5$, $p < 0.001$), by year ($\chi^2 = 42.24$, $df = 2$, $p < 0.001$), and by site ($\chi^2 = 80.22$, $df = 2$, $p < 0.001$). When compared to the control treatment, all treatments except the defoliation only treatment had significantly higher species

richness (pairwise comparisons: $p < 0.01$). The defoliation plus seed treatment more than doubled the species richness when compared to the defoliation only treatment (pairwise comparison: $p < 0.05$). The removal treatment increased species richness when compared to the defoliation treatment by 3.9 times (pairwise comparison: $p < 0.01$). And the swallow-wort removal treatment plus seed addition outperformed all by increasing species richness by 8.5 times when compared to the defoliation treatment, by 6.8 times when compared to the seed treatment, by 6.2 times when compared to the defoliation plus seed addition treatment, and by 4.5 times when compared to the swallow-wort removal treatment (pairwise comparisons: $p < 0.01$) (Fig. 2.3). There was a decrease in species richness from 2021 to 2022, but an increase in species richness from and 2022 to 2023 and an overall increase from 2021 to 2023 (pairwise comparisons: $p < 0.05$). The reduced dense site was significantly higher in species richness by 4.1 times from the reduced moderate site and by 6.8 times from the full treatment site (pairwise comparisons: $p < 0.001$). The reduced moderate site was also 2.6 times more species rich than the full treatment site ($p < 0.05$) (Fig. 2.4).

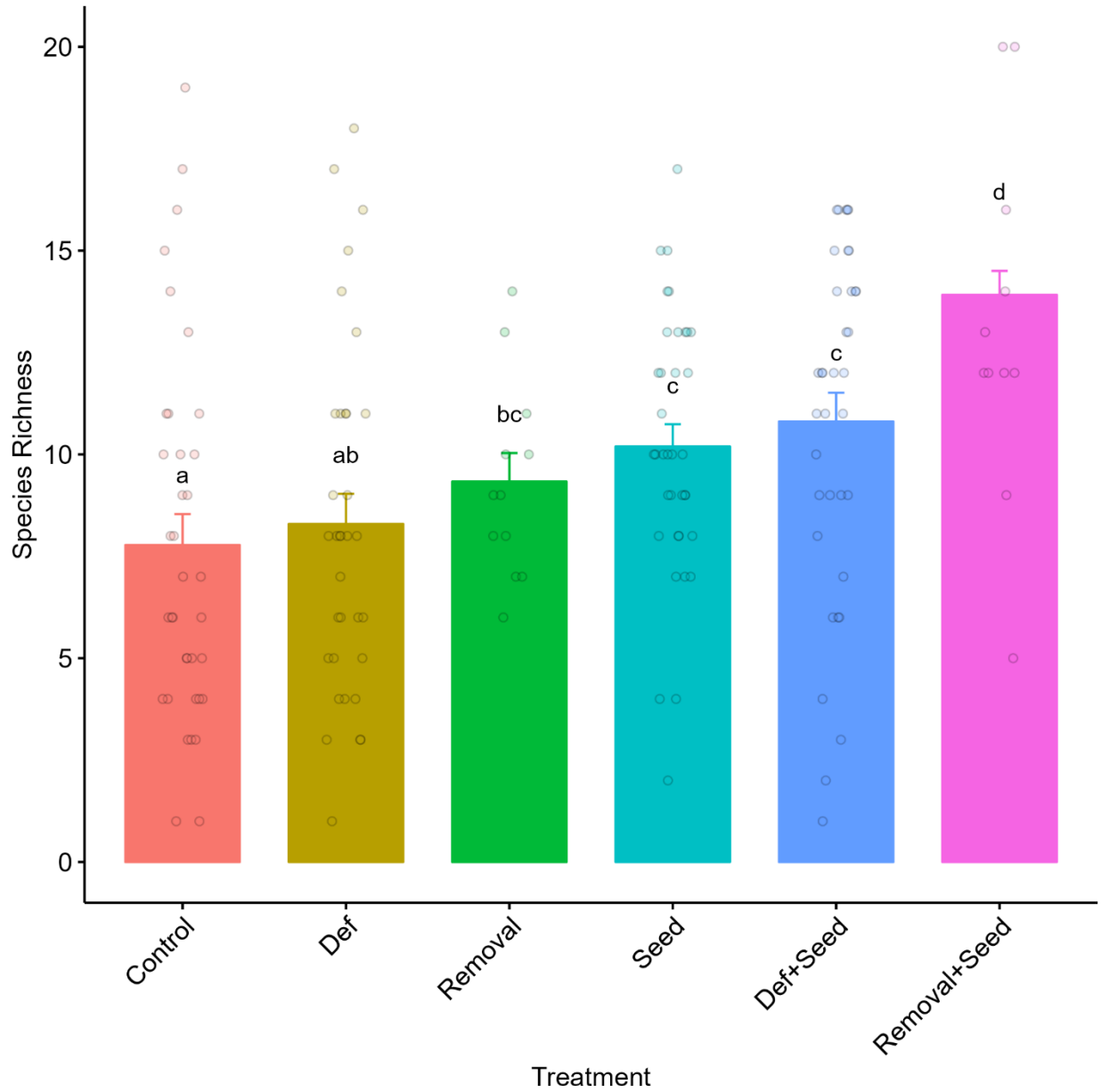


Figure 2.3. Mean and standard error for species richness by treatment (control, swallow-wort defoliation, swallow-wort removal, native seed addition, swallow-wort defoliation + native seed addition, swallow-wort removal + native seed addition). Observations have been jittered to show individual points. Treatments not sharing any letter are significantly different from each other at $p < 0.05$.

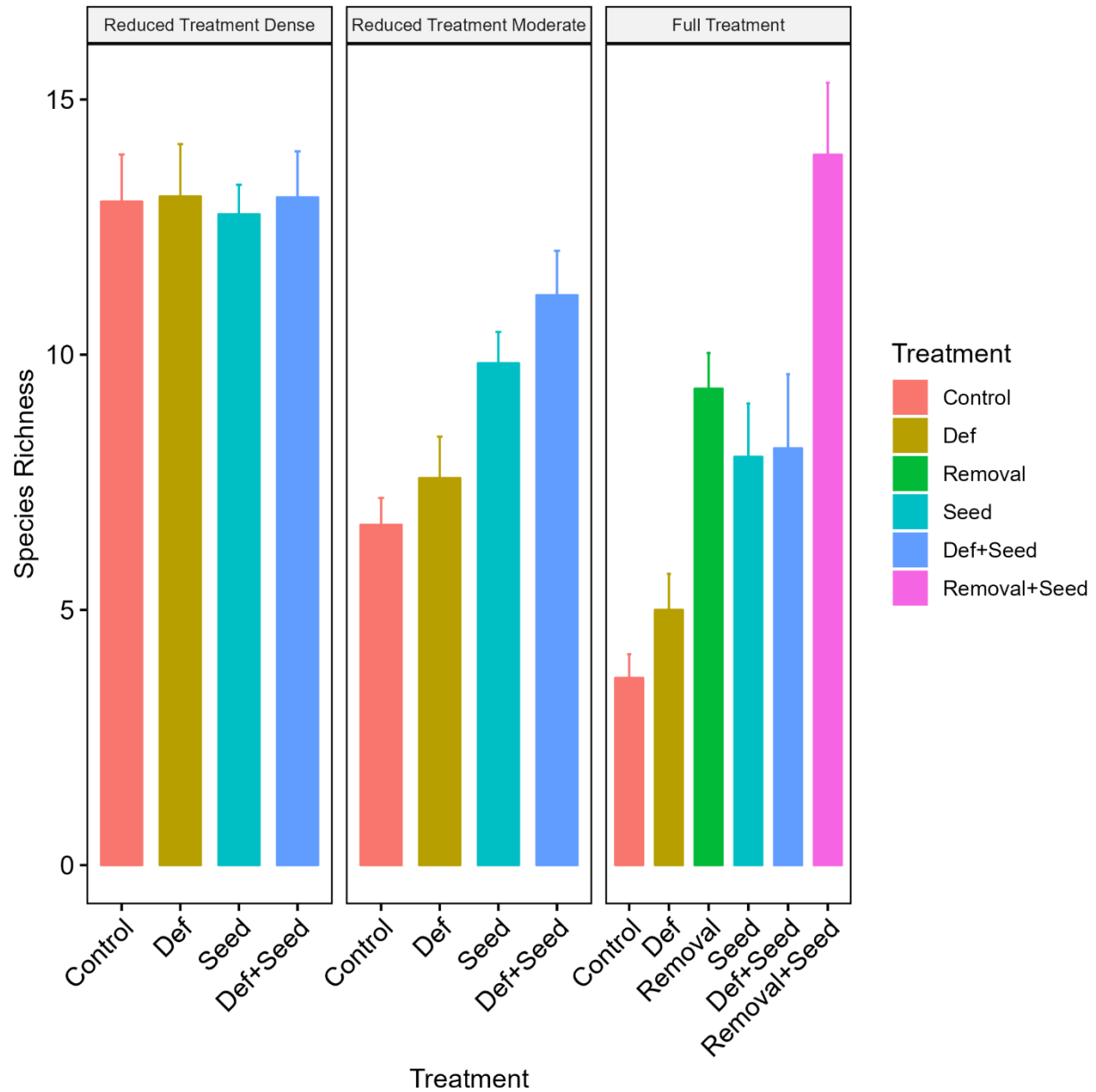


Figure 2.4. Mean and standard error for species richness by treatment (control, swallow-wort defoliation, swallow-wort removal, native seed addition, swallow-wort defoliation + native seed addition, swallow-wort removal + native seed addition). Panels are separated by site (reduced treatment dense swallow-wort site, reduced treatment moderate swallow-wort site, full treatment site). All sites were significantly different from each other ($p < 0.05$).

When species richness was separated by plant status, it varied by status ($\chi^2 = 120.06$, $df = 3$, $p < 0.001$), by year ($\chi^2 = 34.07$, $df = 2$, $p < 0.001$), by site ($\chi^2 = 95.00$, $df = 2$, $p < 0.001$), and the interaction between treatment and status ($\chi^2 = 63.41$, $df = 15$, $p < 0.001$) (Fig. 2.5). However, species richness did not vary by treatment alone ($\chi^2 = 8.46$, $df = 5$, $p = 0.13$). All combinations of status were significantly different from each other (pairwise comparisons: $p < 0.001$) within each treatment with two exceptions. The number of non-native species and unknown species did not significantly differ from each other across all treatments (pairwise comparisons: $p > 0.05$). Additionally, the number of invasive species and native species were not significantly different from each other within the control treatment (pairwise comparison: $p = 0.06$). Between each treatment, all treatments except the defoliation only treatment were significantly different when compared to the control (pairwise comparisons: $p < 0.001$). All other comparisons between treatments were significant ($p < 0.001$), except for the defoliation plus seed addition and removal, defoliation plus seed addition and seed addition, and the removal and seed addition treatments. Similar to the previous model not including status, all pairwise comparisons were significant for site and year (pairwise comparisons: $p < 0.05$).

