Animal Conservation

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Reintroduction of an Endangered Butterfly, the Mottled Duskywing (*Erynnis martialis*)

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ABSTRACT

Many butterfly species around the world face a high risk of extinction, making reintroductions to restored or improved habitats an important tool for enhancing their long-term viability. Despite their potential value, many reintroductions either fail because a suboptimal life stage was used as the founder population, or their effectiveness is difficult to assess because of a lack of postrelease monitoring. Using detailed postrelease surveys from 2021 to 2024, we report on the outcome of the first ever reintroduction of Mottled Duskywing (*Erynnis martialis*), a federally and provincially endangered butterfly, to Pinery Provincial Park, southwestern Ontario, Canada. While reintroducing Mottled Duskywing, we released different life stages to different sites within the park. At one site, after only 4 years, Mottled Duskywing reached densities of > 27 adults/ha through in situ reproduction, which was comparable to densities of the two largest extant Canadian populations > 700 km away. We provide evidence that pupal and adult founders are more effective life stages than larvae for reintroduction of this species, though the use of larval founders requires further testing. Our results demonstrate that Mottled Duskywing can be reintroduced to previously restored habitats in a relatively short period of time, although additional monitoring will be required to confirm the long-term sustainability of the population. Our study provides a framework for future reintroductions of this species and an example of how life-stage experiments can be combined with postrelease monitoring to provide critical information for best practices of butterfly reintroductions.

1 | Introduction

Due to a variety of anthropogenic factors, including habitat loss, degradation, and fragmentation (Forister et al. 2011; Sang et al. 2010; Thomas 2016; Harvey et al. 2020; Sánchez-Bayo and Wyckhuys 2021), many butterfly species are now extant in only a fraction of their historic range (Forister et al. 2011; Thomas 2016; Schweitzer et al. 2018). In response, widespread efforts to restore butterfly habitats are occurring, efforts which are accompanied, in some cases, by the reintroduction of imperiled species (Marttila et al. 1997; Porter and Ellis 2011; Seddon et al. 2014; Kuussaari et al. 2015; Daniels et al. 2018). However, in most cases, butterfly reintroductions either fail (Schultz et al. 2008) or their long-term outcomes remain unknown (Daniels et al. 2018; Demarse et al. 2022). To maximize the impact of butterfly reintroduction efforts and to lessen the risk of extinction for target species, the development of effective reintroduction and postrelease monitoring methods is needed (Schultz et al. 2008; IUCN/SSC 2013; Daniels et al. 2018).

Most butterfly reintroductions include limited postrelease monitoring despite a need to develop effective reintroduction techniques for the group (Schultz et al. 2008; Daniels et al. 2018). The IUCN recommends postrelease monitoring of reintroductions of all taxa at an appropriate timescale to adapt ongoing reintroduction methods based on documented results (IUCN/SSC 2013). Recommendations specific to butterfly reintroductions call for postrelease monitoring of multiple generations to document demography, dispersal/colonization, and ecologically relevant observations (Schultz et al. 2008; Daniels et al. 2018). Though long-term postrelease monitoring of butterflies has been carried out in some reintroductions (e.g., van Langevelde and Wynhoff 2009; Andersen et al. 2014; Kuussaari et al. 2015; Adamski and Ćmiel 2022), most only report monitoring outcomes within 1-year postrelease, and either cease to monitor or fail to report outcomes within 2–5 years postrelease (Demarse et al. 2022). A comprehensive postrelease monitoring program should foster effective, adaptive practices and should contribute to the advancement of the field overall (Schultz et al. 2008, Daniels et al. 2018).

Here, we report on the outcome of years 1–4 of the first ever reintroduction of Mottled Duskywing (*Erynnis martialis*), a medium-sized skipper butterfly. Mottled Duskywing use a variety of habitat types, including sandhills, dry prairies, glades, outcrops, openings in oak woodlands and oak savannas, pine barrens and savannas, as well as alvars (Schweitzer et al. 2018). Important habitat features for the species are sparse canopy cover (< 40%) and the presence of patches of larval host plants, *Ceanothus herbaceous* and *Ceanothus americanus* (Schweitzer et al. 2018). The species' historical range extends from the Florida panhandle to the southern limit of the Canadian Shield and is bounded longitudinally between central New York and central Colorado (Schweitzer et al. 2018). Precipitous declines in populations have been recorded in recent decades, especially in the east, accompanied by local extirpation events (Schweitzer et al. 2018). In Canada, Mottled Duskywing are extant in Ontario and Manitoba and have been extirpated from Quebec (COSEWIC 2012; Linton 2015). Within Ontario, Mottled Duskywing were once found at 20 locations across the Great Lakes Basin but now occur at only seven geographically isolated locations (COSEWIC 2012, C. Schmidt *pers. comm*.). In Canada, the species has been assessed as endangered federally (COSEWIC 2012) and provincially in Ontario (COSSARO 2014). Reintroduction of Mottled Duskywing to formally occupied sites or introduction to new suitable sites is a recommended action within the species' Ontario recovery strategy (Linton 2015).

From 2021 to 2024, we conducted releases of Mottled Duskywing into Pinery Provincial Park (hereafter "Pinery"), in southwestern Ontario, Canada. Pinery comprises > 2500 ha of ancient sand dune complexes along the southeastern shoreline of Lake Huron (Ministry of Natural Resources 1986). Mottled Duskywing was last recorded in oak savanna habitat at Pinery in 1990 and their extirpation has been attributed to an accumulation of habitat stressors including overgrazing of understory plants, including *Ceanothus* spp., by white-tailed deer (*Odocoileus virginianus*), succession of oak savanna habitats due to fire suppression, and the planting of thousands of White (*Pinus strobus*) and Red (*P. resinosa*) Pines to infill oak savannas (Linton 2022), and the historical use of *Bacillus thuringiensis* (*Bt*) pesticide to control invasive spongy moths (*Lymantria dispar dispar*; Linton 2022). In recent decades, the implementation of controlled burns, a reduction of white-tailed deer through annual hunting by local First Nations, and the active removal of pines (*Pinus* spp.) have effectively restored the understory, including *Ceanothus* spp., within the park, creating conditions estimated to be suitable for Mottled Duskywing once again (Linton 2022).

