UNDERSTANDING HOW SHIFTS FROM MIGRATORY TO SEDENTARY BEHAVIOR INFLUENCE PATHOGEN DYNAMICS IN A BUTTERFLY HOST

by

DARA ASHLEY SATTERFIELD

(Under the Direction of Sonia Altizer and John C. Maerz)

ABSTRACT

Seasonal animal migrations can have profound ecological consequences, including for infectious disease dynamics. Migration can often lower infection risk, if animals escape from parasite-contaminated habitats or if strenuous journeys cull infected hosts. Many migratory species are now undergoing shifts or declines in migration. Numerous animal species have responded to environmental changes by forming sedentary populations that remain in the same location. My doctoral research explores how the break-down of animal migrations alters pathogen transmission. Two driving questions guide this work: (1) Do sedentary populations that forego migration face greater infection risk compared to migratory conspecific populations? (2) Do sedentary populations affect the behavior and infection risk of remaining migratory animals? We focused on monarch butterflies (*Danaus plexippus*) and their protozoan parasite (*Ophryocystis elektroscirrha*, OE) as a model system. Most monarchs in North America migrate annually to overwintering areas in Mexico and California, and this journey reduces OE prevalence. In parts of the U.S., however, some monarchs now breed year-round on an exotic milkweed species planted in gardens. We collaborated with citizen scientists to test over 9000 wild monarchs for parasites. Results showed that, relative to migratory butterflies, sedentary monarchs experienced

5- to 9-fold higher infection risk at year-round breeding sites. We next developed a mathematical model to examine host-parasite dynamics at a much smaller scale, within a milkweed patch. Our model indicated that OE spore persistence in the environment led to rising prevalence within a breeding season. This could be particularly important at sedentary sites, where breeding is continuous. Finally, we evaluated potential impacts of sedentary monarchs on migrants, using chemical analyses to distinguish natal origins of wild butterflies. We found that migratory monarchs share habitat with parasitized resident monarchs in the fall and spring in coastal Texas. Migrants sampled at year-round breeding sites showed a greater probability of having OE infections and reproductive activity – both factors that are known to decrease migratory success. Collectively, our findings suggest that human activities that alter animal migrations can influence pathogen dynamics. For this butterfly species, native and seasonal milkweeds (rather than exotic, year-round species) could better support monarch migration and health.

INDEX WORDS: *Danaus plexippus*, animal migration, host-pathogen dynamics**,** *Ophryocystis elektroscirrha*, sedentary population

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DEDICATION

This dissertation is dedicated to my grandfather, George Wannamaker, and to his beautiful daughter, my mother Dawn Wannamaker Satterfield. Their shared mission to serve and love others has shaped my whole life. Their work as scholars and writers has instilled my own affinity for inquiry and learning. Their support has been everything.

Love is indeed the greatest force for the good on earth. –George William Wannamaker

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Animal movement underlies patterns in infectious disease occurrence for many pathogens of concern to wildlife and human health [1–3]. As hosts move through landscapes, they acquire, transmit, avoid, or recover from a diversity of pathogens and in turn drive spatial and temporal dynamics in infection [4,5]. Long-distance animal movements, and seasonal migrations in particular, can alter pathogen spread. Recent work demonstrates that, in numerous cases, long-distance migration operates to reduce infectious disease risk through multiple mechanisms [6], with examples found in bats, fish, butterflies, and ungulates [7–11]. Amid global environmental change, however, long-distance migration has become increasingly challenging for some wild populations. In recent decades, many animal migrations have shifted – in duration, direction, timing, or frequency – in response to human activities [12–14]. Changes in migratory behaviors could alter infectious disease dynamics, but few studies have examined these implications [6]. The aim of this dissertation is to investigate consequences for infectious disease patterns when animal migrations shift in response to human-caused environmental change.

In terms of human pressures causing changes to animal migrations, overharvesting has driven some migrations to disappear altogether, such as for American bison and passenger pigeons [12]. Climate change and habitat alteration have altered migratory timing or routes for other animals, including European birds that have shortened or delayed seasonal movements [12,13,15]. A mounting number of studies have documented shifts from migratory to sedentary behaviors [16–18]. Populations of migratory mallards, trumpeter swans, elk, bustards, and other

species have become more sedentary due to supplemental feeding, barriers in the landscape, or climate change [19–22]. For instance, dark-eyed juncos established an urban population in the 1980s in San Diego, where these year-round resident birds engage in a longer breeding season and forego migration [23]. Changes in migratory behaviors do also occur in the opposite direction (with populations becoming more migratory over time [24]) or in response to natural ecological changes [16]. However, shifts towards sedentary behaviors caused by anthropogenic changes have occurred quickly and across numerous species in the last few decades, and changes in migratory behavior are predicted to continue in the future [13,17,25,26].

Changes in long-distance migrations could prompt changes for host-pathogen dynamics [6]. Recent work suggests a complex relationship between migration and infectious disease risk. On one hand, for instance, seasonal migrations of birds have caused viruses to spread across vast distances [27]. In many other cases, however, migration lowers infectious disease risk for animal populations. This can occur when migration shortens the window of transmission or recovery for animals departing contaminated habitats, removes infected individuals from the population during strenuous journeys, or separates age classes with differential susceptibilities [7,8,11,28,29]. Given the role of seasonal migration in reducing infection prevalence, a crucial question is how the loss of migratory behaviors will affect pathogen transmission and evolution [6]. Sedentary populations could support greater disease burdens due to persistently high host densities and the absence of beneficial mechanisms offered by migration [6]. A handful of studies have investigated this hypothesis [30]. For example, elk in Wyoming given supplemental resources formed high-density, sedentary winter populations with higher brucellosis transmission compared to free-ranging elk [31]. A similar phenomenon may have occurred when early humans transitioned from nomadic to sedentary agricultural societies; these new societies likely

acquired new pathogens and suffered greater parasite burdens [32].Understanding how shifts in host movements could influence pathogen transmission could be critical for predicting and controlling infectious disease in natural and human populations.

My dissertation research investigates how changes in seasonal migration affect host-pathogen interactions, focusing on monarch butterflies (*Danaus plexippus*) and a protozoan parasite (*Ophryocystis elektroscirrha*, OE) as a model system. During the fall, monarchs in eastern North America typically migrate over 3000 km to central Mexico, as their native milkweed host plants die back in the U.S. and Canada. The butterflies overwinter in high-altitude forests for several months and postpone reproduction until the spring [33,34]. Some monarchs, however, now breed year-round in coastal areas of the southern U.S. and California, where they almost exclusively use an exotic milkweed species (commonly planted in gardens) that continues growing during the winter [35]. Previous work showed that monarch migration removes infected butterflies from the population and allows monarchs to escape habitat with infectious parasites for part of the year – both mechanisms that explain lower infection levels among monarch populations with greater migratory propensities [11,36].

Two primary objectives underpin this dissertation work: In Goal 1, I explored whether sedentary populations experience higher infection risk, compared to migratory animals, as a result of the loss of migration. Specifically, I determined whether non-migratory monarchs at sites with year-round exotic milkweed show higher OE infection prevalence compared to migrants (Chapters 2 and 3). In Goal 2, I examined drivers of local transmission dynamics and investigated whether sedentary populations pose risks to migratory individuals that encounter them. Specifically, I investigated how environmental persistence of OE parasites affects host-parasite dynamics for this system (Ch. 4); parasite persistence could be important for sedentary

populations in particular. To evaluate potential impacts of sedentary monarchs on infection dynamics for the larger migratory population, I quantified the extent to which migratory monarchs share habitat with parasitized sedentary monarchs, and if such migrants experience changes in reproduction or infection risk (Ch. 5).

In chapter 1, I measured infection prevalence at winter-breeding sites in the southern U.S. and compared results with that of migratory monarchs, with assistance from 107 citizen scientists through the program *Monarch Health* (Ch. 2; [37]). I also measured parasite virulence for isolates from non-migratory and migratory monarchs, to observe whether the loss of migration was allowing parasites to become more virulent. I hypothesized that higher virulence could evolve when transmission opportunities were no longer interrupted or constrained by host migration. In Chapter 3, I next investigated whether similar patterns in infection occur among migratory and non-migratory monarchs in the western U.S., where monarchs engage in a shorter migration. Volunteers from southern California were also reporting monarch winter-breeding activity on tropical milkweed. I collaborated with citizen scientists through *Monarch Health* and *Monarch Alert* to assess infection risk for California monarchs (Ch. 3; [38]). The key finding of both of these chapters was that sedentary monarchs face extremely high infection risk, multiple times greater than for migratory butterflies.

In both of these studies and in previous work, results suggested that one possible driver of infection dynamics was that parasites accumulated in the environment on milkweed plants [11], which could increase transmission particularly for sites with long or continuous breeding seasons. In Chapter 4, I aimed to understand the role of parasite persistence in the environment on host-parasite dynamics at a local scale. I designed an experiment to observe OE longevity under natural conditions and developed a mathematical model to represent transmission within a

milkweed patch during a single summer-breeding season (Ch. 4). Our model of simple differential equations, with compartments for infected and uninfected monarch adults and larvae and for dormant parasites on milkweed leaves, allowed us to examine how environmental transmission and spore persistence influence host-pathogen dynamics for this system. Key findings showed that transmission stages of this butterfly pathogen are long-lived and indicated that this is a necessary condition for the protozoan to persist in local monarch populations.

To assess whether resident populations pose risks for migratory populations (as part of Goal 2), in Chapter 5 I investigated the degree to which resident populations influence migratory behavior and transmit pathogens to migratory individuals. Results from Ch. 2 and 3 showed that many year-round breeding sites occur in the coastal flyway of migratory monarchs that travel along the Gulf coast *en route* to Mexico. I examined the extent to which resident and migrant monarchs overlap geographically in coastal Texas, and further investigated whether migratory monarchs that encounter sedentary sites are more likely to be reproductive or infected compared to migrants at other stopover sites. This study revealed that migratory and sedentary monarchs share habitats during fall and spring migration, and demonstrated that a small fraction of migratory monarchs remain at sedentary sites after encountering them. I also found evidence suggesting that year-round breeding sites either disproportionately attract reproductive monarch, or induce reproductive activity in migrants.

Collectively, this body of work provides strong evidence that the loss of migratory behaviors increases infection risk for monarchs. My dissertation work suggests that promoting healthy monarch populations requires protecting monarch migration and that milkweeds that are seasonal (not year-round) will better support monarch health by limiting the formation of sedentary populations that foster high infection rates. Milkweed management strategies that

achieve this on a regional scale – through planting native species or removing tropical milkweed growth during the winter – could lower the probability of parasite transmission and accumulation, thus reducing infectious disease burdens for monarchs in the southern U.S.

Beyond monarchs, findings here suggest that human activities that alter animal migrations can influence pathogen dynamics, with implications for wildlife conservation. While sedentary behaviors could reduce parasitism for some animal populations, the loss of migratory behaviors will likely increase infection rates for many species. As more animal populations are predicted to reduce or curtail their migrations in the future, we expect pathogens that have been historically regulated by host migration to pose greater threats to wildlife and human health. Our findings suggest the need for prioritizing the preservation not only of migratory animals themselves, but their behaviors and propensities for migration, which can reduce infectious disease risk and contribute to ecosystem function [6,39].

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CHAPTER 2

LOSS OF MIGRATORY BEHAVIOR INCREASES INFECTION RISK FOR A BUTTERFLY HOST[1]

[1] Satterfield, D.A., Maerz, J.C., and S. Altizer. 2016. *Proceedings of the Royal Society B: Biological Sciences 282* (1804). DOI: 10.1098/rspb.2014.1734. Reprinted here with permission of publisher.

ABSTRACT

Long-distance animal migrations have important consequences for infectious disease dynamics. In some cases, migration lowers pathogen transmission by removing infected individuals during strenuous journeys and allowing animals to periodically escape contaminated habitats. Human activities are now causing some migratory animals to travel shorter distances or form sedentary (non-migratory) populations. We focused on North American monarch butterflies and a specialist protozoan parasite to investigate how the loss of migratory behaviours affects pathogen spread and evolution. Each fall, monarchs migrate from breeding grounds in the eastern U.S. and Canada to wintering sites in central Mexico. However, some monarchs have become non-migratory and breed year-round on exotic milkweed in the southern U.S. We used field sampling, citizen science data, and experimental inoculations to quantify infection prevalence and parasite virulence among migratory and sedentary populations. Infection prevalence was markedly higher among sedentary monarchs compared to migratory monarchs, indicating that diminished migration increases infection risk. Virulence differed among parasite strains but was similar between migratory and sedentary populations, potentially owing to high gene flow or insufficient time for evolutionary divergence. More broadly, our findings suggest that human activities that alter animal migrations can influence pathogen dynamics, with implications for wildlife conservation and future disease risks.

Key words: movement ecology, long-distance migration, infectious disease, virulence evolution, *Danaus plexippus*, neogregarine

INTRODUCTION

Each year, billions of animals migrate long distances to track seasonal changes in resources or climate. These animals comprise a significant portion of global biodiversity and their migratory behaviours have large effects on ecosystem processes [1,2]. In recent decades, numerous migratory species have declined or altered their migratory behaviours in response to anthropogenic environmental change [3-5]; some populations now migrate shorter distances or have transitioned into year-round resident populations [6-8]. For instance, numerous bird species have shown reduced migratory tendency linked to climate warming [6], or established new non-migratory populations owing to habitat loss or supplemental feeding by humans (e.g., at bird feeders) [9]. As one example, Spanish White Storks now forego their traditional migration to Africa each winter and instead subsist on city landfills year-round [10]. Other species are showing similar behaviours, including European Blackbirds, Great Crested Grebes, and grey-headed flying foxes [11-13]. Changes in migration behaviours could influence nutrient transfer in ecosystems, affect pest control and pollination, and in particular, alter infectious disease dynamics [14,2,15].

Long-distance migration influences interactions between animals and pathogens. A crucial question is how the loss of migration and a shift towards sedentary behaviours will affect pathogen transmission and evolution [15]. In some cases, seasonal migration can cause hosts to encounter more diverse pathogen assemblages over heterogeneous habitats [16,17]; acquire infections during periods of dense aggregations [18]; and spread pathogens to geographically distant areas [19]. However, recent work suggests that migration more typically lowers infection risk for migrants by (i) allowing animals to periodically leave behind parasite-contaminated habitats (a process termed *migratory escape* [20]), (ii) weeding out infected individuals during

strenuous, long-distance journeys (*migratory culling* [21,22]), and (iii) separating vulnerable juveniles from infectious adults (*migratory allopatry* [23]). Support for the role of migration in lowering infection risk comes from theoretical models [24] and field studies [25,26; reviewed in 15]. Diminished migrations could enhance pathogen transmission via the loss of migratory escape or migratory culling. Further, reduced migration could allow more virulent pathogen strains to persist by increasing opportunities for pathogen transmission [27]; without the physical demands of migration, infected individuals could survive longer to transmit virulent strains. In sum, sedentary populations could support greater parasite prevalence and virulence than their migratory counterparts [28]. Pathogen dynamics have already shifted in response to changes in migratory patterns for some wildlife populations. For example, the break-down of nomadic movements among fruit bats in Australia likely underpins Hendra virus spillover to horses and humans near sedentary bat colonies in urban centres [29]**.**

Here, we ask whether a shift from migratory to resident behaviour alters the prevalence and virulence of a specialist protozoan pathogen in monarch butterflies (*Danaus plexippus*). Each fall in North America, monarchs migrate up to 2500 km from their summer-breeding range in the eastern U.S. and Canada to overwintering sites in central Mexico (figure 1; [30,31]). In Mexico, migratory monarchs cluster on trees in high-altitude forests in a semi-dormant and non-reproductive state. In spring, these same individuals mate and fly north to recolonize their breeding range over 2-3 generations [32]. Past work showed that long-distance migration annually reduces protozoan infection prevalence in North American monarchs through migratory escape and migratory culling [22].

In recent years, the population size of migratory monarchs in Mexico has severely declined [33,34] in response to deforestation of overwintering sites and intensive agricultural practices

that reduce habitat for milkweeds in the U.S. [35,36]. To counter this decline, some conservation groups have encouraged the public to plant milkweed (monarch host plants) in gardens. Over 100 milkweed species are native to North America [37], however the most commercially available is a single, exotic species known as tropical milkweed (*Asclepias curassavica).* Tropical milkweed does not naturally senesce in the fall like native milkweeds, and in areas with mild climates, continues to produce new foliage and flowers during fall and winter [38]**.** Thus, tropical milkweed provides monarch larval food throughout the year, and reports of monarchs breeding during the winter – rather than migrating or overwintering – have become common in the southern U.S., almost exclusively restricted to sites where tropical milkweed is present [39]. Hereafter, we refer to such areas as winter-breeding sites, where monarch eggs and larvae occur between Dec-Feb (excluding south Florida, as noted in methods). Population dynamics at winter-breeding sites are not well understood, and the origin of immigrant monarchs into these areas is not known. However, a recent study involving cage experiments showed that exposure to milkweed in good condition can induce a percentage of fall migratory monarchs to become reproductively active (and thus, unlikely to migrate) [38]. Although historical data are limited, a search of herbarium records suggests that tropical milkweed occurrence and monarch winter-breeding have become more frequent in the southern U.S. in recent decades (electronic supplementary material). Considered altogether, while the number of migratory monarchs in Mexico has declined largely due to the loss of native milkweeds, the relative number of non-migratory monarchs has increased due to the year-round persistence of exotic milkweed in the southern locations – leading to a net loss of migratory behaviour.

To investigate whether winter-breeding behaviours support greater pathogen prevalence in monarchs, we used a combination of field monitoring and citizen science data. We tested the

prediction that resident monarchs at winter-breeding sites experience higher prevalence of infection with the protozoan *Ophryocystis elektroscirrha* (*OE*), compared to migratory monarchs at overwintering sites or in the summer-breeding range. Next, we experimentally tested whether virulence was greater among parasites collected from winter-breeding sites compared to parasites from migratory monarchs, as would be expected if year-round transmission favours the persistence of more virulent strains.

MATERIAL AND METHODS

(a) Biology of the study system

Adult monarchs infected with the specialist protozoan *OE* emerge from their pupal cases covered with millions of dormant parasite spores on the outside of their bodies [40]. Transmission occurs when infected adults scatter parasite spores onto eggs or milkweed, and larvae ingest spores while feeding [41]. Larva-to-larva transmission does not occur; rather, spores from adults must be eaten by a larva to cause a new infection. Infected monarchs suffer from wing deformities, smaller body size, reduced flight performance, and shorter adult lifespan [21,42]. Infections occur in all monarch populations examined to date, and populations with greater migratory propensity tend to have lower infection prevalence [43,44]. Previous studies of seasonal patterns suggest that parasite prevalence in eastern North American migratory monarchs is reduced annually by migratory culling and migratory escape [22].

(b) Measuring prevalence in migratory and winter-breeding monarchs

We used a combination of field sampling and citizen science data to quantify parasite infection in wild monarchs for two consecutive years (during 2011-2013) at multiple sites (figure 1 and electronic supplementary material, table S1). We focused on four sources: (1) resident monarchs

sampled at winter-breeding sites in the southern U.S.; (2) migratory monarchs sampled across their summer-breeding range (northern U.S. and southern Canada); (3) migratory monarchs sampled at Mexico overwintering sites; and (4) migratory monarchs sampled at coastal overwintering sites in the southern U.S., where a small fraction of eastern North American monarchs overwinter with no breeding activity [45]. We collaborated with citizen scientists through Project *Monarch Health* (*MH*) to quantify infection prevalence at 30 winter-breeding sites in the southern U.S. between Dec-Mar (N=571 monarchs sampled by 36 volunteers), and at 89 summer-breeding sites in the eastern U.S. and Canada between Jun-Oct (N=2566 samples from 69 volunteers). Our lab team sampled additional monarchs at five winter-breeding sites (N=96 samples) and collaborated with J.W. McCord and others to sample five coastal overwintering sites in the southern U.S. (N=254 samples). Winter-breeding sites excluded southern Florida below 27.34° N latitude (Sarasota, FL) where a distinct population of non-migratory monarchs that breeds year-round has long been established [46] and is known to harbour high infection prevalence [43]. Parasite samples from overwintering migratory monarchs at two sites in Michoacán, Mexico were obtained in collaboration with E. Rendón-Salinas, P.F. Jaramillo-López, and WWF-Mexico (N=2390 samples).

We tested monarchs for *OE* infection non-destructively by pressing transparent tape ($1.27cm^2$) against each adult monarch's abdomen and viewing samples at 63X magnification (as described in [43]). Citizen scientists through the *MH* program collected similar samples and mailed these to our laboratory to be scored for the presence/absence of infection. Following [22], we scored samples with >100 spores as *heavily infected*, indicating an acute infection acquired as a larva; in contrast, samples with <100 spores can result from the passive spore transfer between adult monarchs [41,47]**.** Data for each sample included date, sex, location, and monarch

collection stage (adult or larva reared to adulthood). Additional protocols for the *MH* program are described in [22] and at www.monarchparasites.org.