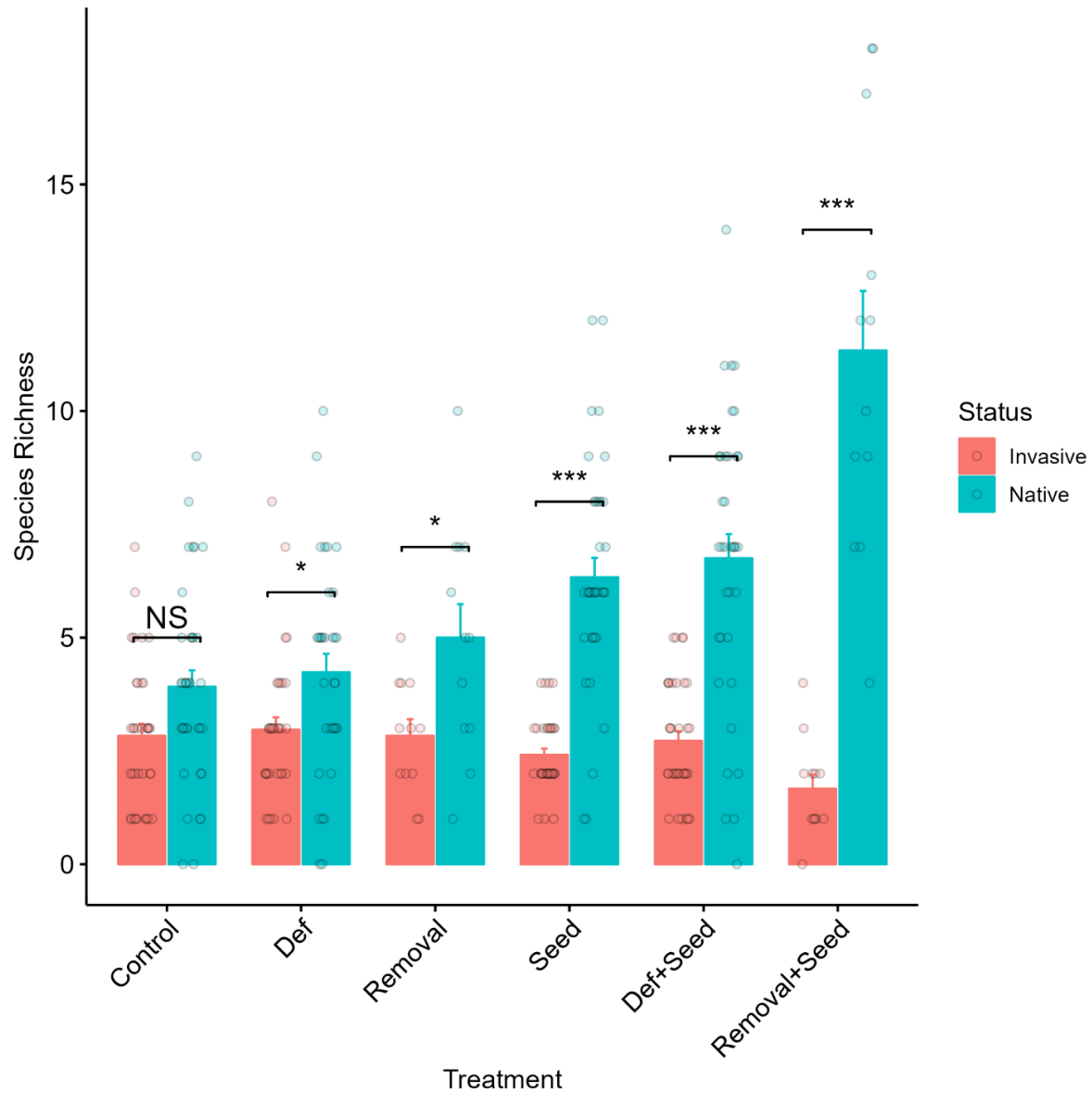


Figure 2.5. Mean and standard error of species richness by treatment (control, swallow-wort defoliation, swallow-wort removal, native seed addition, swallow-wort defoliation + native seed addition, swallow-wort removal + native seed addition) categorized by status (native or swallow-wort). The categories of invasive, nonnative, and unknown for plant invasion status were not included for readability. Observations have been jittered to show individual points. Asterisks indicate significant differences between treatments at the 0.05(*), 0.01(**), 0.001 (***) levels. NS indicates there is not significant difference.

3.3 Swallow-wort Stem Density

Stem density of swallow-wort varied by treatment ($\chi^2 = 116079.88$, $df = 5$, $p < 0.001$), by year ($\chi^2 = 226.65$, $df = 1$, $p < 0.001$), by site ($\chi^2 = 133.72$, $df = 2$, $p < 0.001$), and by the interaction between treatment and site ($\chi^2 = 14955.07$, $df = 6$, $p < 0.001$) (Fig. 2.6). At the full treatment site, all comparisons between treatments were significant (pairwise comparisons: $p < 0.001$), except between the defoliation and the defoliation plus seed addition treatment. At the reduced treatment site with a moderate swallow-wort infestation, all comparisons between treatments were significantly different (pairwise comparisons: $p < 0.001$). At the reduced treatment site with a dense swallow-wort infestation, all comparisons between treatments were significantly different (pairwise comparisons: $p < 0.001$), except between the control and the defoliation plus seed treatment. The year 2022 had a higher swallow-wort stem density than the year 2023 (pairwise comparison: $p < 0.001$)

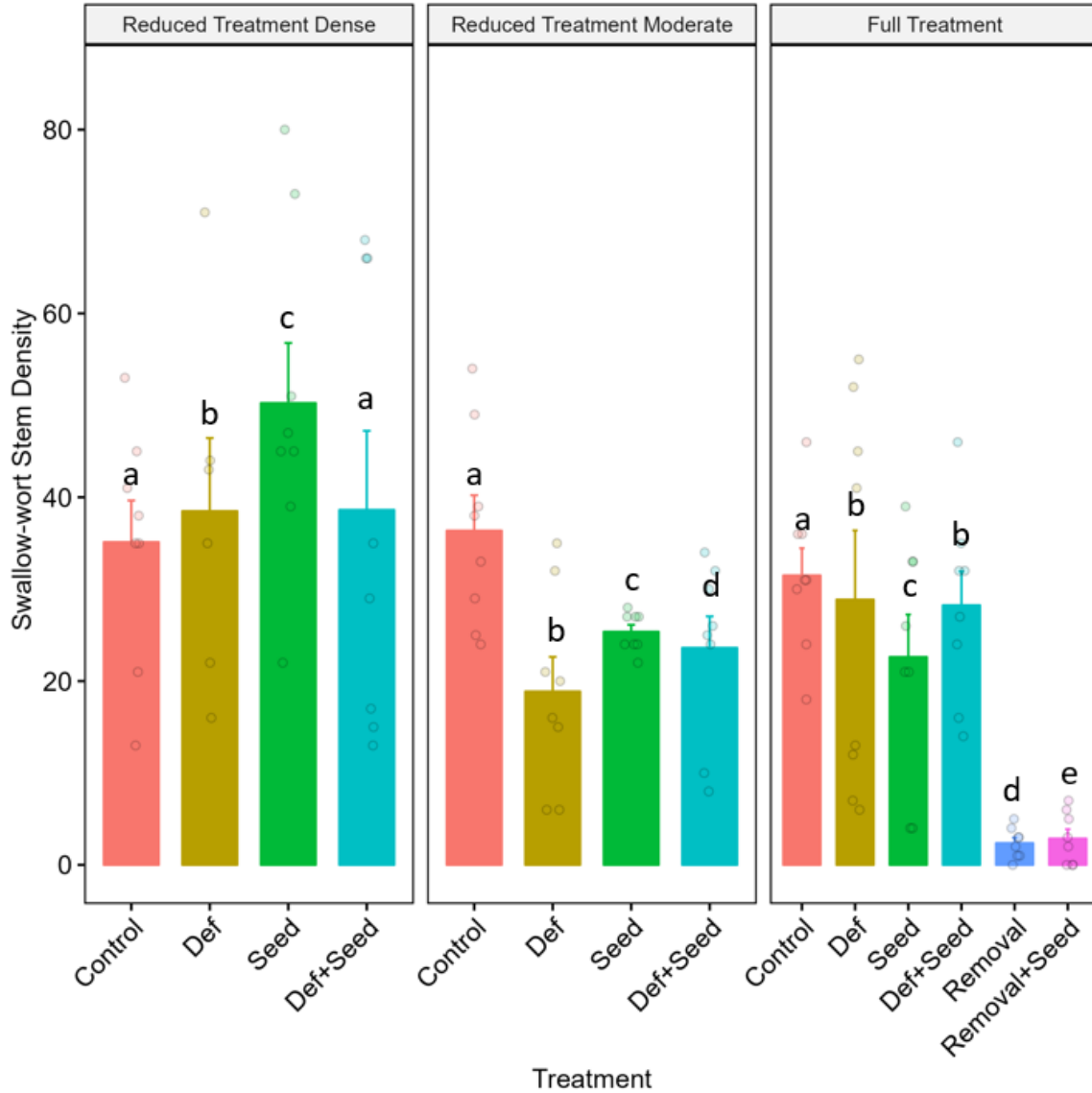


Figure 2.6. Mean and standard error of swallow-wort stem density by treatment (control, swallow-wort defoliation, swallow-wort removal, native seed addition, swallow-wort defoliation + native seed addition, swallow-wort removal + native seed addition). Observations have been jittered to show individual points. Within each panel, treatments not sharing any letter are significantly different from each other at $p < 0.05$.

3.4 Native Planting Survival

Overall, survival of native seedling plugs varied by treatment ($\chi^2 = 16.35$, $df = 2$, $p < 0.001$), by year ($\chi^2 = 6.15$, $df = 2$, $p < 0.05$), and by the species planted ($\chi^2 = 22.96$, $df = 4$, $p < 0.001$). Native plugs planted in the swallow-wort removal plus native seed addition treatment were 2.5 times more likely to survive than plugs planted in the seed addition treatment and 2.7 times more likely to survive than plugs planted in the defoliation plus seed addition treatment (pairwise comparisons: $p < 0.01$). Survival reduced from 2022 to 2023 (pairwise comparison: $p = 0.06$). The plant species *Ageratina altissima* was more likely to survive than *Anemone virginiana* (pairwise comparison: $p < 0.001$), however no other comparisons were significant.

4. Discussion

Overall, we demonstrated that combining any mechanical swallow-wort control with active seeding of native plant species significantly enhanced species richness. Almost all actions, whether single or combined treatments, positively impacted native species richness and reduced swallow-wort cover and stem density. However, changes in percent plant cover were mainly driven by treatments involving the active reduction of swallow-wort biomass. This suggests that both dispersal limitation of native species and competitive pressures from invasive species must be considered to promote native plant diversity in this system.

Our results build on previous work showing that the introduction of native plant species is a critical factor in restoration success. Dispersal limitation can be a significant barrier to restoring native plant communities (Suding et al., 2004). Native plants face challenges in naturally recolonizing areas due to habitat reduction, habitat fragmentation, and population declines (Biswas & Mallik, 2010; Cordeiro et al., 2009). This is well-documented in prairie plant communities (Grman et al., 2015) and is more crucial for restoring forest communities than

understory light competition (Brudvig et al., 2011). In restoring a forest invaded by *Euonymus fortunei*, dispersal limitation, rather than invasive species removal alone, was found to be an important factor (Bauer & Reynolds, 2016). However, evidence for dispersal limitation in invaded forests is sparse, particularly in North America (Pile Knapp et al., 2023). Our study adds to this evidence, showing that in invaded forest communities, dispersal limitation is an essential factor for restoration success. By manually introducing native plants to areas from which they have been extirpated, land managers can bypass dispersal barriers and enhance the speed and success of ecological restoration efforts.