While reintroducing and monitoring Mottled Duskywing at Pinery, we attempted to address a key uncertainty that remained during reintroduction planning—the optimal life-stage(s) that should be released as founders. Uncertainty about optimal founder life-stage(s) exists among invertebrate reintroductions, including butterflies, whose holometabolous life cycle results in four potential founder life-stages: eggs, larvae, pupae, and adults (Sarrazin and Legendre 2000; IUCN/SSC 2013; Jourdan et al. 2019). A review of butterfly reintroductions reported that most projects carried out in the U.K. used adult founders, while projects in the U.S. released almost even proportions of larvae, pupae, and adults (Schultz et al. 2008). A more recent analysis found that founder life-stage was one of the most significant factors influencing butterfly translocation success, with adults outperforming larvae in the short term (1 year) but larvae and adults producing similar results 2–5 years postrelease (Demarse et al. 2022). Notably, these conclusions were limited by the (1) availability of detailed life-stage information about individuals released, (2) limited extent and duration of postrelease monitoring, and (3) scarcity of available reports, including possible underreporting of reintroductions that failed (Demarse et al. 2022). The relative efficacy of founder life-stages has rarely been tested during the reintroduction process (Daniels et al. 2018; Cabrera 2020; Demarse et al. 2022).

Here, we formalize two hypotheses to explain the effect of founder life-stage on butterfly reintroduction success. The *cumulative mortality hypothesis* proposes that pupae and adults will be the most effective founders for reintroduction because they are nearing or within their reproductive window at the time of release, minimizing mortality prior to reproduction. Generally, high mortality (> 70%) occurs during early life stages (egg and larvae; Cornell and Hawkins 1995, Zalucki et al. 2002), potentially leaving few surviving founders to reproduce if individuals are released at these stages. Alternatively, the *environmental cues hypothesis* proposes that larvae will be the most effective founders because they will be exposed to environmental cues during development in situ and are able to engage in in situ behaviors that may optimize reproductive output. Cues such as photoperiod, temperature, rainfall, wind, and host plant quality can impact larval development rate and size (Gotthard 2000; Chen et al. 2018), larval choice to enter diapause (Friberg et al. 2011; Abarca 2019), and pupation and eclosion phenology (Hannam et al. 2018; Davies 2019). Early life-stage founders would be able to select their own pupation sites, which can impact eclosion timing and, therefore, influence mate availability (Odendaal et al. 1985; Brackley et al. 2022). To evaluate the efficacy of founders of different life-stages, we released three Mottled Duskywing life-stages (larvae, pupae, and adults) and assessed (1) postrelease survival and (2) adult abundance in subsequent generations while reintroducing Mottled Duskywing at Pinery.

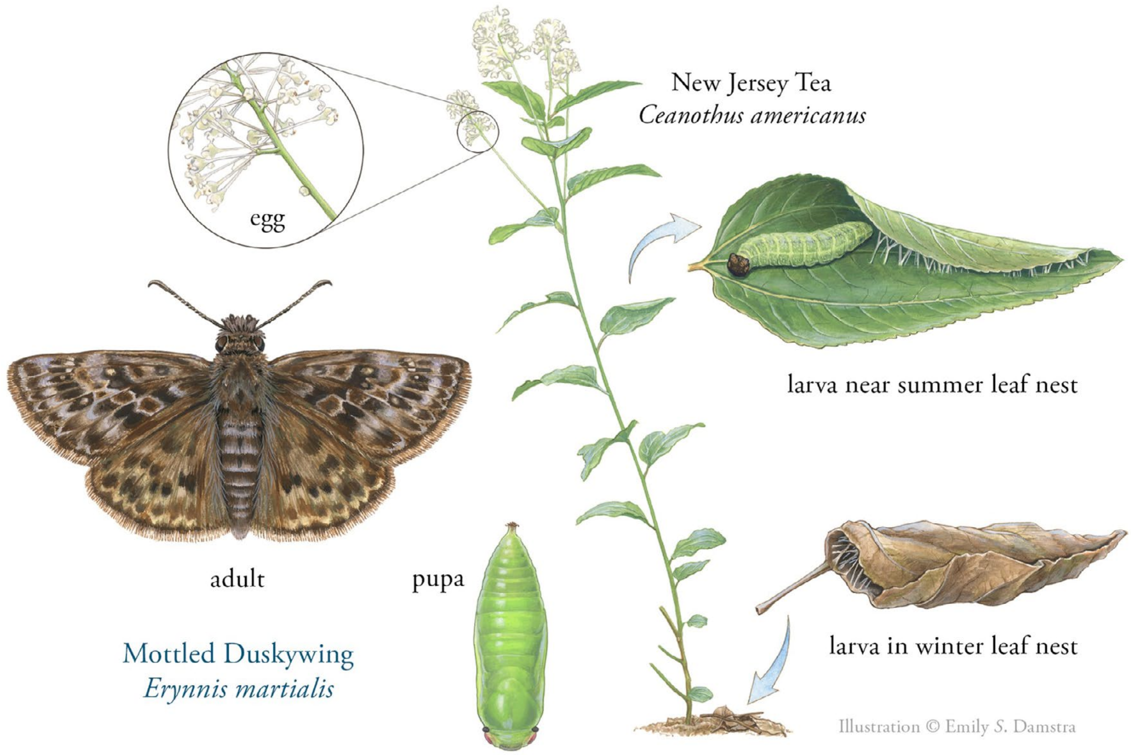

FIGURE 1 | The life cycle of Mottled Duskywing (*Erynnis martialis*). At Pinery Provincial Park, Mottled Duskywing is usually first seen in flight in early to mid-May. Host plants include both New Jersey Tea (*Ceanothus americanus*) and Prairie Redroot (*Ceanothus herbaceous*), and female Mottled Duskywing will lay eggs singly on the plant(s). After hatching from eggs, larvae construct leaf nests on the host plant(s), using silk to secure leaves into folded shelters in which they can often be found resting. Mottled Duskywing larvae undergo six instars of larval growth, constructing new leaf nests throughout their larval development. The species is bivoltine at Pinery, flying once in May/June and again in July/August. While some larvae resulting from the May/June flight period will pupate, eclose, and fly in the July/August flight period, others initiate winter diapause inside of their leaf nest and pupate during the following year's May/June flight period. Illustrated by Emily S. Damstra.

2 | Methods

Field work and related activities were carried out in accordance with a permit under section 17(2)c of the Endangered Species Act (Canada), a Wildlife Scientific Collectors Authorization from the Ministry of Natural Resources (Ontario), and a Science & Research Permit issued by Ontario Parks (Ministry of Environment, Conservation and Parks) according to the Provincial Parks and Conservation Reserves Act (Ontario).