(c) Virulence experiment

We experimentally tested for variation in parasite virulence using isolates collected from wild migratory and resident monarchs. From each of three sources (winter-breeding, summer-breeding, and Mexico overwintering), we chose 17-20 parasite isolates representing temporally and geographically dispersed samples (electronic supplementary material, table S2). Before the experiment, isolates were passed through one monarch generation in the laboratory to obtain viable stocks and a second generation to clone isolates through single-spore infections (following [48,49]).

We randomly assigned monarch larvae from five outbred lineages (half-sib families) to infection by one of 57 parasite clones (10 monarchs/clone). Host lineages were the grand-progeny of wild, uninfected monarchs collected from east-central Texas in April 2012 (representing spring migrants). Larvae were orally inoculated at the second-instar stage following [48]. Control larvae (n=80) were treated similarly but without parasites. Larvae that consumed the inoculum (10 spores/leaf) were transferred to individual 0.47L plastic containers with mesh lids and reared to the adult stage under ambient light at 27-30°C and 32-49% RH. We re-supplied stalks of swamp milkweed (*A. incarnata*) and cleaned containers daily. Treatment groups remained blind to experimenters. We recorded pupal mass and signs of *OE* infection during development, following [49]. After adult eclosion, we recorded sex and tested monarchs with no signs of infection using the tape method described above to verify the absence of infection. Adults were held in individual glassine envelopes at 12°C. We recorded adult longevity (number of days until death), used in prior studies as an inverse measure of *OE*

virulence; shorter adult longevity indicates higher parasite virulence [27,48]. Deceased monarchs were stored at -20°C and quantitative parasite load (a measure of parasite replication) was obtained for infected monarchs by vortexing each abdomen for 5 minutes in deionized water and using a counting chamber to estimate the total number of spores per monarch [48].

(d) Data analysis

We tested for differences in infection across monarch sources (winter-breeding, summer-breeding, Mexico overwintering, and coastal overwintering) in R v.3.0.3 [50] using two approaches. First, we examined predictors of individual monarch infection status (infected/uninfected) using generalized linear mixed models (GLMM) with a binomial error distribution and logit link in package *lme4*. Factors included source, year, collection stage (adult or larva), and sex. Site was a random effect nested within source population. The analysis excluded 151 samples with missing data. We completed model averaging of top models (ΔAICc<10) with package *AICcmodavg* [51]. Second, we analysed site-level prevalence based on the proportion of samples per site that were heavily infected; sites with fewer than 8 samples were excluded from these analyses. Because Moran's I tests and variograms of prevalence data indicated spatial autocorrelation among summer-breeding (I=0.194, p=0.0002) and winter-breeding sites (I=0.279, p=0.01), we accommodated spatial structure in our site-level prevalence analyses. Specifically, we tested the main effect of source population on prevalence per site using a generalized least squares (GLS) model with a Gaussian spatial correlation structure in package *nlme* [52]. The Gaussian structure substantially reduced spatial dependence among sites and improved model fit as evaluated by AIC. We also included a variance structure (*varIdent*), after observing unequal variance in residuals among sources, to allow for heterogeneity without transforming prevalence values [52]. In the GLS model, prevalence per site was calculated

across the entire study period (2011-2013). Finally, we used a linear mixed model (package *nlme*) in a third analysis, which also examined site-level prevalence and accounted for spatial proximity of sites. For this analysis, we assigned sites to sub-regions nested within source and examined effects of source and year (further described in electronic supplementary material).

Analyses for the virulence experiment were completed in SPSS v.22, using a series of general linear models with three response variables: adult longevity (an inverse measure of virulence; $\log_{10}$-transformed), parasite load (a measure of within-host replication; $\log_{10}$-transformed), and pupal mass (to indicate parasite effects on host body size). In each analysis, predictor variables were parasite source (winter-breeding, summer-breeding, or Mexico overwintering) and monarch sex as fixed factors, and monarch lineage and parasite isolate nested within source as random effects. We used Tukey HSD post-hoc tests to examine differences among source means. Pearson correlations tested associations among the three response variables, with the expectation that parasite load would correlate negatively with adult longevity and pupal mass.

RESULTS

(a) Field infection prevalence

Across all field samples (N = 5,877), 16% of monarchs were *heavily infected* with *OE*, with sharp differences in infection measures among sources (figure 2 and electronic supplementary material, figure S2). Across years, infection frequency was 5 to 9 times higher among non-migratory (winter-breeding) monarchs compared to migratory monarchs sampled in Mexico or at coastal overwintering sites. Infection frequency at winter-breeding sites was also 3.6 times higher than for migratory monarchs sampled at summer-breeding sites (figure 2). Analysis of

individual-level infection status (using binomial GLMMs) supported significant effects of source, year, and collection stage on infection status (tables S3 and S4). In particular, winter-breeding monarchs were far more likely to be infected than monarchs at any other source sampled (Tukey contrasts for top model: winter-breeding compared to Mexico overwintering $z=-2.85$, $p=0.02$; summer-breeding $z=-8.11$, $p<0.001$; coastal overwintering $z=-4.20$, $p<0.001$), whereas infection levels among the three migratory sources did not differ significantly. Wild monarchs captured as adults were less likely to be infected compared to those captured as larvae/pupae (figure S3). Monarchs were more likely to be infected in 2012-2013 than in the previous year, 2011-2012, and infection probability was slightly higher for males than females (but NS).

Large differences in infection between sources persisted when we further analysed data at the site level (infection prevalence) and accounted for spatial dependence among sites. Congruent with the previous analysis, the GLS model showed infection prevalence varied significantly among source ($F_{3,74}=9.54$, $p<0.0001$), explaining 69% of the variance in infection among sites (see figure 2 legend for additional details). A linear mixed model for site-level prevalence yielded similar results, with strong effects of source population (electronic supplementary material).

(b) Parasite virulence experiment

Survival of experimental monarchs to the adult stage was within the range observed for prior studies in our laboratory (82.3% for inoculated monarchs, n=570; 87.5% for control monarchs, n=80). Of the inoculated individuals that survived to adulthood, 95.8% became heavily infected with *OE*; no control monarchs were infected. Adult longevity was lower for infected monarchs

(7.9 days ±0.1 SEM) relative to uninfected monarchs (20.9 days ± 0.4 SEM), while pupal mass was similar for infected (1.30 g ± 0.007 SEM) and uninfected individuals (1.31 g ± 0.017 SEM).

Among infected monarchs, measures of adult longevity (figure 3), parasite load, and pupal mass were similar across parasite source populations. Adult longevity, an inverse measure of virulence, did not depend on source population ($F_{2,437}$=0.24, p=0.79) but did co-vary with sex, such that infected males lived on average 0.7 days longer than infected females ($F_{1,438}$=6.20, p=0.013). Adult longevity also varied significantly among parasite isolates nested within source population ($F_{54, 383}$=1.77, p=0.001; figure 3), with average longevity per isolate ranging from 6.0 to 10.7 days, depending on monarch lineage ($F_{4,435}$=8.15, p<0.001).

Quantitative parasite load was similar across source populations, with an average of $10^{6.1}$ spores (±$10^{4.7}$ SEM) per infected monarch. Parasite load differed significantly among isolates ($F_{54,384}$=2.55, p<0.001) and monarch lineages ($F_{4,436}$=4.857, p=0.001), but did not depend on sex. As observed in previous studies, monarchs with higher parasite loads lived shorter lives (Pearson r= -0.492, N=451, p<0.001). Counter to our expectations, pupal mass was positively correlated with spore load (Pearson r= 0.183, N=446, p<0.001). Pupal mass was higher for males (1.36 g ±0.01 SEM) than for females (1.26 g ±0.01 SEM; $F_{1,502}$=124.30, p<0.001). Pupal mass also varied significantly across monarch lineages ($F_{4,499}$=28.28, p<0.001) but did not depend on parasite source population ($F_{2,433}$=0.58, p=0.57).

DISCUSSION

Non-migratory monarchs sampled in tropical milkweed gardens showed markedly higher infection rates compared to migratory monarchs. This difference suggests that loss of the traditional migration leads to greater infection risk, likely owing to the absence of processes by

which migration removes infected individuals and interrupts parasite transmission. Despite higher infection rates at winter-breeding locations, we found no evidence that parasites isolated from winter-breeding monarchs were more virulent than parasites from migratory monarchs. Instead, virulence and replication (quantitative parasite load) varied more widely among parasite isolates *within* each source population than between source populations.

High prevalence of infection at winter-breeding sites could occur through several mechanisms including the absence of migratory culling and migratory escape. There is ample evidence for migratory culling in monarchs; heavily infected monarchs fly less well [21] and experience shorter lifespans [42], leading to the disproportionate removal of infected monarchs during strenuous migratory journeys. Consistent with this idea, *OE* prevalence has been shown to decline during the long-distance fall migration and is lowest when monarchs reach Mexico, at the end of their fall journey [22]. In contrast, infected monarchs that remain in the U.S. to breed during the winter bypass the physical demands of migration and overwintering, and can reproduce shortly after eclosing. Prior studies on monarchs also offer support for migratory escape. Infection rates within the migratory monarchs' breeding range are lowest early in the breeding season and increase later in the season [22]; this suggests that successive cycles of host breeding allow infectious parasite stages to accumulate [41]. Migratory monarchs leaving behind contaminated patches during the fall will experience a temporary reprieve from parasite transmission and return to parasite-free host plants in the spring. In contrast, continuous breeding allows *OE* transmission cycles to continue uninterrupted. High larval monarch density could be another factor increasing infection rates in winter-breeding monarchs. We have noted average larval densities of up to 10 eggs/larvae per milkweed plant at winter-breeding sites [D. Satterfield, personal observation], several times higher than in the summer-breeding range [22].

Past field and experimental work showed that parasite transmission and host susceptibility increase with monarch larval density [53].

Because winter-breeding sites are distributed along the southern U.S. coast whereas summer-breeding sites occur farther north, latitudinal differences could confound comparisons of non-migratory vs. migratory behaviours. However, a previous study using citizen science data showed that infection prevalence was low for migratory monarchs breeding in the southern U.S., which occurs as migrants return from Mexico in the spring. Our findings from monarchs in the coastal southern U.S. also provide evidence that migratory and breeding behaviours, rather than geography, affect parasite transmission most strongly. A small fraction of the eastern monarch population overwinters, but does not breed, in the southern U.S., while the vast majority of their conspecifics overwinter in Mexico [45]. Results here showed that these coastal overwintering adults (not showing breeding activity) experienced low infection prevalence, similar to that of Mexico overwintering monarchs and much lower than winter-breeding monarchs at neighbouring locations. This finding has two important implications: Migration from summer-breeding grounds to southern coastal areas can produce some level of migratory culling; and year-round breeding enabled by the planting of tropical milkweed is the primary driver of the high parasite transmission reported in parts of the southern U.S.

In addition to large variation among regional sources, our findings demonstrate wide variation in infection prevalence among local sites. For instance, across all winter-breeding sites, prevalence ranged from 0 to 100%. In the GLMM analyses, variance explained by the model (R^2) more than doubled when site was included as a random effect (table S4). Other site-level factors not measured in this study, such as host density, local host genotypes, temperature, precipitation, and patch size, likely play a role in infection dynamics. Sensitivity to

environmental variables at local spatial scales is common in host-pathogen systems [54-56] and merits additional study. Importantly, prevalence differences among migratory and non-migratory monarchs remained strong despite heterogeneity at smaller scales.

Multiple studies to date on monarch parasites and other host-pathogen systems show that greater transmission opportunities tend to favour increased pathogen virulence [27,49,57]. Contrary to this trend, parasite isolates from winter-breeding sources were not more virulent than parasites from migratory sources. Our finding was consistent with another recent study showing no differences in the virulence of *OE* strains from eastern migratory versus south Florida resident monarchs [58]. We expected that highly virulent strains would be selectively removed among migratory monarchs, especially those sampled at overwintering sites in Mexico, whereas such selection would be relaxed at winter-breeding sites. We found high heterogeneity in virulence among parasite isolates within each source, suggesting that genetic variation for virulence exists, as found in earlier work [27,49]. One explanation for the lack of evolutionary divergence is that the parasite has experienced too few transmission cycles to produce a response to selection, especially because many winter-breeding study sites were established only recently [D. Satterfield, personal observation].

Another explanation for lack of parasite differentiation (as suggested by [58]) is that high gene flow of parasites between resident and migratory monarchs constrains evolutionary divergence. During the fall, migratory monarchs likely pass through locations inhabited by winter-breeding monarchs [59]. Moreover, migratory monarchs might lay eggs at winter-breeding sites in the spring when they travel north from Mexico to reproduce in the Gulf coast states [60]. If migratory monarchs oviposit on milkweed previously visited by winter-breeding butterflies, their progeny would be exposed to the same parasite strains as winter-breeding

monarchs. A corollary to this scenario is that tropical milkweed patches that support winter-breeding monarchs are likely to create sources of infection that increase parasite prevalence across the entire migratory monarch range. Other host-parasite systems demonstrate this possibility. As one example, sedentary salmon reared in aquaculture enclosures can become infested with sea lice. Farmed salmon pens are often located along migratory routes where wild juvenile salmon enter the sea from their freshwater hatching grounds. As a result, wild juvenile salmon become infected with sea lice during a stage when they are highly vulnerable, decreasing wild salmon survival [61,62]. In a similar way, infection of migratory monarchs passing through the southern U.S. each spring could offset the effects of migration in lowering parasite prevalence during the fall. In support of this idea, *OE* prevalence has increased nearly three-fold in eastern migratory monarchs since 2002 [S. Altizer and J. de Roode, unpublished data]. Future work is needed to investigate the extent to which migratory and non-migratory monarchs transmit *OE* and share habitat.

Our study indicates that by planting exotic tropical milkweed in southern coastal areas, humans are providing a consistent resource that allows monarchs to forego long-distance migration, breed year-round, and suffer high parasite transmission. Such human-provided resources have altered pathogen transmission in other wildlife hosts by encouraging more sedentary behaviour and higher host aggregations around food sources. For example, supplemental feeding of elk during winter in the Greater Yellowstone Ecosystem increased host exposure to brucellosis and gastrointestinal nematodes [63,64]. Importantly, without considering infectious disease, winter-breeding monarchs could represent a reserve population to augment the numbers of eastern migratory monarchs in the face of steep declines. However, because these same winter-breeding monarchs support high parasite transmission, their potential role as a

source of infection for migratory monarchs during seasonal periods of mixing is cause for concern. The widespread declines of migratory monarchs in North America have been widely publicized [33], with most attention focused on habitat loss as a major cause [35,36]. Shifts towards year-round breeding on tropical milkweed resulting in high rates of *OE* infection could pose an additional emerging threat to the long-term viability of migratory monarchs.

Year-round breeding in the southern U.S. may be relatively new as a widespread phenomenon. While records of winter-breeding occurred anecdotally in earlier decades (i.e., 5 reports from 1939-1960, excluding south Florida; electronic supplementary material), winter-breeding appears to have become more common in recent years (95 reports from 2002-2010 [39]). Winter-breeding behaviours could also be enhanced by milder winters associated with global climate change. In the future, monarch activity along the Gulf and southern Atlantic coasts could increasingly resemble that of south Florida, where a long-established resident monarch population (not sampled in this study) experiences consistently high infection rates (>70%) and breeds year-round [43,46]. An online herbarium search shows a modest temporal increase in the frequency of records of tropical milkweed, but quantitative data are lacking (see electronic supplementary material). Recent commercial demand for milkweed has stimulated tropical milkweed sales, often to the exclusion of native milkweeds [65]. To reduce monarch winter-breeding and its associated disease risk, gardeners and land managers need wider access to native milkweeds (which naturally senesce in the fall), especially in coastal areas with mild winters.

In conclusion, we provide evidence that transitioning from migratory to non-migratory behaviours coupled with a shift to year-round breeding on introduced host plants dramatically increases the prevalence of a debilitating parasite for North American monarchs. Our results add

to a growing number of studies that show migratory species worldwide are shifting the timing and spatial patterns of movement in response to human activities, with consequences for disease transmission and virulence evolution. While sedentary behaviour could reduce parasitism for some animal populations, the loss of migratory behaviours will likely increase infection rates for many species. As more animal species are predicted to shift or reduce their migrations in the future, we expect pathogens that have been historically regulated by host migration to pose greater threats to wildlife and human health.

Ethics

Authorisation for interstate movement of monarchs obtained from USDA APHIS permit P525P-11-04112.

Data Accessibility

Data available in Dryad (doi:10.5061/dryad.s4dv0).

Authors' contributions

DS collected field data, processed and coordinated citizen science data, conducted the virulence experiment, ran statistical analyses, participated in the design of the study, and drafted the manuscript. JM contributed to the design of the experiment, suggested and interpreted statistical analyses, and revised the manuscript. SA conceived of the study and helped to design the experiment, coordinated citizen scientists, collected field data, assisted with statistical analyses, and offered important revisions and helped to draft the manuscript. All authors approved final submission.

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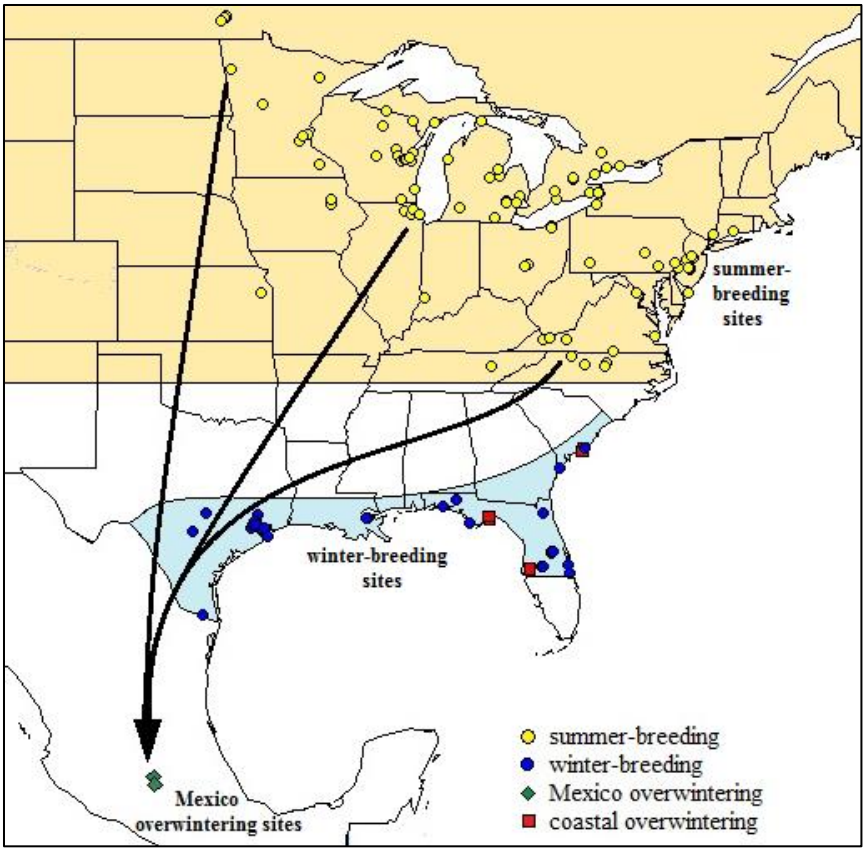

Figure 1. Sampling locations (symbols) and major fall migratory routes (arrows) in eastern North America. In fall, migratory monarchs travel from the summer-breeding range (extending from the southern U.S. into Canada) to overwintering sites in high-altitude fir forests in the transvolcanic mountains in central Mexico. In the spring, the same individuals fly north from Mexico into the southern U.S. [32], where they lay eggs on milkweed to produce the next generation. Symbols show sampling locations used to compare infection prevalence for summer-breeding (yellow circles) and Mexico overwintering sites (green diamonds) of migratory monarchs. Also shown are winter-breeding sites (blue circles) for non-migratory monarchs sampled in locations where tropical milkweed grows year-round, and coastal overwintering sites where adults but no breeding activity are observed (red squares). Sample sizes for each source and year are provided in electronic supplementary material.