Another key component of restoration success in invaded systems is the active control or biomass reduction of the invader. Species richness was enhanced by swallow-wort removal with or without seed addition, and defoliation with seed addition. All treatments that included a swallow-wort biomass reduction method (defoliation or removal) reduced the percent cover of swallow-wort, with removal having the most dramatic effect. This is consistent with previous studies showing that swallow-wort removal can significantly reduce its coverage (Collings et al., 2023). Defoliation, either alone or combined with seed addition, decreased swallow-wort biomass and increased native species richness compared to the control. Biomass reduction of swallow-wort by either method is likely beneficial by alleviating the competitive pressures that invasive plants impose on the community. Invasive plants outcompete native plants for resources such as light, water, and nutrients, hindering native plant establishment. Numerous studies have demonstrated that invasive species can alter soil chemistry and disrupt mutualistic relationships, sometimes continuing these processes even after removal (Vilà et al., 2011; Levine et al., 2003). By implementing control measures to reduce the presence and spread of invasive species, competitive pressure on native plants is alleviated, allowing them to reclaim their natural

habitats. Projects targeting vine invasions, such as those involving *Vincetoxicum rossicum*, require such approaches due to vines' ability to rapidly dominate spaces and impede native plant recovery (Ditomaso et al., 2005; Livingstone et al., 2020).

The combined effects of native seed addition and swallow-wort control methods were the most effective in reducing swallow-wort and enhancing species richness simultaneously. The removal of swallow-wort with native seed additions was the most effective treatment for all measurements, indicating minimal legacy effects from swallow-wort infestation. The defoliation plus seed addition treatment was not significantly different from defoliation treatment in percent plant cover, nor from the removal treatment and seed addition treatment in species richness. Despite this, the defoliation plus seed addition treatment outperforms both stand-alone treatments when we consider both swallow-wort reduction and native species richness simultaneously. It was more effective than defoliation alone, which did not significantly improve species richness, and the seed-only treatment, which failed to substantially reduce swallow-wort cover compared to the control. When we compare biological control to manual removal, biological control has several advantages, although it is a less immediate solution. It is less labor-intensive, potentially saving on labor costs. Additionally, biological control agents can naturally spread across barriers that humans cannot, accessing difficult-to-reach areas through natural spread or nearby releases. With this information, practitioners can weigh the pros and cons of both treatments to make a practical decision fitting their goals and available resources.

The combination of native species establishment through active dispersal of seeds and invasive species control benefits restoration through various mechanisms. Native plants may occupy resources and space that would otherwise be taken by invading species, preventing what is commonly called the 'treadmill effect', referring to the continual cycle of new invasive species

colonizing areas after the initial invasive species have been controlled (Lake et al., 2014; Pile Knapp et al., 2023; Schuster et al., 2018). Established native plants can prevent reinvasion or new invasions and potentially suppress the existing invasive plant population. This combined approach can create a synergistic effect, leading to a more resilient and self-sustaining ecosystem (Lake & Minter, 2018). Invasive species often create feedback loops that further facilitate their dominance, such as altering nutrient cycling in ways that disadvantage native plants (Ehrenfeld, 2003). By removing or reducing invasive species and reintroducing native plants, these negative feedback loops can be disrupted, and positive interactions among native species can be restored, leading to a more robust and dynamic ecosystem (Falk et al., 2006).

Additionally, considering which species of seeds were most successful is important. In seed addition treatments, specific native plants succeeded, with native grasses being the earliest established. The number of forbs in seed addition treatments increased each year, dominating the percent cover in combination treatments but not in seed-only treatments. Species in the family Asteraceae established early, similar to native grasses. To understand this success and enhance resistance to invasions, it is beneficial to create native plant communities with functional diversity, including species that share traits with invasive plants. This approach aligns with the concept of "limiting similarity" (Funk et al., 2008; Shea & Chesson, 2002; Pile Knapp et al., 2023). Research into the traits of native forbs in the family Asteraceae and swallow-wort could reveal if limiting similarity could be a factor in their success, allowing prioritization of such species in swallow-wort restorations.

This experiment emphasizes that combining invasive plant control with native seed additions can mitigate swallow-wort prevalence and bolster native species richness, despite some site-specific exceptions. The consistent decrease in swallow-wort and increase in native species

richness across treatments points to the potential of combining management approaches. Our results highlight the importance of addressing dispersal limitation and competition in restoration approaches across various sites and the significance of conducting research that spans multiple years of control and observation. We intend to track the long-term outcomes by continuing data collection for at least an additional year, with aspirations for two more years of observation. This extended observation period could gather supplementary information, including the effects of treatments on arthropod diversity and the reproductive potential of swallow-wort. Future research should explore combining various restoration tactics with biological control across diverse environments and spatial arrangements, considering their effects on the biological control agent. This approach could enhance the impact and efficacy of biocontrol agents.

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APPENDIX

Table 2.1. A list of all native species in the seed mix from seed addition treatments.

Plant Type	Latin Name	Common Name
Grass	<i>Bromus purgans</i>	Hairy Wood Chess
Sedge	<i>Carex cristatella</i>	Crested Sedge
Sedge	<i>Carex grayi</i>	Common Bur Sedge
Sedge	<i>Carex sprengelii</i>	Long-beaked Sedge
Grass	<i>Elymus canadensis</i>	Canada Wild Rye
Grass	<i>Elymus hystrix</i>	Bottlebrush Grass
Grass	<i>Elymus villosus</i>	Silky Wild Rye
Grass	<i>Elymus virginicus</i>	Virginia Wild Rye
Grass	<i>Glyceria striata</i>	Fowl Manna Grass
Rush	<i>Juncus tenuis</i>	Path Rush
Forb	<i>Ageratina altissima</i>	White Snakeroot
Forb	<i>Allium tricoccum</i>	Wild Leek
Forb	<i>Anemone canadensis</i>	Canada Anemone
Forb	<i>Anemone virginiana</i>	Tall Thimbleweed
Forb	<i>Aquilegia canadensis</i>	Wild Columbine
Forb	<i>Arisaema triphyllum</i>	Jack-in-the-pulpit
Forb	<i>Aster cordifolius</i>	Heart-leaved Aster
Forb	<i>Blephilia hirsuta</i>	Hairy wood mint
Forb	<i>Campanula americana</i>	Tall Bellflower
Forb	<i>Caulophyllum thalictroides</i>	Blue Cohosh
Forb	<i>Eupatorium purpureum</i>	Sweet Joe Pye Weed
Forb	<i>Geranium maculatum</i>	Wild Geranium
Forb	<i>Helianthus grosseserratus</i>	Saw-toothed Sunflower
Forb	<i>Impatiens capensis</i>	Spotted Touch-me-not
Forb	<i>Lobelia siphilitica</i>	Great Blue Lobelia
Forb	<i>Monarda fistulosa</i>	Wild Bergamot
Forb	<i>Oenothera biennis</i>	Common evening-primrose
Forb	<i>Penstemon digitalis</i>	Foxglove Beardtongue
Forb	<i>Polygonatum canaliculatum</i>	Great Solomon's Seal
Forb	<i>Rudbeckia lacinata</i>	Golden Glow
Forb	<i>Rudbeckia triloba</i>	Brown-eyed Susan
Forb	<i>Smilacina racemosa</i>	False Solomon's Seal
Forb	<i>Solidago caesia</i>	Blue-stemmed Goldenrod
Forb	<i>Solidago flexicaulis</i>	Zigzag Goldenrod
Forb	<i>Solidago rugosa</i>	Rough Goldenrod
Forb	<i>Thalictrum dioicum</i>	Early Meadow Rue
Forb	<i>Veronicastrum virginicum</i>	Culver's Root
Forb	<i>Zizia aurea</i>	Golden Alexander

Table 2.1. (cont'd)

Annual Nurse Crop	<i>Avena sativa</i>	Seed Oats
Annual Nurse Crop	<i>Lolium multiflorum</i>	Annual Rye

CHAPTER 3

Exploring demographic and genetic factors for the establishment of *Hypena opulenta* for the biological control of invasive swallow-wort vines (*Vincetoxicum spp.*)

Abstract

About 40% of exotic herbivorous insects introduced for biological control of invasive weeds do not establish. Demography, such as release size and the frequency of introductions is a well-known factor in mediating establishment success in diverse taxa. Genetic diversity of the released populations and their chances of establishment are also positively correlated in some species. However, most evidence for the effects of demography and genetics in establishment success comes from laboratory studies. First releases of newly approved biological control agents can be used to test the importance of these factors for establishment and persistence in the field. We conducted field releases of *Hypena opulenta*, a biological control agent against invasive swallow-wort vines (*Vincetoxicum spp.*), to test the roles of demography and genetics in establishment in the field. In 2020, releases were conducted at 18 field sites in southern Michigan using *H. opulenta* populations with two distinct genetic backgrounds (a laboratory and outbred population) and at two different release sizes (5 or 20 pairs of adults) using a factorial design. Monitoring later in the season in 2020, the following two years in 2021 and in spring of 2022 indicated that these releases were not successful. Thus, we could not assess the combined effects of demography and genetics in establishment. In 2022, we released replicated *H. opulenta* populations with three distinct genetic backgrounds (lab, field, and outbred) at 15 field sites to test how genetic background may affect establishment. For the 2022 releases, *H. opulenta* was released all through the season, starting in early June until August using both adult and larval stages for a total of 34-37 females, 32-35 males and 37 larvae per site. In the release cages, the presence of any larval feeding and the extent of defoliation was measured. The outbred

population exhibited similar performance to the field population, although the outbred population had the highest mean defoliation overall. As of spring 2024, establishment success could not be confirmed. Given that the build-up of biocontrol agent populations to densities where they are consistently detectable can take multiple years continued monitoring of the release sites will be necessary to evaluate the importance of genetic background for establishment success.

Nevertheless, this study provides guidance how biological control agent releases could be used to test various mechanisms that can mediate establishment with the ultimate goal of increasing the success and impact of new biocontrol programs.

1. Introduction

The long-term management of invasive species is most efficiently achieved through the introduction of natural enemies that have co-evolved with them in their native habitats. Despite the long history of the use of classical biological control agents against invasive species, little is known about what factors influence their successful establishment. Roughly 40% of weed biocontrol agents fail to establish and only 24% of established agents have a high impact on the target weed (Schwarzländer et al., 2018). Several factors can affect the successful establishment of species colonizing novel environments including environmental conditions and random stochastic events, as well as the genetic background and demography of introduced populations. Propagule pressure—defined as the cumulative size and frequency of introductions—emerges as the most reliable indicator of successful establishment (Blackburn et al., 2015; Lockwood et al., 2005; Simberloff, 2009). While the influence of genetic background in establishment success in the field is largely unknown.

Laboratory experiments using insects as model organisms have confirmed field observations that larger initial populations enhance the likelihood of successful establishment (Hufbauer et al.

2013; Szűcs et al. 2014; Szűcs et al. 2017). Similarly, few field trials in weed biocontrol systems have shown that larger releases of control agents tend to result in better establishment and persistence (Grevstad, 1999; Grevstad, 2006; Memmott et al. 2005; Memmott et al. 1998). For example, establishment success of a psyllid released for the control of *Arytainilla spartiophila* in New Zealand generally increased with increasing release sizes that ranged from 2-270 individuals but some of the smallest releases led to establishment as well (Memmot et al., 2005). This outcome may stem from several reasons, such as diminished Allee effects, and increased resiliency to environmental stochasticity in larger populations (Boyce et al. 2006; Lande, 1993). Moreover, a larger founding population often encompasses greater genetic diversity, potentially mitigating inbreeding and enhancing adaptability to new environments, thereby promoting the long-term survival of the population (Drake, 2006; Forsman, 2014; Rius and Darling, 2014). Despite these trends, there are instances where small introductions succeed, as noted above in the psyllid biocontrol agent, and where large-scale releases involving 65,000 individuals failed to establish for a planthopper biocontrol agent (Grevstad et al., 2011). In instances of unsuccessful establishment despite high release size, a critical, yet overlooked aspect of the habitat may affect establishment, such as with planthoppers (Grevstad et al., 2011). Similarly, incidences of establishment at low release sizes indicate that propagule pressure is not the only driving factor behind establishment.