2.1 | Study Species

Adult Mottled Duskywing are low-flying butterflies with a wingspan of 25–33 mm and are often found near their host plants *Ceanothus americanus* and *C. herbaceous* (Figure 1; Schweitzer et al. 2018). Females oviposit directly onto host plant leaves, laying eggs singly (Schweitzer et al. 2018; Henault et al. 2022). After hatching, larvae construct leaf nests on the host plant, spending time feeding both within and outside of the leaf nest as they develop (Figure 1). When they complete development, larvae may either pupate and eclose for a second flight period in the same season (Mottled Duskywing are bivoltine in all but the northeastern portion of their range) or enter diapause and overwinter as larvae within the leaf litter (Schweitzer et al. 2018). In southwestern Ontario, Mottled Duskywing have a first flight period from May to June and a second flight period in July–August (COSEWIC 2012).

2.2 | Reintroduction Sites

We reintroduced Mottled Duskywing to three sites within Pinery (labeled A, B, and C in Figure 2). The sites were separated by features such as the Old Ausable Channel and closed-canopy forest, as well as campgrounds, roads, and trails. The Euclidean distance between sites was 1–3 km. Precise locations of release sites are withheld because of the endangered status of the species. Site A consisted of 7.48 ha of oak savanna habitat with sandy soils, wide swaths of open habitat containing flowering plants, intermittent woody shrub growth (mainly *Rhus aromatica* and *Prunus virginiana*), and sparse canopy cover (Linton 2022). The site was subjected to a high-intensity prescribed burn in 2001, resulting in few canopy trees (mainly Black Oak, *Quercus velutina*) and many standing snags (Linton 2022). Both *C. herbaceous* and *C. americanus* were present as both individual plants and contiguous patches measuring up to 752 m^2 (Figure 2). Site B was 5.25 ha and was characterized by open, sandy soils and scattered stands of small white pine (*Pinus strobus*) and eastern red cedar (*Juniperus virginiana*) (Linton 2022). Site B was subjected to a prescribed burn in 1990 (Linton 2022), and very few canopy

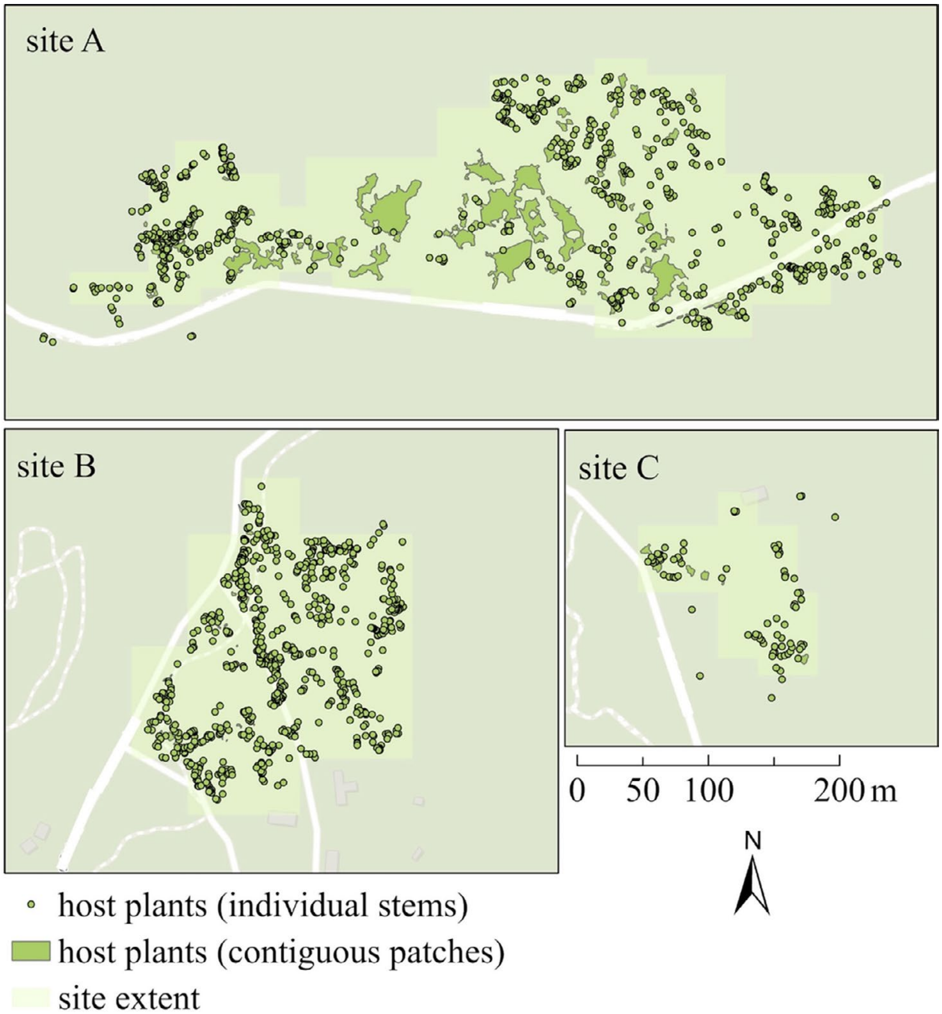

FIGURE 2 | Three release sites for Mottled Duskywing at Pinery Provincial Park, Ontario, Canada. Mottled Duskywing host plants are *Ceanothus americanus* and *Ceanothus herbaceous*. Site A is 7.48 ha, site B is 5.25 ha, and site C is 1.11 ha. Contiguous patches represent plant(s) with an area of groundcover > 1 m^2.

trees currently exist. At site B, *C. herbaceous* and *C. americanus* were present mostly as individual plants with a small number of patches measuring up to 32 m^2 (Figure 2). Site C, the smallest site at 1.11 ha, was burned in 2007 and contained hilly sections of unforested habitat bordered by oak-dominated forest. Site C is a clearing at the base of a decommissioned downhill ski slope adjacent to a series of connected gravel parking lots extending up to 150 m from the site boundary. *C. herbaceous* and *C. americanus* at site C were found both as individual stems and in contiguous patches measuring up to 105 m^2 (Figure 2).

2.3 | Captive Rearing

To produce individuals for reintroduction, larvae, pupae, and adults were raised in captivity using a previously established captive rearing protocol (Brewster and Young 2020). Adult females were collected from an extant donor population in southcentral Ontario (location withheld) and brought to Cambridge Butterfly Conservatory, Cambridge, Ontario, where they were housed with host plant cuttings in an oviposition cage. The larvae that hatched from the resulting eggs were then reared in captivity for release. The number of individuals produced from the captive rearing program varied year to year, which influenced the quantity of released animals as well as the proportion of animals released in each life stage. The annual variation in captive raised individuals was affected by the quantity of females available from the source population, the success of captive oviposition efforts, and the developmental pace of the resulting larvae.