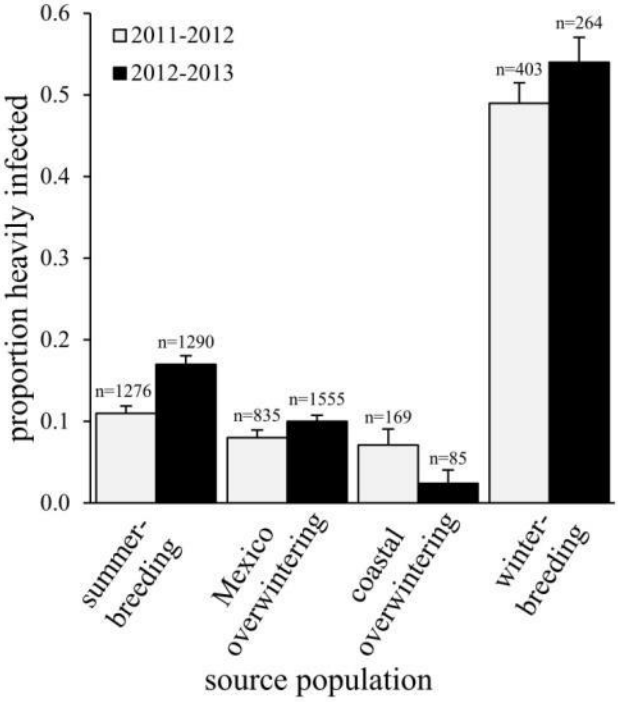

Figure 2. Proportions of monarchs heavily infected with *OE* parasites across sources and years of sampling. Comparison of means in the GLS model showed that prevalence was significantly higher among non-migratory monarchs sampled at winter-breeding sites in the southern coastal U.S. (50.8% infected on average) compared to migratory monarchs sampled at Mexico overwintering sites (9.3% infected; t_{19}= -5.08, p<0.00001) or coastal overwintering sites (5.5% infected; t_{21}=-5.03, p< 0.00001). Prevalence at winter-breeding sites was also higher than for migratory monarchs sampled at summer-breeding sites (14.1% infected; t_{70}= -4.36, p<0.0001). Proportions shown are averaged across all samples (regardless of sample size per site) across the entire source (site locations shown in figure 1). Error bars represent standard error.

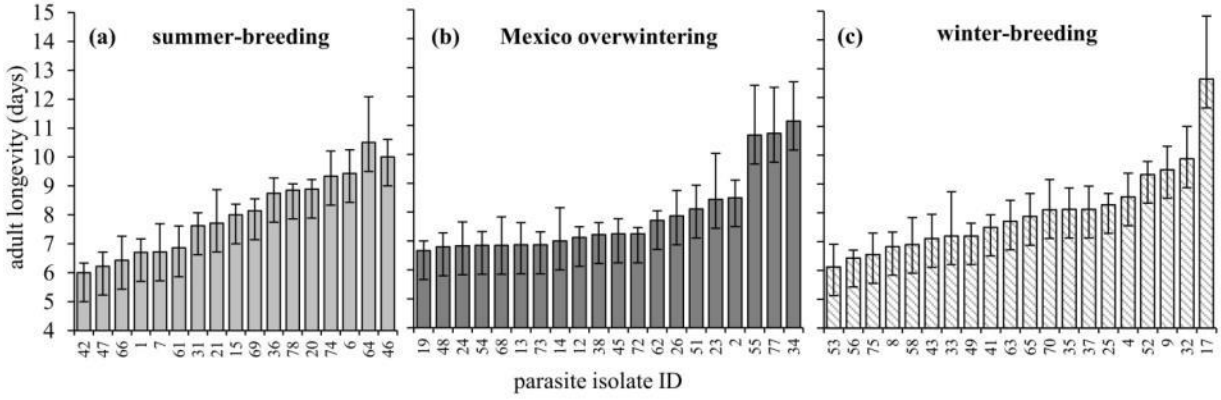

Figure 3. Monarch longevity in days for adults heavily infected with *OE*. Shorter longevity indicates higher parasite virulence. Averages for parasite clones are shown, representing three sources: (*a*) summer-breeding sites, 17 clones; (*b*) Mexico overwintering sites, 20 clones, and (*c*) winter-breeding sites, 20 clones. Origins of parasite isolates are noted in electronic supplementary material. Error bars represent standard error.

CHAPTER 3

MIGRATORY MONARCHS WINTERING IN CALIFORNIA EXPERIENCE LOW INFECTION RISK COMPARED TO MONARCHS BREEDING YEAR-ROUND ON NON-NATIVE MILKWEED[2]

[2]Satterfield, D.A. Villablanca, F.X., Maerz, J.C., and S. Altizer. 2016, *Integrative and Comparative Biology 56*(2): 343-362. Reprinted here with permission from publisher.

ABSTRACT

Long-distance migration can lower infection risk for animal populations by removing infected individuals during strenuous journeys, spatially separating susceptible age classes, or allowing migrants to periodically escape from contaminated habitats. Many seasonal migrations are changing due to human activities including climate change and habitat alteration. Moreover, for some migratory populations, sedentary behaviors are becoming more common as migrants abandon or shorten their journeys in response to supplemental feeding or warming temperatures. Exploring the consequences of reduced movement for host-parasite interactions is needed to predict future responses of animal pathogens to anthropogenic change. Monarch butterflies (*Danaus plexippus*) and their specialist protozoan parasite *Ophryocystis elektroscirrha* (*OE*) provide a model system for examining how long-distance migration affects infectious disease processes in a rapidly changing world. Annual monarch migration from eastern North America to Mexico is known to reduce protozoan infection prevalence, and more recent work suggests that monarchs that forego migration to breed year-round on non-native milkweeds in the southeastern and south central U.S. face extremely high risk of infection. Here, we examined the prevalence of *OE* infection from 2013-2016 in western North America, and compared monarchs exhibiting migratory behavior (overwintering annually along the California coast) with those that exhibit year-round breeding. Data from field collections and a joint citizen science program of *Monarch Health* and *Monarch Alert* showed that infection frequency was over 9 times higher for monarchs sampled in gardens with year-round milkweed as compared to migratory monarchs sampled at overwintering sites. Results here underscore the importance of animal migrations for lowering infection risk and motivate future studies of pathogen transmission in migratory species affected by environmental change.

Key words: *Danaus plexippus*, animal migration, host-pathogen dynamics**,** *Ophryocystis elektroscirrha,* sedentary population

INTRODUCTION

Migratory animals have intrigued humans for thousands of years and motivated scientific inquiry since Aristotle (Berthold 2001). More recently, scientists have begun to recognize that animal migrations can influence ecological processes in profound ways (Bauer and Hoye 2014). A question of particular interest is how seasonal migration affects the spread of infectious disease (Altizer et al. 2011). While birds and other migrants are capable of spreading pathogens vast distances and recent disease outbreaks highlight such instances (e.g., Koehler et al. 2008; Cohen et al. 2015), in numerous systems, long-distance migration actually has the opposite effect of reducing opportunities for pathogen transmission. Because the extent and frequency of many seasonal migrations are changing due to human activities (Wilcove and Wikelski 2008), a critical task is to examine the consequences of these changes for host-parasite interactions, in part to predict future risks for wildlife and human health.

Modeling and empirical work in caribou, butterflies, bats, and birds shows that migration can lower disease risk through at least three mechanisms (Hall et al. 2014; reviewed in Altizer et al. 2011). Strenuous long-distance migrations can remove infected individuals, a process termed 'migratory culling' (Bradley and Altizer 2005; Altizer et al. 2011). Migration can allow animals to leave behind habitats where parasites otherwise accumulate over time, a process called 'migratory escape'(Loehle 1995; Folstad et al. 1991). Further, migratory life cycles can spatially and temporally separate susceptible juvenile age classes from infected adults, a mechanism referred to as 'migratory allopatry' (Krkošek et al. 2007). Through these processes, migration

has been linked to lower pathogen occurrence in galaxiid fish, Arctic charr, Pacific salmon, caribou, bats and other species (Poulin et al. 2012; Bouillon and Curtis 1987; Krkošek et al. 2007; Folstad et al. 1991; Akbar et al. 2012).

Anthropogenic change is affecting many migratory species (Wilcove and Wikelski 2008). Habitat loss, barriers to movement, and climate change have altered the timing, frequency, and routes for many animal migrations (Visser et al. 2009; Both et al. 2006; Sutherland 1998). In particular, warmer temperatures and supplemental feeding have prompted shifts from mobile to more sedentary behaviors. For instance, Great Crested Grebes in the Netherlands have become increasingly sedentary since 1980 (Adriaensen et al. 1993), and European Blackbirds have established resident populations in urban areas of Latvia and Estonia in recent decades (Evans et al. 2012). White Storks, Common Cranes, Red Kites, grey-headed flying foxes and numerous other species show similar changes towards non-migratory behaviors (Sutherland 1998; Van Der Ree et al. 2006; Fiedler 2003). Milder winters in particular are predicted to reduce migratory behavior for species that are already partially migratory (i.e., in which some individuals act as migrants and some as residents, as observed in many birds; Berthold 1999; 2003).

Monarch butterflies (*Danaus plexippus*) and their specialist protozoan parasite *Ophryocystis elektroscirrha* (hereafter *OE*) provide a model system for examining how long-distance migration affects infectious disease processes in a rapidly changing world (Bartel et al. 2011). Across wild monarch populations, which are globally distributed, infection prevalence decreases with greater migratory propensity (Altizer and de Roode 2015; Altizer et al. 2000). Monarch migration from the eastern U.S. and Canada to central Mexico has been demonstrated to annually reduce protozoan infection prevalence through migratory escape and migratory culling (Bartel et al. 2011). Recent work focused on the eastern U.S. showed that monarchs

breeding year-round on exotic milkweed along the Gulf and southern Atlantic coasts face significantly higher risk of protozoan infection compared to migrants that breed at more northern latitudes and migrate to Mexico (Satterfield et al. 2015). Monarchs also migrate annually in western North America, traveling from spring- and summer-breeding areas west of the Rocky Mountains to wintering sites along a 1000-km stretch of the California coast (Stevens and Frey 2010; Nagano et al. 1993). Here, we examine western monarchs to ask whether similar increases in infection prevalence have occurred in response to year-round breeding on human-propagated tropical milkweed in southern California. Monarchs typically migrate from northern or inland areas of the western U.S. (long-distance migrants) or from coastal southern areas (short-distance migrants; Yang et al. 2015; Dingle et al. 2005) to overwintering locations, where the butterflies roost in trees for several months and postpone reproduction until the spring (Frey and Schaffner 2004; CNDDB 2002).While most western monarchs follow this annual cycle, observers have previously noted a year-round breeding population of monarchs (thought to be mostly non-migratory) in and around the Los Angeles basin.

It is not well understood when the year-round monarch population became established in southern California, or to what degree the population is comprised of residents versus short-distance migrants. Williams (1958) noted that monarchs were overwintering but "certainly not breeding" during the winter in southern California. Urquhart (1960) reported a single record of monarch larvae and pupae in Santa Monica, CA (in the L.A Basin) in winter 1957-1958. Ten years later, Urquhart (1970) referred to a resident population in southern California where monarchs "breed throughout the entire year." Urquhart postulated, based on laboratory and field studies, that in conditions when temperatures remain relatively stable, "breeding becomes continuous, assuming the presence of the host plant" (Urquhart et al. 1970). More recently,

between 2010-2015, citizen scientists for the program Journey North reported 31 winter-breeding records (i.e., larvae, eggs, or pupae in Jan-Feb) in southern California (Journey North 2016). These winter-breeding behaviors appear to be similar to those described for monarchs in the southeastern U.S. (Howard et al. 2010). Year-round breeding typically occurs on a popular garden plant, tropical milkweed (*Asclepias curassavica*), which is not native to the U.S. and grows year-round in warm locations (unlike most native milkweeds), thus allowing monarchs to breed throughout the winter (Batalden and Oberhauser 2016) and ultimately providing the condition that Urquhart et al. (1970) proposed would lead to continuous breeding.

We collaborated with citizen scientists in California to investigate whether year-round breeding behavior alters infectious disease risk for monarchs compared to migratory behavior. Over two years (Dec 2013-Feb 2016, including three winters) in coastal California, we measured *OE* infection prevalence (1) during the winter in roosting clusters at overwintering locations, where monarchs are migratory and postpone reproduction during the winter, (2) across seasons at year-round breeding sites, where monarchs (hereafter, "residents") breed during the winter (defined as Dec-Feb), and (3) during the fall or spring at seasonal breeding sites, where monarchs breed during the traditional breeding season but not during winter. We predicted that monarchs from year-round breeding sites would face higher risk of parasite infection because the loss of the migration eliminates the benefits of migratory escape and migratory culling that reduce disease levels. We also investigated how infection prevalence varied by season and whether milkweed diversity and abundance predicted prevalence within year-round breeding sites.

MATERIALS AND METHODS

(a) Biology of the study system

Monarchs infected as larvae with the specialist protozoan *OE* emerge as adult butterflies covered with millions of dormant parasite spores on the outside of their bodies (McLaughlin and Myers 1970). Transmission occurs in the breeding grounds, when infected adults scatter parasite spores onto eggs or milkweed, and larvae then ingest spores when feeding (Altizer et al. 2004). Adults can passively transfer spores to other adults, but new infections only occur when larvae consume parasites. Mild or moderate infections shorten adult life span or weaken flight performance; the most severe infections can be fatal, causing butterflies to get stuck in the chrysalis or exhibit wing deformities (de Roode et al. 2007; Bradley and Altizer 2005; Altizer and Oberhauser 1999). Infections occur in all monarch populations examined to date around the globe, and populations with greater migratory propensity tend to have lower infection prevalence (Altizer et al. 2000; Altizer and de Roode 2015).

(b) Measuring prevalence in migratory and resident monarchs

We used a combination of field sampling and citizen science data to measure parasite infection in wild monarchs from Dec 2013 to Feb 2016 (spanning three winters) in California at year-round breeding sites, overwintering sites, and seasonal breeding sites (Figure 1). Specifically for this project, in 2013 we created the *Western Monarch Initiative*, a joint citizen science program of *Monarch Health* (University of Georgia) and *Monarch Alert* (California Polytechnic State University), to quantify infection prevalence at 30 year-round breeding sites (N=1109 monarchs sampled by 33 volunteers) and at 4 seasonal breeding sites (N=39 samples from 3 volunteers) in California. Our lab team sampled monarchs at 12 additional year-round breeding sites (N=181 samples) and 3 additional seasonal breeding sites (N=19 samples). Parasite samples from

overwintering migratory monarchs at 8 sites in coastal California (N=2135 samples) were obtained in collaboration with a team of researchers and volunteers, including Jessica Griffiths, Alexa Fritzsche McKay, Wendy Caldwell, Kelly Nail, Justin Wright, Mallory Claassen, Danielle Patterson, Allison Watson, and Ann Wasser.

We tested monarchs for *OE* infection non-destructively by pressing transparent tape ($1.27cm^2$) against each adult monarch's abdomen and viewing samples at 63X magnification (as described in Altizer et al. 2000). Wild adult monarchs were obtained by either net-capturing butterflies or rearing wild larvae until adulthood. Wild larvae were fed unwashed milkweed stalks collected from outdoors and typically from the same site, to expose larvae to naturally occurring parasites in their natal environment. Citizen scientists through the *Western Monarch Initiative* collected samples using the same method and mailed these to our laboratory to be scored for the presence/absence of infection. Following Bartel et al. (2011), we scored samples with >100 spores as heavily infected, indicating an acute infection acquired as a larva; in contrast, samples with <100 spores can result from the passive spore transfer between adult monarchs (Altizer et al. 2004; de Roode et al. 2009)**.** Data for each sample included date, sex, location, and monarch collection stage (adult or larva/egg reared to adulthood). We shared results with volunteers via email. Detailed protocols for parasite testing in the citizen science program are described in Bartel et al. (2011) and at www.monarchparasites.org. Volunteers also responded to a site survey providing information about monarch activity throughout the year, milkweed species identity and abundance, outward signs of *OE* disease (such as adult wing deformity), garden age, and other characteristics of their sampling location. We classified each monarch breeding location as either a year-round breeding site (including monarch breeding during the winter, as shown by the presence of larvae, pupae, or eggs in Dec.-Feb.) or a seasonal

breeding sites (without winter-breeding). These were confirmed with volunteers through survey responses, email correspondence, or volunteer-submitted data.

(c) Data analysis

We tested for differences in infection across source population (year-round-breeding compared to overwintering sites) in R v.3.2.3 (R Core Team 2016). We did not include seasonal-breeding monarchs in this analysis because of a limited sample size. We examined predictors of individual monarch infection status (infected/uninfected) using a generalized linear mixed model (GLMM) with a binomial error distribution and logit link in package *lme4* (Bates et al. 2015). Factors included source population, year, monarch sex, and a source-by-year interaction. Site was a random effect nested within source population. The analysis and all sample sizes reported here excluded 209 samples with incomplete data.

We also examined predictors of infection status within the subset of monarchs sampled at year-round breeding sites (n=1290 monarchs in total). With infection status as a response variable, we used a GLMM with binomial error structure to test whether season (winter, spring, summer, or fall) and monarch stage at collection (larva/egg or adult) predicted infection. We included site as a random variable. Finally, to investigate whether garden characteristics influenced infection prevalence, we used a GLMM on infection status for the subset of monarchs from year-round breeding locations for which we had full site information (n=1089 monarchs from 34 sites). Fixed factors included milkweed diversity (treated as an ordinal variable with two categories: 1-3 milkweed species, or 4 or more species) and milkweed abundance (treated as an ordinal variable with three categories: 1-30 plants, 31-60 plants, or >60 plants). Site was treated as a random factor. Tukey contrasts in package *multcomp* were used to examine significant

factors (Hothorn et al. 2008). In all models, non-significant terms were backwards-eliminated and model fit was evaluated based on AIC.

RESULTS

Infection prevalence was approximately 9 times higher among monarchs sampled in year-round breeding gardens (n=1290) compared to migratory monarchs sampled at overwintering sites (n=2135) across years (2013-2016; Figure 2). Analysis showed significant effects of source (overwintering or year-round breeding; χ^2=24.80, df=1, p<<0.0001), with year-round breeding monarchs significantly more likely to be infected (74% infection prevalence) than monarchs at overwintering sites (8% prevalence). Infection prevalence varied by year (χ^2=9.32, df=2, p=0.009), depending on source (source-year interaction: χ^2=5.99, df=2, p=0.05). Year-round breeding monarchs experienced lower infection prevalence during the third winter of the study (2015-2016, in which sample sizes were limited), whereas monarchs at overwintering sites experienced significantly higher infection that same year compared to the first year. Overwintering monarchs were sampled at more southern locations, including two sites in the Los Angeles area, during the third winter (2015-2016). Monarch sex was not a significant predictor of infection status (χ^2=2.79, df=1, p=0.095, NS). Among the monarchs sampled at seasonal breeding sites (n=58 butterflies), prevalence was 52%.

To understand predictors of infection among the subset of year-round breeding monarchs, we examined the influence of season, collection stage, and milkweed variables on infection status. A GLMM indicated that infection varied by season (χ^2=35.76, df=3, p<<0.0001), with monarchs sampled during the spring experiencing significantly lower infection prevalence

compared to monarchs from other seasons (Figure 3). Monarch collection stage was not significant (χ^2=0.12, df=1, p=0.73, NS).

Citizen scientists returned survey responses for 29 out of 34 volunteer-monitored sites. Volunteers noted that most milkweed gardens were relatively young, with 18 gardens planted in the past five years and 11 planted more than five years ago. Citizen scientists also indicated changes in their gardens in the previous three years: 11 sites acquired more native milkweed species, 7 acquired more individual milkweed plants, and one had fewer milkweed plants; 6 reported no change. For 18 sites, volunteers reported observing outward signs of *OE* disease in monarchs (other than diagnostic results from our laboratory). Specifically, through site surveys (n=9 volunteers) or notes recorded on datasheets (n=9 volunteers), citizen scientists reported seeing crumpled or deformed adults, monarchs remaining stuck in the chrysalis, or pupae with dark spots. These are commonly observed signs of OE disease, although other causes are possible. Five volunteers reported observing spores under a microscope at their home.

Field visits and site survey responses from volunteers indicated that 38 of 40 year-round breeding locations had tropical milkweed (*A. curassavica*). The remaining two sites had only native milkweeds (*A. fascicularis* and *A. eriocarpa*), which supported monarch larvae in early winter before dying back in late winter (e.g., in January); these sites thus fall under our definition of year-round breeding (i.e., larvae occur in Dec.-Feb.), although breeding was not continuous. There were two additional year-round breeding sites (for a total of 42) for which volunteers did not provide milkweed information. Interestingly, three citizen scientists indicated on site surveys that they cut back the tropical milkweed at their year-round breeding garden during the fall or winter. Cutting back milkweed during the winter has been suggested (by the authors in Satterfield et al. 2015 and others) as a potential management strategy – which still requires

formal hypothesis testing – to remove accumulated parasites from exotic milkweed and reduce *OE* disease by discouraging monarch winter-breeding behaviors in the southern U.S. At the three locations reporting cut milkweed (n=51 monarchs), *OE* prevalence was 73%; this was similar to prevalence at sites where milkweed was not cut (72%). Many volunteer gardens had other species of milkweed as well, commonly *A. fascicularis* or *A. speciosa*. Our analysis of year-round breeding sites indicated no significant effect of milkweed diversity (χ^2=0.01, df=1, p=0.93, NS) or milkweed abundance (χ^2=1.27, df=2, p=0.53, NS) on infection.

