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Patterns and causes of oviposition in monarch butterflies: Implications for milkweed restoration

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ABSTRACT

Effective habitat restoration requires an understanding of species habitat preferences and the associated mechanisms driving those preferences. We examined the patterns and causes of oviposition preference in the monarch butterfly, a rapidly declining species, in southwestern Ontario at both landscape and milkweed patch spatial scales. Additionally, we measured the abundance of invertebrate predators, parasitoids and parasites across these same spatial scales. Oviposition preference was dependent on both the size of the milkweed patch and the density of milkweed within the patch, as well as landscape type. Small (< 16 m^2), low-density (0.1–2 milkweed per m^2) milkweed patches in agricultural landscape had the highest egg density compared to all types of milkweed patches in non-agricultural and roadside landscapes. Medium-sized patches had the highest predator abundance. Variation in the abundance of parasitoids, and occurrence of parasites of monarch eggs and larvae did not appear to coincide with preferred egg laying habitats. Our results suggest that investing heavily in milkweed restoration in roadside habitats should be done cautiously. Instead, a better strategy may be for managers to develop incentive programs with landowners to plant and maintain milkweeds in agricultural landscapes, which could complement other pollinator initiatives or ecosystem service programs in agricultural landscapes that focus on increasing nectar availability. Our results have important implications for restoring milkweed as an approach to counteract monarch butterflies declines.

1. Introduction

Habitat loss is one of the leading causes of species decline and extinction worldwide (Wilcove et al., 1998; Pimm and Raven, 2000; Ceballos and Ehrlich, 2002; Kerr and Cihlar, 2004; Venter et al., 2006). Although not applicable to all species, one way to mitigate the negative effects of habitat loss is through active habitat restoration (Kareiva and Wennergren, 1995; Fahrig, 1997; Wisdom et al., 2002). However, realizing optimal gains in restoring habitat requires detailed and accurate knowledge of species habitat preferences. It is well known that mobile animals make decisions about where to settle based on multiple spatial scales, from landscapes to microenvironments, with the animal relying on different cues to identify a suitable site (Johnson, 1980). Even if it is known what type of habitat a species prefers and at what spatial scale (Åström et al., 2013; Camaclang et al., 2015; Foit et al., 2016), the spatial configuration of the habitat can also influence settlement patterns (Pulliam et al., 1992; Lewis et al., 1996; Huxel and Hastings, 1999). For example, patch area (Freemark and Merriam, 1986; Davis, 2004; Winter et al., 2006), patch shape (Davis, 2004; Weldon and Haddad, 2005), connectivity (Schadt et al., 2002; O'Brien et al., 2006), fragmentation (Hunter et al., 1995; Pereboom et al., 2008), and habitat heterogeneity (Freemark and Merriam, 1986; Hunter et al., 1995; Heikkinen et al., 2004) have all been shown to influence individual choice (Bergin et al., 2000; Misenhelter and Rotenberry, 2000; DeCesare et al., 2014) and, in some cases, settlement preferences. Additionally, the preference of a species for particular habitat or habitat feature can also depend on the larger spatial scale in which it exists (Mazerolle and Villard, 1999; Boyce et al., 2003; Quevedo et al., 2006; Mayor et al., 2009). Knowledge of what factors can influence species habitat preferences is important for effective restoration.

The eastern North American population of monarch butterflies (*Danaus plexippus* L.; Lepidoptera: Danainae) has declined by 95% in the last 20 years (Brower et al., 2012) and the population is at a high risk of extirpation (Semmens et al., 2016). Butterflies of the last generation of the summer migrate up to 4000 km to the overwintering sites in central Mexico where they congregate in massive clusters in oyamel fir (*Abies religiosa*) forests (Urquhart and Urquhart, 1976; Brower, 1996). In the

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spring, the same individuals mate and migrate north to breeding grounds, and over successive generations that follow, repopulate northern areas (Cockrell et al., 1993; Malcolm et al., 1993; Miller et al., 2012; Flockhart et al., 2013). Summer breeding individuals that live for 2–5 weeks travel comparatively shorter distances in search of nectar, mates, and egg-laying locations (Oberhauser, 2004). Monarchs oviposit – lay their eggs – exclusively on milkweeds of the subfamily Asclepiadoideae (milkweeds), typically singly on the undersides of leaves (Urquhart, 1960) and most commonly one per plant (Zalucki and Kitching, 1982a). Milkweed provides both food and a chemical defense for the developing larvae (Parsons, 1965; Rothschild et al., 1966; Brower, 1984).

While a number of factors have been proposed for the population decline of monarchs, recent population models have shown that monarch abundance is more sensitive to the decline of milkweed, the obligate larval host plant, on the breeding grounds compared to deforestation or rising temperatures on the overwintering grounds in Mexico (Flockhart et al., 2015; Semmens et al., 2016; Pleasants et al., 2017 but see Inamine et al., 2016). The most significant reduction of milkweed has occurred in agricultural fields due to the use of glyphosate herbicides to kill weeds (Pleasants and Oberhauser, 2013). The increase in the use of glyphosate herbicides follows the adoption of genetically modified (GM) crops, notably corn and soybean, altered to be glyphosate-tolerant (Padgette et al., 1996; Duke and Powles, 2008). This has reduced the number of milkweed in North America, most severely in the central midwestern United States (Hartzler, 2010; Pleasants and Oberhauser, 2013; Pleasants, 2017), a significant region of monarch production (Wassenaar and Hobson, 1998; Oberhauser et al., 2001; Pleasants and Oberhauser, 2013; Flockhart et al., 2017a). For example, one study estimated that the 2.2 billion milkweeds present on the landscape in the central Midwest in 1999 had declined by almost 40% by 2014 (Pleasants, 2017). Another study estimated changes in agricultural weed management in Illinois led to an estimated 68% loss of available milkweed for monarchs in the last two decades (Zaya et al., 2017). To counteract the loss of milkweed on the breeding grounds, habitats could be restored to increase the availability of egg laying sites. Thus, it is imperative to understand the causes of monarch butterfly oviposition preference in different landscapes and the spacing of milkweed plants to determine the most effective restoration strategy on the breeding grounds.

To date, studies examining female preferences for oviposition sites have largely consisted of counting eggs and larvae on milkweed in agricultural and non-agricultural landscapes (Oberhauser et al., 2001; Pleasants and Oberhauser, 2013; Kasten et al., 2016). Agricultural landscapes have been shown to contain a higher number of eggs per plant than non-agricultural landscapes (Oberhauser et al., 2001; Pleasants and Oberhauser, 2013). Roadsides, previously categorized as non-agricultural landscape with natural areas, have been proposed as a potentially suitable area for milkweed restoration due to the abundance of roads and availability of land on road margins (Hartzler and Buhler, 2000; Taylor and Shields, 2000; Oberhauser et al., 2001; Hartzler, 2010; Pleasants and Oberhauser, 2013). However, a recent study showed that roadsides have significantly lower egg per plant densities than non-agricultural areas, which included gardens, natural areas, pastures, and old fields (Kasten et al., 2016). There has yet to be a comprehensive study to compare all landscapes concurrently in the same region.

In addition, the mechanisms driving the oviposition preference among landscapes are not well understood. Females may prefer to oviposit in agricultural landscapes over non-agricultural landscapes and roadsides because agricultural landscapes may have fewer invertebrate predators. This pattern could arise from the use of agro-chemicals, specifically insecticides targeted to kill insects, as well as herbicides, which could reduce habitat for invertebrate predators. Conversely, females may prefer non-agricultural landscapes to oviposit due to the greater availability of nectar sources, which may lead to lower foraging times, better lipid reserves and, ultimately, a larger number of eggs laid (Brower et al., 2015).