Genetic background and initial population release size of the colonizing populations stand out as pivotal, yet underexplored, determinants of success (Szűcs et al., 2014; Szűcs et al., 2019). Incorporating new genetic material into initial populations can mitigate the effects of inbreeding, boost adaptive capabilities, and generate new genotypes better adapted to unfamiliar settings (Barker et al., 2019; Sinclair et al., 2019; Stewart et al., 2017; Szűcs et al., 2017). One of the

mechanisms leading to heterosis is the masking of fixed recessive deleterious alleles by dominant fitter alleles in sexually reproducing species (Dlugosch et al., 2015). Laboratory research demonstrated that populations created from genetic admixture benefit from enhanced establishment rates and increased survival (reviewed in Szűcs et al., 2014; Szűcs et al., 2017). For example, laboratory experiments with *Tribolium castaneum* not only found a general positive effect of outbreeding on the establishment and persistence of populations, but they also found that small founding sizes reduced fitness (Szűcs et al., 2017). Additionally, genetic mixing in pheasant populations has triggered a 'catapult effect', wherein a temporary boost in fitness in early generations arises from heterosis or 'hybrid vigor', thereby aiding in the population's endurance (Drake, 2006). Few studies investigated the significance of genetic variation on establishment success in the field and there remains a substantial gap in the literature concerning the combined and individual roles of demography and genetic background on establishment.

Biocontrol agents often suffer the negative effects of genetic drift and inbreeding that can lead to reduced genetic diversity, lower fitness and limit adaptive potential (Hufbauer and Roderick, 2005; Szűcs et al., 2019). These effects stem from the common practice of sourcing biological control agents in small numbers from native populations and mass-rearing them for release over tens or hundreds of generations, potentially diminishing their adaptive ability to new environmental conditions (Bertin et al., 2017; Frankham, 2005; Szűcs et al., 2019). Empirical evidence from laboratory studies supports the hypothesis that increased genetic diversity and larger founding sizes can increase establishment and persistence, but these studies are not conducted with any biocontrol agents (Hufbauer et al., 2013; Szűcs et al., 2014). Initial releases of newly approved biocontrol agents lend themselves to experimentation to test mechanisms that can mediate establishment success and persistence in novel environments. Insights obtained from

studies on biocontrol agents can inform our understanding of invasion dynamics, as well as conservation and restoration efforts for other colonizing species (Szűcs et al, 2019).

We conducted releases of *Hypena opulenta* Christoph (Lepidoptera: Erebidae), a biological control agent deployed against swallow-wort vines (*Vincetoxicum spp.*) in an explicitly experimental way to test the roles demography and genetics may play in establishment success and their impact on swallow-wort. We created genetically distinct populations by crossing individuals from a long-term laboratory reared population (a lab population) and individuals from a laboratory reared population that has been established in the field for 10 generations (a field population) to create an outbred population. The genetic differentiation between the parental and outbred populations and the increased heterozygosity of the outbred population was confirmed through molecular analyses (see Chapter 1). In 2020, we released two genetically distinct populations of *H. opulenta* (the lab population and the outbred population) in two different release sizes (low and high) at 18 field sites in Michigan. We hypothesized that the outbred population would exhibit higher levels of establishment than the lab population. And that higher release sizes would result in higher levels of establishment than low release size. After we failed to detect any signs of establishment in 2020 or 2021, we released again in 2022 using a modified experimental design. In 2022, we omitted release size to solely focus on how genetic background alone might influence establishment and impact. We expected that the outbred population would have higher or at least similar establishment success as the field population and that the lab population would have the lowest rates of establishment.

2. Methods

2.1 Study System

Invasive swallow-wort vines from the *Vincetoxicum* genus, including black swallow-wort (*Vincetoxicum nigrum* (L.) Moench) and pale swallow-wort (*Vincetoxicum rossicum* (Kleopow) Barber.), have emerged as significant ecological threats in their non-native ranges, especially in the northeastern United States and southeastern Canada. Originating from Europe, these hardy and adaptable perennial vines disrupt native ecosystems by forming dense mats that outcompete native flora, reduce biodiversity, and alter habitats for local fauna. Their presence is notably problematic for agricultural lands and poses risks to livestock due to their toxicity (Ditommaso et al., 2005; Ditommaso et al., 2018; Ernst & Cappuccino, 2005; Weed & Casagrande, 2010). Managing these invasive species is challenging due to their robust root systems and resistance to herbicides, making mechanical removal and chemical treatments ineffective (Ditommaso et al., 2005; Lawlor & Raynal, 2002; Weed & Casagrande, 2010). Introduced to North America with regulatory approval and after comprehensive testing, the moth species *H. opulenta* has been released as an alternative control method (Bourchier et al., 2019).

The biological control agent *Hypena opulenta* received approval from the United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS) for release in the US in 2017 (Stewart, 2017). This agent targets swallow-wort across all five of its larval stages by feeding on the plant's leaves before transitioning to pupation either in the soil or within leaf structures. Emergence is asynchronous and diapause is triggered primarily by photoperiod (Weed & Casagrande, 2010). Host-specificity trials have confirmed that *H. opulenta* poses no risk to economically significant or native North American species (Hazlehurst et al., 2012). Research involving artificial defoliation demonstrated that repeated leaf removal

significantly diminishes biomass and seed output in both *Vincetoxicum rossicum* and *Vincetoxicum nigrum* under low-light conditions within greenhouse settings (Milbrath, 2008). Field studies over two years observed a direct correlation between the degree of leaf clipping on swallow-wort and a reduction in seed production (Doubleday & Cappuccino, 2011). Further evaluations revealed that as few as two larvae could substantially decrease above-ground biomass and reproductive capability of *V. rossicum*, alongside diminished floral production in *V. nigrum*, within controlled laboratory conditions (Weed & Casagrande, 2010). The capacity of *H. opulenta* to undergo two generations annually, with evidence indicating generational overlap, provides optimism that this agent could effectively impair the growth and reproductive success of *V. rossicum* and *V. nigrum* over extended periods (Weed & Casagrande, 2010). However, changes in photoperiod of the release environments from the natal environment and high predation rates have emerged as barriers to establishment and persistence (Jones et al., 2020; Jones et al., 2022).

Before shipment to Michigan, the first lab population of *Hypena opulenta* was created from wild caught individuals in Donetsk, Ukraine in 2006 from four pupae and 32 larvae (Weed et al., 2011). Population maintenance started at CABI EU-CH (Delémont, Switzerland) before shipment to the University of Rhode Island (URI) which then started quarantine and population rearing in 2008 (Hazlehurst et al., 2012). URI provided a larval shipment to Michigan State University (MSU; East Lansing, MI) in June of 2018 which founded the MSU population with 18 females and 22 males.

In 2020, additional *H. opulenta* specimens from a Canadian field population, which was established from the same initial stock as the MSU population but six years earlier in 2012, were sent to MSU (Bourchier et al., 2019). After successful field releases in Canada in 2013 and

establishment by May 2015, specimens collected in 2019 underwent one generation of rearing at URI before being shipped to MSU as pupae in May 2020, founding a population with 15 females and 13 males. During the summer of 2020, reciprocal crosses were setup between the laboratory-raised and the Canadian field-collected *H. opulenta* populations. The original MSU population from 2018 is referred to as the "lab population," while the population from Canada is termed the "field population." Crosses between these two groups is referred to as the "outbred population" (see Chapter 1 for details).

For moth rearing, swallow-wort plants collected from various infestations in Michigan, including Grand Rapids and Oakland County, were transplanted into 1.14 L pots with SUREMIX® perlite mix and grown in a greenhouse at $26.6\pm 4.9^{\circ}\text{C}$ from March to September. In the fall, these plants were moved outdoors at the MSU Entomology Farm and returned to the greenhouse in March/April of the following year for repotting and use in rearing. Rearing occurred in mesh cages (40 x 40 x 60 cm, Restcloud) in a lab maintained at 22-25°C, with each cage containing six potted plants on a tray of soil, under a 16:8h light-dark photoperiod with supplemental lighting. Adult moths were fed a honey-water mixture, with the feeding solution changed regularly and cages misted daily.

From March to August annually, up to five pairs of adult moths were introduced into each cage for oviposition. Larvae fed on the potted plants and supplementary cut swallow-wort stems inserted into water picks. Initially, these stems were sourced from potted plants, transitioning to field-collected stems from late May. Larvae were moved to ventilated plastic containers for pupation after reaching the 3rd-5th instar stage. Pupae collected from these containers and the cages were sexed and placed in small containers with moist vermiculite under controlled photoperiods. To induce diapause, pupae containers were kept in incubators where the initial 20

C rearing temperature was reduced by 5°C each monthly between September and December and kept at 5°C from December to April/May until the rearing of the next generation started.

2.2 Experimental Field Releases in 2020

To evaluate the influence of genetic and demographic factors on the establishment success of *H. opulenta*, we conducted field experiments in 2020 across 18 locations, utilizing populations of *H. opulenta* with two genetic backgrounds: 1) a lab population, and 2) an outbred population. The lab population had been reared for approximately eight generations at MSU before release and the outbred population constituted second generation hybrids. We applied a factorial design, pairing these two genetic backgrounds with two release sizes—either 5 or 20 pairs of adults—resulting in 4-5 replications per treatment combination. The 18 field sites were divided into four blocks based on geographic proximity. The distribution of treatments across the sites followed an unbalanced block design. In the first three blocks, each of the four treatments (5-pair lab, 5-pair outbred, 20-pair lab, 20-pair outbred) was implemented once. Block 4 lacked the 20-pair outbred treatment, and block 5 omitted the 20-pair lab treatment due to site availability constraints. The releases occurred within 1m² quadrats enclosed by mesh cages to facilitate mating and egg-laying, with cages removed after two weeks to allow larvae to disperse. Releases were conducted on June 24th, 2020. The experiments took place in Oakland County with sites mostly located in forested areas ranging from full shade to edge habitats on private properties or beside trails. All locations were infested with pale swallow-wort (*Vincetoxicum rossicum*).

To monitor population level effects of *H. opulenta*, eight 0.5m² permanent quadrat plots were established at each site, with half marked to be treated with insecticide to serve as controls and the other half marked to be left untreated to allow for *H. opulenta* feeding. These plots were randomly arranged around the release areas within a 10m radius of the release site. To monitor

impact of *H. opulenta* on the individual plant level five swallow-wort plants were marked within each plot. Within each plot the number of swallow-wort stems were counted at the start and end of the growing season, and the percent defoliation was estimated on 10 stems. For the individually marked plants the number of seed pods were counted at the end-of-season. Plots and individual plants were monitored two times following the releases in 2020, monthly during 2021 and in May in 2022.

2.3 Experimental Field Releases in 2022

Field releases were repeated in a modified design by omitting the release size treatment in 2022 after establishment failed from the 2020 releases. To test the effect of genetic background on establishment field releases took place at 15 locations, using three populations of *H. opulenta*: 1) the lab 2) the field, and 3) the outbred populations. Before release the lab population had been reared for approximately 14 generations at MSU, the field population for eight generations, and the outbred population constituted seventh generation hybrids. An unbalanced block design was used whereas the first block contained three replicates of each treatment and the second block contained two replicates of each treatment ($n = 5$) (Fig. 3.1). The 15 sites used for releases in 2022 were the same where the 2020 releases took place that were deemed unsuccessful. Changes in treatment assignments at each location from 2020 to 2022 can be found in Table 3.1. In general, an attempt was made to match the genetic background of releases at given sites between the 2020 and 2022 releases to account for the possibility of establishment from the first release that might have gone undetected. The outbred population was released at sites that were assigned to the ‘low’ release size of 5 pairs of adults in 2020 because establishment from the first releases were least likely at these sites that might have interfered with the later releases. The releases occurred within 1m^2 quadrats enclosed by mesh cages to facilitate mating and egg-laying. Both

adults and larval stages were released from June through August. Cages were kept on until August 2nd. Release times and release sizes are detailed in Table 2. In sum, 37 adult females, 32 males and 37 larvae were released at each site for the field and outbred populations, and 34 females and 35 males for the lab population. We attempted to release equal numbers at the same time for each population treatment but were restricted due to the availability and emergence time of individuals, particularly from the lab population. For this reason, the timing and release sizes of the field and outbred populations are in good agreement but there is a delay and initially lower release sizes for the lab population (Table 3.2).