2.4 | Testing Optimal Life-Stages for Release

While our priority during release planning was to reestablish Mottled Duskywing at Pinery, we also designed our release protocol to test the relative efficacy of different life stages through assessment of postrelease survival and adult abundance in subsequent generations. Our intention was to release different life stages (larvae, pupae, and adults) at the three release sites in Pinery using random assignment in the first year (2021). However, our release plan was partially compromised based on real-time decisions about the habitat conditions, suitability, and colonization status of the sites. In the first year of reintroduction, we had only two sites available

TABLE 1 | Releases of Mottled Duskywing made reintroduction sites (A, B, and C) in Pinery Provincial Park, Ontario, Canada between 2021 and 2023. First flight period releases occurred in May–June and second flight period releases occurred in July–August. Released life stages include larvae, pupae, and adults. All released individuals were captive reared and resulted from females collected at an extant population of Mottled Duskywing near Marmora, Ontario.

Year	Date range	Flight period	Site	Life-stage	Quantity
2021	July 21–August 16	2nd	A	Adults	172
	July 21–August 16	2nd	A	Pupae	138
	July 23–August 6	2nd	C	Larvae	382
2022	July 19–August 22	2nd	C	Adults	92
	July 21–August 17	2nd	B	Larvae	291
2023	May 16–June 20	1st	A	Adults	62
	July 29–August 29	2nd	A	Adults	67
	August 29	2nd	A	Pupae	5

for release and elected to carry out both pupae and adult releases at our largest site. Subsequent decisions about release life stage/site combinations were made with consideration of the colonization and habitat condition status of release sites and the impact of existing animals at a site on our ability to monitor release treatment outcomes. Additionally, the number of animals released in treatments was determined by the captive-rearing program, limiting our ability to standardize treatment sizes. Despite these limitations, we collected data that allowed us to compare the relative efficacy of founder life stages throughout our project.

We released larvae, pupae, and adults at different times over 3 years of the study (2021–2023) as detailed in Table 1. Late-instar larvae (4th, 5th, and 6th instar) were released within a leaf nest of their own construction (Figure 1), which was placed among the living leaves of a host plant and secured using a 53 cm wire flag (Figure 3a,b). Pupae were released after being attached to 30 cm long wooden dowels using hot glue (Figure 3c), with dowels then placed inside segments (15 cm length, 5 cm diameter) of polyvinyl chloride piping that were partially buried at the release site with one end protruding from the soil (Figure 3d). The PVC pipes had plastic mesh (with 2 cm^2 openings) secured with rubber bands to the top to discourage predation while still allowing eclosed butterflies to exit the PVC pipe, and window screen material secured to the bottom with hot glue to allow rain to filter through the pipes (J. Daniels, *unpubl. data*). Adults were chilled and then marked with small dot(s) of nail polish on one wing (Figure 3e; Demarse et al. 2023) and released from small popup mesh enclosures placed into patches of host plants (Figure 3f).

2.5 | Postrelease Surveys

To assess the outcomes of the releases, we conducted various surveys intended to document (1) postrelease survival and (2) adult abundance in subsequent generations. A summary of survey type by site and year is provided in Table 2. Larvae and pupae surveys were made in any weather conditions deemed safe for field work. Surveys for adult butterflies were carried out in weather conditions that met accepted criteria for butterfly counts (Pollard 1977) : at > 13°C and not raining, at 13°C–17°C but only when the sun was shining, and at > 17°C no matter the level of cloud cover.

2.5.1 | Documentation of Postrelease Survival

Larval survey methods in 2021 included general searches to relocate larvae and document behavior, with no defined subset of larvae searched for. In 2023, two subsets of larvae were checked daily postrelease to document their survival, developmental status and behaviors. Pupae were checked daily postrelease to determine their survival and development status. Monitoring of adults for postrelease survival occurred during all adult survey types (see below). Adult founders were released with marks on their wings to distinguish them from butterflies resulting from either in situ reproduction or the release of other life-stages (Otis and Linton 2016).

2.5.2 | Documentation of Adult Abundance in Subsequent Generations

To monitor abundance, we carried out a variety of survey types targeting adult butterflies at all sites during Mottled Duskywing flight periods from 2021 to 2024. For each observation of an adult, we recorded time and date, UTM coordinates, wing markings, wing wear, and behavior (such as nectaring, mating, or oviposition). When required, individuals were netted to confirm species and sex and for the collection of nonlethal DNA samples for concurrent research.

2.5.2.1 | Targeted Searches. When adults were present at low densities or adult presence was unconfirmed at a site during a given flight period, targeted searches were carried out. Targeted searches involved thoroughly searching relevant habitat (e.g., hilltops, nectar sources, and areas with a high concentration of host plants) and were carried out by one or two surveyors for a duration of at least 20 min, usually on alternate days. If targeted searches revealed that adults

FIGURE 3 | Photographs of Mottled Duskywing release methods used at Pinery Provincial Park, Ontario, Canada from 2021 to 2023. (a) Larvae were released by pushing the stem of a wire flag through the larval leaf nest. (b) Flags were then planted into the ground so that the larval leaf nest was in contact with host plant leaves. Inset: Mottled Duskywing larva. (c) Pupae were affixed to wooden dowels prior to release by gluing their cremaster to the dowel. (d) Wooden dowels with pupae were then placed into vertical sections of PVC pipes which were partially buried in the soil among host plants. The PVC pipes had plastic mesh affixed to both ends to allow drainage and prevent predation. (e) Adults were marked with small dots of colored nail polish on their wings prior to release. (f) Adults were released from mesh enclosures placed on the ground among host plants. Enclosures had zippered doors that were opened to allow individuals to exit.

were consistently observed, then formal surveys (see below) would begin.

2.5.2.2 | **Sighting Route Surveys.** In 2021 and 2022, a mark/resighting protocol was used (see Supporting Information) but did not produce sufficient data to estimate abundance (0 marked butterflies were resighted in 2022 despite extensive marking and resighting effort). In 2023, we substituted distance sampling for mark/resighting (see below). However, we continued to survey along the sighting route outlined in the abandoned mark/resighting protocol whenever adults were consistently observed in flight at a site. Sighting surveys were performed throughout the entirety

TABLE 2 | Postrelease monitoring performed at Mottled Duskywing reintroduction sites (A, B and C) in Pinery Provincial Park, Ontario, Canada between 2021 and 2024. First flight period surveys occurred in May–June and second flight period surveys occurred in July–August. Survey types include larval monitoring, pupae monitoring, targeted searches, mark/resighting, sighting route surveys and distance sampling.