Monarch oviposition preference could also be influenced by the spatial configuration of habitat, such as the size or density of the milkweed patch. Low-density milkweed patches and single individual milkweed plants have been shown to contain a higher number of eggs per plant than high-density milkweed patches both in agricultural fields (Oberhauser et al., 2001; Pleasants and Oberhauser, 2013) and in natural areas (Zalucki and Kitching, 1982a; Zalucki and Suzuki, 1987). However, this pattern in natural areas has only been shown in Australia where monarchs have been introduced and breed year-round in some regions, and it is not known whether the same pattern would occur in the eastern North American population in a different ecosystem containing different milkweed species. While valuable, these studies also do not explain the possible mechanisms behind these patterns. Females may seek small milkweed patches to avoid natural enemies because large patches may be easier for predators, parasitoids, and parasites to find and could support their populations better than a smaller patch (Zalucki and Kitching, 1982b). A protozoan parasite that monarchs are susceptible to is *Ophryocystis elektroscirrha* (OE), which in heavily infected individuals can result in short adult lifespans, reduced body size, lower mating success, decreased flight ability, and failure to eclose, emerge as an adult properly (Altizer and Oberhauser, 1999; De Roode et al., 2007). The occurrence of OE in monarchs has not been examined in relation to the size of the milkweed patch they inhabit. The rate of OE infection in monarchs could be higher in larger milkweed patches that are frequented by more adult butterflies, potentially increasing the spread of OE to other adults or to milkweed leaves. Investigating which features in the landscape drive oviposition selection could help guide where restoration efforts should be focused.

Here, we examined the factors that drive monarch butterfly oviposition preference by monitoring the number of eggs and larvae in different landscapes (agricultural, non-agricultural, and roadsides) in patches of milkweed, *Ascelpias syriaca*, of varying sizes and densities, and by measuring the abundance of invertebrate predators and parasitoids and the occurrence of the protozoan parasite, OE, in adults that emerged from collected fifth instars. Our hypotheses were considered at two spatial levels: the 'landscape' and 'patch' level. At the landscape level, previous literature suggests that agricultural landscape contains a higher number of eggs per plant than non-agricultural landscape (Oberhauser et al., 2001; Pleasants and Oberhauser, 2013) that may arise because females avoid invertebrate predators, parasites, and parasitoids. We predicted that egg densities would therefore be higher in agricultural landscape compared to non-agricultural landscape and roadsides. Following this same hypothesis, we also predicted that invertebrate predators, parasitoids, and rate of OE infection would be lowest in agricultural landscapes and highest in non-agricultural landscapes due to reduced vegetation biodiversity because of the use of agro-chemicals. At the patch level, prior evidence suggests that low-density patches, single and small milkweed patches, contain higher egg densities than high-density milkweed patches in both agricultural fields (Oberhauser et al., 2001; Pleasants and Oberhauser, 2013) and natural areas (Zalucki and Suzuki, 1987) due to fewer predators, parasitoids, and parasites locating and breeding in small and low-density patches. Thus, we predicted that number of eggs per milkweed would be negatively related (i) to milkweed density in a patch and (ii) to patch size, as measured by monitoring milkweed patches of different sizes and densities in different landscape types. In addition, we predicted that estimated abundance of invertebrate predators and parasitoids, as well as the rate of infection of OE, would be positively related to milkweed density in a patch and to patch size.

2. Methods

2.1. Study sites & experimental design

We conducted our study from Jul 13–Aug 21, 2015, Jul 11–Aug 19, 2016 in Norfolk, Oxford, and Brant Counties in southwestern Ontario,

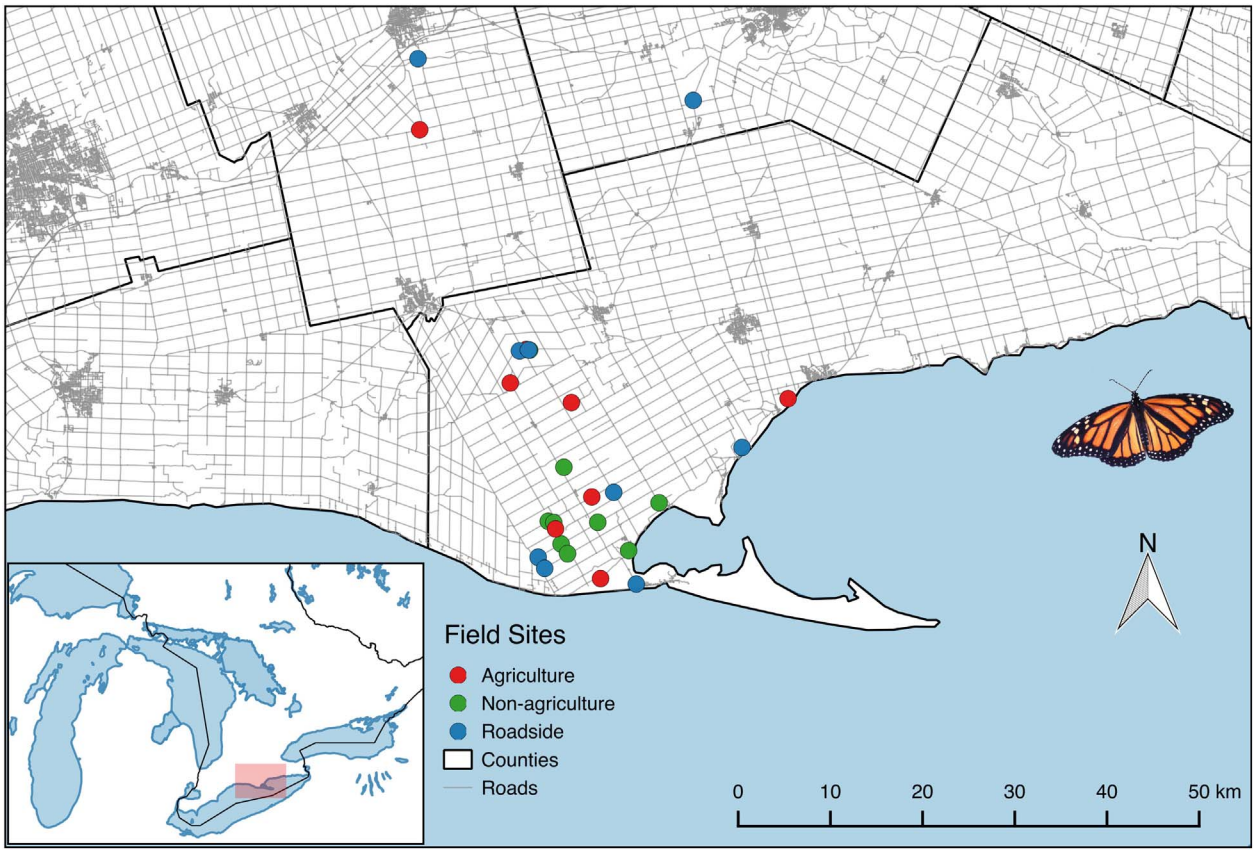

Fig. 1. Map of study area showing field site locations color coded by landscape type and the location of the study area within Ontario, Canada (red box in the inset map). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Canada (Fig. 1). The focal area, Norfolk County, borders the north shore of Lake Erie, which is a major migration pathway for monarchs (Gibo and Pallett, 1979; Brower, 1995). Study sites (n = 26 total; 2015: n = 7; 2016: n = 19) were located in one of three landscape types based on land use: agricultural crop fields (n = 8 total; 2015: n = 3; 2016: n = 5), non-agricultural fields (n = 9 total; 2015: n = 1; 2016: n = 8), and roadsides (n = 9 total; 2015: n = 3; 2016: n = 6). No sites were sampled in multiple years. Agricultural fields contained either herbicide-treated corn or soybean crops and landowners were contacted through Syngenta Canada. Milkweeds sampled in agricultural fields were located both within fields, up to three crops rows in, and on field borders. Non-agricultural fields consisted of restored meadows (n = 6) and private gardens and lawns (n = 3). Roadside sites were stretches of public land between roadways (county highways, regional, and municipal roads) and property borders (agricultural, natural, and residential). At each site there could be multiple milkweed patches (number of patches per site: mean = 4, range = 1–21).