Defoliation by offspring of the released adults and from the larval releases were assessed twice: once early in the season to capture defoliation from the first two releases and once late in the season to capture defoliation from the last two releases. This was done by selecting ten random stems within each release cage and estimating the average defoliation per stem. A subsequent release occurred following the removal of the cage, with late-season defoliation assessments carried out about one month after release in the same area previously enclosed by the cage. The cages were removed due to the observation that intense feeding within some cages had led to a decrease in available swallow-wort biomass for further feeding. Stem density counts were taken in the release cages at the time of the first release to account for any effects in food availability. To account for site differences, sites were categorized by their habitat type. Sites were categorized as ‘roadside’ if they were within 5m of a road with motorized vehicles. If a site was within 5m of a walking trail or campsite, it was categorized as an ‘edge’ site. Sites that were more than 5m from any trail or roadside were categorized as ‘rural’.

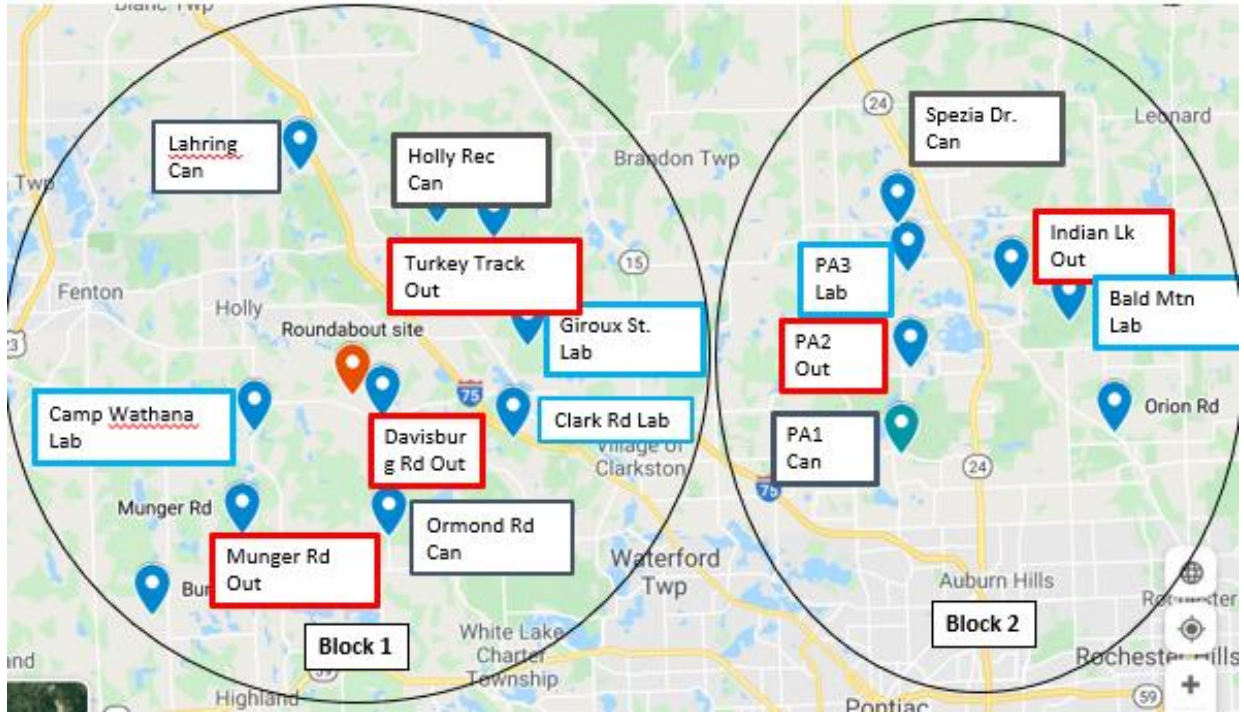


Figure 3.1. Map of the 2022 release sites. Boxes are placed next to the pinned release locations with three border colors to indicate the populations released at that site. Boxes in blue indicate releases of the lab population. Boxes in gray indicate releases of the field population. Sites within the circles belong to either of two spatial blocks.

Table 3.1. Sites used for field releases in 2020 and in 2022. The genetic background and release size treatments are indicated for the 2020 releases. The new genetic background treatment assignment is indicated for the 2022 releases. Habitat types are given for each site (edge, roadside, or rural). If sites were dropped from 2020 to 2022, their habitat type is classified as Not Applicable or NA.

Site	Location	2020 Block Assign- ment	2020 Treatment	2022 Block Assign- ment	2022 Treatment	Habitat Type
Bald Mountain	42.785675, -83.196377	4	Outbred, 20	2	Lab	Edge
Burkhart	42.6742565, -83.6733341	3	Lab, 5	Dropped	Dropped	NA
Camp Wathana	42.736288, -83.61545	3	Outbred, 5	1	Lab	Edge
Clark Road	42.741395, -83.485015	5	Outbred, 20	1	Lab	Rural
Davisburg Road	42.749908, -83.553234	5	Outbred, 5	1	Outbred	Roadside
Giroux Street	42.776174, -83.477617	1	Lab, 20	1	Lab	Rural

Table 3.1 (cont'd)

Site	Location	2020 Block Assign- ment	2020 Treatment	2022 Block Assign- ment	2022 Treatment	Habitat Type
Holly Recreational Area	42.823514, -83.524798	1	Outbred, 5	1	Field	Edge
Indian Lake Road	42.797983, -83.226714	4	Lab, 5	2	Outbred	Roadside
Lahring Road	42.843221, -83.596098	1	Lab, 5	1	Field	Roadside
Munger Road	42.7052562, -83.6256498	3	Lab, 20	1	Outbred	Rural
Orion Road	42.743146, -83.172950	4	Outbred, 5	Dropped	Dropped	NA
Ormond Road	42.703515, -83.54906	5	Lab, 5	1	Field	Rural
Polly Ann Trail North	42.804251, -83.280229	2	Outbred, 5	2	Lab	Edge

Table 3.1 (cont'd)

Site	Location	2020	2020	2022	2022	Habitat Type
		Block Assignment	Treatment	Block Assignment	Treatment	
Polly Ann Trail Center	42.769441, -83.278723	2	Outbred, 20	2	Outbred	Edge
Polly Ann Trail South	42.734927, -83.283541	2	Lab, 5	2	Field	Edge
Turkey Track	42.817365, -83.495402	1	Outbred, 20	1	Outbred	Rural
Roundabout	42.761100, -83.567001	3	Outbred, 20	Dropped	Dropped	NA
Spezia Drive	42.8219639, -83.2867473	2	Lab, 20	2	Field	Rural

Table 3.2. The number and time of adult and larval releases at each of 15 sites for three genetically distinct populations of *H. opulenta* in southern Michigan in 2022.

Release Date	Life Stage	Lab		Field		Outbred	
		Female	Male	Female	Male	Female	Male
6/1/2022	Adult	6	5	19	10	19	10
6/9/2022	Larva	0		37		37	

Table 3.2 (cont'd)

Release Date	Life Stage	Lab		Field		Outbred	
		Female	Male	Female	Male	Female	Male
6/27/2022	Adult	16	14	6	6	6	6
8/25/2022	Adult	12	16	12	16	12	16
Total	Adult	34	35	37	32	37	32
Total	Larva	0		37		37	
Total		69		106		106	

2.4 Statistical Analyses

All analyses were performed in R 4.3.0 (R Core Team, 2023). We used the *DHARMA* package to calculate the residuals to assess best model fit for all non-parametric models (Hartig, 2022). Models were also assessed using the Anova functions from the *car* package (Zeileis & Hothorn, 2002). All post-hoc pairwise comparisons were performed using the *emmeans* package (Lenth et al., 2021).

For the 2022 releases, feeding success of the different populations was evaluated by categorizing release cages as "0" if no feeding was observed and "1" if feeding activity was present. A generalized linear mixed model (GLMM) with a binomial distribution, implemented using the *lme4* package (Bates et al., 2015), was used for analysis. The model included an interaction between genetic background (field, lab, and outbred) and season (early or late) plus habitat type as fixed effects. Stem density nested within site was used as a random effect.

To compare feeding activity among populations with different genetic backgrounds for the 2022 releases we analyzed total defoliation per stem as a proportion (ranging from 0 to 1) using a

GLMM with a beta distribution in the *glmmTMB* package in R (Brooks et al., 2017). This approach incorporates a zero-inflation parameter to accommodate data values of zero, which are possible despite beta distributions being theoretically bound between values greater than zero and less than one. The zero-inflation parameter was adjusted based on fixed and random effects. The model included an interaction between genetic background (field, lab, and outbred) and season (early or late) plus habitat type as fixed effects. Stem density nested within site was used as a random effect.

3. Results

No sign of feeding was detected from the 2020 release experiment. For the 2022 field releases, feeding success varied significantly across populations ($\chi^2 = 20.83$, $df = 2$, $p < 0.001$), seasons ($\chi^2 = 24.39$, $df = 1$, $p < 0.001$), and by habitat type ($\chi^2 = 10.93$, $df = 2$, $p < 0.01$) (Fig. 3.2). Early in the season, feeding damage was more likely to be present at sites where the field and outbred populations had been released compared to sites with the lab population (pairwise comparisons: $p < 0.001$). This effect can be attributed to the variation in release numbers for the lab population. However, later in the season there were not significant differences in the presence of feeding among populations (pairwise comparison: $p > 0.05$). The different populations showed different feeding success across the season (population*season interaction: $\chi^2 = 15.13$, $df = 2$, $p < 0.001$). The lab population increased feeding success from the early to late season (pairwise comparison: $p < 0.001$), likely due to release number variation. The outbred population also increased feeding success from early to late season (pairwise comparison: $p < 0.01$) but the field population did not show significant differences in feeding between seasons (pairwise comparison: $p = 0.521$). Across all populations and seasons, releases at rural sites were more

successful than releases at either edge sites (pairwise comparison: $p < 0.01$) and roadside sites (pairwise comparison: $p < 0.05$) in terms of showing any feeding success.