Year	2021	2022		2023		2024	
Flight period	2nd	1st	2nd	1st	2nd	1st	2nd
Survey type	Sites applied						
Larvae monitoring	C	—	B	—	—	—	—
Pupae monitoring	A	—	—	—	—	—	—
Targeted searches	—	—	B	B, C	B, C	B, C	B, C
Mark/resighting	A, C	A, C	A, C	—	—	—	—
Sighting route	—	—	—	A	A	A	A
Distance sampling	—	—	—	A	A	A	A

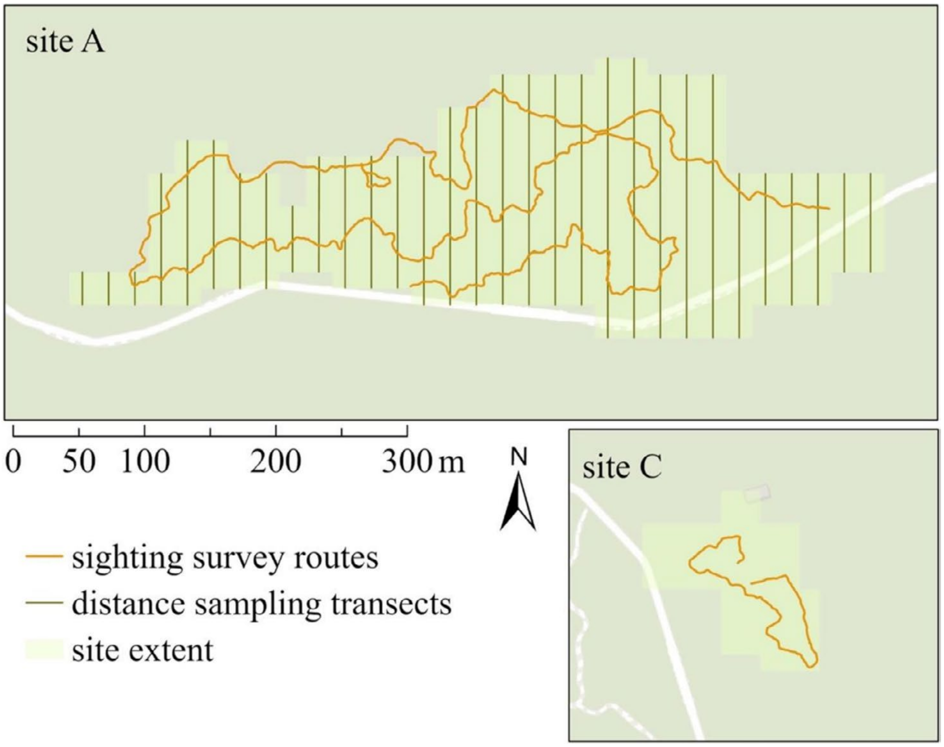

FIGURE 4 | Sighting survey routes and distance sampling transects used to assess adult Mottled Duskywing abundance at sites A (2021–2024) and C (2021–2023) in Pinery Provincial Park, Ontario, Canada. Site A (7.48 ha) contained 31 distance sampling transects that run north–south through the site, and a sighting route that was 1881 m long. Site B (not shown, 5.25 ha) did not have transects or sighting routes. Site C (1.11 ha) contained a sighting route that is 324 m long. Site B (not shown, 5.25 ha) did not have transects or sighting routes. For a summary of the monitoring methods used in each year and flight period across all three sites, see Table 2.

of our study, including at site A in 2021–2024 and at site C in 2021–2022 (site B did not have a sufficient number of adults to initiate surveys). The length of the sighting route at site A was 1.88 km and at site C was 0.32 km. For each sighting route survey, two surveyors walked a set route (Figure 4) at a slow and consistent pace.

2.5.2.3 | Line-Transect Distance Sampling. Distance sampling was implemented at site A in 2023 and 2024 to replace mark/resighting efforts (Brown and Boyce 1998, Isaac et al. 2011, Henry and Anderson 2016). Distance sampling was carried out along 31 parallel linear transects spaced 20 m apart (Figure 4). Transects were surveyed repeatedly throughout the two flight periods, with a random selection of transects surveyed day. Transects were walked by two surveyors, with a lead person responsible for detecting adults and the second person responsible for navigation and record keeping. The lead person carried a 2 m aluminum pole, which they swung through vegetation while walking, flushing resting butterflies to increase detection (Hicks 2025). When necessary, butterflies encountered during surveys were captured to determine species and sex. When an adult Mottled Duskywing was detected, the perpendicular distance between the point of detection and the survey transect was recorded.

2.5.2.4 | Dispersal Monitoring. The dispersal of adults from one site to another was monitored by following likely dispersal routes with suitable habitat that linked sites A, B, and C. Because newly released adults were marked with site-specific colors, their origin would be known if they were encountered. Subsequent generations had no markings, so the site of origin could not be determined. That said, no Mottled Duskywing was recorded along the surveyed dispersal routes, so results of dispersal surveys are not reported below.

2.5.2.5 | Incidental Observations. We also recorded incidental observations of any life stage of Mottled Duskywing made throughout the park (see Supporting Information).

2.6 | Analyses

2.6.1 | Larval and Pupal Monitoring

The amount of data collected from larvae and pupae monitoring was not sufficient to warrant formal analysis, though anecdotal evidence of postrelease survival is described in our results.

2.6.2 | Sighting Route Surveys

Sighting route data were transformed into encounter rates (number of individuals per km of survey effort). Data were divided into 6-day sampling periods, and the mean encounter rate of each sampling period was calculated, allowing the encounter rate from sighting route surveys to be compared to density and abundance estimates from distance sampling.

2.6.3 | Distance Sampling

Distance sampling data were divided into 6-day periods and were used to calculate the average per-day density across the 6 days. Density estimates were then converted to abundance estimates based on the size of the release site. Abundance estimates provide an average per-day number of adults in flight each day through the sampling period. Six-day periods were used to balance the need between sufficient data (quantity of detections) and the need to account for the dynamic nature of butterfly abundances over the year (phenology of abundance).