2.2. Egg and larva monitoring

In both years, we counted eggs and larvae by checking and counting all common milkweeds (*Ascelpias syriaca*) in a patch for all sites during the breeding season (Jul 11–Aug 21). To maximize the number of observations without double counting eggs or missing larvae, milkweed patches (n = 111 total; 2015: n = 43; 2016; n = 68) were checked for eggs and larvae every 7 d (Prysby, 2004). Monarch larvae hatch 4–6 d after oviposition and have five instars, with each instar lasting from 2 to 5 d depending on ambient temperature (Zalucki, 1982). The fifth instar pupates into a chrysalis from which the adult will eclose 9–14 d later (Zalucki, 1982). Larval instars were identified by measuring the head capsule and tentacle lengths with a ruler (Oberhauser and Kuda, 1997). '*Egg density*' at a given site was calculated by the number of eggs counted divided by the total number of milkweeds checked. A milkweed patch was defined by a cluster of milkweed stems (hereafter milkweed stems referred to as 'milkweed') that were at least 10 m away from any other surrounding milkweed stems (Matter, 1996). Milkweed '*patch size*' (m^2) was measured by either using a 1 m × 1 m sampling quadrat or by walking the perimeter of the clustered stems using a Global Positioning System (GPS – GPSMAP 64st model ± 5 m accuracy; Hartzler, 2010). Single stems were assigned a patch size of 1 m^2 (Hartzler and Buhler, 2000). Milkweed density within a patch, hereafter '*milkweed density*', was calculated as the number of individual stems divided by the total area of the patch. To determine milkweed density in a patch, an individual milkweed stem was defined as any stem that was separated from another stem of the same milkweed species by soil (Kasten et al., 2016). The area and milkweed density of the patch was measured each time eggs were counted (every 7 d) to have an accurate representation of the patch area and milkweed density at the time of oviposition because milkweed plants may have emerged or died over time.

Patch size was recorded as a continuous variable but there was some evidence that some landscape categories had mostly small (e.g. agriculture) or large (e.g. non-agriculture) patch sizes (Fig. A-1). To capture this variation in patch area across landscapes we conducted an initial analysis to determine discrete patch size categories to ensure that we had sufficient sample sizes for each landscape. To do so, we used a generalized linear mixed model to explain egg density using Julian date, year (2015, 2016), and landscape type (agricultural, non-agricultural, roadside), which included the number of plants checked as an offset. Patch ID was included as a random effect because the same patches were checked each week over the breeding season. The model was then iterated to cycle through all possible patch area combinations among three patch sizes (small, medium and large). We recorded the AIC (Akaike Information Criterion; Burnham and Anderson, 2002)

Table 1
Number of monitored milkweed patches in each size category, by landscape type over 6-week periods in both 2015 and 2016.

Landscape	Milkweed patch size categories		
	Small (< 16 m^2)	Medium (16–28 m^2)	Large (29–472 m^2)
Agricultural	276 (63%)	42 (39%)	12 (11%)
Non-agricultural	99 (23%)	27 (25%)	36 (32%)
Roadside	65 (15%)	39 (36%)	65 (58%)
Total	440 (100%)	108 (100%)	113 (100%)

value of each model iteration and then used the size categories reported in the model with the lowest AIC value. From this preliminary analysis, the patch size categories were determined to be 'small' 1–15 m^2, 'medium' 16–28 m^2, and 'large' 29–472 m^2 and were used in all further statistical models (Table 1; see Statistical analyses, below).

2.3. Invertebrate predator and parasitoid abundance

The abundance of invertebrate predators and parasitoids was estimated using pan traps that were placed inside 86 (2015: n = 18; 2016: n = 68) of 111 monitored milkweed patches. Standard yellow insect pan traps were used because they have been shown to attract the widest diversity of insects (Kirk, 1984) although trap color sampling bias may exist (Vrdoljak and Samways, 2012), and monarch eggs and larvae are known to be subject to an array of invertebrate predators and parasitoids (Oberhauser et al., 2015). Predators include lacewing larvae (Chrysopidae; Oberhauser et al., 2015), lady beetles (Coccinellidae; Koch et al., 2003; Koch et al., 2005), true bugs (Hemiptera; Zalucki and Kitching, 1982b; De Anda and Oberhauser, 2015), ants (Formicidae; Calvert, 2004; Prysby, 2004) and paper wasps (Vespidae; Rayor, 2004; Oberhauser et al., 2015). Monarchs are also susceptible to parasitism by parasitoid Hymenoptera (Oberhauser et al., 2015; Stenoien et al., 2015) and tachinid flies (Tachinidae; Arnaud, 1978; Borkin, 1982; Oberhauser, 2012). The traps were placed such that they were flush with the soil surface and to drown the attracted insects filled halfway with a solution made with 4 teaspoons of salt and 5 drops of unscented dishwashing detergent per litre of water. Pan traps were placed in a patch for 48 h, then reinstalled every week in 2015 or every other week in 2016. Data collected in 2015 were subset to only include samples from every other week to be consistent with 2016 sampling. The contents of each pan trap were strained, rinsed, and put into a glass vial containing 75% ethanol. All invertebrates were identified to family level or below in the laboratory using a microscope at 35 × and dichotomous keys (Triplehorn and Johnson, 2005; Marshall, 2007; Marshall, 2012).

2.4. Parasitism of fifth instars

To evaluate the occurrence of parasitism by tachinid flies and the protozoan parasite, *Ophryocystis elektroscirrha* (OE), fifth instars from monitored patches were collected and reared until eclosion (n = 106 total; 2015: n = 45; 2016: n = 61). OE can be spread by vertical transmission from female to offspring with spores on the surface of the egg capsule or milkweed leaves being ingested by the emerging larvae (McLaughlin and Myers, 1970; Leong et al., 1997). Additionally, OE can spread by horizontal transmission between adults during mating or other interactions such as on flowers or roosting (Altizer and Oberhauser, 1999). Spores must be ingested by the host to cause new infections (Leong et al., 1997). To minimize the transfer of spores to larvae, we followed the sampling procedure outlined by Project Monarch Health (University of Georgia; http://monarchparasites.org). Each larva was kept in an individual plastic container with mesh fabric held in place with rubber bands as a lid. Enclosures were cleaned daily by removing frass and old milkweed leaves. The containers were cleaned using a 20% chlorine bleach-water solution. Larvae were given fresh milkweed daily with a moist paper towel placed on the bottom of the enclosure to reduce dehydration of leaves. Milkweed leaves were obtained from non-monitored milkweed patches and soaked in 10% chlorine bleach-water solution for 20 min to kill OE spores that may have been on the leaves followed by rinsing and soaking for another 20 min in water prior to being given to larvae.