Among the 7 sites with seasonal monarch breeding, five sites contained native species of milkweed including *A. eriocarpa*, *A. fascicularis*, and *A. speciosa*. Prevalence among monarchs from seasonal breeding sites with native milkweed (n=40) was 35%. Several of these sites occurred in close proximity to year-round breeding locations in southern California (Figure 1).

DISCUSSION

Infection risk was extremely high for monarchs in southern California gardens with year-round breeding. The large majority of monarchs at these locations were heavily infected with *Ophryocystis elektroscirrha*. Volunteers at these locations reported monarch wing deformities or death during eclosion, both commonly associated with OE infection. In contrast, migratory monarchs at overwintering sites experienced significantly lower infection risk. These results support earlier findings that the loss of migratory behaviors and continuous breeding that occurs at locations with winter milkweed enhance parasite transmission (Satterfield et al. 2015; Altizer et al. 2000). Site surveys indicated that tropical milkweed (*A. curassavica*) was present at all but two year-round breeding sites for which milkweed data were available. Thus, tropical milkweed enabled winter-breeding by providing food for monarch larvae during the southern California

winter. Infection prevalence at these locations remained high throughout the year but did vary by season, with prevalence showing a modest decline during the spring.

It is important to note that most (but not all) sites with resident monarchs monitored here occurred at more southern locations compared to overwintering or seasonal breeding sites. The warmer winters at these low-altitude and low-latitude sites likely facilitated the year-round persistence of tropical milkweed. It is possible that a different factor that co-varies with latitude (aside from year-round milkweed availability) caused the high infection prevalence, although we are aware of no plausible factor that would generate such a strong and positive relationship.

Findings here are consistent with previous evidence that year-round breeding increases disease risk for monarchs. The infection patterns observed in this study – with high infection prevalence among non-migratory monarchs and low prevalence among migratory monarchs – appears strikingly similar to a recent study of winter-breeding monarchs in the south eastern and south central U.S., despite differences in climate, geography, and migratory routes between eastern and western monarchs (Satterfield et al. 2015). Likewise, resident monarchs breeding year-round in south Florida, where non-migratory monarchs have long been established, show high *OE* prevalence maintained over many years (Altizer et al. 2000).

High infection prevalence could occur through several processes. Whereas migratory monarchs in eastern North American periodically escape from infectious parasite stages in the environment as they depart breeding grounds at the end of summer (when infection prevalence is highest; Bartel et al. 2011), non-migratory monarchs experience continual exposure to parasites that accumulate on year-round milkweed. Further, whereas migration removes infected butterflies and reduces infection prevalence (as observed among eastern monarchs; Bartel et al. 2011; Altizer et al. 2015), year-round breeding could increase infected monarchs' opportunities

for reproduction – and thus, for parasite transmission. Migratory monarchs in the western U.S. likely benefit from migratory escape and migratory culling in a similar way as eastern monarchs, albeit migratory culling could be less prominent because the western migration is thousands of kilometers shorter (Altizer et al. 2000). Additionally, some western migrants are known to travel particularly short distances, on the order of hundreds of miles, from southern coastal natal areas to overwintering sites (Yang et al. 2015). Finally, high local monarch densities at year-round breeding sites could enhance transmission. For instance, we observed an average of 3.8 larvae and/or eggs per *A. curassavica* plant at four year-round breeding sites in Dec 2013; in contrast, citizen scientists for the Monarch Larva Monitoring Project reported <1 larva/egg per plant at four seasonal breeding locations in California in 2014 (Monarch Larva Monitoring Project, MLMP). High larval densities in combination with parasite accumulation on year-round plants and the loss of migratory culling likely increase host-parasite contact rates as well as the transmission of infectious spores. Additionally, larval overcrowding could introduce starvation risk, with implications for pathogen susceptibility (Lindsey et al. 2009; Fritzsche McKay et al., in revision). Despite strong differences in infection risk, the relative fitness of resident versus migratory monarchs is not known and should be explored.

Among year-round breeding sites, infection prevalence remained high throughout the year but was significantly lower during the spring. Three potential processes, none of which have been tested, could explain this pattern. First, milkweed growth accelerates and produces new, parasite-free leaves in the spring. This could lower infection risk temporarily for monarch larvae consuming new growth. Second, abnormally high larval densities resulting in complete defoliation of milkweed plants at year-round breeding sites may account both for the high prevalence in winter (as spores are consumed) and subsequently low prevalence in spring (as

defoliation prompts new growth). Third, an influx of migratory monarchs – most of which are free of parasites – could decrease infection prevalence if migrants provide a pulse of uninfected eggs at year-round breeding sites in the spring. However, if migrants' offspring are exposed to parasites in these habitats, this raises the possibility that year-round breeding sites could act as sources of infection for the larger migratory population. Future research could investigate the degree to which migrants use year-round breeding sites and incur infection risk for offspring.

Contrary to our expectations, milkweed abundance did not influence infection prevalence at breeding sites. We had expected that gardens with larger milkweed patches would show lower infection rates, if greater milkweed availability reduces the density of parasites and host larvae per plant (on average) and thus makes host-parasite contact less likely. It is possible that larger gardens attract more adult monarchs (and thus parasite deposition) than gardens with fewer milkweeds, such that host-parasite contact rates are similar on average, irrespective of garden size. Further, monarch preference for *A. curassavica* could influence infection dynamics and potentially obscure other garden effects. In some experiments, monarchs prefer to oviposit on *A. curassavica* over certain other milkweed species (Malcolm and Brower 1986), and monarchs infected with *OE* in particular prefer to lay eggs on *A. curassavica,* to offer some chemical protection to their offspring exposed to parasites (Lefèvre et al. 2010). Anti-parasitic properties in the toxic cardenolides of *A. curassavica* can reduce parasite load and increase lifespan of infected monarchs (Sternberg et al. 2012). As such, despite monarchs' potential for trans-generational medication, gardens in southern California with *A. curassavica* may particularly attract infected monarch butterflies and prolong infected monarch lifespan, allowing for greater opportunities for transmission. It is not clear whether self-medication is expressed at the year-

round breeding locations, however, the exceedingly high infection probability suggests that any protection from this behavior, while plausible, is limited.

Our study indicates that tropical milkweed (*A. curassavica*) was the primary milkweed enabling winter-breeding behavior in southern California. This was similar to a study in the southeastern and south central U.S. (Satterfield et al. 2015), in which all reported winter-breeding activity occurred exclusively on *A. curassavica* (Satterfield, unpublished data). Thus, in both the southern and western U.S., the year-round presence of tropical milkweed is associated with high levels of protozoan disease. Here, all but two sites with year-round breeding monarchs contained *A. curassavica*. While some milkweed species native to California have been observed growing into January during mild winters (Villablanca, pers. obs.), most native milkweeds senesce (even if this occurs in late winter) before spring re-growth. In effect, winter-breeding did not likely become a widespread phenomenon in the Los Angeles basin until tropical milkweed was planted. It is not known when tropical milkweed began to support this year-round monarch population. Previous work in the eastern U.S., based on limited historical data, suggests that resident monarchs persisting on exotic milkweeds likely became more common in the last few decades (Satterfield et al. 2015). Many nurseries in southern California now exclusively sell *A. curassavica*. In northern or inland locations with colder winters (e.g., at one of our seasonal breeding locations), *A. curassavica* is reported to die back in response to winter freezes and does not support year-round breeding.

Recently, efforts to reduce winter-breeding behaviors and *OE* disease among monarchs in southern coastal areas have included planting native milkweed or cutting back tropical milkweed to limit its availability during the winter. In our study in California, infection was still common among monarchs at five seasonal breeding sites with only native milkweed (35% infection

prevalence), although infection risk was lower than for monarchs at year-round sites. In addition, infection remained extremely high at three breeding sites at which tropical milkweed was cut back; at these three locations, prevalence appeared similar to sites with un-cut tropical milkweed. Further work should be conducted to confirm this finding. However, this early result raises a concern regarding habitat management. In particular, it is possible that monarchs in gardens with native milkweed or managed tropical milkweed (cut back) that live within the vicinity of uncontrolled, year-round breeding sites could still experience high infection risk as a result of pathogen spread from neighboring locations. Infected monarchs that originate from year-round milkweed sites could readily transport parasites to nearby gardens with native or seasonal milkweeds. If this is occurring, management options that are adopted on a regional scale could be more effective in reducing monarch disease levels. For example, a community of gardeners that implements a strategy to make milkweed unavailable during the winter (e.g., cutting back on a certain date) may be able to reduce the spatial spread of parasites more so than a single gardener. Indeed, the control of many infectious diseases of humans and animals require coordinated, large-scale efforts. This approach has not been investigated for the monarch-protozoan system.

Collectively, our work suggests that milkweeds that are seasonal (rather than perennial) will better support monarch health. Efforts to plant native milkweed species that tend to not grow perennially (above ground) and thus are more closely synchronized with the monarch migratory cycle might reduce infection levels in southern California. Alternatively, *A. curassavica* could be managed on a regional scale such that it mirrors the phenology of native plants. Specifically, making *A. curassavica* regionally unavailable to monarchs during the winter by cutting back or covering plants could correct for the life history differences between exotic and native

milkweeds and could potentially support healthier monarchs during the traditional breeding season in California (March-November). While currently untested, regional management could be important; without coordinated efforts, monarchs from unmanaged sites may quickly spread parasites to managed sites. These strategies – planting native milkweeds or removing tropical milkweed growth during the winter, and doing so on a regional scale – could terminate parasite accumulation and lower the probability of transmission and reintroduction, thus reducing infectious disease burdens on California monarchs.

Populations of birds, bats, mammals, and fish are now shifting their migratory behaviors in response to human-caused changes to habitats or migratory routes. Partially migratory populations (i.e., those that include migrant and resident individuals) may be more likely to undergo shifts towards non-migratory behaviors (Berthold 2003; 1999). By this definition, monarch butterflies in North America now exhibit partial migration similar to many bird populations in temperate regions. Partial migration and the ability to switch between resident and migratory strategies can be advantageous in allowing animals to respond to changing environmental conditions (Fiedler 2003; Gilroy et al. 2016); however, changes in migratory behaviors are not always adaptive (e.g., Sutherland 1998). In the case of monarchs breeding on year-round *A. curassavica*, shifts towards sedentary behaviors are associated with significantly higher parasitism.

Our work adds to a growing body of literature suggesting that changes in movement behaviors can alter infectious disease dynamics for wildlife (Altizer et al. 2011; Bartel et al. 2011). Scientists increasingly recognize the complexity of the relationship between infectious disease and animal migration. For monarch-*OE* interactions, the role of migration in reducing disease risk is well demonstrated. A fuller understanding of the range of disease consequences

resulting from alterations in animal movement is urgently needed to inform management options that preserve migration or mitigate infection risk for wild populations. As our work underscores, citizen science approaches that allow for monitoring animals with large geographic ranges could be critical to these efforts.

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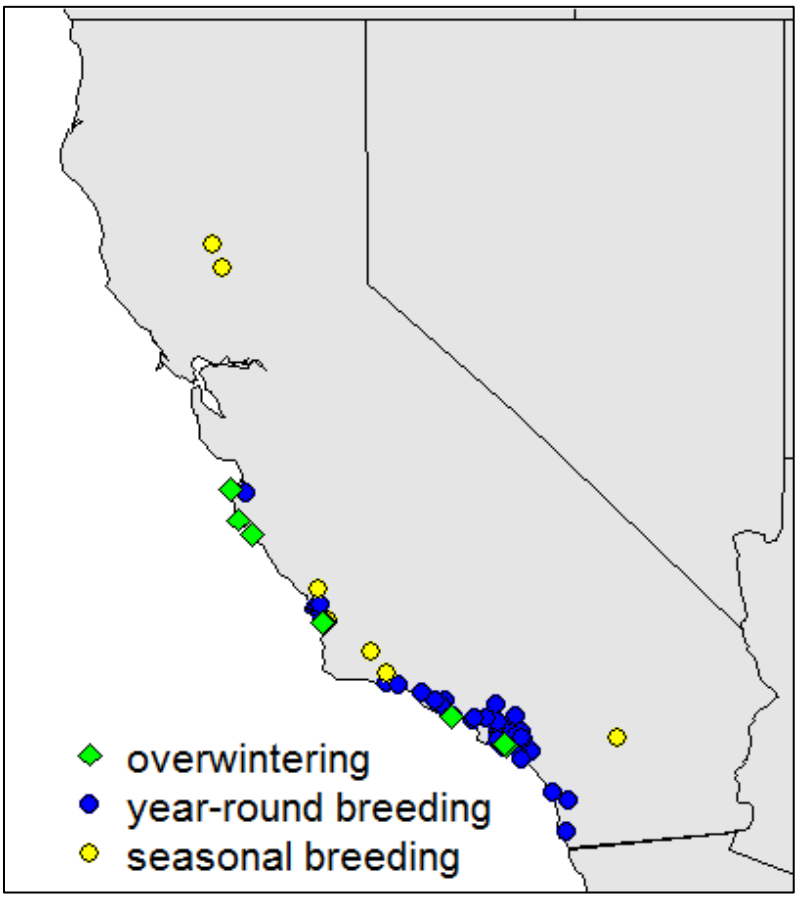

Figure 1. Sampling locations in California. Symbols show sampling locations used to compare infection prevalence in monarchs at 42 year-round breeding sites (blue circles), 7 seasonal breeding sites (yellow circles), and 8 overwintering sites (green diamonds). Overwintering sites by name in order from most northern to most southern locations are: Pacific Grove (Pacific Grove, CA; n=520); Andrew Molera State Park (Big Sur, CA; n=265); Esalen (Big Sur, CA; n=380); Pismo State Beach (Pismo Beach, CA; n=67); Halcyon Hill (Halcyon, CA; n=668); Ramirez Canyon (Malibu, CA; n=81); Norma Gibbs Park (Huntington Beach, CA; n=48); and Huntington Beach Central Park (Huntington Beach, CA; n=106).

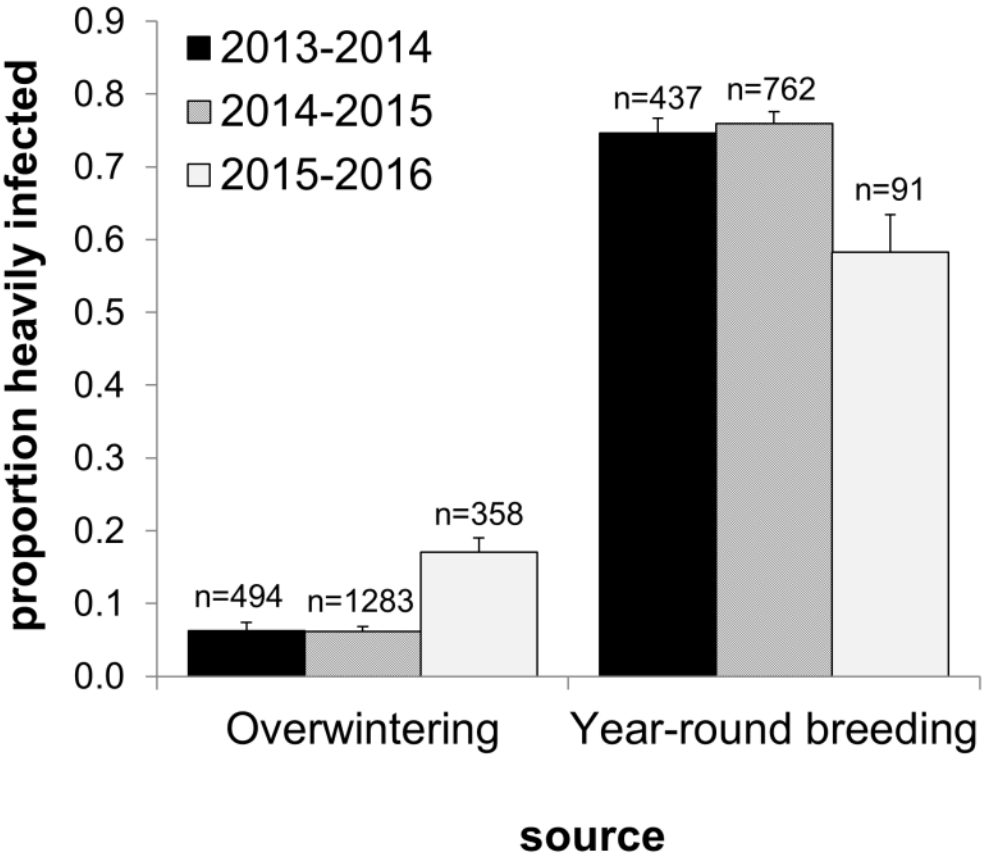

Figure 2. Proportion of monarchs heavily infected with *OE* parasites at overwintering sites compared to year-round breeding sites across years: Dec. 2013-November 2014, Dec. 2014-November 2015, and Dec. 2015-Feb. 2016. Note that samples from 2015-2016 include only winter data. Error bars represent standard error.

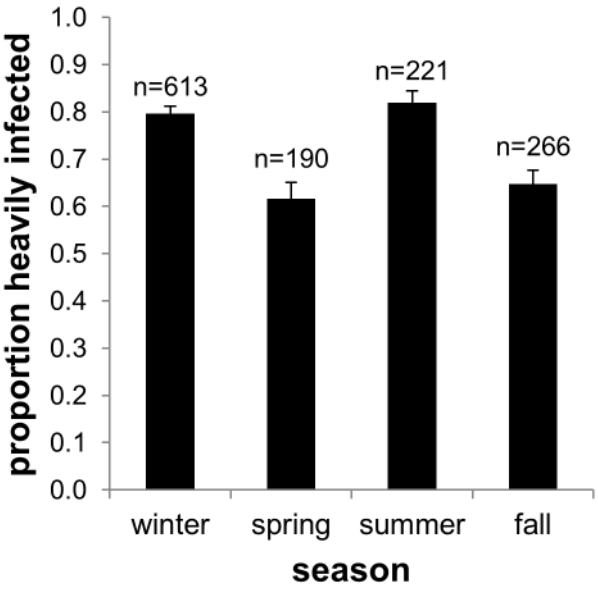

Figure 3. Proportion of monarchs heavily infected with *OE* parasites at year-round breeding sites by season. Spring monarchs experienced significantly lower infection (62% ± 4% SEM) compared to winter monarchs (80 ± 2%; $p<0.001$) and summer monarchs (82 ± 3% ; $p<0.001$). Error bars represent standard error.

CHAPTER 4

ENVIRONMENTAL PERSISTENCE INFLUENCES INFECTION DYNAMICS FOR A BUTTERFLY PATHOGEN[3]

[3] Satterfield, D.A., Altizer, S., Williams, M.K., and R.J. Hall. Submitted to *PLOS ONE*, 05/31/16.

ABSTRACT

Many pathogens, including those infecting insects, are transmitted via infectious stages shed into the environment, where they must persist until encountering a susceptible host. Understanding how abiotic conditions influence environmental persistence, and how these factors influence pathogen spread, are crucial for predicting patterns of infection risk. Here, we explored the consequences of environmental transmission for infection dynamics of a debilitating protozoan (*Ophryocystis elektroscirrha*) that infects monarch butterflies (*Danaus plexippus*). We first conducted an experiment to observe the persistence of protozoan spores exposed to natural conditions. Experimental results showed that, contrary to our expectations, pathogen doses remained highly infectious even after 16 days in the environment, although pathogens did cause less severe infections after environmental exposure. Because pathogen longevity exceeded the time span of our experiment, we developed a model to better explore environmental persistence for this host-pathogen system. The local transmission model indicated that typical spore doses shed onto milkweed plants must remain viable for at least 3 weeks for prevalence to increase appreciably during the summer-breeding season, and to match levels of infection commonly reported from the wild. Longer spore persistence led to higher prevalence and slightly smaller monarch population sizes. Our findings showed that transmission stages of this butterfly pathogen are long-lived and indicated that this is a necessary condition for the protozoan to persist in local monarch populations.

Key words: monarch butterfly, *Danaus plexippus*, spore longevity, environmental transmission, host-pathogen dynamics**,** *Ophryocystis elektroscirrha*

INTRODUCTION

Many pathogens achieve transmission by being shed into the environment and persisting long enough to encounter and infect a new host. Scientists have long recognized environmental transmission, particularly among insect pathogens, with Louis Pasteur being among the early investigators to describe this strategy when a *Nosema* outbreak devastated the French silkworm industry in the 1860s [1]. Environmental transmission is now known to be common among diverse insect pathogens, including viruses, fungi, protozoa and nematodes [2]. Mathematical models have explored the influence of environmental transmission on invertebrate host and pathogen population dynamics and stability [3, 4, 5]. Despite these advances and the pervasiveness of this transmission mode, the ecological implications of environmental transmission are less well understood compared to direct transmission between hosts.