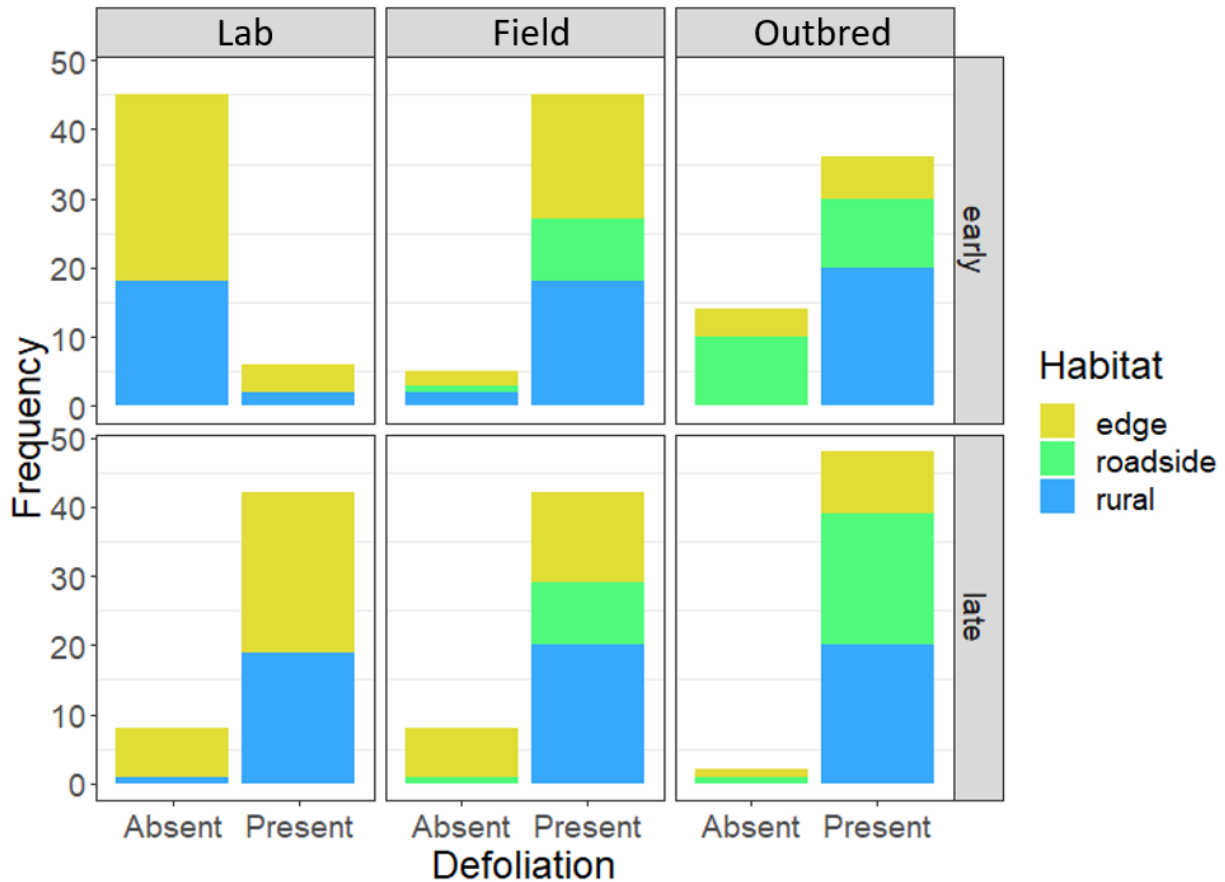


Figure 3.2. The frequency at which stems were recorded as having some defoliation (present) or no defoliation (absent) for each population (lab, field, outbred) and season (early or late) in 2022. Each bar is divided by habitat type (edge, roadside, or rural). Please see Table 3.1 for a list of the total number of sites per habitat and population, which varied. At each site, ten stems were recorded.

Defoliation levels did not differ significantly between populations ($\chi^2 = 4.60$, $df = 2$, $p = 0.10$) but did vary by season ($\chi^2 = 13.60$, $df = 1$, $p < 0.001$) and habitat type ($\chi^2 = 10.74$, $df = 2$, $p < 0.01$) (Fig. 3.3). Early in the season, the field and outbred population had nearly identical

feeding rates. However, the lab population had significantly lower defoliation rates compared to the field population (pairwise comparison: $p < 0.05$) and the outbred population (pairwise comparison: $p < 0.05$). Again, this can be attributed to variations in release size of the lab population. These differences disappeared later in the season. The different populations showed marginally different feeding patterns across the season (population*season interaction: $\chi^2 = 5.67$, $df = 2$, $p = 0.059$). By season's end, both the lab and outbred populations had doubled their total defoliation rates (pairwise comparisons: $p < 0.05$), whereas the field population did not show significant variation in feeding from early to late season (pairwise comparison: $p = 0.279$). Across all populations and seasons, releases at rural sites had higher defoliation rates than releases at edge sites (pairwise comparison: $p < 0.01$) but only negligibly so at roadside sites (pairwise comparison: $p = 0.07$).

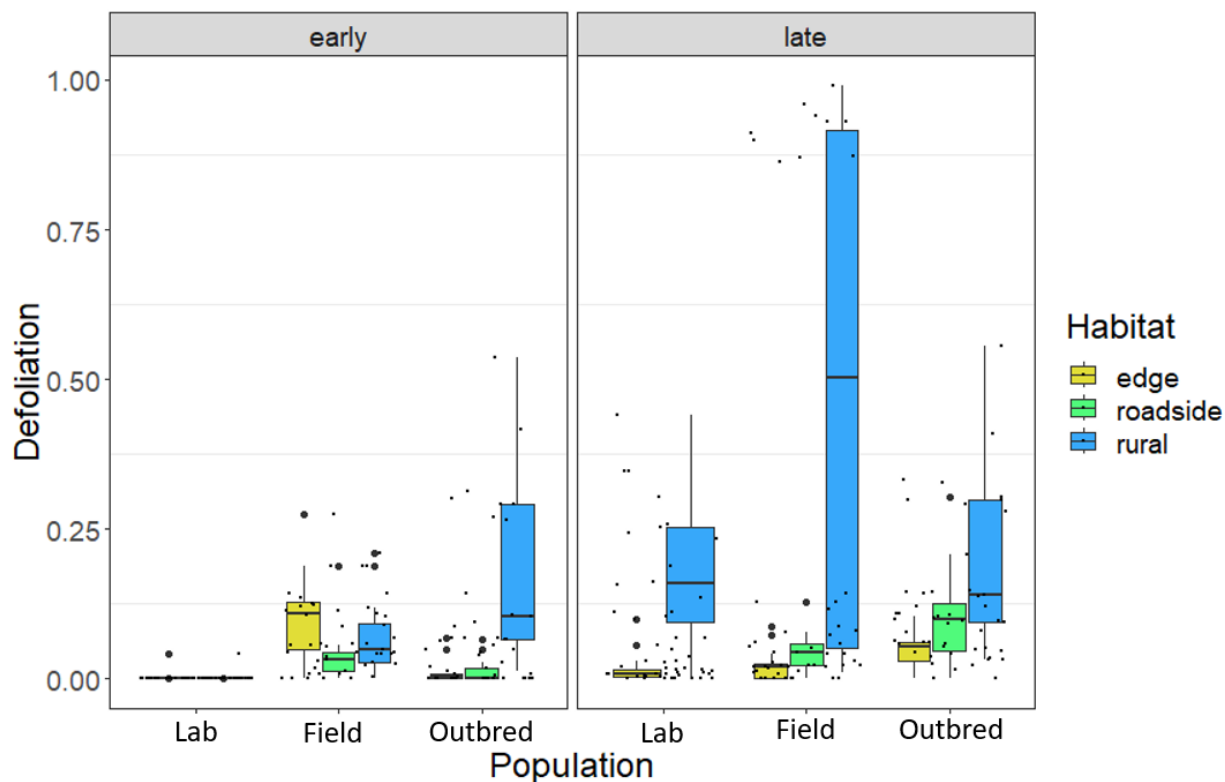


Figure 3.3. Defoliation of stems from each of the three populations (lab, field, and outbred) over two seasons (early and late). For each season and population, separate boxes are given for habitat type (edge, roadside, or rural). Note that the lab population had no sites that were categorized as roadside. Large dots indicate outlier observations and small dots indicate the individual values. The horizontal line indicates the median with the box representing the interquartile range, and vertical lines are 1.5 times the interquartile range.

4. Discussion

Our conclusions are constrained due to the inability to verify the overwintering success following the releases in both 2020 and 2022. To date, *H. opulenta* establishment has only been confirmed at one site in Ottawa, Ontario, Canada where approximately 2000 larvae were released between 2013 and 2014 (Bourchier et al., 2019). Even with these numbers, it took until 2015 to successfully confirm establishment (Bourchier et al., 2019) so it is unsurprising that we

did not detect overwintering success from our relatively small releases within 3 years. The lack of establishment to date may stem from a complex interplay of factors such as predation, climate, and photoperiod, all of which significantly impact the survival and efficacy of *H. opulenta* (Jones et al., 2020; Jones et al., 2022; Weed & Casagrande, 2010). The impact of predation on *H. opulenta* is an important consideration for its successful establishment and effectiveness in target areas. Predation can significantly influence the survival rates of *H. opulenta* at various life stages, from eggs to adults, potentially affecting its population density and control efficiency (Jones et al., 2022). Studies on biological control agents often highlight the role of native predators in shaping the outcomes of biocontrol introductions (Louda et al., 2003). For instance, the presence of generalist predators, such as certain species of ants and beetles, can lead to higher mortality rates in biocontrol agents, thereby requiring careful assessment of the ecological context prior to release (Van Driesche et al., 2008). Predation presents a considerable challenge, as evidenced by Jones et al. (2022), who found that *H. opulenta* pupal mortality rates soared to 70% during summer due to predation, contrasting with the lower winter predation mortality. However, non-predation mortality was notably high (around 66%), especially during colder winters. This variance underscores the vulnerability of *H. opulenta* to predation and climate which can adversely affect its population density and control effectiveness.

Photoperiod plays a pivotal role in the life cycle of *H. opulenta* by triggering diapause (Weed & Casagrande, 2010). The critical photoperiod, or critical threshold, for *H. opulenta* is 15 h 35 min, triggering 50% of individuals to enter diapause. In the introduced range, photoperiod does not reach the same maximum length as it does in Donesk, Ukraine (16 h 2 min) where *H. opulenta* was originally collected from (Jones et al., 2022). However, where *H. opulenta* has established in Ottawa, Ontario the photoperiod is still above the critical threshold (15 h 40 min)

(Jones et al., 2022). Whereas the photoperiod in East Lansing, Michigan is below the critical threshold (15 h 20 min) (Jones et al., 2022). The probability of diapause at the maximum day length in Michigan is still approximately 75% (Jones et al., 2022). In Michigan, two generations of *H. opulenta* are feasible, with releases conducted prior to the summer solstice being more advantageous than those made post-solstice to minimize the early entry of *H. opulenta* into diapause (Alred et al., 2022). Minimizing early diapause is two-fold. Firstly, premature diapause can heighten vulnerability to predation and environmental conditions, highlighting the importance of understanding these mechanisms to improve the utilization of *H. opulenta* as a biological control method (Jones et al., 2022; Louda et al., 2003; Van Driesche et al., 2008). Secondly, high diapause frequency detracts from the impact of the biocontrol agent (Jones et al., 2020). In 2020, we released very close to the summer solstice. While adults in this release were exposed to the maximum photoperiod in Michigan, larvae from those releases were exposed to decreasing daylight which could have induced early diapause. In 2022 we amended this and conducted releases early in June so that first generation larvae would be exposed to the most daylight possible. Releases later in the season were made to attempt to supplement a second generation if most of the *H. opulenta* from the first release went into diapause. Although we have not detected any signs of establishment from the 2022 releases, continued monitoring might still reveal establishment if 2023 levels were below detection.

The second experimental release provided limited data to how genetic background may have influenced the presence and rates of feeding. The lower feeding rates of the lab population early in the season could have been due to their later emergence in the laboratory rearing that resulted in lower initial release size. However, by the end of the season the release sizes of the three different *H. opulenta* populations were similar and at that time point there was no difference in

their feeding rates. The field and outbred populations had similar feeding rates, which was generally consistent with results from a field cage experiment (see Chapter 1). Although the field population exhibits significantly lower genetic variation than the outbred population, it appears that environmental effects, that is pre-adaptation to the field environment, masks any negative effects of lower genetic diversity may have had on performance. Seasonal defoliation patterns, particularly the increase from early to late season in the outbred population, suggest a cumulative effect, possibly driven by the emergence of a second generation that went undetected in our experiments. Similar outcomes have been observed in past field cage studies involving *H. opulenta*, where the emergence of a second generation resulted in significant increases in defoliation rates later in the season (Alred et al., 2022). Consistent with previous studies, we found that *H. opulenta* were more likely to succeed at sites that were primarily shaded and more isolated (rural sites) (Alred et al., 2022). This is an important factor to consider when attempting to minimize site effects.