Eclosed adults were tested for OE 10 h or more after emergence. Following previous studies (Altizer et al., 2000; Satterfield et al., 2015; Altizer et al., 2015), OE spores were collected using a clear mailing sticker (2.54 cm diameter) wrapped around the sides of the abdomen and removed and then placed on a white index card. Spores were viewed and counted using a microscope at 65 ×. Samples were assigned to parasite load classes according to the following ordinal scale: 0: no spores, 1: one spore, 2: 2–20 spores, 3: 21–100, 4: 101–1000 spores, 5: > 1000 spores (Altizer et al., 2000). Following previous studies (Altizer et al., 2000; Bartel et al., 2011; Satterfield et al., 2015; Altizer et al., 2015), these 6 parasite load classifications were then further generalized to a binary scale, with 0–3 = '*moderate to no infection*', and 4–5 = '*heavily infected*'.

Chrysalises and larvae that appeared abnormal and deceased were kept for an additional 7 d past typical emergence date to check for presence of emerging parasitoids.

2.5. Statistical analyses

To understand the factors that drive monarch butterfly oviposition preference, a generalized mixed-effects Poisson model was fitted using maximum likelihood (Laplace approximation) through the 'glmer' function in the package lme4 (Bates et al., 2015) in R v. 3.3-1 (R Development Core Team, 2017). Because the response variable, egg count, was dependent upon the number of milkweed monitored, an offset of the number of milkweed monitored in the patch was included in the model such that fixed-effects parameter estimates were scaled on a per milkweed basis. Julian date, year (2015, 2016), landscape type (agriculture, non-agricultural, roadside), patch size (small, medium, large), and milkweed density were all included as fixed-effects. We included patch ID as a random effect because patches were checked each week over the breeding season. A two-way interaction between landscape type and patch size was also included to account for the possibility that the effect of patch size on the number of eggs per milkweed differed by landscape type. Significance of fixed effects was assessed using type III ANOVA with Wald chi-square tests (Bolker et al., 2009). The model evaluation approach used for all models was a backwards model selection based on a priori hypotheses to select the best fitting model using Akaike Information Criterion (AIC) values (Burnham and Anderson, 2002). Parameter estimates from the top model were used to predict the egg density across landscape type, patch size, and milkweed density to inform land managers of the most effective planting strategy depending on the site location and milkweed distribution.

To assess the effect of abundance of invertebrate predators and parasitoids on monarch oviposition, we performed two separate generalized mixed-effects Poisson models fitted using maximum likelihood (Laplace approximation) through the 'glmer' function in the package lme4 (Bates et al., 2015) in R v. 3.3-1 (R Development Core Team, 2017). Firstly, a model was used to explain the abundance of invertebrate predators (the number of predators present per trap per sampling period), and a second model to explain the abundance of parasitoids (the number of parasitoids present per trap per sampling period). Julian date, year (2015, 2016), landscape type (agriculture, non-agricultural, roadside), patch size (small, medium, large), and milkweed density were all included as fixed-effects in all models. We included patch ID as a random effect because the same patches were sampled every other week over the breeding season. Significance of

fixed effects was assessed using type II ANOVA with Wald chi-square tests (Bolker et al., 2009).

To understand the consequences of oviposition in regards to OE parasitism, a binomial generalized linear model (GLM) was used to predict the binary response variable, OE infection status (not infected/infected) of collected fifth instars. Julian date, year (2015, 2016), landscape type (agriculture, non-agricultural, roadside), patch size (small, medium, large), and milkweed density were all included as fixed-effects.

3. Results

3.1. Egg density

A total of 30,069 milkweed stems were counted and checked for monarch eggs over two years (2015: n = 6526; 2016: n = 23,543). Monitored milkweed patches were distributed between landscape types with agricultural landscape having the most 'small' patches (< 16 m^2) and the fewest 'large' patches (> 28 m^2), while the opposite was found in roadside landscape (Table 1). Mean milkweed density in a patch was 2.7 ± 5.3 milkweed/m^2 (mean ± SD) (range = 0.1–58.0 milkweeds/m^2) across all landscapes with agricultural landscape having the highest average milkweed density (3.6 ± 7.3 milkweed/m^2; $\chi^2 = 6540$, df = 2, $p \leq 0.0001$) (Fig. 2).

From the monitored milkweed patches, a total of 1988 eggs were counted over two years (2015: n = 1071; 2016: n = 917). Although eggs were laid in all landscape types, there were no eggs counted in 21 patches (19%) (2015: n = 5; 2016: n = 16). Egg density, the number of eggs per milkweed in a given patch, was 0.1 ± 0.4 (max. = 4) across all landscapes (Fig. A-1).

The best supported generalized mixed-effects Poisson model for egg density was the global model that included Julian date, year, landscape type, milkweed density, patch size, and the interaction between landscape type and patch size (Table A-2). All fixed effects were significant predictors of egg density (Table 2). Egg density tended to increase over the breeding season and was higher in 2015 compared to 2016. Agricultural landscape milkweed patches had significantly higher egg densities compared to roadside landscape patches. However, milkweed patches in non-agricultural landscape did not differ significantly from agricultural landscape in predicting egg density. Small milkweed patches (< 16 m^2) had higher egg density than medium patches (16–28 m^2) and large patches (> 28 m^2). As expected, egg density decreased as milkweed density increased (Fig. 3). In this model, there was also a significant negative interaction between landscape type and patch size suggesting that the effect of patch size on egg density differed by landscape type (Fig. 3). In agricultural landscapes, small patches had the highest egg density followed by medium patches and lastly large patches (Fig. 3). In non-agricultural landscapes, medium patches tended to have fewer eggs per milkweed than small and large patches (Fig. 3). In contrast, in roadside landscape, more eggs were laid per milkweed in medium patches than small patches, with fewest eggs laid in large patches (Fig. 3).

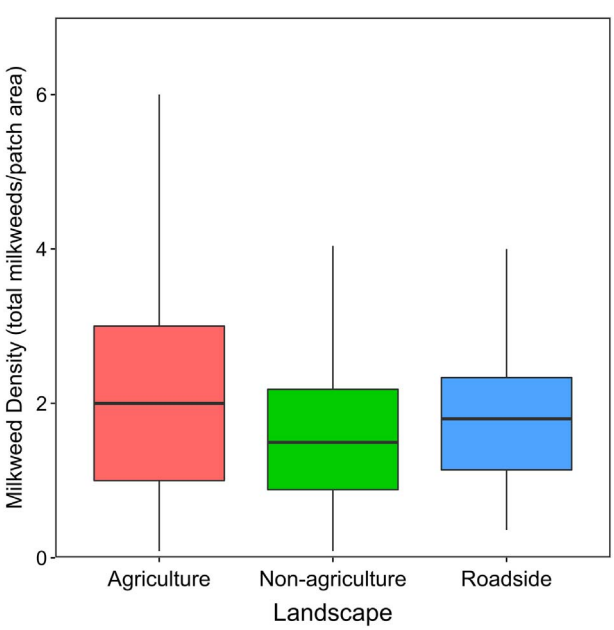

Fig. 2. Milkweed density (milkweed/m^2) in patches of milkweed in each landscape type (agriculture: 3.6 ± 7.3 milkweed/m^2 (mean ± SD), n = 330; non-agriculture: 1.6 ± 1.1, n = 162; roadside: 1.9 ± 1.1, n = 170). Outliers not shown (see Fig. A-2). Box and whiskers plot are composed of lower and higher quartiles (boxes), non-outlier ranges (whiskers), and medians (middle lines).