The window of opportunity for pathogen transmission depends crucially on the longevity of infectious stages in the environment, thus pathogen persistence outside the host – which can range from days to decades (e.g., [6, 7]) – should strongly influence infection dynamics. In support of this, a model of nucleopolyhedrovirus (NPV) infection in gypsy moths showed that viruses with reduced longevity caused less severe infections and shorter epidemics [8]. Numerous pathogens used for biological control of insects are environmentally transmitted, with some of the most effective having long persistence times [9]. Using genetic engineering or protective adjuvants to lengthen environmental persistence has improved the efficacy of some biological control agents, such as granulosis virus in codling moths [2]. Pathogen persistence can also affect transmission among beneficial insects; recent work on pollinator pathogens showed that even parasites with limited environmental longevity (surviving <3 hours) deposited onto flowers can lead to new infections in foraging bees [10]. As these studies highlight,

understanding the extent and effect of pathogen persistence in the environment can help evaluate control strategies for pest insects and predict consequences of infections for beneficial insects.

Monarch butterflies (*Danaus plexippus*) and their protozoan pathogen *Ophryocystis elektroscirrha* (*OE*) provide a well described system that is useful for examining the constraints and consequences of environmental transmission. This specialist pathogen can cause debilitating disease in wild monarchs [11]. Infections develop internally in monarch larvae and pupae, and adult monarchs emerge covered with millions of dormant spores on their exterior [11]. Transmission occurs when infected butterflies shed infectious spores onto eggs or milkweed leaves and spores are consumed by monarch larvae [12]. Infection can shorten monarch lifespan, diminish flight performance, or cause death during eclosion [13, 14, 15]. Previous work suggests that *OE* infection removes some monarchs from the population during the butterflies' long-distance migration from eastern North America [16]. This protozoan has been detected in all monarch populations examined to date, and prevalence varies by location, season, and year [17].

Monarchs in eastern North America migrate over 2500 km each fall to overwintering sites in central Mexico [18,19]. Migratory monarchs remain non-reproductive until the spring, when they return to the their breeding range to lay eggs on newly emerging milkweed plants [20]. During April-September, monarchs undergo 3 to 4 successive breeding generations that expand the monarchs' range into the northern U.S. and southern Canada [21, 22]. The transmission of *OE*, which depends on larvae ingesting spores, primarily occurs during this breeding season. Infection prevalence increases as the breeding season progresses and peaks just before the fall migration [16]. This pattern suggests that parasite spores accumulate on milkweed host plants over time, and if these spores remain viable, larvae born late in the season face a higher risk of infection than earlier cohorts.

OE spores have a thick, amber-colored wall that appears to offer some protection from environmental stress [11]. Previous work indicates that *OE* spores remain infectious for several months under laboratory conditions [S. Altizer, personal observations]; however, spores shed onto milkweed could be damaged during exposure to high summer temperatures and UV light or could be displaced by wind or rain, likely reducing their longevity. Quantifying environmental persistence would allow researchers to better predict variation in infection risk across the monarch's annual migratory cycle. This knowledge could be useful for evaluating the population impacts of a pervasive pathogen that often removes monarchs from the migratory population.

Here we designed a study to measure *OE* spore longevity under field conditions and investigate its effect on infection patterns. First, we experimentally tested spore decay in a natural setting. We deposited infectious parasite doses onto milkweed plants, exposed the plants to two outdoor environmental treatments (sun vs. shade), and measured pathogen infectivity over time. Counter to our expectations, spores showed no significant loss of infectivity over the two-week span of the experiment. This limited our ability to quantify spore decay but did demonstrate that parasite doses exposed to the environment caused less severe infections over time – thus providing evidence that some spores within an infectious dose are lost or killed in the environment. Second, we developed a mathematical model to better understand the role of parasite longevity in pathogen dynamics. While the experiment was not sufficiently long to provide pathogen decay rate as a model parameter, we used our model to investigate how pathogen persistence time affects infection prevalence within a milkweed patch during a typical summer-breeding season. We also examined the effect of spore deposition rate on infection dynamics. Our modeling work indicated that environmental persistence approaching 3 weeks was necessary for pathogens to persist in the monarch population through the breeding season

and for prevalence to reach levels commonly observed in the wild. Our study provides the first test of environmental persistence for this naturally occurring protozoan pathogen and introduces a simple model to better understand key drivers of infection in this butterfly-pathogen interaction.

MATERIALS AND METHODS

(a) Environmental persistence experiment

We experimentally tested the longevity of *OE* spore doses following exposure to two environmental treatments over 16 days. Prior lab findings demonstrated that *OE* spores are sensitive to ultraviolet radiation and heat [S. Altizer, unpublished data]. Wind and rain could also remove spores and further reduce the probability of infection over time. Thus, we predicted that both the infectivity of spores (ability to cause an infection upon exposure to larvae) and infection severity (quantitative pathogen load) would decrease with longer exposure time, and that spores exposed to sunlight and rainfall would decay more rapidly than spores on shaded, sheltered plants.

To set up the experiment, we manually deposited pathogen spores onto marked leaves of greenhouse-grown, potted swamp milkweed plants (*Asclepias incarnata*). We obtained spores by swabbing lab-reared adult monarchs previously infected with one of three pathogen isolates. Isolates were originally derived from wild monarchs in eastern North America and chosen for this study to represent different levels of virulence (as measured in [23]). We used multiple isolates because previous studies documented variation among isolates for pathogen morphology and virulence [23, 24]; additional details for isolates are provided as in S1 Supporting Information. To mimic the behavior of an infected female monarch shedding *OE* spores while

ovipositing, we transferred approximately 200 spores onto the underside of each milkweed leaf using a glass wand (as described in [15]); spores were manually counted at 63X magnification. This starting dose is within the range of the number of spores that a heavily infected female deposits onto a leaf during oviposition, as found in a laboratory study [25]. Prior work showed that 100 'fresh' spores caused 100% infection probability in second-instar larvae [23]. We chose 200 spores as an initial dose because we assumed that some spores would be removed from leaves or lose viability, and we aimed to maintain sufficiently large sample sizes of infected monarchs to accurately measure infection severity at the end of the experiment. Further, our experiment used older, third instar larvae, which tend to be less susceptible to infection than the second instar larvae tested in earlier work, and thus require higher spore doses to acquire infections [S. Altizer, J. de Roode, M. Strand, unpublished data].

We inoculated and marked 5 to 6 leaves per plant. Inoculated plants were placed outdoors in Athens, GA for either 6, 11, or 16 days in a "sun" treatment on a grass lawn or in a "shade" treatment under a fiberglass screen enclosure covered with a tarp roof that also shielded rainfall (N= 2 plants per isolate per environmental and time treatment, for a total of 36 plants). During the experiment (June 6 - 21, 2014), outdoor temperatures ranged from a 19 to 42ºC in the sun treatment (average daily temperature 29.6 ºC) and 17 to 37 ºC in the shade treatment (average daily temperature 26.6 ºC). Six precipitation events occurred. Plants were kept in trays of water and time and isolate treatment groups were spatially interspersed. We also deposited spores from each isolate onto 6 plants that were not exposed to outdoor conditions ("day 0" plants); these were fed to larvae the same day that spores were applied to leaves. Finally, we fed milkweed leaves without spores to 20 additional monarchs to confirm that larvae were not acquiring infections due to laboratory contamination.

To test the infectivity of spores after environmental exposure, we fed individual inoculated leaves to lab-reared monarch larvae (1 leaf per larva) in the early third instar, when monarchs are large enough to consume an entire leaf yet still susceptible to *OE* infection. Larvae were kept in petri dishes for up to 48 hours until the leaf was consumed. Monarch larvae were grand-progeny of wild uninfected monarchs collected in Savannah, GA during spring 2014. Larvae that consumed the leaf were reared individually in 0.47L plastic containers with mesh screen lids under ambient light at average minimum and maximum temperatures of 27.4º and 30.8ºC, respectively. Larvae were given fresh *A. incarnata* stalks daily. We reared a total of 200 larvae, with 25 larvae for each of six time-by-environment treatment groups, 30 larvae for the "day 0" plants, and 20 larvae for the control (uninoculated) group.

We recorded signs of *OE* infection during pupal development following [23]. For monarchs that showed no sign of infection as pupae, we verified infection status after eclosion by pressing transparent tape ($1.27cm^2$) against the butterfly's abdomen and observing the sample for spores at 63X (following [17, 16]). *Infection status* (infected or uninfected) was noted for each monarch based on the presence of spores. We recorded sex and held all adults in individual glassine envelopes prior to freezing at -20ºC. To measure *infection severity*, we quantified pathogen load of infected butterflies by vortexing each abdomen for 5 minutes in 5 mL deionized water and used a counting chamber to estimate the total number of spores per individual [15]. Past work showed that more severe infections (with higher pathogen load) reduce adult monarch lifespan (i.e., cause higher virulence) [15, 26].

We tested how environmental exposure affected monarch infection status and infection severity. First, we used a generalized linear model (GLM) with a binomial link function to test the main effects of exposure time (0, 6, 11, or 16 days, treated as a continuous variable),

exposure treatment (sun versus shade), and pathogen isolate on binary infection status (infected/uninfected). We used a GLM with a Gaussian link function to test the same effects on infection severity (log-transformed quantitative pathogen load) among infected monarchs (with uninfected monarchs excluded). Because monarchs inoculated with spores in the day-0 group were not placed in sun versus shade treatments, we randomly assigned an environmental treatment group to these individuals for the purposes of statistical analysis. This approach allowed us to assess time and environmental treatment in the same analyses across all experimental monarchs and time points. To ensure this approach did not alter experimental conclusions, we also ran the analyses without day-0 monarchs and found similar results. Data from plants assigned to pathogen isolate E13 in the day-6 exposure group (n=17) were excluded from analyses, as these leaves did not receive the full pathogen dose due to experimental error. Analyses were conducted in R v. 3.0.3 [27].

(b) Model development

We next used a mechanistic model to examine the ecological consequences of environmental persistence of pathogens. We constructed a simple stage-structured model to describe infection dynamics within a milkweed patch at a Midwestern site during a typical summer-breeding season (approximately 100 days, June-August). Monarch hosts were subdivided according to *OE* infection status and life stage, such that S_L and I_L represented the abundance of susceptible vs. infected pre-adult monarchs (larvae, eggs, and pupae) and S_A and I_A represented uninfected vs. infected adults (Fig 1). Pathogen infectious stages in the environment were described as the number of milkweed leaves with an infectious dose of spores (W). Experimental work has shown that doses of 10 to 100 spores are highly infectious causing between 70 to 100% of inoculated second instar larvae to acquire infection [24]. We assumed that monarch eggs were produced by

adults at per capita rate *b* and developed into adults at rate *g*; we further assumed that monarch larvae experienced per capita density-independent mortality at rate μ_0 and density-dependent mortality at rate μ_1. Adults experienced per capita mortality at rate μ_A.

Pathogen transmission was modeled via two pathways. *Vertical transmission* (from parent to offspring) occurs when infected adult monarchs transfer spores directly onto eggs or onto the milkweed surface surrounding the egg during oviposition; we modeled vertical transmission by assuming a fraction of eggs laid by an infected female become infected. In this case, we assumed that vertical transmission occurs at rate *b* (the host birth rate) based on prior experimental work showing that infected female monarchs infect over 90% of larvae [12]. *Environmental* or *horizontal transmission* (between unrelated hosts) occurs when susceptible larvae consume milkweed leaves contaminated with spores shed by unrelated adults. We modeled environmental transmission as the product of the larval consumption rate, *c*, of milkweed leaves and the probability of encountering contaminated leaves, *W*/*M* (where *M* is the total number of leaves in the milkweed patch). Fitness costs of infection occur after pupation and were modeled as a reduced probability of eclosion and reproduction, p_E, and increased adult mortality rate, μ_I, relative to uninfected adults. Under these assumptions, infection dynamics in hosts were described by the following system of ordinary differential equations:

$$\frac{\mathrm{d}S_L}{dt} = bS_A - \left(\mu_0 + \mu_1 \frac{S_L+I_L}{M}\right) S_L - gS_L - \frac{cW}{M} S_L$$

$$\frac{\mathrm{d}I_L}{dt} = bI_A - \left(\mu_0 + \mu_1 \frac{S_L+I_L}{M}\right) I_L - gI_L + \frac{cW}{M} S_L$$

$$\frac{\mathrm{d}S_A}{dt} = gS_L - \mu_A S_A$$

$$\frac{\mathrm{d}I_A}{dt} = p_E g I_L - \mu_I I_A$$

We assumed that leaves are exposed to infectious doses when adults deposit spores while nectaring or ovipositing on milkweed plants at rate λ. Leaf exposure also depended on the probability that a visited leaf did not already have spores (1-*W*/*M*). Two processes removed pathogens from milkweed. Spores could die or fall off, causing environmentally exposed leaves to lose infectivity at rate μ_w. Additionally, exposed leaves were consumed by larvae at a rate proportional to larval abundance (S_L+I_L), the larval consumption rate *c*, and the probability that the leaf consumed had spores (*W*/*M*). Therefore, the dynamics of the environmental stage of *OE* were described as

$$\frac{dW}{dt} = \lambda\left(1 - \frac{W}{M}\right) I_A - \mu_w W - \frac{cW}{M}(S_L + I_L)$$

Model parameters are outlined in Table 1. Estimates of most parameters in our model were derived from previous studies or personal observations (see S2 Supporting Information); however, the pathogen dose decay rate μ_w (or inversely, pathogen persistence) in the environment and the pathogen shedding rate (λ) remain unknown. Our experiment, while originally designed to estimate pathogen persistence, was not long enough to detect decay rate of pathogen doses. Thus, we conducted a sensitivity analysis, varying the duration of environmental pathogen persistence ($1/\mu_w$, or the inverse of pathogen decay rate) from 1 to 80 days and the pathogen shedding rate (λ) from 1 to 300 leaves per day per adult monarch. We expected that infected monarchs might shed spores on up to 300 leaves per day, based on observations of wild monarchs visiting an average of 70 milkweed stalks per hour while nectaring or ovipositing [A. Majewska, personal communication]. We observed effects on infection prevalence and host population size during the 100-day breeding season. We used the *deSolve* package in R v. 3.0.3 to solve the system of differential equations [27].

RESULTS

(a) Spore persistence on milkweed leaves

Ninety percent of both experimental (inoculated) and control monarchs (un-inoculated) survived to adulthood. Among inoculated monarchs, 74% acquired *OE* infections. Average pathogen load for infected monarchs was $10^{5.55}$ spores ($\pm$ $10^{4.43}$ SEM). No control monarchs became infected.

Exposure time did not significantly affect infection probability (χ^2=0.16, df=1, p=0.69). Pathogen doses exposed for 0 days infected 81% of inoculated monarchs, and pathogen doses exposed for 16 days infected 75% of monarchs (Fig 2A). Thus, our experiment showed that spores remained highly infectious even after 16 days in the natural environment. There was no significant difference in infection rate between sun and shade exposure treatments, although infection probability tended to be lower for spores exposed to the sun compared to the shade (Fig 2A; χ^2=1.6, df=1, p=0.21, NS). Infection probability varied for the three pathogen isolates, with isolate E3 resulting in significantly higher infection rates (90% across all larvae) compared to isolate E10 (64% infection rate; χ^2=9.7, df=2, p=0.008).

Among the subset of infected monarchs, total pathogen load declined with greater exposure time to the environment (Fig 2B; $F_{1,106}$=6.96, p=0.01). This suggests that some spores on each inoculated leaf lost viability during environmental exposure, resulting in smaller effective doses (details appear in S1 Supporting Information file). Spores exposed to the sun caused less severe infections compared to spores from the shade treatment (Fig 2B; $F_{1,82}$=4.33, p=0.04). Pathogen load varied among isolates but this effect was small and non-significant ($F_{2,82}$=2.74, p=0.07, NS).

(c) Modeling results

We first examined how host population size and infection prevalence varied over time within a breeding season in relation to pathogen shedding (λ) and persistence ($1/\mu_W$). For moderate to

high environmental persistence times, ($1/\mu_W$ > 20 days) and pathogen shedding rate (λ > 150 leaves/day/monarch), our model captured the steady increase in prevalence observed among wild summer monarchs as the breeding season progresses [16]. Similar to wild infection data, our model showed that prevalence peaked at approximately 15% at the end of the season (Fig 3A). Abundance of monarch larvae and adults increased throughout the breeding season, and declined weakly with greater pathogen environmental persistence (Fig 3B).

We quantified how final infection prevalence at the end of the breeding season depended on the duration of environmental persistence ($1/\mu_W$; Fig 4). When assuming a high pathogen shedding rate (λ=300 leaves/day), model analyses showed that *OE* spores must persist on milkweed leaves for a minimum of 15 days for infection prevalence to increase above initial conditions (Fig 4A), and spores must persist for 24 days or longer for prevalence to reach the upper range of values observed in wild monarchs by the end of the breeding season [16]. When assuming a slightly lower value for pathogen shedding rate (λ=250 leaves/day), pathogen spores must persist for 40 days for prevalence to reach the upper range of values observed in the wild (Fig 4A). In general, prevalence at the end of the season increased as environmental persistence of pathogens increased. However, beyond a persistence time of 50 days (depending on other parameter values), the end-of-season prevalence remained similar (Fig 4A), likely because the proportion of contaminated milkweed leaves in the patch (*W/M*) saturates at around 80%. Longer spore persistence in the environment mildly decreased final adult population size, such that the adult population was 10-15% smaller when pathogens were long-lived (e.g., persist 50 days) compared to when pathogens were short-lived (Fig 4B).

Other factors beyond spore persistence were important for host-pathogen dynamics. Although the shedding rate in the wild of infectious doses onto milkweeds (λ) is not known, this

parameter can influence infection dynamics (Fig 4). High shedding rates increased infection prevalence at the end of the season (Fig 4A) and the rate at which prevalence rose during the breeding season (Fig 3A). Higher shedding rates also reduced the influence of spore persistence on end-of-season infection prevalence, such that even infectious doses persisting for approximately 24 days resulted in late-season prevalence greater than 15% (Fig 4A).

DISCUSSION

Our study suggested that transmission stages of *Ophryocystis elektroscirrha* remain infectious for multiple weeks in natural conditions, and that spore persistence in the environment is essential for *OE* to invade and persist in local monarch populations. Experimental results showed that pathogen doses remained almost as infectious after 16 days of environmental exposure as after 0 days. While the experiment did not allow us to measure pathogen decay quantitatively, a reduction in infection severity (i.e., pathogen load) over this 16-day period, and particularly in the sun treatment, suggested that environmental exposure killed or removed some pathogens within each dose. We developed a transmission model to examine ecological consequences for a range of pathogen persistence values. The transmission model indicated a threshold for environmental persistence above which the pathogen can invade and infection prevalence will increase during the summer-breeding season. Depending on the pathogen shedding rate, spore doses must remain viable for 24-80 days (or 4-12 weeks) to allow prevalence to reach the upper range of that observed in summer-breeding milkweed patches in eastern North America [16]. In addition to pathogen persistence, the model showed that pathogen shedding rate by adult monarchs was an important determinant of late-season infection prevalence.

Previous work on the dynamical consequences of environmental persistence for insect

pathogens showed that persistent environmental transmission stages can cause population cycles on a multiyear scale (e.g., [28, 29, 4]). Our study provides evidence that environmental persistence also influences short-term patterns in infection within a single season. Specifically, the model showed that longer environmental persistence drove faster and higher increases in prevalence, with infection rates increasing as the breeding season progressed, comparable to patterns in the wild [16]. This increase in infection prevalence was driven by an increase in the proportion of leaves contaminated with spores, indicating that spore longevity causes pathogens to accumulate on milkweed leaves, as previously suggested by field and experimental work [16].

Like many entomopathogens, *OE* can spread through both vertical and environmental (horizontal) transmission. Our model suggests contrasting roles for these modes of transmission which sustain host-pathogen interactions. Here, we found that environmental transmission was critical to pathogen persistence during the breeding season. Vertical transmission alone (modeled in the bI_A term, representing parent-to-offspring transmission) was not sufficient for the pathogen to establish in the host population, consistent with prior modeling work [30, 31]. However, the biology of this system suggests that vertical transmission is likely crucial for long-term *OE* persistence *between* breeding seasons, when monarchs overwinter in Mexico before resuming reproduction in the spring. Because pathogen transmission is limited to the breeding season, only those pathogens that survive on the overwintering adult monarchs' bodies for several months can be successfully transmitted to the next generation in the spring. We expect that the monarchs' migratory cycle thus selects for even greater spore longevity, compared to populations of monarchs that breed year-round. Thus, both vertical and environmental transmission require pathogen spores to persist for substantial periods of time, with vertical transmission causing new

infections across years and environmental transmission contributing to pathogen increase during the breeding season.