Using Distance software version 7.5, Release 2 (Thomas et al. 2010), we tested the fit of various detection function models (proposed linear equations of curves to represent detectability) and adjustment series. During analysis, distance sampling data were grouped into 50 cm bins to account for rounding error and were truncated at 350 cm, removing observations ($n = 24$) beyond that distance to improve the accuracy of the analysis (Buckland et al. 2001). We tested a uniform model with cosine adjustments, a half-normal model with Hermite adjustments, and a hazard rate model with simple polynomial adjustments (Buckland et al. 2015), used Kolmogorov–Smirnov and Cramér–von Mises tests within program Distance to assess model fit, and Akaike's Information Criterion (AIC) to select the best fitting model for the data. The best fit detection function was a hazard rate model with no adjustments. We then applied this function to six-day subsets of distance sampling data to create estimates of the average per-day density (n/ha) in each six-day period and multiplied by the size of the site to provide estimates of abundance.

Because Pinery hosts six species of duskywing (*Erynnis* spp.) in addition to Mottled Duskywing, some of which can be difficult to identify to the species level during visual surveys, detections of "unknown" *Erynnis* spp. comprised 15% ($n = 129$) of observations made during distance sampling. To accommodate for the likelihood that some of these detections were Mottled Duskywing, select observations were assigned as Mottled Duskywing observations post hoc. We calculated the proportion of detections of Mottled Duskywing vs. detections of individuals identified as other *Erynnis* spp. within each sampling period and randomly assigned that proportion of the period's "unknown" detections as Mottled Duskywing.

2.6.4 | Abundance Estimates From Sighting Route Survey Data via Backcasting

At site A, we had 4 years of encounter rate data from sighting route surveys (2021–2024) and concurrent density estimates from distance sampling in 2 years (2023–2024). Thus, we created a linear regression between the encounter rates (n/km) and density estimates from distance sampling (n/ha) for six-day sampling periods in 2023–2024. From these data, we found that encounter rates effectively predicted density estimates ($R^2 = 0.74$). Thus, we used the equation of this linear relationship ($y = 2.61x + 0.12$) to transform the encounter rates at sites A and C in 2021 and 2022 into estimates of density.

3 | Results

Overall reintroduction results are summarized by site and year in Figure 5. At site A, we observed Mottled Duskywing in every flight period from initial releases in 2021 to the end of the reporting period in 2024. Peak abundance estimates of 208 individuals were reached at site A from May 11 to 16 during the first flight period of 2024, with highs of 188 and 192 also made during the two subsequent six-day sampling periods. At site B, we observed only incidental sightings in 2023 (1 adult) and 2024 (12 larvae). At site C, Mottled Duskywing were observed at low abundances in 2021 and 2022 (< 10 adults in each year) and too few Mottled Duskywing were observed in 2023 and 2024 to allow abundance estimates to be made, although 12 larvae were recorded during the second flight period in 2024.

3.1 | Evidence of the Relative Efficacy of Founder Life-Stages

3.1.1 | Larval Releases: Postrelease Survival

At site C, 382 larvae were released between July 23 and August 6, 2021. During subsequent surveys from July 26 to August 20 (Table 2), we documented larvae feeding on host plants, entering diapause, and the pupation of 4 individuals. However, locating larvae after release proved difficult and most released

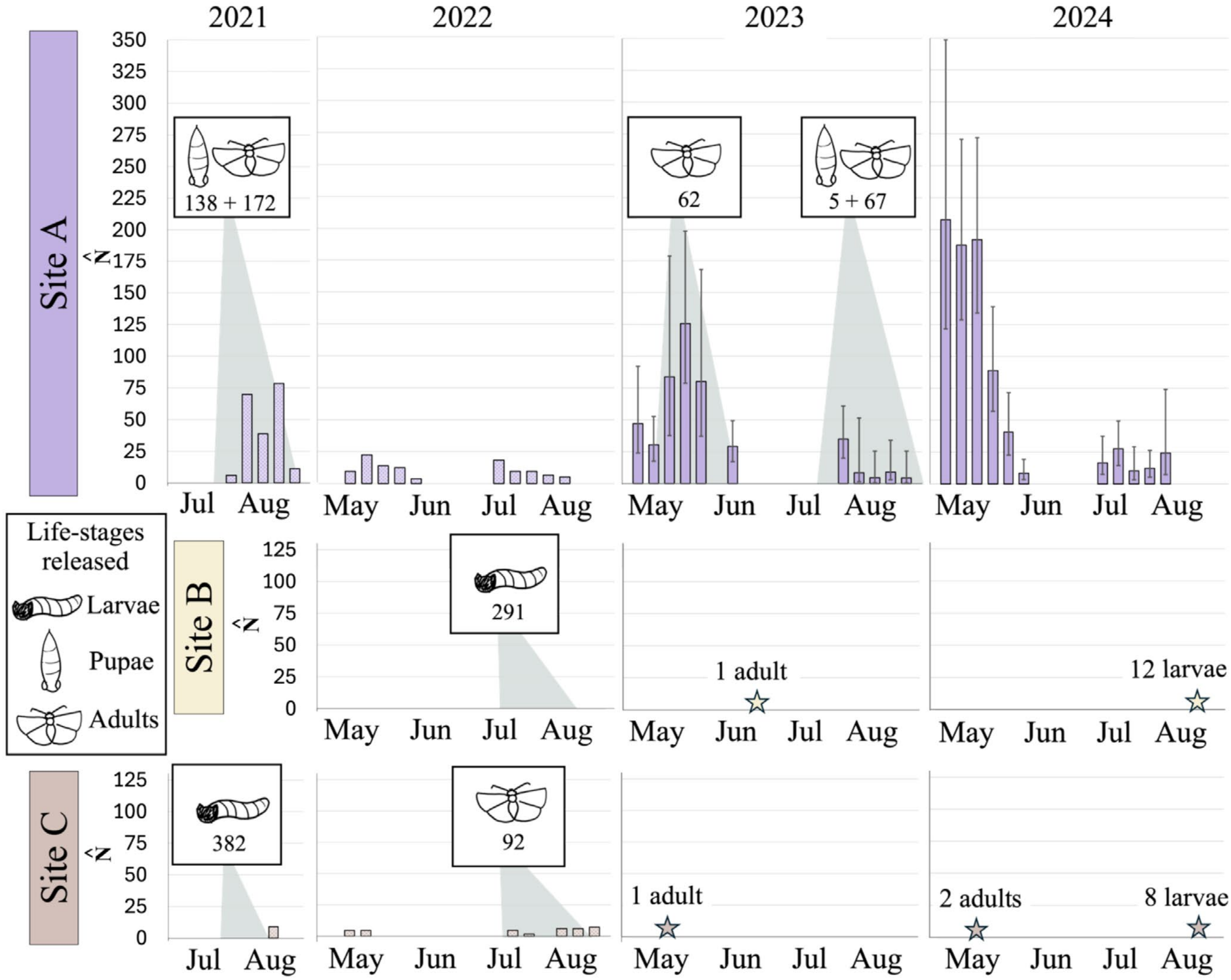

FIGURE 5 | Estimates of mean abundance per six-day sampling period in 2021–2024 at three release sites for Mottled Duskywing in Pinery Provincial Park, Ontario, Canada. Released individuals and their life stage of release are shown in inset boxes, with greyscale triangles indicating the release date range. Crosshatched bars show population estimates created by backcasting onto sighting route data (2021 and 2022). Population estimates in 2023 and 2024 were created using distance sampling data (site A). Error bars show 95% confidence intervals of abundance estimates from distance sampling. Stars indicate observations made during targeted searches or incidentally, from which abundance estimates could not be created.