Table 2
Parameter estimates from the top mixed effects generalized linear model (based on AIC model selection, see Table A-2) to explain egg density based on landscape type, Julian date, year, patch size, milkweed density, and patch ID. Note the intercept value represents the predicted egg density in large agricultural patches in 2015. We report 95% confidence intervals.

Parameter	Estimate ± SE	z	95% confidence interval	SD
Random effect				
Patch ID				0.85
Fixed effects				
Intercept[a]	− 2.51 ± 0.60	− 4.16	− 3.73, − 1.30	
Julian date[a]	0.22 ± 0.025	8.68	0.17, 0.26	
Year 2016[a]	− 0.58 ± 0.12	− 5.46	− 0.80, − 0.37	
Non-agricultural	− 0.13 ± 0.70	− 0.19	− 1.55, 1.25	
Roadside[a]	− 1.70 ± 0.66	− 2.59	− 3.05, − 0.39	
Small patch	0.50 ± 0.62	0.81	− 0.80, 1.74	
Medium patch[a]	− 1.71 ± 0.68	− 2.51	− 3.14, − 0.39	
Milkweed density[a]	− 0.30 ± 0.09	− 3.35	− 0.48, − 0.13	
Non-agricultural: small patch	− 0.51 ± 0.74	− 0.69	− 2.00, 0.99	
Roadside: small patch	0.30 ± 0.74	0.40	− 1.17, 1.84	
Non-agricultural: medium patch	− 0.012 ± 0.92	− 0.013	− 1.84, 1.88	
Roadside: medium patch[a]	3.18 ± 0.77	4.13	1.41, 4.79	

[a] Represents parameters that do not overlap with zero.

3.2. Invertebrate predator abundance

From the 86 patches monitored (2015: n = 18; 2016: n = 68), 3167 invertebrate predators were identified and counted (Table 3). The mean abundance of invertebrate predators (count of predators per trap per sampling period) was 12.9 ± 26.1 across all landscapes. Collectively, 15 different invertebrate predator families were sampled (max. per sample = 5) (Table A-1).

The best model predicting invertebrate predator abundance included Julian date, year, and patch size but not landscape type (Table A-3). Predator abundance tended to decrease over the breeding period (Table 4) and medium milkweed patches (16–28 m^2) tended to have higher predator abundance compared to small (< 16 m^2) and large patches (> 28 m^2) (Fig. 4). Although year was in the top model, it was not a significant predictor of invertebrate abundance.

3.3. Parasitoid abundance

The same 86 monitored patches (2015: n = 18; 2016: n = 68) were used to sample parasitoid abundance (Table 3). From the collected samples, 704 parasitoids were identified and counted (Table A-1). The abundance of parasitoids (count of parasitoids per trap per sampling period) was 2.9 ± 5.2 across all landscapes. Collectively, four different

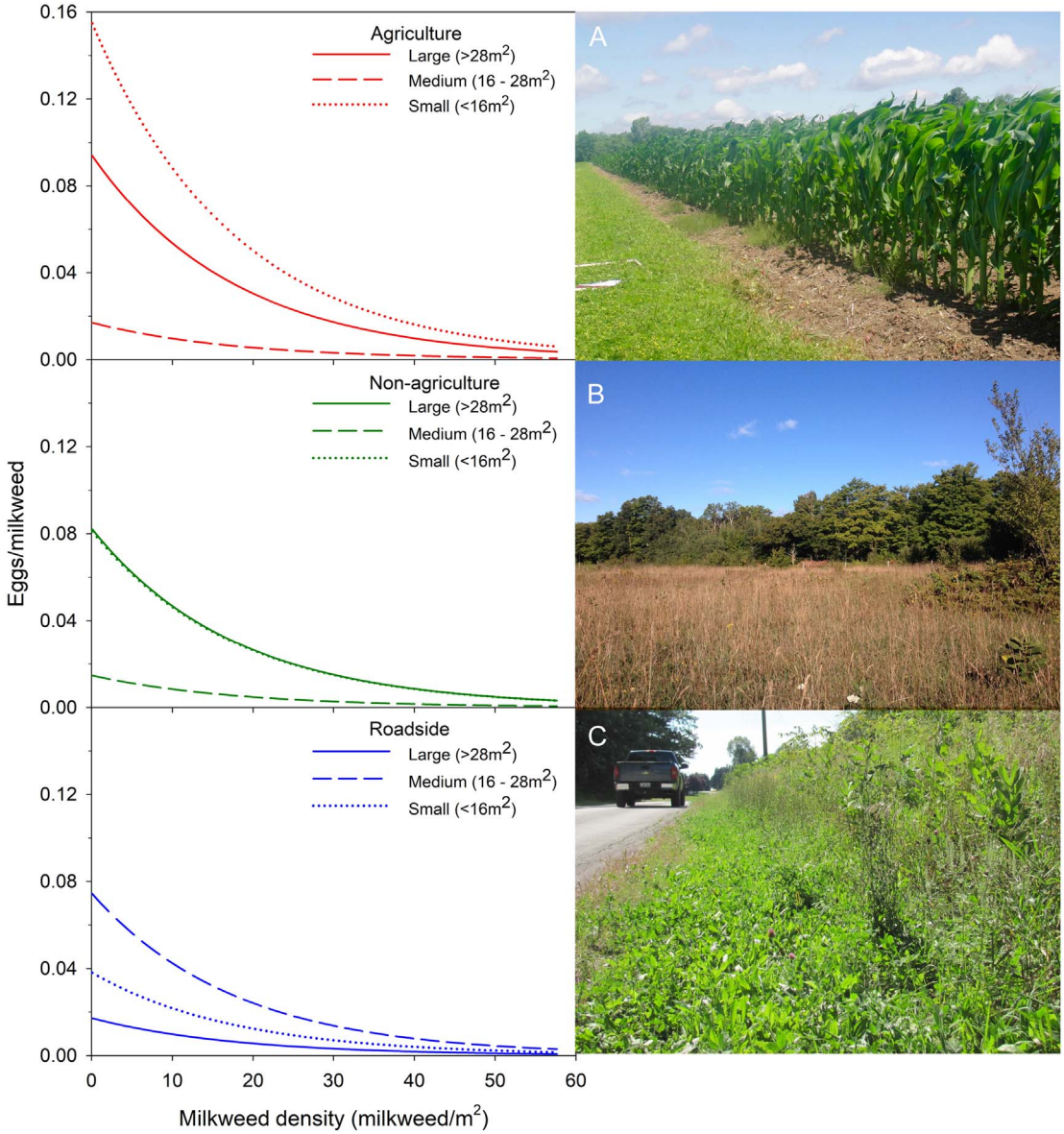

Fig. 3. Predicted egg density (eggs/milkweed stem) in three landscape types (A, agriculture; B, non-agriculture; C, roadside) by patch size and milkweed density (milkweed stems/m^2) predicted from parameter estimates from the top model. Adjacent to each panel figure is a photographic example of a site in each associated landscape type.

Table 3
Summary of the total number of samples collected for invertebrate predator and parasitoid abundance sampling from 86 (2015: n = 18; 2016: n = 68) of 111 monitored milkweed patches, among milkweed patch sizes and landscape types. Sampling was conducted every other week with a given patch being sampled a maximum of 3 times over the total 6-week period.

Landscape	Milkweed patch sizes		
	Small (< 16 m^2)	Medium (16–28 m^2)	Large (29–472 m^2)
Agricultural	72 (48%)	16 (36%)	6 (12%)
Non-agricultural	50 (33%)	13 (29%)	17 (35%)
Roadside	29 (19%)	16 (36%)	26 (53%)
Total	151 (100%)	45 (100%)	49 (100%)

parasitoid families were present in the pan trap samples (max. per sample = 2).