Our model emphasized that the rate at which infected adult monarchs shed pathogens onto leaves, as determined by butterfly visitation of milkweed leaves, strongly increased infection prevalence. Because monarchs land on milkweed plants to obtain nectar as well as to oviposit, we expect monarch-milkweed interactions and pathogen shedding rate to increase when milkweeds are flowering. Pathogen shedding rate might be higher for locations where monarchs rely heavily on milkweed for nectar, such as in coastal areas of the southern U.S., where some monarchs continue breeding and nectaring on exotic milkweeds into the winter [32] when other nectar sources are limited. Conversely, locations with a high diversity of nectar sources could lower milkweed visitation rates and decrease opportunities for pathogen spread.

Although our experimental results motivated our model development, we did not observe a decline in infectivity of leaves as predicted over two weeks of environmental exposure, precluding the opportunity to parameterize pathogen decay rate in the model. However, some degree of pathogen decay was supported, as spore doses exposed to the environment caused infections with lower final pathogen loads. This suggests that the number of viable spores declined through time due to increased exposure to harmful abiotic conditions. The presumed loss of viable spores was more apparent among sun-exposed pathogen doses than shade-exposed pathogens, consistent with previous laboratory work showing that UV exposure can destroy protozoan spores [S. Altizer, unpublished], a phenomenon that is well described for many other insect pathogens [9, 33].

One assumption of our model is that all infected monarchs transmit pathogens at the same rate and experience the same costs of infection. However, prior field and laboratory work

showed that pathogen load per butterfly varies among infected monarchs, and that pathogen load influences the probability and rate of pathogen shedding, the probability of infection for larvae consuming the spores, and the severity of the resulting infection [15]. Thus, heterogeneity in spore load on infected adults and spore dose consumed by susceptible caterpillars could influence infection outcomes, and might partially explain the range of infection prevalence (between 0 and 100%) observed in wild monarchs in different geographic regions and with different migratory propensity [17]. The model also motivates future empirical work to refine model parameter estimates and improve its predictive power. In particular, a longer-term experiment with lower initial spore doses could be informative to estimate pathogen decay rate (μ_W) under natural conditions. A lower initial spore dose could also improve estimation of pathogen dose decay. Estimates of milkweed visitation rates of wild monarchs are important for parameterizing pathogen shedding rate (λ), which was shown to strongly influence end-of-season infection prevalence.

Beyond insects, our study contributes to a growing body of work that highlights how pathogen persistence in the environment influences infectious disease dynamics across diverse systems. Modeling approaches demonstrated that environmental transmission enables the spread and persistence of avian influenza in water bird populations [34], causes population cycling of red grouse controlled by nematode infections [35], and enhances the persistence of hantavirus in wild rodents [36]. There is increased interest in modeling environmental transmission of pathogens [37] in the face of global environmental change. This is particularly relevant to insect-vectored pathogens, where host and pathogen survival and distribution depends critically on temperature, precipitation, and UV exposure [38, 39].

Migratory monarchs in eastern North America have recently experienced severe declines, mostly attributed to habitat loss at breeding and overwintering sites [40, 41, 42]. The extent to which mortality associated with pathogen infection has affected monarch population declines remains unclear. Our model predicted that mild reductions in the number of late-breeding season adults (on the order of a 16% reduction) could result from higher pathogen shedding and spore persistence, an effect that was likely limited by the relatively low infection prevalence observed in the model. Conditions that might crowd larvae, such as habitat fragmentation, could further increase infection prevalence and lead to stronger pathogen-mediated declines. Importantly, recent field monitoring documented extremely high infection prevalence in year-round breeding patches in the southern U.S. [33], a result not captured by our within-season model that extended to only 100 days. Further work integrating empirical data and modeling approaches is needed to understand drivers of spatial heterogeneity in infection and to predict future pathogen impacts on wild monarch populations.

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Table 1. Parameters of the model, including definitions, units, and values. See S2 Supporting Information for derivation and data sources for parameter estimates.

Parameter	Definition	Units	Value
b	Host fecundity rate	eggs/adult/day	15
g	Immature host development rate (egg to adult)	1/days	0.0385
c	Larval consumption rate of milkweed	leaves/day	1.35
p_E	Probability of infected larva eclosing and mating successfully		0.72
μ_0	Density-independent *per capita* larval mortality rate	1/day	0.08
μ_1	Density-dependent *per capita* larval mortality rate based on a larval density of 0.25 larvae/plant	1/day	1372
μ_A	Mortality of uninfected adult monarchs	1/day	0.0417
μ_I	Mortality rate of infected adult monarchs	1/day	0.05
λ	Shedding rate of infectious doses onto leaves	leaves/day	1-300
μ_w	Decay rate of infectious doses on milkweed leaves	1/day	0.0125-1.0
S_0	Initial uninfected adult monarch population	adults	18
I_0	Initial infected monarch population	adults	2
M	Total number of milkweed leaves in patch	leaves	25000
T	Length of breeding season	days	100

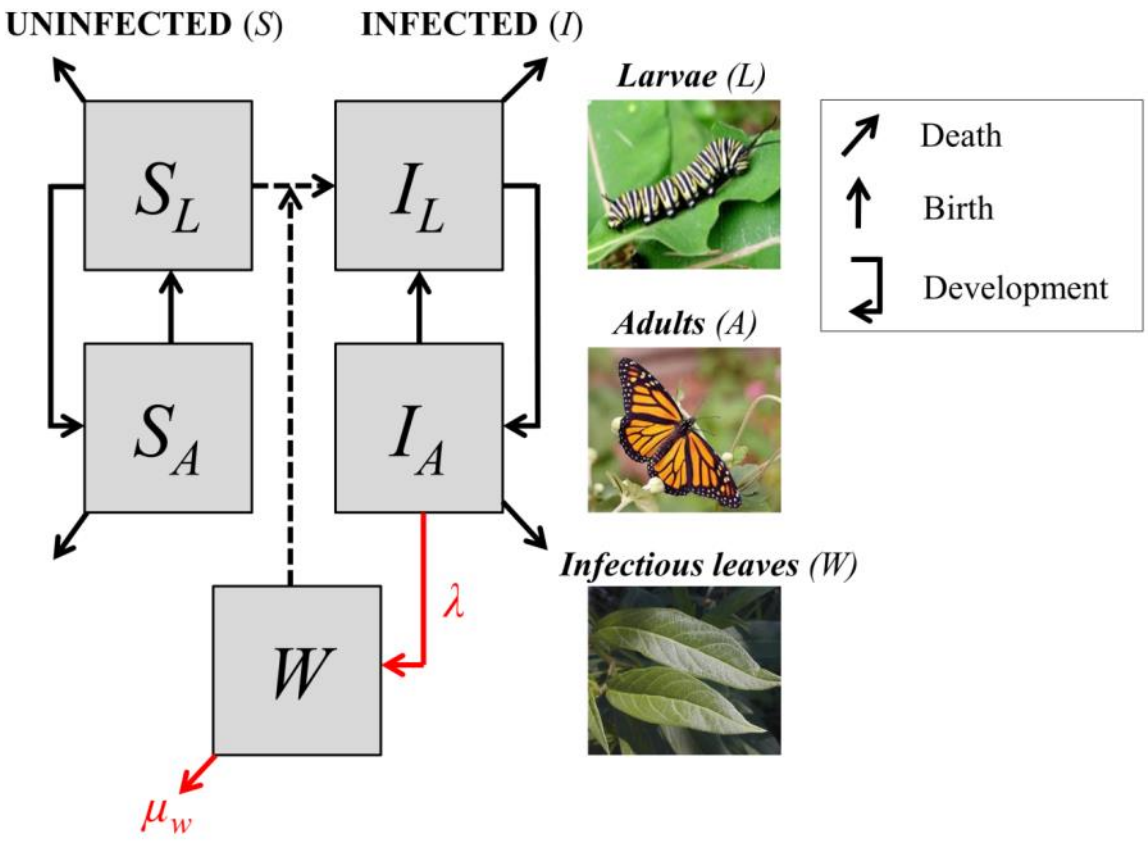

Figure 1. Schematic of model describing protozoan pathogen transmission in monarch hosts. Monarchs are represented as uninfected or infected immature stages (S_L or I_L) or adults (S_A or I_A). Milkweed leaves with spores (W) arise when infectious doses are deposited by adult monarchs (at rate λ), and are lost through larval consumption (c) or through decay in dose viability (at rate μ_W). All larvae produced by infected females are assumed to become infected (perfect vertical transmission) and uninfected larvae become infected by consuming leaves with spores (environmental transmission, dashed line).

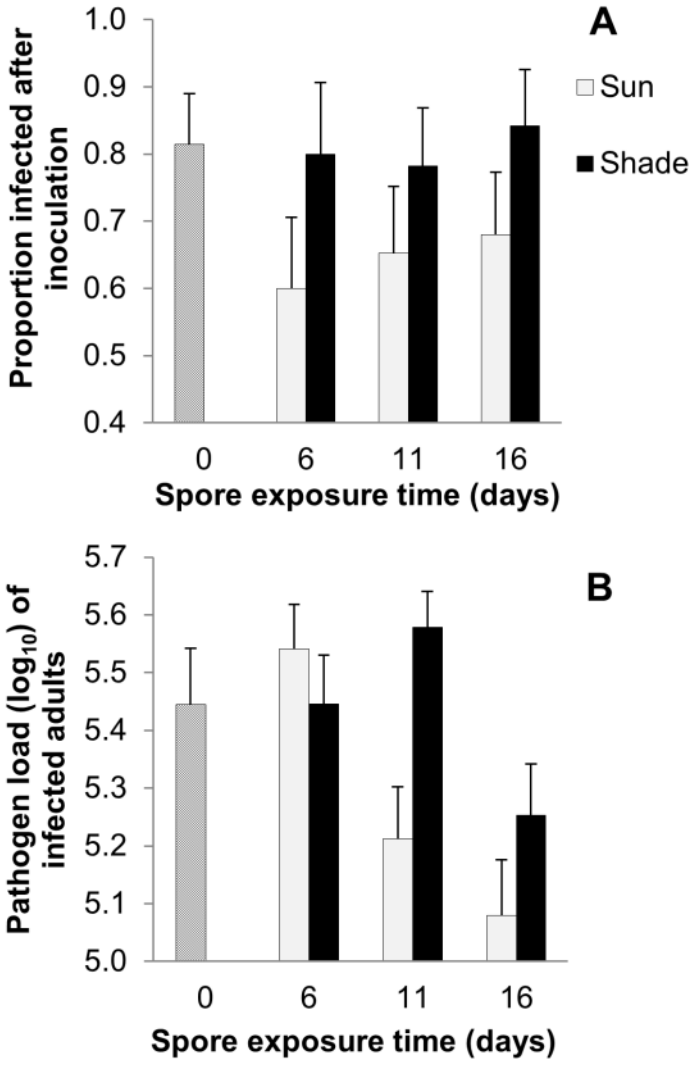

Figure 2. Experimental results on infection probability and pathogen load following spore exposure to sun or shade treatments for 0, 6, 11 or 16 days. (a) Proportion of monarchs infected following inoculation with spores on milkweed leaves exposed for 0 days (n=27); 6 days, in either sun (n=15) or shade conditions (n=15); 11 days in sun (n=23) or shade (n=24); or 16 days in sun (n=25) or shade (n=19). (b) Pathogen load (log-transformed) of monarchs infected with spores exposed for 0 days (n=22); 6 days, in either sun (n=10) or shade conditions (n=11); 11 days in sun (n=15) or shade (n=19); or 16 days in sun (n=17) or shade (n=16). Error bars show standard error of the mean.

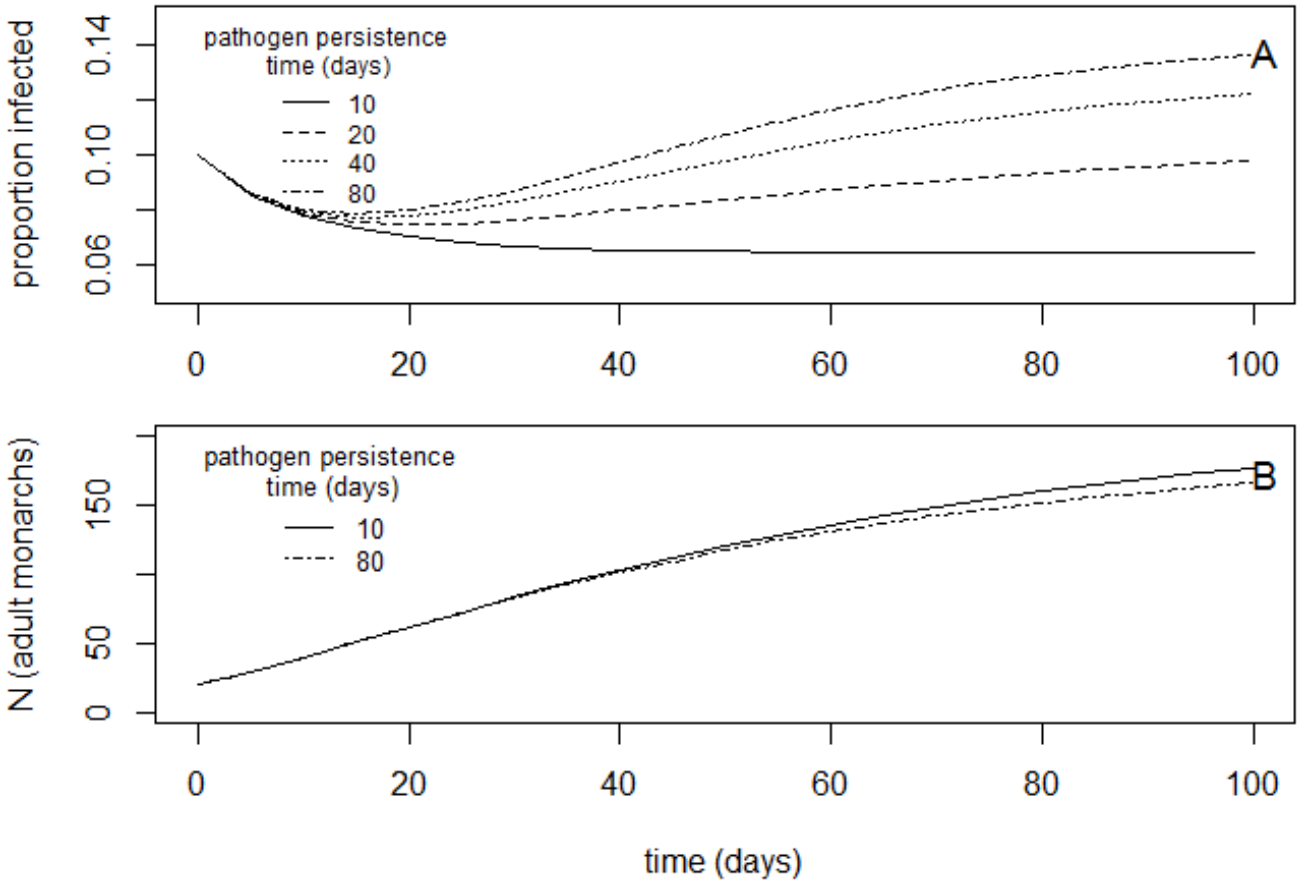

Figure 3. Results from the transmission model showing within-season dynamics in (a) infection prevalence and (b) adult population size within the milkweed patch for a range of pathogen persistence times (in days, $1/\mu_w$). Longer pathogen persistence produced higher infection prevalence and slightly lower adult population size. The dynamics shown assume an intermediate value for leaf visitation rate (λ=150 leaves/day/adult); all other parameter values are listed in Table 1.

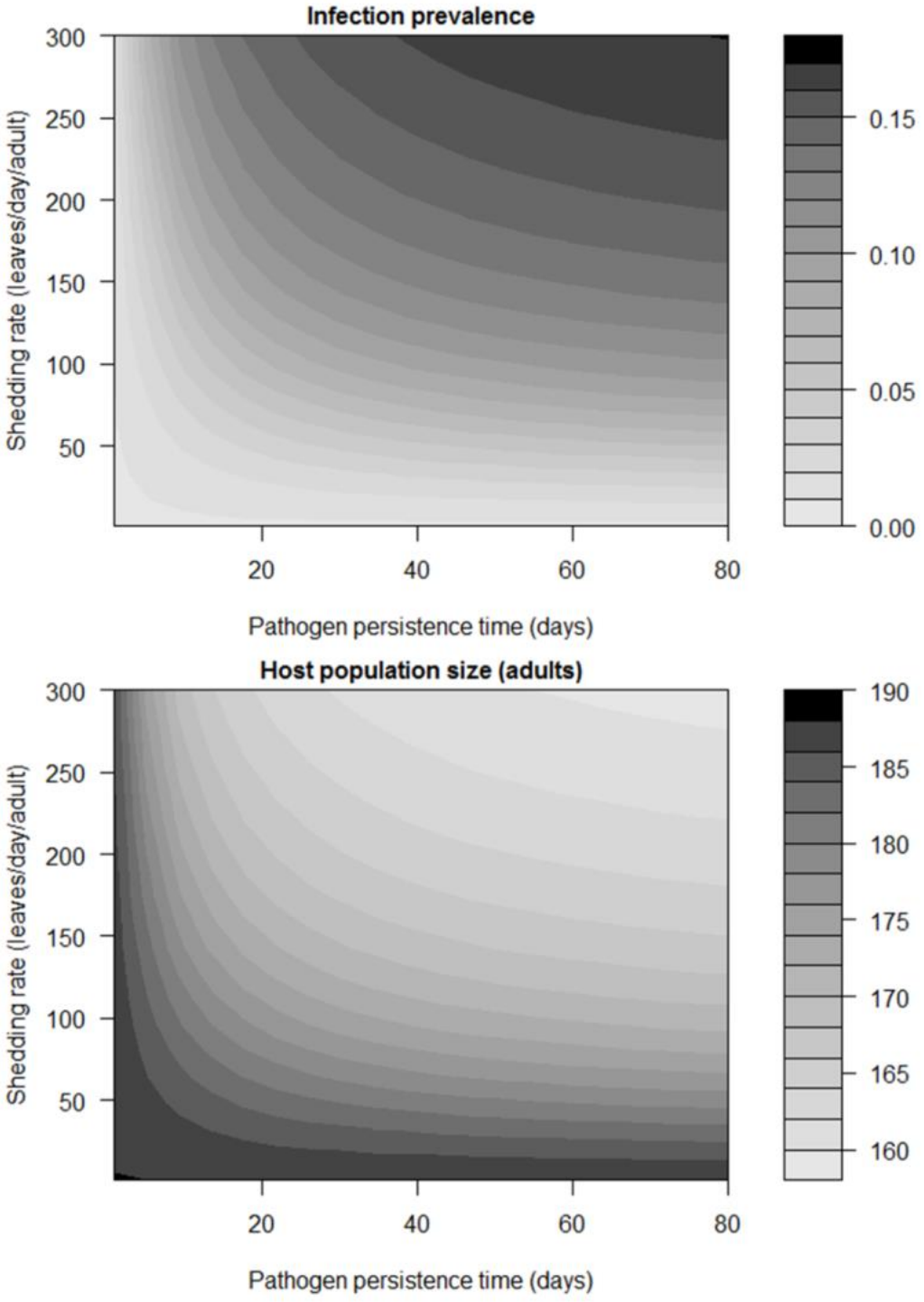

Figure 4. Results from the transmission model for a range of pathogen persistence times ($1/\mu_W$) and shedding rates (λ). (A) End-of-season infection prevalence and (B) adult monarch population size (T=100 days; scale bars).

CHAPTER 5

MIGRATORY MONARCHS THAT SHARE STOPOVER HABITAT WITH SEDENTARY CONSPECIFICS SHOW GREATER REPRODUCTIVE ACTIVITY AND INFECTION RISK[4]

[4] Satterfield, D.A., Maerz, J.C., Hunter, M.D., Flockhart, D.T.T., Hobson, K.A., Norris, D.R., Streit, H., Fritzsche McKay, A., de Roode, J. and S. Altizer. To be submitted to *Ecology Letters*.

ABSTRACT

Most migratory species include both migrant and resident individuals. In response to environmental changes, numerous partially migratory populations are now shifting to become more sedentary. Of particular concern is whether and how sedentary populations influence the health and behavior of migratory conspecifics that encounter them. Resident populations can experience higher parasite infection prevalence compared to migrants, for which long-distance migration often reduces parasitism levels. Residents that share habitat with migrants could act as sources of infection, or further, could potentially influence migrants to alter migratory behaviors or the physiological states that facilitate migration. Here, we focus on monarch butterflies (*Danaus plexippus*) and their protozoan parasite *Ophryocystis elektroscirrha* to investigate the frequency and consequences of interactions between heavily infected resident butterflies and migrants. Whereas most monarchs from eastern North America migrate to Mexico each fall, some monarchs in recent years have shown sedentary behavior, breeding year-round in the southern U.S. on an exotic milkweed species. We used chemical analyses to distinguish wild monarchs from Texas as migrants or residents. This allowed us to determine the degree to which migrants and residents share habitat and whether migrants encountering resident sites were more likely to experience changes that decrease migratory success. We found that migrant and resident monarchs shared habitat at year-round breeding sites during the fall and co-occurred in breeding habitats in the spring, although to a lesser extent. Migrants sampled at year-round breeding sites were more likely to show infection and to be reproductive compared to monarchs at stopover sites. This pattern could potentially be a result of tropical milkweed gardens either inducing these states or attracting migrants that are already infected or reproductive.