larvae were not observed again. Adults were observed at site C after larvae releases, confirming that some larvae had survived to the reproductive life stage, and a peak mean daily abundance of 9 adults was estimated for site C from August 9 to 14, 2021 (Figure 5). Since larvae released in 2021 could have initiated diapause postrelease, pupating and eclosing the following spring, the presence of adult Mottled Duskywing at site C in the first flight period of 2022 is considered further evidence of larval founder survival rather than evidence of recruitment. Mean daily abundance of adults at site C reached an estimated peak in 2022 of 5 adults, which occurred between May 17 and 28 (Figure 5).

At site B, 291 larvae were released from July 21 to August 17, 2022. Two subsets of released larvae were monitored daily after release. One day postrelease, 30% of the first set and 56% of the second set could not be located. At the conclusion of monitoring, 90% (after 22 days) and 81% (after 9 days) of the larvae could not be located. No adults were observed at site B in the flight period that larvae were released. One adult was observed at site B in the following flight period (June 2023), which is the sole evidence of larval founder survival to the adult life stage that was documented at that site.

3.1.2 | Larval Releases: Adult Abundance in Subsequent Generations

At site C, marked adult founders were released during the second flight period of 2022 (see below) but, because of wing markings, were distinguishable from butterflies resulting from larval founder reproduction. The only definitive evidence of reproduction resulting from the larvae releases of 2021 is the incidental observation of one unmarked butterfly in a parking lot near the boundary of site C, on August 6 during the second flight period of 2022. At site B, there was no definitive evidence of Mottled Duskywing persistence in the flight periods following larvae releases. In 2024, 12 larvae were found in leaf nests at site B, but we are unable to eliminate the possibility that these larvae resulted from the dispersal of adults from another release site.

3.1.3 | Pupal Releases: Postrelease Survival

At site A, 138 pupae were released from July 21 to August 16, 2021. The estimated survival rate of the released pupae to eclosure was 82% ($n=113$). The remaining pupae (18%, $n=25$) were either missing entirely or had damaged pupal cases, both of which were taken as indicators of predation. Adults were also released at site A in 2021 but had wing markings applied prior to release, allowing them to be distinguished from butterflies resulting from pupal founders. Pupal founders made up 48% ($n=26$) of the butterflies observed at site A during sighting route surveys postrelease in 2021. The mean daily abundance of butterflies at site A reached an estimated high of 79 adults during that flight period, from August 9 to 14 (Figure 5), but this estimate includes butterflies from both pupal and adult releases. The 5 pupae released at site A in 2023 were not monitored postrelease.

3.1.4 | Pupal Releases: Adult Abundance in Subsequent Generations

Mottled Duskywing persisted in every flight period after the initial release of pupae (and adults) at site A, reaching a peak mean daily abundance estimate of > 200 butterflies in 2024 (3 years after pupae were first released there). However, we cannot draw conclusions about the contribution of pupal founders relative to adults because both life stages were released at this site.

3.1.5 | Adult Releases: Postrelease Survival

At site A, 172 marked adults (Figure 3e) were released from July 21 to August 16, 2021. Released adults made up 52% ($n=28$) of the butterflies observed on sighting route surveys during that flight period. Mean daily abundance of Mottled Duskywing postrelease reached a peak estimate of 79 from August 9 to 14, 2021 (Figure 5), but this figure includes individuals resulting from pupae and adult releases. Subsequent releases of marked adults were made at site A in 2023, including 62 adults released during the first flight period (May 16–June 20) and 67 adults during the second flight period (July 29–August 29). These new releases constituted 4% and 20% of the butterfly observations made on sighting route surveys during those flight periods, respectively.

At site C, 92 marked adults were released from July 19 to August 22, 2022. The decision to release adults at that site was made after larvae releases (2021) resulted in low abundances (< 10 individuals) of Mottled Duskywing adults at site C in 2022. After adults were released at site C, mean daily abundance reached a peak estimate of 7 individuals, with marked adult founders being the only Mottled Duskywing observed during surveys.

3.1.6 | Adult Releases: Adult Abundance in Subsequent Generations

In 2024, 3 years after the release of pupae and adults began at site A, mean daily abundance there reached a peak estimate of > 200 butterflies. However, we cannot draw conclusions about the contribution of adult founders relative to pupae because both life stages were released at this site.

4 | Discussion

Our results provide evidence that the establishment of self-sustaining populations of Mottled Duskywing is possible through their reintroduction to formerly occupied areas. While we are cautious about making strong conclusions regarding the long-term viability of Mottled Duskywing at Pinery, we are encouraged by the high abundance estimated at site A in the final year (2024) of analysis. Peak abundance was estimated at 208 butterflies, which is comparable to estimates at currently occupied sites that our team has been monitoring in eastern Ontario, ~700 km from Pinery (Demarse et al. 2023). All Mottled Duskywing observed in 2024 originated from in situ reproduction by founders released in 2023 or earlier because we did not conduct releases in 2024. Also encouraging were numerous incidental sightings in 2024 at other locations where we had not observed Mottled Duskywing in the 3 preceding years. These included public campsites adjacent to site A, as well as locations > 1750 m from site A, and > 900 m from any other release site in our study. This "spillover" suggests that site A may have been at or near carrying capacity, thereby initiating dispersal away from the site. Taken together, the success achieved at Pinery provides compelling evidence that reintroduction could be an effective tool to improve the conservation outlook of this endangered species.