The best model to explain parasitoid abundance included year and landscape type, and excluded milkweed density and patch size (Table A-4). Year and landscape type were significant predictors of parasitoid

Table 4
Parameter estimates from the top mixed effects generalized linear model (based on AIC model selection, see Table A-3) to explain invertebrate predator abundance based on Julian date, year, patch size, and patch ID. Note the intercept value represents the predicted predator abundance in large patches. We report 95% confidence intervals.

Parameter	Estimate ± SE	z	95% confidence interval	SD
Random effect				
Patch ID				1.05
Fixed effects				
Intercept[a]	5.66 ± 0.44	12.98	4.79, 6.52	
Julian date[a]	− 0.021 ± 0.0015	− 14.01	− 0.024, − 0.018	
Year 2016	0.32 ± 0.29	1.13	− 0.24, 0.89	
Small patch (< 16 m^2)[a]	0.45 ± 0.18	2.47	0.093, 0.81	
Medium patch (16–28 m^2)[a]	0.68 ± 0.24	2.96	0.21, 1.16	

[a] Represents parameters that do not overlap with zero.

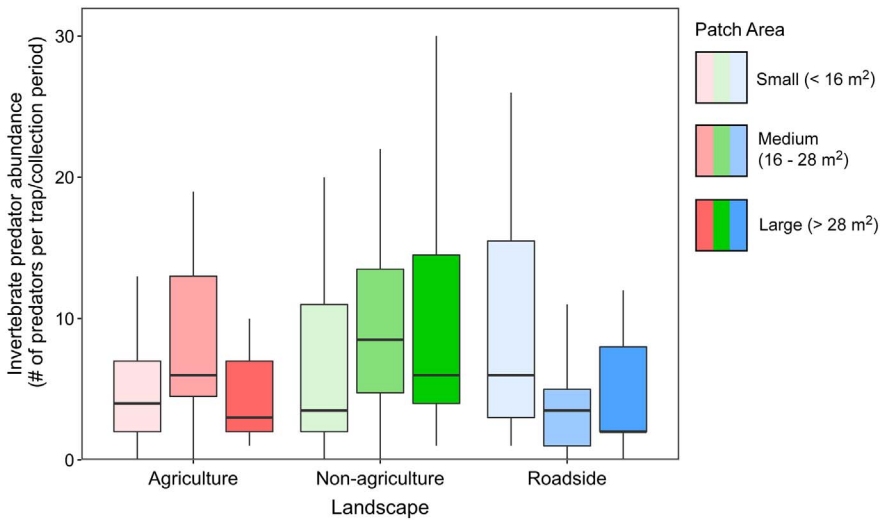

Fig. 4. Invertebrate predator abundance (number of predators per trap sampled every other week) per each patch size and landscape type (agriculture, non-agriculture, roadside). Outliers not shown (see Fig. A-3). Box and whiskers plot are composed of lower and higher quartiles (boxes), non-outlier ranges (whiskers), and medians (middle lines). For each landscape, the light-to-dark shading presented left to right represents small, medium and large patches.

Table 5
Parameter estimates from top mixed effects generalized linear model (based on AIC model selection, see Table A-4) to explain parasitoid abundance based on landscape type, Julian date, year, and patch ID. Note the intercept value represents the predicted predator abundance in agricultural landscapes. We report 95% confidence intervals.

Parameter	Estimate ± SE	z	95% confidence interval	SD
Random effect				
Patch ID				0.58
Fixed effects				
Intercept	0.74 ± 0.70	1.06	− 0.65, 2.12	
Julian date	0.0034 ± 0.0032	1.08	− 0.0029, 0.0097	
Year 2016[a]	− 0.59 ± 0.20	− 3.02	− 0.98, − 0.20	
Non-agricultural	− 0.030 ± 0.18	− 0.16	− 0.40, 0.34	
Roadside[a]	− 0.55 ± 0.19	− 2.92	− 0.94, − 0.18	

[a] Represents parameters that do not overlap with zero.

abundance. Parasitoid abundance was greater in 2015 than 2016, and roadside landscapes had a lower abundance of parasitoids compared to agricultural and non-agricultural landscapes (Table 5; Fig. 5).

3.4. Parasitism by *Ophryocystis elektroscirrha*

From the 106 fifth instars collected, 18 (2015: n = 11; 2016: n = 7) were heavily infected (> 100 spores) with the protozoan parasite, OE (Table 6). Two of the collected fifth instars were parasitized by tachinid flies and, therefore, were not able to be tested for OE parasitism. The highest OE infection rate was found in roadside landscapes (Table 6). The best fitting binomial generalized linear model, based on AIC comparisons, included Julian date, year, landscape type, and milkweed density as predictors of OE (Table A-5). However, the confidence interval of Julian date (β ± SE: − 0.0047 ± 0.024, Z = 0.20, 95% CI: [− 0.040, 0.054]), year (β ± SE: –0.53 ± 0.56, Z = − 0.97, 95% CI: [− 1.65, 0.57]), landscape type (non-agricultural β ± SE: − 0.96 ± 1.17, Z = − 0.83, 95% CI: [− 4.00, 1.02]; roadside β ± SE: 0.82 ± 0.66, Z = 1.26, 95% CI: [− 0.49, 2.12]), and milkweed density (β ± SE: − 0.26 ± 0.19, Z = − 1.39, 95% CI: [− 0.74, 0.024]) overlapped with zero.

4. Discussion

We provide evidence that monarch butterfly oviposition patterns are related to both the size and density of the milkweed patch, as well as the landscape in which the milkweed patches reside. Small (< 16 m^2) and low-density (0–2 milkweed stems per m^2) milkweed patches in agricultural landscapes had the highest egg density compared to larger milkweed patches and higher milkweed densities found in non-agricultural and roadside landscapes. Consistent with previous literature (Zalucki and Kitching, 1982a; Zalucki and Suzuki, 1987; Pleasants and Oberhauser, 2013; Stenoien et al., 2016), we also found that low-density milkweed patches had greater egg density across all landscape types and patch sizes than high-density milkweed patches. In two of the three landscape types (agriculture and non-agriculture), we found that predator abundance was highest in patch sizes where egg density was lowest. In contrast, we found no support for parasitoid abundance driving egg-laying patterns by patch size. Furthermore, we did not find evidence that rates of OE parasitism varied by landscape type, milkweed density, or patch size.

Our results have important implications for restoration of milkweeds for conserving monarch populations. Given the option, agricultural fields appear to be the most effective landscape to plant and maintain milkweeds to attract egg-laying females. Milkweed stems in agricultural landscape sampled in our Ontario-based study averaged 3.5 times more monarch eggs than milkweed stems in non-agricultural landscape, comparable but slightly lower than that found by Pleasants and Oberhauser (2013) in Midwest USA from 1999 to 2010. One important implication, therefore, is that it will be vital to develop incentive programs working with landowners to plant and maintain milkweeds in agricultural landscapes. Programs for milkweed restoration could be conducted in collaboration with other pollinator initiatives or ecosystem service programs in agricultural landscapes that focus on increasing nectar availability (e.g. Alternative Land Use Service, ALUS; Conservation Reserve Program, CRP). Ideal areas for planting milkweed patches could be in crop margins, field corners, or other marginalized land within close proximity to crop fields. Consideration should be made for areas where milkweed will not be trampled by machinery or livestock or sprayed by herbicides during the monarch breeding season.