The endeavor to establish *Hypena opulenta* as a biological control agent for invasive swallow-wort vines illuminates the complex interplay of ecological, environmental, and biological factors essential for its success. Predation, climate conditions, and the critical timing of photoperiods are significant challenges, impacting the survival and efficiency of *H. opulenta*. Further monitoring may help elucidate the genetic factors that drive the successful establishment and persistence of colonizing populations. Despite facing setbacks in initial experiments, these studies contribute to a growing body of knowledge, reinforcing the necessity of a multifaceted approach in biological control strategies. They also highlight the gaps in our understanding, particularly regarding the interplay of genetic and demographic factors that affect establishment. By refining our strategies to account for these factors, we can enhance the potential of *H.*

opulenta to serve as an effective biological control agent, paving the way for more sustainable management of invasive species.

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CHAPTER 4

Navigating the ecological trap: monarch butterfly (*Danaus plexippus*) behavior in the presence of invasive swallow-wort vines (*Vincetoxicum spp.*)

Abstract

The monarch butterfly (*Danaus plexippus*) is faced with declining populations of its milkweed host plants across its range. At the same time, populations of invasive species, such as swallow-wort (*Vincetoxicum spp.*) vines are increasing that are related to milkweeds. Oviposition choices of herbivorous insects, including those of monarchs can be maladaptive, especially when closely related plants are available en masse, and may not reflect survival probability of their offspring. We investigated oviposition choices of the monarch butterfly in Michigan, focusing on its preference for common milkweed over swallow-wort in the field. In addition, we attempted to test in a laboratory experiment how larval exposure to swallow-worts during development may affect the oviposition choice of adult monarchs, and whether the presence of the biocontrol agent, *Hypena opulenta*, may deter oviposition. Field surveys found a marked preference for milkweed, however, monarchs laid 8% of their eggs on swallow-wort across sites. There were marked site differences with up to 17% of monarch eggs were laid on swallow-wort locally. The laboratory experiments that aimed to test some underlying mechanisms for oviposition were not successful. However, we documented a 16% mortality rate among larvae that consumed swallow-wort alongside of common milkweed, suggesting that non-host chemical signals could misleadingly attract monarchs and elicit feeding. This reveals an ecological trap for monarch butterflies. Our findings contribute to a growing body of knowledge about the interplay between monarch reproductive strategies, invasive plant species, and biocontrol agents, emphasizing the need for comprehensive conservation strategies that address these complex ecological interactions.

1. Introduction

The iconic monarch butterfly (*Danaus plexippus*), celebrated for its remarkable migration from Mexican wintering grounds to breeding sites across North America, is currently facing a significant conservation crisis. Recent population studies have highlighted a dramatic decline, especially in the Midwest, where numbers have decreased by up to 58% (Pleasants & Oberhauser, 2013). This decline is attributed to habitat destruction, increased application of herbicides, and the rise of glyphosate-resistant crops, compounded by the invasion of non-native species, all of which substantially reduce the availability of milkweed, the monarch's host plant (Pleasants & Oberhauser, 2013; Zaya et al., 2017). Invasive swallow-wort vines (*Vincetoxicum spp.*) compete with native flora including milkweeds but more importantly they are also related to milkweeds and monarchs mistakenly lay eggs on them (Alred et al., 2022; Casagrande & Dacey, 2007). In response to this urgent conservation challenge and the complex interplay between monarchs and invasive plants, there has been a growing focus on understanding the monarch's oviposition behavior to enhance habitat support along its migratory route.

The preference-performance hypothesis is thought to explain oviposition behavior, where females select host plants for oviposition based on the maximization of survival and fitness of their offspring (Greenstein et al., 2022; Thompson, 1988). However, plants and insects are involved in a complex evolutionary arms race, attempting to improve survival and reproduction (Ferrer-Paris et al., 2013; Labandeira, 1998; van der Linden, 2021; Whitfield et al., 2012). A result of this dynamic is an ecological trap set for monarch butterflies (Alred et al., 2022; Casagrande & Dacey, 2007). Oviposition behaviors are shaped by evolutionary and ecological mechanisms and can lead monarchs to choose suboptimal oviposition sites. The monarch butterfly has a propensity to deposit eggs on non-host plants, notably on swallow-wort species,

with as much as 15-25% of eggs being laid on these unsuitable hosts (Alred et al., 2022; Casagrande & Dacey, 2007). Despite both swallow-worts and milkweeds belonging to the Apocynaceae family and sharing morphological traits, swallow-worts fail to support monarch larvae to maturity, suggesting that shared oviposition cues may misguide monarchs (Endress & Bruyns, 2000; Mattila & Otis, 2003). These chemical cues can cause egg laying on less than suitable or non-host plants leading to reduced fitness or mortality after hatching. The pipevine swallowtail (*Battus philenor*) is known to oviposit on non-host plants that emit similar volatile compounds as its host pipevine (*Aristolochia*), leading to larval mortality upon hatching (Pellmyr, 1986). Non-host plants may share chemical cues with the host plants of butterflies, leading to mistaken oviposition, due to a complex interplay of evolutionary strategies and ecological interactions. This phenomenon can be understood through the lens of mimicry, convergent evolution, and co-evolutionary dynamics, where plants and insects engage in an ongoing arms race for survival and reproductive success. Mimicry, for instance, is a strategy where non-host plants evolve to emit chemical signatures that resemble those of genuine host plants. This adaptation serves as a defensive mechanism, reducing the likelihood of being consumed by herbivorous larvae (Berenbaum & Zangerl, 1996). Convergent evolution also plays a role, where unrelated plants independently develop similar chemical compounds as a result of evolving under analogous environmental pressures. These similarities can inadvertently attract butterflies that rely on specific chemical cues to identify suitable oviposition sites (van der Linden, 2021; Whitfield et al., 2012). Additionally, closely related plant species may share chemical profiles due to their shared evolutionary history, potentially confusing butterflies that have evolved to cue into specific chemical signals for host plant identification (Ferrer-Paris et al., 2013; Labandeira, 1998). These strategies highlight the delicate balance of co-evolution, where

plants and insects continually adapt to each other's defenses and counter defenses. Understanding these interactions is crucial for conservation efforts, especially for species like the monarch butterfly, whose populations are in decline.

In the evolutionary arms race, not only do plants engage in complex strategies, but insects partake as well. The concept of phenotypic plasticity—where distinct genotypes yield varied phenotypes in different environmental contexts (Pigliucci et al., 2006)—plays a pivotal role in shaping insect behavior. This adaptability is illustrated in fruit flies (*Drosophila melanogaster*), which adjust their behaviors based on environmental signals and past experiences, exemplifying the essence of phenotypic plasticity (Dukas, 2008). Similarly, research on monarch butterflies has shed light on how their varying preferences for different milkweed species are a result of phenotypic plasticity and learning, enabling them to navigate the diverse habitats along their migratory paths (Jones & Agrawal, 2019; Snell-Rood & Papaj, 2009). This adaptability, however, can come with trade-offs in fitness, influenced by the female's previous experiences with host plants. While it allows monarchs to lay eggs on a variety of milkweed species—beneficial in times of scarcity—it also poses the risk of choosing host plants unsuitable for larval development (Jones & Agrawal, 2019). Contrasting outcomes in earlier experiments, with some finding monarch oviposition on swallow-wort species and others not, underscore the complexity of such behaviors (Alred et al., 2022; DiTommaso & Losey, 2003; Mattila & Otis, 2003; Casagrande & Dacey, 2007). Variations between studies could be attributed to differences in the origin of the monarch populations examined or their prior exposure to swallow-worts, hinting at a learned component to their oviposition choices (Alred et al., 2022; DiTommaso & Losey, 2003; Mattila & Otis, 2003; Casagrande & Dacey, 2007). This learning behavior suggests that monarchs encountering swallow-worts alongside milkweeds might change their oviposition

strategy, a hypothesis supported by the observed plasticity in host preference (Casagrande & Dacey, 2007; Jones & Agrawal, 2019).

We investigated oviposition behavior of monarch butterflies in Michigan under natural conditions at field sites where milkweeds and swallow-worts co-occur. In a lab study, we aimed to test how exposure to swallow-worts during larval development may influence the oviposition behavior of adult monarchs on swallow-wort plants that either had larvae of the biocontrol agent, *Hypena opulenta* Christoph (Lepidoptera: Erebidae) larvae and feeding present or not. We hypothesized that adult monarchs that had been exposed to swallow-worts during development would be more likely to lay eggs on swallow-worts. Furthermore, we expected that the presence of another defoliating caterpillar in the form of the biocontrol agent *H. opulenta* on swallow-worts may deter oviposition to avoid larval competition and egg cannibalism (Alred et al. 2022, Jones & Agrawal 2019).

2. Methods

2.1 Field Survey

To evaluate the frequency with which monarch butterflies deposit their eggs on swallow-wort vines in Michigan, an egg survey was carried out across seven locations in Oakland County (Table 4.1). These sites featured both pale swallow-wort (*Vincetoxicum rossicum*) and varieties of milkweed, specifically common milkweed (*Asclepias syriaca* L.) and swamp milkweed (*Asclepias incarnata* L.). The survey involved inspecting 50 randomly chosen stems each of milkweed and pale swallow-wort, examining every part of the plant from the top down, including flowers, seed pods, stems, and both surfaces of leaves. This detailed egg scouting took place at the end of July in 2023, spanning four days. The chosen field sites encompassed a

diverse range of habitats, including forest peripheries, trail borders, and open areas alongside roads.

Table 4.1. Monarch egg survey locations given by the site name and the associated latitude and longitude.

Site	Latitude	Longitude
Indian Lake Road	42.797983	-83.226714
Davisburg Road	42.749908	-83.553234
Maureen Lane	42.7087042	-83.5732006
Munger Road	42.7052562	-83.6256498
Clark Road	42.741395	-83.485015
Turkey Track	42.817365	-83.495402
Wood Trail	42.804251	-83.280229

2.2. Lab Experiment

In 2023, a series of experiments were conducted to determine how exposure of larvae to swallow-wort during development may affect monarch oviposition and to examine whether the presence of *H. opulenta* larvae and feeding on these plants influenced their oviposition decisions. In brief, monarch eggs and early instar larvae were collected in the field and were subjected to two rearing treatments during development to adulthood. The two treatments involved milkweed-only or the combination of milkweed and swallow-wort plants next to each other in the rearing cages. Then the adults emerging from these two rearing treatments were offered two plant treatments for oviposition: 1) a fresh, undamaged swallow-wort plant alone, or 2) a swallow-wort plant with feeding damage by *H. opulenta* and with *H. opulenta* larvae present

alongside of a fresh, undamaged swallow-wort plant. The adult monarchs were allowed to oviposit on either of these swallow-wort plant treatments if they chose to and after 72 h of exposure, they were moved onto a milkweed plant to check egg laying capability to validate if the lack of position on swallow-worts was due to choice or physiological constraints.