Our release strategy was partly designed to test the efficacy of founders in the larval, pupal, and adult life stages and, in the process, examine the cumulative mortality hypothesis and environmental cues hypothesis. While we were unable to complete a full factorial design to provide robust tests of predictions from these hypotheses, we did collect some data that provided important insights into the relative efficacy of founders. When we consider postrelease survival, larvae performed poorly relative to pupae and adult founders. Postrelease survival of larvae was apparently low, with only 1%–3% of larval founders observed as adults, indicating that very few of the released larvae had opportunities for reproduction. Additional evidence of low postrelease survival can be drawn from our postrelease monitoring of larvae (see Supporting Information) at site B: larvae seemed to quickly disappear after release, with most of the larvae missing from their release plants (within 9 and 22 days for each of two monitored subsets). The relative isolation of the individual plants that larvae were placed onto, combined with the low abundance of adults subsequently observed, suggests that absences were largely due to mortality rather than voluntary larval movement. A high rate of mortality among larval founders would be consistent with mortality rates expected for exophytic holometabolous insect larvae (Cornell and Hawkins 1995). Comparatively, postrelease survival of pupae (site A) was high, with 82% of pupal founders documented as surviving to eclosion during postrelease pupal monitoring (see Supporting Information). Pupae and adults seemed to have comparable postrelease survival at site A, as they each made up approximately half of the animals subsequently observed during sighting route surveys (48% and 52%, respectively). Considered together, pupal and adult founders reached a peak estimate of mean daily abundance during the flight period of releases at site A (2021) that was equal to 25% of the number of animals released, suggesting that a high proportion of founders had opportunities to reproduce. Adult releases at site A in 2023 during both flight periods resulted in additional evidence of postrelease survival, as new releases constituted 4% and 20% of the butterfly observations made on sighting route surveys during those flight periods, respectively. Evidence of more moderate postrelease survival of adult founders was observed after adult releases at site C in 2022, where estimated mean daily abundance reached a peak equal to 8% of the released adults.

When considering adult abundance in subsequent generations as the criteria for success, larvae again performed poorly in comparison to pupae and adults. Abundance following larvae releases at two different sites (B and C) was low, with only one butterfly that was suspected to be the progeny of larval founders observed (at site C in the second flight period of 2022). After pupae and adult releases, abundances were generally higher, with site A reaching mean daily abundance estimates of >200 animals after both pupae and adults were released there. Adult releases at site C, however, resulted in low abundance in subsequent generations, and persistence at that site is uncertain.

Our results lead us to the preliminary conclusions that pupae and adults are viable release options and to caution against the release of larvae as founders for Mottled Duskywing reintroduction. Apparent high postrelease mortality of larvae relative to pupae and adults provides some support for the cumulative mortality hypothesis and refutes the environmental cues hypothesis. If larval founders experienced environmental cues and behavioral opportunities that increased their reproductive output, the effect seems to have been negated by extreme larval mortality prior to reproductive opportunities, preventing any benefits of early life-stage release from being reflected in the abundance of subsequent generations. Pupae and adults apparently performed similarly, with both life stages showing similar postrelease survival. While we had no way of determining whether pupal and adult founders contributed equally to the subsequent generation, we see no biological reason why their contribution to recruitment would have been radically different. Cabrera (2020) drew similar conclusions, showing that pupal and adult Miami Blue butterflies displayed similar survivorship, fecundity, and likelihood of reproductive success when released as founders.

Though larvae performed poorly by both criteria, we caution against drawing a firm conclusion about not using the larval stage for reintroduction because we were not able to perform a full factorial experiment. Larvae were not trialed at site A, which hosted the highest density of host plants and where a viable population was established from pupae and adult releases. Through our detailed GPS mapping, site A contained $>6600\,m^2$ of host plants (density of $890\,m^2$/ha), while site B had $362\,m^2$ of host plants (density of $69\,m^2$/ha) and site C contained $546\,m^2$ (density of $492\,m^2$/ha). Similarly, while the release of pupae and adults at site A suggests that these life stages are likely the most effective for reintroduction, we have also shown that the release of adults at site C was not effective and we did not conduct adult or pupae releases at site B. We suspect that the likely explanation for poor performance of adult founders at site C is the amount and/or configuration of host plants that were available. Thus, a conservative conclusion at this stage of the project is that the release of pupae and adults is a viable option for Mottled Duskywing reintroduction, providing there is sufficient host plant at the release site. A more accurate estimate of the minimum host plant area needed by Mottled Duskywing will likely arise with conservation translocations that are currently underway to restored sites outside of Pinery.

While we acknowledge that our shift from mark-resighting surveys to distance sampling for adult Mottled Duskywing compromised the uniformity of our methods for estimating abundance, we are confident that we devised an appropriate bridge between the two sampling methods. We carried out frequent surveys of the sighting route(s) in all years (2021–2024), creating a large dataset that spanned all sampling periods. From this, we were able to thoroughly assess the relationship between the outputs of the sighting route and concurrent distance sampling estimates in 2023 and 2024, resulting in an R^2 value of 0.74 between the two methods. Additionally, while we took precautions to minimize habitat disturbance and negative impacts on Mottled Duskywing during sampling, we believe that our impact was minimal and that the outcomes of our research justify any potential impact on reintroduction outcomes or release sites.

Imperiled butterfly species reintroductions should prioritize the optimization of successful reproduction by founders. In this paper, we have provided evidence that Mottled Duskywing can be reintroduced, with year-over-year site persistence, using pupae and adults as founders. We suggest that the success of pupal and adult founders is due to the relatively short time window of mortality risk intervening their release and in situ reproduction. We also posit that limited exposure to environmental cues and in situ behaviors experienced by pupal and adult founders is not detrimental enough to offset their lower mortality risk relative to larvae. We did not find evidence that larval founders were effective for Mottled Duskywing reintroduction at Pinery, although it is difficult to draw strong conclusions because a full factorial design with all life-stage/site combinations was not executed.

Additionally, we provide an example of a butterfly reintroduction and monitoring scheme that is informative and adaptive, addressing some recognized gaps in butterfly reintroduction methodology (Schultz et al. 2008; Daniels et al. 2018; Demarse et al. 2022). We developed postrelease monitoring methods for Mottled Duskywing reintroduction sites that effectively captured changes in abundance over time, and produced several key natural history observations (see Supporting Information). As Mottled Duskywing is reintroduced to other habitats within its former range, knowledge about which life stages are effective for founding new populations and implementing appropriate postrelease monitoring techniques can be used to optimize reintroduction success while contributing to the development of techniques for other butterfly species reintroductions as well.

Author Contributions

M.P., J.E.L., D.R.N. responsible for study conception and design. D.R.N., J.L., A.B., N.K., A.S.M. acquired funding. M.P. and J.E.L. performed field work and data collection and preparation. M.P. and D.R.N. performed data analysis, figure preparation and manuscript writing. All authors reviewed and approved the manuscript.

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Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** acv70038-sup-0001-Supinfo.docx.