If milkweed restoration in agricultural landscapes is difficult to implement or not feasible, our results suggest that non-agricultural landscapes may be the next most effective landscape for attracting egg-laying females rather than roadsides. Non-agricultural milkweed patches are commonly large as they are left to naturalize and are not subject to pesticides or vegetation management. Large milkweed patches may be particularly important to consider for restoration because they tend to house a higher density of male monarchs searching for mates. Previous work has provided evidence of a male-biased sex ratio around large, high-density milkweed patches, while showing that females tend to reside outside of these patches (Zalucki and Kitching, 1984; Bull et al., 1985). Small milkweed patches in non-agricultural

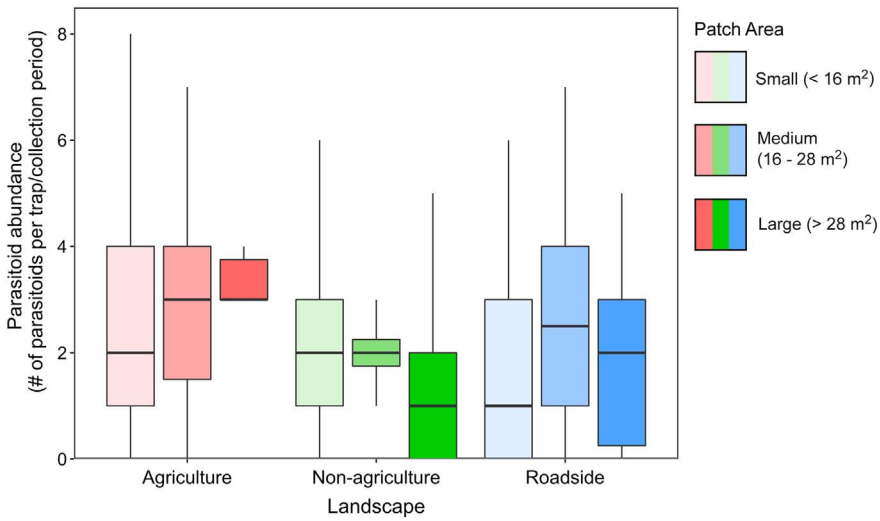

Fig. 5. Parasitoid abundance (number of parasitoids per trap sampled every other week) per each patch size and landscape type (agriculture, non-agriculture, roadside). Outliers not shown (see Fig. A-4). Box and whiskers plot are composed of lower and higher quartiles (boxes), non-outlier ranges (whiskers), and medians (middle lines). For each landscape, the light-to-dark shading presented left to right represents small, medium and large patches.

Table 6
Infection rate of *Ophryocystis elektroscirrha* (OE), a protozoan parasite, from adults eclosing from collected 5th instars (N = 106) originating from different landscape types. The parasite loads classified on a binary scale, with 0–3 (0–100 spores) = moderate to no infection, and score 4–5 (> 100) = heavily infected.

Landscape	Heavily infected (4–5)	Moderate to no infection (0–3)	Infection rate
Agricultural	12	66	15%
Roadside	5	11	31%
Non-agricultural	1	9	10%
Total	18	86	17%

landscapes, such as in gardens and urban parks, while not as preferred as agricultural patches can still provide usable egg-laying habitat and are usually in close proximity to other nectar sources that can provide lipid energy for adults. Restoration efforts in urban areas include registering and certifying monarch waystations (Monarch Watch, University of Kansas, www.monarchwatch.org), to encourage creating habitat that includes milkweeds and nectar sources to support monarchs both during the breeding season and migration. Additionally, the new 'Butterflyway Project', organized by the David Suzuki Foundation (www.butterflyway.davidsuzuki.org), works on creating a network of wildflower patches across large urban cities in Canada.

Despite the fact that roadside habitat is abundant throughout North America, milkweed patches in this landscape received half the number of eggs that were laid in agricultural landscape patches. One possible reason for this could be that females are often not reaching roadside milkweed because of high mortality from vehicle collisions while flying along roadside habitat (Munguira and Thomas, 1992; McKenna et al., 2001; Ries et al., 2001). Even if some roadsides are appealing to females for oviposition due to availability of host plants, nectar sources, and sodium, the restoration of milkweeds in these locations could result in low survival of larvae. Roadside habitats can be detrimental for developing larvae because of heavy metal contamination from cars that can leach into the soil and vegetation (Lagerwerff, 1970; Scanlon, 1987), regular mowing milkweeds that contain eggs and larvae, and road salt runoff (Snell-Rood et al., 2014). Road salt runoff can affect neural investment that result in larger eyes in female monarchs and increased muscle mass in male monarchs (Snell-Rood et al., 2014). Monarchs reared on roadside-collected milkweed leaves also had lower survival than monarchs reared on prairie-collected milkweed leaves (Snell-Rood et al., 2014). In this sense, it is possible that some roadsides could act as ecological traps (Dwernychuk and Boag, 1972; Ries et al., 2001; Battin, 2004). We have noted multiple factors that affect monarch persistence in roadside habitats, however it is not well understood from an evolutionary perspective how effects such as increased mortality or roadside avoidance may drive natural selection and the resulting consequences on the population (Brady and Richardson, 2017). These factors, combined with our results, suggest that investing heavily in milkweed restoration in roadside habitats should be met with some caution.

While the overall differences in oviposition preference between the three landscape types we studied are consistent with previous literature reporting that monarchs lay more eggs in agricultural areas (Oberhauser et al., 2001; Pleasants and Oberhauser, 2013), it is still unclear as to why agricultural landscapes are more attractive for oviposition compared to other landscapes. One explanation for why agricultural landscapes are attractive could be the use of fertilizer, which would run off into areas along field margins where milkweeds are growing and result in more attractive milkweed (Pleasants, 2015). Host plants with higher nitrogen levels have been shown to increase development in some species of Lepidoptera (Slansky and Feeny, 1977; Tabashnik, 1982; Taylor, 1984), and increase larval survival (Myers and Post, 1981; Myers, 1985; Clancy, 1992). There is some equivocal evidence that plant nitrogen content could also influence oviposition preference in other species of Lepidoptera. Cabbage white butterflies (*Pieris rapae*) prefer to oviposit on plants that have higher nitrogen content (Myers, 1985). In contrast, neither copper butterflies (*Lycaena tityrus*; Fischer and Fiedler, 2000) nor monarchs in Australia (Oyeyele and Zalucki, 1990) showed oviposition preference for plants with higher nitrogen.

Another proximate factor driving oviposition preference in agricultural landscapes could be that the chemical signals used to locate milkweeds are easier for females to distinguish in monoculture fields versus milkweed that are embedded in more complex plant communities (Pleasants and Oberhauser, 2013). In our study area, non-agriculture landscapes had the most diverse vegetation communities followed by roadsides and agricultural fields. In support, we found highest egg densities in agriculture landscapes but we found that roadsides had the lowest egg densities. Using chemical receptors on antenna (Thorsteinson, 1960), insects recognize host plants by comparing ratios of host plant volatiles against the volatiles of surrounding plants (Bruce et al., 2005). Thus, detection of a host plant is thought to be more difficult when surrounded by a high diversity of other plants (Tahvanainen and Root, 1972; Finch and Collier, 2000). However, if the surrounding plants are all one species, as in a monoculture crop field, this could make a milkweed's chemical signal easier to detect by females seeking to lay eggs.