For the rearing experiment, monarch eggs and 1st-2nd instar larvae were collected around East Lansing and in Oakland CO., Michigan starting on June 20, 2023, with subsequent collections made as necessary to compensate for hatching failures or early larval losses. Collection involved detaching milkweed leaves directly from stems in the field and placing them in water picks for hydration. Field-collected larvae, sourced from areas without swallow-wort (East Lansing area), were designated for the milkweed-only rearing process and those collected from mixed stands of swallow-wort and milkweeds (Oakland CO.) were assigned to the combined plant treatment to ensure that larvae that had hatched before entering the lab had not had any exposure to swallow-wort. All experiments were conducted at ambient temperature and humidity in the laboratory with supplemental lighting set at 16:8h L:D photoperiod (4ft, 80 Watt; Barrina®; Zhongshan, Chin). Each treatment initially received twenty-five replicates, undergoing replacements due to hatch failures or larval demise, with three replacements in the milkweed-only group and five in the combined plant treatment. Upon hatching, larvae were individually housed in small mesh cages (30.5 x 30.5 x 30.5 cm, Restcloud) equipped with a water pick containing a milkweed leaf. For those in the combined plant treatment, cages also contained a water pick with a fresh pale swallow-wort stem alongside milkweed. The water picks were stabilized in 1.14 L square plastic pots filled with SUREMIX® all-purpose perlite mix (Michigan Grower Products Inc., United States). In the combined plant treatment, milkweed and swallow-wort were placed adjacently within the same pot. Milkweed leaves were changed daily, with additional leaves provided as

larval appetite increased in later stages. Swallow-wort stems were similarly maintained, with new daily cuttings to ensure freshness and replacements were made upon signs of wilting.

Within their respective enclosures, larvae transitioned into the chrysalis stage, with the cut stems preserved until the adult monarchs emerged. Throughout their development, adults were provided with Gatorade dispensed to 2 oz cups with cotton wicks, refreshed every other day to ensure hydration. Upon emergence, adults were grouped into large mesh cages (61 x 40 x 40 cm, Restcloud) equipped with a potted mum plant for resting to facilitate mating. Group sizes were carefully managed to include no more than two females and three males, or at minimum, one female and two males. Mating was initiated as soon as an adequate number of adults were available, allowing a 48-hour period for mating. Following this mating period, females were relocated to separate tall mesh cages. Here, they were presented with the following setup: one fresh cut stem of swallow-wort (at least 30 cm tall) and another from swallow-wort with *H. opulenta* feeding damage, alongside two 4th to 5th instar *H. opulenta* larvae. Preparation for the feeding damage involved allowing two *H. opulenta* larvae to feed on a swallow-wort stem in a cage for 24 hours, after which the stem, along with the larvae, was introduced to the cage containing the adult female monarch. Swallow-wort stems were each placed into individual potting containers filled with soil, positioned at opposite ends of the cage. Females were given a 72-hour window to lay eggs before being transferred to a milkweed-exclusive environment to verify egg-laying capability. Egg-laying observation cages were set up similarly, featuring a cut milkweed stem of at least 30 cm in a water pick placed into a soil-filled pot for stability. Egg-laying activity was monitored every 24 hours during these 72-hour periods. Due to high mortality, just ten females from the larva exposed to the milkweed only rearing treatment survived to the egg laying phase, four of which died before the 72-hour egg laying period

finished. Only three females from the combined plant treatment survived to the egg laying phase, and two of those died before the 72-hour egg laying period had finished. In summary, six monarch adults from the milkweed only treatment and one monarch adult from the combined treatment were able to be assessed.

2.2 Statistical Analysis

To assess the prevalence of monarch oviposition on common milkweed and swallow-wort in the field, a generalized linear model was used with a negative binomial distribution from the *lme4* package in R (Bates et al., 2015; R Core Team, 2023). Plant species (either milkweed or swallow-wort) and site were used as fixed effects. After assessing that common milkweed and swamp-milkweed had no significant differences in monarch oviposition, they were batched together as ‘milkweed’ for comparison to swallow-wort. Model evaluations were completed using Anova from the *car* package (Zeileis & Hothorn, 2002) and residual testing from the *DHARMA* package (Hartig, 2022). Post-hoc pairwise comparisons were completed using estimated-marginal means in the *emmeans* package with a Tukey adjustment (Lenth et al., 2021). No formal statistical models were used on the lab experiment data as adult monarchs did not lay any eggs.

3. Results

3.1 Field Survey

Monarch oviposition varied by plant species ($\chi^2 = 59.01$, $df = 1$, $p < 0.001$) and site ($\chi^2 = 38.85$, $df = 6$, $p < 0.001$). On the 350 common milkweed and swamp milkweed stems examined across 7 sites, 78 monarch eggs were found or a 22% oviposition rate ($z = -3.95$, $p < 0.001$). On the 350 swallow-wort stems assessed, 7 monarch eggs were found, or a 2% oviposition rate ($z = -2.43$, $p < 0.001$). Monarchs were 11 times more likely to lay eggs on milkweed than swallow-

wort (pairwise comparison: $p < 0.001$) but were shown to lay up to 8% of their eggs on swallow-wort across all sites. However, there were notable site-specific effects. At only three sites did monarchs lay eggs on swallow-wort, two of which had the highest combined egg counts (29 and 20 eggs). The other site had just 6 eggs on both swallow-wort and milkweed combined. Other sites where monarchs laid eggs on only milkweed had moderate to low egg counts (equal to or below 12). The site with the highest egg count on swallow-wort contributed to 71% of all eggs found on swallow-wort across all surveyed sites and monarchs laid 17% of their eggs on swallow-wort at this site.

In the laboratory experiment, adult female monarchs did not lay eggs on either swallow-wort or milkweed. Despite this setback, we observed one behavior of interest in monarch larvae. There was no mortality in larvae reared in the milkweed only treatment. However, there were 6 larvae that died in the swallow-wort exposure treatment (24% mortality rate). Four of which were observed having actively fed from the swallow-wort leaves in their last few instars, despite being provided with sufficient fresh milkweed (16% mortality rate) (Figure 4.1). Five adult monarchs from the milkweed only treatment failed to emerge from their chrysalis or died during or shortly after emergence (20% mortality rate). Just ten of the nineteen monarchs in the swallow-wort exposure treatment emerged (47% mortality rate). At the time of emergence, the cumulative mortality rate in the swallow-wort exposure treatment was 40%, double that of the milkweed only treatment. Since this behavior was not anticipated, we believe that some feeding on swallow-wort went undetected initially and it is possible that larvae that fed on swallow-wort made it to the chrysalis stage before dying.



Figure 4.1. The left image shows a monarch larva resting on a fresh milkweed cutting after feeding on a swallow-wort leaf (left). The right image shows a monarch larva resting underneath a swallow-wort leaf with defoliation present on the opposite leaf.

4. Discussion

The field study revealed a marked preference of monarch butterflies for common milkweed compared to pale swallow-wort. Observations from natural swallow-wort infestations in Michigan suggest that the proportion of eggs laid on swallow-wort by monarchs could be as high as 17% at certain locations. Site-specific variables, however, suggest that additional factors influence the oviposition choices of monarchs. Although our lab experiment did not yield the expected outcomes to elucidate these behaviors further, we uncovered a previously unknown larval behavior. Specifically, we observed a 16% mortality rate among monarch larvae attributed to consuming swallow-wort, a figure that is presumably underestimated since monitoring larval consumption of swallow-wort was not an initial focus of the study.

Previous field research using potted swallow-wort indicated that up to 25% of monarch eggs might be laid on swallow-wort in Michigan (Alred et al., 2022) and at approximately 6% in pasture surveys in Rhode Island (Casagrande and Dacey 2007). Our results suggest overall lower rates of oviposition across swallow-wort infestations in nature at 2%, yet strong special differences exist with one site reaching 17% of egg laying on swallow-worts. Monarch butterflies do exhibit spatial patterns in their oviposition behavior, largely influenced by the distribution and availability of their primary host plant, milkweed (Pleasants & Oberhauser, 2013). Studies have shown that monarchs tend to lay eggs more frequently on milkweed plants situated in open or edge habitats rather than in densely vegetated or shaded areas (Zalucki & Lammers, 2010). This preference is thought to maximize the survival chances of their offspring, as these habitats often offer higher quality host plants and reduced competition and predation (Jones & Agrawal, 2019; Kessler & Baldwin, 2001; Zalucki & Lammers, 2010). Furthermore, within a suitable habitat, monarchs may exhibit selectivity towards individual plants based on plant size, age, and health, which can influence the nutritional quality of the plant for the developing larvae (Ladner & Altizer, 2005). This approach to monarch oviposition is assumed to maximize the reproductive success of their offspring by choosing sites that maximize survival. Studies have shown that factors at the landscape level, such as habitat fragmentation and the existence of agricultural areas, can influence where monarch butterflies choose to lay their eggs by impacting the availability and accessibility of milkweed (Flockhart et al., 2015; Oberhauser et al., 2017). Future research could benefit from expanding the diversity and number of sites surveyed to further understand the site-specific factors that drive monarch oviposition patterns in Michigan. Pale swallow-wort, which prefers shaded or semi-shaded areas and creates dense thickets, presents an environment that is not typically favored by monarchs for egg-laying

(Ditomaso et al., 2005; Ditoomaso et al., 2018). Moreover, the low oviposition rates observed at many of our sites suggest that the number of stems examined may have been insufficient to accurately capture monarch egg-laying behavior on swallow-wort. It is unclear from the field survey the extent of role of chemical cues and learned behavior play in monarch oviposition, except that they must play some role since there is prevalence of mistaken oviposition in monarchs in Michigan. Understanding this aspect could be crucial in deciphering the nuances of monarch butterfly oviposition decision-making process.

Our experiment did not shed light on the learning behavior in monarch butterflies due to the absence of egg-laying activity among the female monarchs involved in the study. This outcome suggests that the adult butterflies might have belonged to the migratory generation, which does not reproduce, or that an environmental variable during laboratory rearing inhibited their oviposition. A preliminary trial involving a single adult female monarch, which successfully laid eggs on milkweed, leads us to speculate that the generation of monarchs used in our main experiment was not inclined to lay eggs under natural conditions either. However, we were able to observe an undocumented behavior in monarch larvae. About 16% monarch larvae mortality in our experiment can be attributed to direct consumption of swallow-wort. This finding supports the idea that swallow-wort might produce chemical signals that attract monarchs, a theory previously considered primarily in the context of egg-laying behavior and larval death due to the larvae's inability to travel far from where they hatch in their early stages (Oberhauser & Solensky, 2004). Yet, our observations indicate that larvae might also be misled by the chemical signals of non-host plants, even when their host plants are available. Research by Greenstein et al. (2022) showed that up to 62% of monarch larvae might attempt to consume a low-performance host (plants that considerably decrease survival), leading to a mere 3.5% survival

rate. These findings, from no-choice experiments, did not clarify whether nutritional deficiencies or toxins were responsible for the high mortality rates. Despite having the option between suitable and non-suitable plants in our study, larvae still opted for the latter, hinting that chemical signals could be a significant factor in their erroneous feeding behavior. Both species of *Asclepias* and *Vincetoxicum*, along with other plants like foxgloves (*Digitalis*), produce cardiac glycosides, a compound monarch larvae accumulate for their defense (Groen & Whiteman, 2021; Sliumpaite et al., 2013). The specific makeup of cardenolides can differ based on the plant source, potentially leading to variations in toxicity upon ingestion (Greenstein et al., 2022). Investigating how cardiac glycosides influence the feeding behavior of monarch larvae on swallow-wort could be a valuable direction for future research.

This study contributes to our knowledge on the oviposition behaviors of monarch butterflies, confirming a pronounced preference for common milkweed over pale swallow-wort, albeit with a notable oviposition rate on swallow-wort in natural settings. Despite the absence of egg-laying in our laboratory experiments that could have further illuminated the mechanisms behind these behaviors, our field observations offer a glimpse into the complex interplay of environmental, spatial, and chemical factors influencing monarch choices. Particularly, the documentation of a significant mortality rate among larvae due to swallow-wort consumption underlines the potential deceptive role of chemical cues emanating from non-host plants. These insights underscore the importance of considering both habitat characteristics and plant chemical properties in understanding and supporting monarch conservation efforts, highlighting the need for expanded research to capture the full spectrum of factors impacting monarch reproductive success in diverse environments.

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