Key words: partial migration, resident-migrant interactions, long-distance migration, infectious disease, *Danaus plexippus*, deuterium, isoscapes

INTRODUCTION

Migratory animals often show substantial variation in their propensity to move. Many migratory species include both migrant and resident individuals, with some portion of the population migrating seasonally between habitats, and other individuals remaining in the same area throughout the year [1,2]. This phenomenon of mixed movement strategies, known as partial migration, has been observed in birds such as song sparrows (*Melospiza melodia*) in North America, marine animals such as bull sharks (*Carcharhinus leucas*) in coastal Australia, and in terrestrial mammals such as wildebeest (*Connochaetes taurinus*) in eastern Africa [3–5].

Numerous partially migratory populations are now shifting to become more sedentary. In recent decades, species of birds, bats, and ungulates have established or expanded resident populations in response to human activities including habitat alteration or novel food resources (e.g., bird feeders) [6–9]. For instance, a partially migratory population of great bustards (*Otis tarda*) has become increasingly sedentary, with resident birds in Spain shifting from 17% of the population to 45% in recent years; one factor underlying this shift was the high mortality of migrants on power lines [10]. As another example, white storks (*Ciconia ciconia*) breeding in Europe historically migrated to Africa each winter, but part of the population now forages in city landfills in Spain year-round [11,12]. Canada geese (*Branta canadensis*), trumpeter swans (*Cygnus buccinators*), flying foxes (*Pteropus poliocephalus*), and other species are showing similar behaviors [13–15]. Past work has demonstrated that residency can evolve in just a few generations [16]. Under future climate change scenarios and habitat alteration, residency is

predicted to increase in some migratory populations [17–19]. Modeling work supports these predictions, assuming environmental changes affect migrant and resident demographic rates similarly [20].

The ecological consequences of changes in migration remain largely unknown. Of particular concern is whether and how resident populations influence the health and behavior of migratory conspecifics. Migratory animals, which rely on resources and routes across a geographic range, already face numerous threats. Many migratory populations are in decline due to habitat loss, overharvesting, human-constructed barriers, and climate change [21]. Quantifying the extent to which migrants interact and respond to growing resident populations could inform conservation plans and population projections for migratory animals.

One critical question is whether resident populations increase the risk of pathogen infection for migratory animals that encounter these populations. Theoretical models and empirical studies of mammals, fish, and birds have demonstrated significantly greater infection risk for residents compared to migrants [22–26]. This contrast likely occurs because residents do not experience the mechanisms through which migration can reduce parasite infection risk [reviewed in [27]. Migration can lower transmission by periodically allowing migrants to leave behind parasite-contaminated habitat (migratory escape) [28–30]; by removing infected individuals from the population (migratory culling) [29]; by enabling migrants to recover from infections during their journeys (migratory recovery) [31]; or by separating susceptible and infected age classes (migratory allopatry) [32]. Without these advantages, resident populations might experience higher parasite infection prevalence and act as sources of infection for migrants. As one case in point, elk (*Cervus elaphus*) in Greater Yellowstone Park area that have become sedentary due to supplemental feeding are associated with a higher prevalence of

brucellosis, which increases opportunities for transmission to migratory elk [24,33]. Resident mallard ducks, which often subsist on rice fields, were found to have higher prevalence of particular subtypes of avian influenza in California that were transmitted to migratory mallards (*Anas platyrhynchos*) [34].

Resident populations could potentially induce migrants to alter the behaviors or physiological states that facilitate migration. Migrants' sensitivities to environmental cues are complex and conditional, such that they respond to (or ignore) environmental information differently depending on the phase of their migratory cycle [35,36]. During migration, animals are thought to delay responding to favorable resources even when encountered mid-journey; indeed this ability to "switch off" initial responses to resources *en route* has been used to define migratory behavior [35]. As migrants reach the end of their journeys, however, they become highly attuned and responsive to resources [37]. It is possible, then, that resident sites near wintering or breeding habitats (at their end of migrants' journeys) could affect migrants' behaviors or physiological states. Migrants commonly prepare for their journeys by allocating resources away from reproduction or non-essential functions, a mechanism that can enhance survival during strenuous migrations [35]. Environmental cues help to control "migratory syndromes," and highly attractive resources at resident sites could potentially modify these states. Migrants could also respond to the presence of resident conspecifics. Dispersing kittiwakes, for instance, are thought to use other conspecifics as a cue of breeding habitat quality (conspecific attraction hypothesis; [38]).

The magnitude of influence of sedentary populations on migrant health and behavior will depend on the degree to which residents and migrants share habitat. Some resident populations are periodically inundated with migrants, as with resident blackcaps (*Sylvia atricapilla*) that

overlap with their migratory counterparts on wintering grounds in Europe [39]. In the tropics, the seasonal influx of migrants can double the number of birds in an area [40]. Quantifying these interactions, however, has historically been difficult due to challenges in distinguishing residents and migrants [1]. As David Lack noted, partially migratory populations "reveal a problem of remarkable complexity" [41]. Recent advances in chemical ecology and tracking techniques offer new opportunities to distinguish migratory from resident animals. In particular, stable isotope analysis of natal origins and chromatographic methods to quantify diets have become powerful tools to identify migratory animals.

Here, we focus on monarch butterflies (*Danaus plexippus*) to investigate the degree to which migrants and residents share habitat, and to ask whether migrants encountering sedentary populations experience changes in behavior or parasite infection risk. Traditionally, monarchs in eastern North America migrate up to 3000 km each fall to overwinter in Mexico in a state of reproductive dormancy [42]. In recent decades, however, a growing number monarchs have shown sedentary behavior, foregoing migration to breed year-round in the southern U.S. as a result of extensive planting of tropical milkweed (*Asclepias curassavica*) in gardens [43,44]. Non-migratory monarchs in the southeastern and western U.S. experience up to five times higher infection risk by the specialist protozoan parasite *Ophryocystis elektroscirrha* (hereafter, OE) compared to migrants [44,45]. Infection can shorten adult monarch lifespan, weaken flight, or cause wing deformities or death during eclosion from the chrysalis [46–48]. Past work indicated that monarch migration reduces infection risk through both migratory culling and migratory escape [29].

Migratory monarchs likely encounter parasitized resident monarch populations during their fall and spring migrations, particularly as they move through Texas on the way to and from

their wintering sites in central Mexico. Some scientists are concerned that such encounters could increase infection risk for migrants or their offspring or could prompt migrants to break reproductive diapause and halt their migration [43]. In this study, we conducted field sampling of wild monarchs in eastern Texas during the fall and spring migration to ask: (1) To what extent do migrant and resident monarchs overlap in habitat use? (2) Do migrants incur higher infection risk in areas with resident monarchs? (3) Are migrants more likely to break reproductive diapause in areas with resident monarchs? We used chemical assays based on host plant cardenolide fingerprints and isotopic natal origin assignment to determine the proportion of residents and migrants at sampling locations (following [49–52]). Monarchs were further examined for infection status and reproductive activity. We predicted that monarchs of migratory and resident status would overlap at year-round breeding sites in Texas during the spring and fall migration, and that migratory monarchs sampled at year-round breeding habitats would (a) show evidence of higher infection risk, and (b) demonstrate a higher frequency of reproductive status (i.e., evidence of having broken reproductive diapause) than those sampled at other stopover sites. We hypothesized that year-round breeding sites with continuously growing tropical milkweed would encourage reproductive development, as the presence of young milkweed has been shown to play a role in controlling diapause for monarchs [53].

MATERIALS AND METHODS

(a) Biology of the study system

Monarchs in eastern North America migrate annually between breeding grounds in the US and Canada and wintering grounds in Central Mexico [42,54]. During fall migration (Aug-Nov), monarchs travel primarily along either the central flyway, extending from the Midwest through

central Texas, or the eastern flyway, running down the east and Gulf coasts through coastal Texas [55]. Monarchs fly only during daylight hours and feed on nectar at stopover sites during the journey [56]. Most monarchs that reach Mexico remain in reproductive diapause (a state of stalled reproductive development) throughout the winter, enabling them to conserve energy reserves and prolong lifespan [57,58]. In the spring, monarchs break diapause, mate, and return to the southern US to lay eggs on milkweed. Their progeny and grand-progeny continue to migrate northward to recolonize the northern limits of the breeding range [51].

Some monarchs, which we term 'sedentary monarchs,' breed continuously throughout the year in the southern coastal U.S. and do not appear to migrate [43,45,59]. Anecdotal reports of year-round monarch breeding first appeared in the literature in the 1950s [60,61]. Reports have become more common in recent years, based on citizen science data from 2002-2010 documenting winter sightings of larval monarchs in the south [62]. Surveys of citizen scientists in the southern U.S. [Satterfield, unpublished] and California [45] indicate that sedentary monarch populations form almost exclusively around gardens with tropical milkweed (*Asclepias curassavica*), an exotic plant often sold at commercial nurseries. Unlike native milkweeds, tropical milkweed does not die back in the fall in mild climates and thus provides a year-round food source for larval monarchs [43–45]. These year-round breeding sites appear to be separate from the non-migratory population that has long been established in south Florida [63,64].

Sedentary monarchs along U.S. coasts experience infection risk by the *Ophryocystis* protozoan up to 9 times higher than that for migratory monarchs [44,45]. This is consistent with previous findings that migration reduces parasitism by allowing monarchs to escape parasite spores that build up during the summer and by removing infected butterflies during migration, as evidenced by a drop in prevalence post-migration [29,64,65]. OE transmission occurs when

parasitized adult butterflies (covered with millions of dormant spores on the outside of their bodies) scatter parasites onto milkweed leaves or eggs and then the spores are ingested by a monarch larva [66]. Infected adults can also passively transfer spores to other adults (e.g., during mating), however, a new infection only occurs when spores are consumed by a larva [66].

(b) Field collections and capture-mark-recapture study

To investigate migrant-resident interactions during the fall, we conducted field studies in Texas during October-December 2014 at 7 sites categorized as either: (a) *stopover sites* along the central flyway or coastal flyway where there was no history of monarch winter-breeding, which we sampled during the peak migration period; or (b) *year-round breeding sites*, with monarch winter-breeding activity (Dec-Feb) on tropical milkweed along the coastal flyway which we sampled before, during, and after the peak migration (Figure 1A). We collected 201 wild adults at stopover sites and 258 adults at year-round breeding sites in Oct-Nov 2014. To investigate interactions during the spring, we collected both adult and pre-adult monarchs in Texas during Apr 2015 at 2 stopover sites (N= 12 adults, 29 eggs or first-instar larvae from native milkweeds, *A. viridis* or *A. asperula*) and 3 year-round breeding sites (N = 40 adults and 27 pupae or fifth-instar larvae). After consuming their natal leaf, larvae from stopover sites were reared in individual containers and fed greenhouse-grown, parasite-free milkweed until pupation. Of these, 13 larvae from stopover sites survived to adulthood for infection assessment. Larvae from year-round breeding sites pupated soon after collection and exclusively ate the milkweed from which they were collected.

To observe the proportion of migrants that stopped migration after encountering sites with tropical milkweed, we conducted a small capture-mark-recapture study at three year-round breeding sites. We captured, tagged, and released a total of 141 adults before, during, and after

the peak migration period to estimate site fidelity. Additional details are provided in the electronic supplementary information (ESM).

(c)Morphological measurements

For all captured monarchs (N=511 adults and N=40 pre-adults), we determined sex and measured forewing length. We also assessed wing wear (on an ordinal scale of 1-5), wing damage (the number of wings, 0-4, with tears or holes), and abdomen fatness (on an ordinal scale of 1-4), following [67,68]. We obtained mass for most monarchs using a field scale. For collected individuals with at least one intact forewing, we scanned the dorsal side of the forewing in best condition with a flatbed scanner and used the FoveaPro plugin (Reindeer Graphics, Inc.) in Adobe Photoshop to calculate wing area, length, and aspect ratio (length to width) [69–71].

(d) Infection assessments

We tested all adult monarchs non-destructively for OE infection by pressing a clear sticker against the abdomen, as in [64]. Sticker samples were viewed at 60X to observe the presence of absence of parasites. Samples with 100 or more parasite spores are considered infected, indicating that the monarch experienced a true internal infection acquired as a larva. Monarchs with less than 100 spores could have acquired spores through the passive transfer of parasites from another source; they are considered uninfected [66,72]. Infection prevalence was calculated as the proportion of monarchs infected of the total sampled per site or time interval. Monarchs collected as pre-adults were assessed for infection after reaching adulthood.

(e) Reproductive assessments

We evaluated reproductive status for monarchs collected in the fall (N=296 butterflies for which we also obtained natal origin information). Wild females were dissected to observe the presence

or absence of mature eggs in the ovaries within 5 days after capture [73]. Wild males were placed in cages either outdoors (N=166) or in incubators set to outdoor conditions (N=34) to observe matings (with a separate group of mostly lab-reared females) for 8 to 10 days, or until outdoor monarchs had at least 7 days at >21.1ºC (70F). Females with mature eggs and males that mated in enclosures were considered reproductively active. Additional details appear in the ESM.

(f) Distinguishing residents and migrants

Monarch and resident assignments of wild monarchs depended on information from stable isotope and cardenolide concentration and composition in wing tissue. First, we used stable hydrogen (δ^2H) isotope composition from wing tissue to help infer the geographic region from which monarchs originated as larvae [49,65,74,75]. Wing chitin δ^2H values are informative because mean δ^2H patterns in precipitation (δ^2Hp; amount-weighted mean growing season values) decline with increasing latitude; these patterns are passed on to plant tissue and, in turn, to chitin in monarch wing membranes [50]. Wing samples from all collected adults were stored at -20°C until preparation as described in [49]. Briefly, after rinsing the right hindwing twice with 2:1 chloroform-methanol and air-drying, we loaded wing pieces into silver capsules and weighed the wing tissue (0.34 ± 0.015 mg). Pressed capsules were combusted in a Eurovector elemental analyser (Milan, Italy) interfaced with a Micromass continuous flow mass spectrometer (CFIRMS). We report all stable isotope values in the typical delta (δ) notation as parts per thousand (‰) deviation from the VSMOW-SLAP scale for δ^2H. We accounted for exchangeable δ^2H in the laboratory atmosphere using a comparative equilibration technique based on within-run and identically treated laboratory standards (CBS: -197 ‰, SPK: -121 ‰;

KHS: - 54.1 ‰) [49]. Based on within-run replicates of standards (n=5), we estimated measurement precision to be ± 2‰ δ^2H.

Next, we used cardenolide assays to infer whether monarchs fed as larvae on tropical milkweed (*A. curassavica*) or another species (non-*A. curassavica*). In North America, monarch larvae typically consume one of dozens of milkweed species with varying concentrations and diversities of toxic cardenolides [76,77]. Because cardenolides are retained in the integuments of adult monarchs, chromatography has been used to determine natal host plant species [51,78], and in our study, informed resident and migrant classifications. Tropical milkweed (*A. curassavica*), the larval food source for resident monarchs at year-round breeding sites, has greater concentrations of highly toxic (non-polar) cardenolides compared to most native milkweed species that support the vast majority of migrants [79]. We quantified cardenolide concentration, non-polarity (retention time for each cardenolide peak), and diversity for each butterfly with ultra-high-pressure high-performance liquid chromatography (HPLC; similar to in [80]), with details provided in the ESM. We then used non-metric multidimensional scaling (NMDS) to reduce the dimensionality of these data to two Cartesian coordinates per sample. NMDS analysis was not possible for monarchs with cardenolide concentrations of 0; these were assigned as migrants in both approaches described below, as these butterflies originated from non-*A. curassavica* milkweed (and thus could not be residents).

We classified wild monarchs as residents or migrants with two approaches: (1) a two-stage discriminant analysis where discriminant functions were determined from δ^2H, cardenolide concentration, cardenolide NMDS scores, and wing length measurements from known residents and migrants, and then unknown butterflies were classified using those discriminant functions; (2) a series of threshold decision rules based on previous knowledge about δ^2H, cardenolides,

and monarch biology from prior studies. We conducted subsequent statistical analyses on individuals with classification agreement from both approaches (N=377).

In the decision-rules method, we first assumed that all monarchs originating from northern latitudes were migratory monarchs, as they would have had to travel a considerable distance southward to reach our collection sites. Thus, all butterflies with <-110.86 ‰ δ^2H, which occurs at locations north of Texas (on previously described isoscapes), were designated as migrants. Importantly, known resident monarchs had a mean δ^2H of -90.57‰ (range: -75.64‰ to -103.70‰); we placed the threshold for migrants to be 3 standard deviations above this. These results were consistent with previous work based on mean annual precipitation (http://www.waterisotopes.org) and on a calibration algorithm linking monarch wing chitin δ^2H with δ^2Hp [50].

We then examined the remaining monarchs from southern latitudes (δ^2H more positive than -110.86‰). Some migrants that travel to Mexico originate from the southern U.S., thus these monarchs could be either residents or migrants. We used cardenolide information to further distinguish these specimens. We assigned as residents any monarchs with NMDS coordinates occurring within a tightly defined polygon, previously constructed from lab-raised and field monarchs known to be from *A. curassavica* (N=132 monarchs; see ESM, Fig. S3). We assigned as migrants any monarchs with NMDS values occurring well outside of this cluster (specifically, at distances > 3 standard deviations from the cluster means), indicating that these butterflies likely originated from a native, non-*A. curassavica* plant. We confirmed that monarchs from native milkweeds occur outside of this polygon, testing this assumption with lab-raised and field monarchs from 11 native milkweed species (N=214 monarchs, with none in the polygon). Other southern monarchs with ambiguous cardenolide profiles (with NMDS coordinates outside the

polygon but within 3 standard deviations) were assigned to an uncertain status (N=11), as these could be either migrants of southern origin, or resident monarchs that fed on low-cardenolide milkweed plants.

For the discriminant analysis approach, we first grouped monarchs as residents if they showed *A. curassavica* cardenolide profiles (inside the NMDS polygon) and δ^2H values from southern origins (>-110.86‰, see above). To classify the remaining monarchs (N=198), we used a linear discriminant analysis that collated information from δ^2H, total cardenolide concentration, cardenolide NMDS coordinates, and wing length to predict probable group. We determined discriminant functions based on the 25 known residents and 84 known migrants collected from Mexico overwintering sites in 2013. These "training" data (N=109) indicated that total cardenolide concentration was the strongest predictor of resident vs migrant status (Wilk's lambda= 0.52, $F_{1,107}$=262.5, p<<<0.001) and δ^2H value was also informative although not significant (Wilk's lambda= 0.15, $F_{1,107}$=2.00, p=0.16, NS). Wing length and cardenolide NMDS coordinates were not significant. Testing the discriminant functions showed that known residents (25 monarchs sampled as larvae from tropical milkweed sites in Texas) and migrants (85 monarchs sampled as adults from wintering sites in Mexico in Feb 2013) were correctly classified. Applying the discriminant analysis to unknown Texas butterflies placed monarchs into groups with very high posterior probabilities (>0.9 in most cases). Any monarch with a posterior probability <0.7, which applied to only one butterfly, was not classified. Twelve butterflies with assignment discrepancies between the discriminant and decision-rules analyses were considered "uncertain" and excluded from further analysis.

(g) Data analyses

Classifications based on chemical analyses allowed us to quantify the proportion of migrants and resident monarchs at each site and to observe dynamics in these proportions throughout the fall at year-round sites (Figure 1). We quantified morphological differences between monarchs assigned to migrant vs resident status, using a multiple analysis of variance (MANOVA) to compare mass, wing condition (treated as a continuous variable), and wing characteristics including forewing length, area, and aspect ratio (for N=171 monarchs). We conducted a second, similar MANOVA on a subset of the data to compare morphology for migrants (N=137) from the coastal versus central flyways.

We examined differences in migrants and residents for reproductive activity (N=287) and infection status (N=330). We used a generalized linear mixed model (GLMM) with binomial error distribution to test effects of migratory status, sex, and a migratory and sex interaction on reproductive status (reproductive or in diapause), with site included as a random variable. A second GLMM with the same factors assessed predictors of infection status (infected or uninfected). Non-significant terms were eliminated and models re-evaluated based on AIC. We examined significant differences with post-hoc Dunnett's tests.