Despite the fact that monarchs lay more eggs in low-density

milkweed patches, our results suggest that this cannot be explained by a lower abundance of predators, parasitoids, or parasites. An alternative explanation could be that females are attracted to low-density milkweed patches because the quality of milkweed in these patches is higher than in high-density patches. Plants growing in high-density would likely experience increased competition for resources compared to plants growing in a low-density spatial arrangement. Plant nutrient uptake is proportional to the root length density (Reich et al., 2003; Raynaud and Leadley, 2004; Craine et al., 2005), which could be restricted and reduced when growing more closely to surrounding stems. Milkweed stems of higher nutrient quality growing in a lower competitive environment could be preferred by females, to provide larvae with higher quality host plants.

Although egg density was highest in agricultural milkweed patches, our results suggest this cannot be attributed to a lower abundance of invertebrate predators in this landscape. In agreement with our findings, 74% of studies reviewed by Bianchi et al. (2006) showed that populations of predatory invertebrates were higher in complex, non-crop habitats compared to simplified agricultural landscapes. In the present study, we did not quantify the abundance or diversity of surrounding vegetation. However, while the abundance of predators did not differ between landscape types, predator abundance was influenced by the size of the milkweed patch and Julian date. We found that medium patches had the highest predator abundance compared to small and large patches, coinciding with medium patches having the lowest monarch egg density in both agricultural and non-agricultural landscapes.

While predator abundance was not influenced by landscape type, parasitoids were least abundant in roadside milkweed patches where females laid the fewest eggs compared to agricultural and non-agricultural landscapes. An overall lower abundance of insects in roadside landscapes could be due to reduced vegetation in the landscape (Murdoch et al., 1972; Southwood et al., 1979; Lawton, 1983) or collisions with vehicle (Munguira and Thomas, 1992; McKenna et al., 2001; Ries et al., 2001), therefore limiting available hosts for parasitoids. Roadsides in our study area mostly consisted of planted non-native grasses (*Poa pratensis*) as well as common roadside flowering species (e.g. *Trifolium repens*, *Lotus corniculatus*, *Cichorium intybus*), many of which are invasive and may not support sufficient food sources to meet their nutritional needs. We expected agricultural landscapes to have higher monarch egg density in part due to fewer parasitoids as we would expect agricultural landscape to not be able to support parasitoid populations because of their low plant diversity.

There was no support that OE rates differed by landscape type, patch size, or milkweed density in a patch, which could be due to the overall low abundance of OE in this region (Flockhart et al., 2017b) or resultant low statistical power. Testing for differences in OE parasitism between habitats was limited by the number of fifth instars detected as large sample sizes are necessary to test for differences among landscape types or habitats (Bradley and Altizer, 2005). Low OE prevalence in the northern range of the eastern North American monarch population is therefore unlikely to result in any reduction in lifetime fecundity of females (Altizer and Oberhauser, 1999). Thus OE prevalence is unlikely to influence laying patterns in this portion of the breeding range.

It is important to note that conclusions drawn about the effect of patch size on egg density should be made with some caution because of the uneven distribution of patch sizes that were sampled across the landscape types. Differences in the distribution of patch sizes across landscapes were likely due to differences in vegetation structure and management practices. In non-agricultural (e.g. meadows and fields) and roadside landscapes, milkweed is commonly not managed and left to naturalize with patches growing larger over time, rendering small milkweed patches relatively uncommon in these landscapes. Conversely, large milkweed patches are rare in agricultural landscapes, which is likely due to both the use of herbicides and the widening of crop fields, which reduces field margins where milkweed commonly grows. In addition to patch size, there were also different mean values of milkweed patch density between landscape types. Non-agricultural patches tended to have lower milkweed density than agricultural and roadside patches. The difference in mean values of patch densities could be due to non-agricultural landscapes commonly having a greater availability of land and providing more space for milkweeds to grow compared to agricultural and roadside habitats, which are often restricted by property borders and crops. While one obvious solution to uneven distribution of patch sizes and densities between different landscape types would be to plant specific sized patches and densities, this would be challenging to execute. Creating patches of milkweed plants would include growing plants and transplanting plants and waiting for multiple growing seasons for the patches to become established with a mixture of mature and young plants. Female's oviposition preference may be affected by the age of the plant, but it also important to have mature plants to secure the establishment of the patch in future growing seasons. Furthermore, establishing large (e.g. $> 28\ m^2$) milkweed patches in agricultural landscapes might be difficult due to space restrictions.

While we have provided evidence that egg-laying preferences in monarchs are influenced by patch size, milkweed density, and landscape type, we acknowledge that there may be additional factors affecting female choice of sites. One of these factors could be the proximity of milkweed patches to each other on the landscape and how monarchs perceive these distances with respect to oviposition. Using a simulated egg-laying model, Zalucki and Lammers (2010) showed that when small milkweed patches are removed from the matrix (the area between larger patches), search time for milkweeds increased, resulting in reduced lifetime potential fecundity by ~20%. However, there are no empirical data to support this hypothesis and the sensory basis behind female preferences for oviposition is not well understood despite the importance it might have for milkweed restoration efforts. A second factor to consider could be the quality of the milkweed plants, such as height, age, and leaf quality. Females have been shown to prefer young plants that have newly emerged leaves (Zalucki and Kitching, 1982a; Alcock et al., 2016), and taller plants that are closer to flowering with intermediate levels of cardenolides (Cohen and Brower, 1982; Malcolm and Brower, 1986; Oyeyele and Zalucki, 1990) in a variety of milkweed species.

Although we have provided evidence for factors that influence the preference of egg-laying sites, a key question remains: do these same characteristics influence the subsequent survival of larva? It is possible that even though oviposition preference is higher in small agricultural patches, survival could be relatively low in these patches due to the use of agro-chemicals and predators. From our results the abundance of predators in agricultural landscapes was comparable to that of non-agricultural and roadside landscapes, suggesting that agricultural patches do not act as a prey refuge for monarchs. Monarchs are susceptible to many predators and have a very high mortality rate, ~88–98% during egg and early larval instars (Borkin, 1982; Zalucki and Kitching, 1982b; Prysby, 2004; Nail et al., 2015), therefore having a significant impact on population growth. How agrochemicals, such as neonicotinoids, affect larval survival is not well understood. Neonicotinioid insecticides (e.g. clothianidin) are the most widely used class of insecticide (Goulson, 2013). These compounds are water-soluble (Tomizawa and Casida, 2005), allowing for uptake into plants (Krupke et al., 2012) including milkweeds (Pecenka and Lundgren, 2015). Some research has suggested that monarch larvae on milkweeds in agricultural fields may be exposed to neonicotinoids (Pecenka and Lundgren, 2015). It is unknown what sublethal effects neonicotinoid exposure might have on monarch behaviour during foraging and navigation. It is crucially important to understand the factors that affect oviposition behaviour and larval survival in relation to habitat in order to aid in monarch population recovery.

In summary, we found that monarch egg density was highest in small, low-density milkweed patches in agricultural landscape. Small

milkweed patches also yielded lower invertebrate predator abundance than medium patches. Based on these findings it will be important to develop programs with landowners and other pollinator initiatives or ecosystem service programs to actively restore milkweed in agricultural landscapes. Ideal areas for planting milkweed patches are crop margins, field corners, and other marginalized cropland within close proximity to crop fields. Small and large patches in non-agricultural landscapes provide the next most effective landscape for attracting egg-laying females. Large patches may also be important for providing a location for male monarchs to search for mates. Roadside patches which received half the number of eggs compared to agricultural landscapes, may potentially pose a number of threats to monarchs because of vehicle collision and accumulation of noxious chemicals; restoration should, therefore, be approached with some caution. Detailed planning and immediate action is needed to continue to help protect this vulnerable and rapidly declining monarch butterfly.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocon.2017.10.019.

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