To evaluate risks for monarchs that encounter residents and year-round tropical milkweed sites, we examined whether migrants were more likely to be reproductive or parasitized at year-round breeding compared to stopover sites. We assessed predictors of reproductive status for migrants (N=238 with reproductive assessments) with a GLMM including fixed factors for site type (year-round breeding or stopover), sex, and a sex by site type interaction term. We also included hydrogen value (a proxy for latitude) to understand from which regions reproductive

migrants originated. Site was treated as a random variable. Migrants were analyzed with an additional GLMM on infection status (N=274), using the same structure.

To examine how long migrants at year-round breeding sites remained at these locations, we evaluated Cormack-Jolly-Seber models on capture-mark-recapture data to estimate site fidelity of presumed migrants versus residents and to observe duration of stay at these locations. Additional methods are reported in the ESM. All statistical analyses were conducted in R 3.2.3.

RESULTS

(a) Co-occurrence of residents and migrants

Across all sites and sampling periods, we detected 291 migrant and 112 resident monarchs. During the fall, migrant and resident monarchs shared habitat at year-round breeding sites (Figure 1A), where our analyses classified 57% of sampled monarchs as migrants and 43% as residents (N=130 total monarchs). Dynamics in these proportions throughout October-December showed a wave of migrants arriving at year-round sites and later departing as most moved southwards (Figure 1B). The greatest geographic overlap occurred at the most coastal site (Galveston, TX). In contrast, fall migrants and residents did not interact at stopover sites, where butterflies were exclusively migratory at both inland and coastal locations (N=199).

During the spring, we observed some migrants and residents sharing habitat, although their co-occurrence was less common than in the fall (Figure 1D). Sample sizes were substantially smaller during the spring, when migratory monarchs disperse broadly across Texas and do not funnel into major flyways. At year-round breeding sites, our analyses classified 24% of spring-sampled monarchs as migrants and 76% as residents, out of a small collection (N=38).

At the single stopover site sampled for adults in the spring, we detected primarily migrants (8 individuals of 10) but also two residents.

(b) Reproductive activity

Among fall monarchs with classifications and reproductive assessments (N=287), migratory status was a significant predictor of reproductive activity (χ^2=6.04, df=1, p=0.014), with resident monarchs more likely to be reproductively active (47%) than migrants (18%). Most reproductive monarchs were male. Across samples, males were significantly more likely to be reproductive relative to females (χ^2=7.52, df=1, p=0.006). Sex differences were especially strong among migrants, with 4% of females and 26% of males being reproductive; among residents, sex differences were less pronounced (Figure 2; migratory status and sex interaction: χ^2=4.61, df=1, p=0.03). A second analysis focused on predictors of reproductive activity among fall migrants only (N=238). Migrants sampled at year-round breeding sites were 3 times more likely to be reproductive (35%) compared to monarchs at stopover sites (11%; Figure 2; χ^2=4.94, df=1, p=0.026). This contrast remained whether examining migrants at stopover sites along the central flyway (13% reproductive across 3 sites) or coastal flyway (4% reproductive at 1 site). Sex had a strong significant effect on reproduction (χ^2=9.98, df=1, p=0.002). Neither hydrogen values nor the interaction term between site type and sex were predictors of reproductive status.

(c) Infection risk

In the fall, residents showed extremely high infection prevalence (95%), as observed at year-round breeding sites in previous studies [44,45]. Migratory status was the strongest predictor of infection status in the fall (χ^2=30.93, df=1, p<<0.001). Whereas the vast majority of residents were heavily infected with OE, only 9% of migrants had infections. As uninfected migrants arrived at and departed year-round breeding sites with parasitized residents, the proportion of

infected monarchs (prevalence) at these sites decreased decreased and then rebounded (Figure 1C). Among the subset of migrants, monarchs were significantly more likely to be infected at year-round breeding sites than at stopover sites (Figure 3; χ^2=14.03, df=1, p=0.0002). Infected monarchs were more likely to be from southern latitudes (less negative δ^2; χ^2=16.12, df=1, p<0.001).

In the spring, resident adults continued to exhibit high infection prevalence (71%, N=31) relative to migrants (24%, or 4 infected out of N=17), but sample sizes were limited. Infection prevalence among a small number of pre-adult monarchs demonstrated that infection risk is higher for monarch offspring at year-round breeding sites, where 41% of larvae collected showed infection (N=27), than stopover sites, where 0% of eggs/larvae (N=13) were infected.

(d) Morphological differences between residents and migrants

Migrants had significantly more worn forewings than residents, with lower condition scores (a composite of wing wear and damage scores; $F_{1,169}$=14.63, p=0.001). Wing condition is a proxy for monarch age, with more scale loss over time. Migratory monarchs in the coastal flyway had longer forewings (mean=52.00 mm) and greater forewing area (mean=910.6 mm^2) than migrants in the central flyway (mean length=51.19 mm and mean area=885.8 mm^2; $F_{1,135}$=10.06, p=0.002 for length; $F_{1,135}$=9.93, p=0.002 for area). Coastal flyway monarchs were also slightly more worn than central migrants ($F_{1,135}$=3.97, p=0.048).

(e) Monarch behavior at year-round breeding locations

Our capture-mark-recapture (CMR) study in the fall allowed us to estimate site fidelity and duration of stay for monarchs at year-round breeding sites (Table 1). For a subset (N=24) of recovered butterflies, we confirmed migratory status with chemical analyses. However, because we could not determine natal origins for unrecovered monarchs, we proceeded with CMR

analyses under the assumption that infected butterflies were likely residents and uninfected butterflies likely migrants. Of 102 infected butterflies (presumably residents) in the CMR study, we recovered 40%, with on average 10 days between the first and last capture. Of 39 uninfected butterflies (presumably migrants), most were never recovered and thus stopover duration was no more than a few days. However, three uninfected individuals remained at the year-round breeding site (of original capture) for two weeks or more; two of these butterflies stayed 20 days and were later determined to be migrants through chemical analysis. Chemical analyses also later confirmed that 9 additional migrants, which were infected, remained at the locations 10 days or more. Of these, 7 were from southern latitudes and 2 from northern. This very limited dataset suggests that most migrants continue moving southwards, yet some small fraction of butterflies halt their journeys and get recruited to year-round milkweed sites. In this case, among the uninfected monarchs suspected to be migrants, 8% (or 3 of N=38) remained at year-round sites.

DISCUSSION

At the peak of their southward migration during the fall, migratory monarchs flooded into locations in coastal Texas where they encountered exotic milkweed and resident monarchs with high loads of protozoan parasites. Our analyses indicated that migrants at these year-round tropical milkweed locations were more likely to show infections and to be reproductively active compared to other migrants, the large majority of which are uninfected and in reproductive diapause. While most migrants that visited tropical milkweed gardens continued to migrate to Mexico, a small number remained (staying 7 to 20 days). In the spring, when overwintering monarchs from Mexico returned to Texas to lay eggs, we observed (with a limited sample size) that migrants shared milkweed habitat with residents, although to a lesser extent than in the fall,

both at sites with native and exotic year-round milkweed.

While migratory monarchs typically postpone reproduction to conserve energetic reserves, migratory monarchs at year-round breeding locations were more likely to be reproductive, compared to migrants at other stopover sites. We emphasize that this finding with site type was of modest statistical significance (with $p=0.026$) and that monarch sex was a stronger predictor of reproduction. However, male migrants collected from year-round sites showed a distinctive pattern for higher reproductive activity than at stopover sites (Figure 2). Cage effects on reproduction were possible, however, we expect any such effect would be similar for migrants at either site type. One potential explanation for the pattern in migrant reproduction could be that exotic milkweed in good condition in the fall (in contrast to most native milkweeds) could induce butterflies to break reproductive diapause, as some investigators have hypothesized [43]. Previous experiments have found that milkweed age, in addition to the day length and temperature, play a role in controlling diapause [53]. Most migrants in our study remained at tropical milkweed sites for only short periods of time, however, and it is unclear whether such temporary exposure could induce a physiological change as strong as reproductive development [43]. A second, more likely explanation could be that year-round breeding sites along the coastal flyway attract migrants that are already reproductively active. Past work showed that while most migrants are in diapause by mid-fall, a small portion are reproductive (e.g., [60]). Year-round breeding sites with warm temperatures and continuously available host plants may recruit these migrants.

A higher proportion of migratory monarchs at year-round breeding sites were infected compared to other migrants. This could result from butterflies acquiring spores passively, for example, while in contact with milkweed leaves covered in OE spores; for instance, we observed

8 migrants nectaring on tropical milkweed. As an alternative explanation, higher infection prevalence among migrants at these sites could occur if tropical milkweed gardens particularly attract parasitized migrants. Previous laboratory studies demonstrated that infected females preferred to oviposit on tropical milkweeds, which offer highly toxic cardenolides that can reduce parasite load in larval offspring [81]. Tropical milkweed gardens could, then, disproportionately draw in infected monarchs.

Infection risk to migrants' offspring during the spring, when migratory butterflies are returning to Texas to lay eggs, is of particular concern if residents and migrants share milkweed habitat. We detected migrants and residents co-occurring and ovipositing at milkweed habitats in Spring 2015 (N=48 spring adults), both at year-round monarch-breeding sites and at one stopover site. We observed 6 resident and 4 migrant females ovipositing on *A. curassavica* at year-round sites; one resident and two migrant females oviposited on *A. asperula* at the stopover site. Migrants that lay eggs in year-round tropical milkweed gardens will expose offspring to high OE infection risk. Our study showed that 41% of larvae and pupae collected from year-round sites became infected. This is consistent with findings from citizen science data [44]. As we noted with two individuals, residents from year-round sites also dispersed to inland habitats with native milkweed in the spring (where we measured 0% infection prevalence from pre-adults). This could represent another route for pathogen transmission from residents to migrants, although additional data are needed to assess resident movements. If migrants and residents share breeding habitat frequently, infection levels could increase among the first generation of spring monarchs, most of which are produced in Gulf coast states and are critical to the larger migratory population, colonizing 90% of the breeding range [51,75].

Migratory monarchs face numerous threats and have undergone a 90% decline in North America in the past two decades [82]. Recent studies highlighted evidence for significant losses of monarchs during the fall migration, with drivers remaining unidentified [83,84]. Any effect of resident monarchs on migration is not likely one of the major drivers. Yet understanding the types of habitats through which migrants travel on their journeys and how these habitats influence monarch health, behavior, or migratory success is critical. Here, we investigated potential consequences for migrants that share habitat with resident conspecifics. Our study presents the possibility that year-round breeding sites could disproportionately attract reproductive or infected migrants, in which case any offspring produced by the migrants at these sites will face extremely high infection probability. Alternatively, year-round tropical milkweed sites may induce monarchs to break diapause, in which case migrants can halt their migrations altogether – as we directly observed for multiple migrants – or will be unlikely to survive the overwintering period. The explanatory mechanism remains unknown. In either case, however, tropical milkweed sites in the southern coastal areas do not appear to be significantly supporting the migratory monarch population. Additional evidence comes from the known-migrant data in our study, showing that 1% of migrants in Mexico originated from *A. curassavica.* Similarly, among the unknown Texas monarchs we examined, only 0.8% of monarchs classified as migrants originated from *A. curassavica.* Rather than contributing to the migration, some scientists have raised the question of whether year-round tropical milkweed could even remove monarchs from the migration. Our capture-mark-recapture data demonstrated that these locations recruited a small number of butterflies classified as migrants to delay or halt migration. The direct cause of such behavior changes, unusual among the monarchs we studied, could be the presence of year-round exotic milkweed or could be an unrelated driver. Even with such

questions remaining, the confluence of several recent studies alongside our findings here suggests that seasonal (rather than year-round) milkweeds best support migratory monarchs.

Beyond monarchs, understanding the extent and risks of migrant-resident co-occurrence could be useful for predicting or preventing consequences for migrants across taxa. Many migratory species are in decline, due to overharvesting and habitat alteration [21], or are undergoing shifts towards more sedentary behaviors, facilitated by human activities [10–15,85]. As a result of these changes, migrant-resident interactions may become more common in the future, and could be consequential for migratory populations already facing multiple stressors. For partially migratory species (including monarchs), migrant encounters with residents have intrigued and, historically, eluded ecologists. Methods in chemical ecology enabled us to distinguish migratory strategies among monarch butterflies and assess the frequency and effects of their geographic overlap. This study enhances our understanding of implications of these interactions for migrants' reproductive behavior and infectious disease risk. Our findings underscore growing scientific support for prioritizing the preservation not only of migratory animals themselves, but their behaviors and propensities for migration, which can reduce infectious disease risk and contribute to ecosystem function [27,86].

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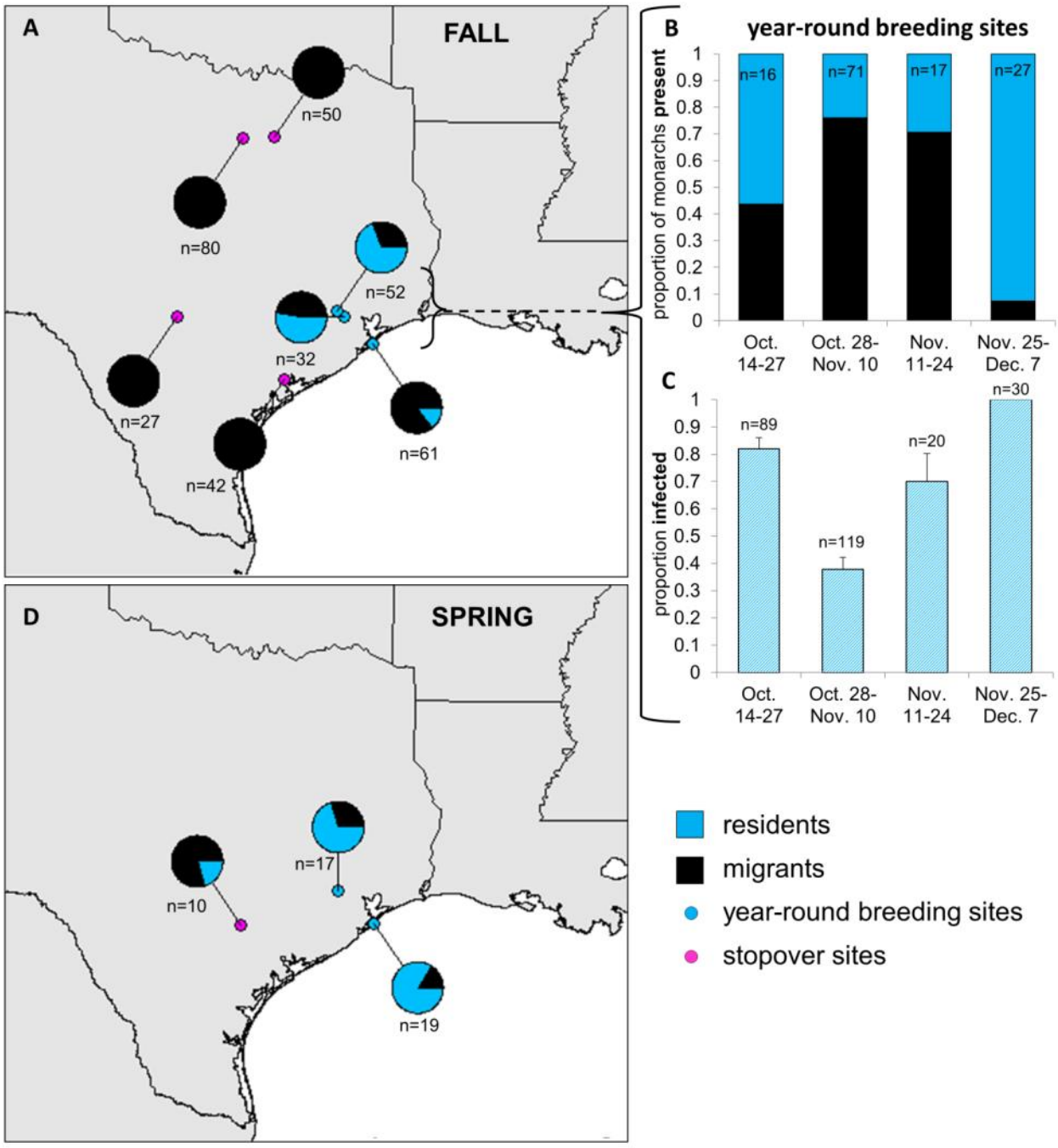

Figure 1. Migrant and resident monarchs mixed geographically during migration in Texas.

(A) Map of sampling locations in Fall 2014 with the proportion of sampled adult monarchs that were determined to be migrants (black) or residents (blue) at year-round breeding sites (blue points) and stopover sites (pink points).

(B) Dynamics in the proportion of migrants vs. residents at year-round sites during the fall.

(C) Dynamcis in infection prevalence of adult monarchs at year-round sites during the fall.

(D) Map of sampling locations in Spring 2015 with proportions of sampled adult monarchs.

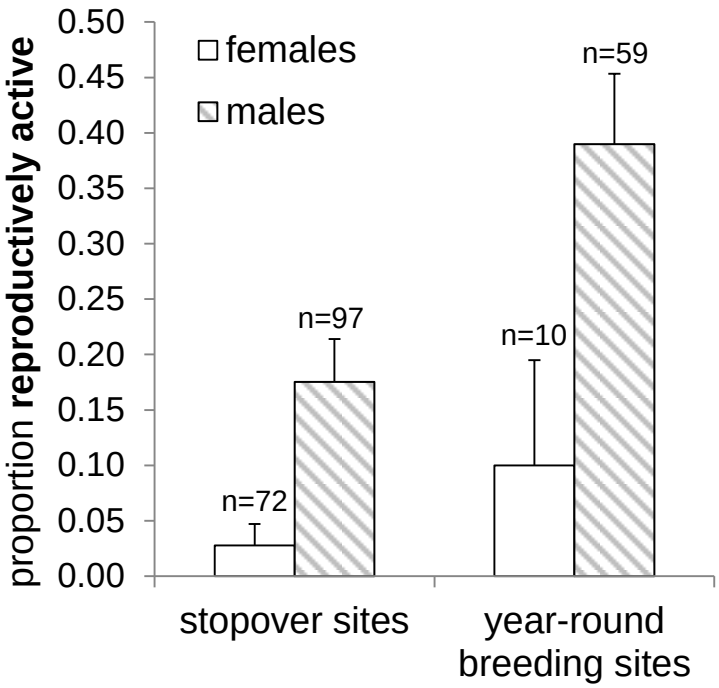

Figure 2. Proportion of migrant monarchs by sex that were reproductively active across site types. Males were significantly more likely to be reproductive, regardless of site type. Migrants were more likely to be reproductive at year-round locations compared to stopover locations.

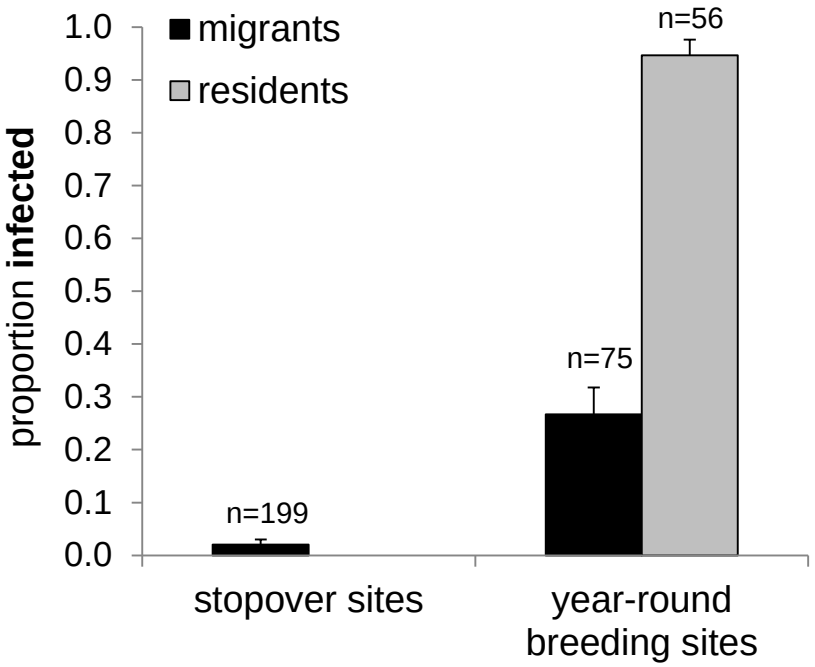

Figure 3. Infection prevalence by migratory type in Fall 2014, at stopover sites and year-round breeding sites. Resident monarchs were more likely to be infected than migrants, as expected based on previous work. Migratory monarchs sampled at year-round breeding sites were significantly more likely to show OE infections than migrants at other stopover sites.

Table 1. Summary of capture-mark-recapture sample sizes, recovery rates, and estimated parameters from the top-ranked (by DIC) Cormac-Jolly-Seber model. Note that because of small sample sizes at site 1, only sites 2 and 3 were used in the model. Additional details about the model are in the ESM.

	Uninfected monarchs (likely migrants)	**Infected monarchs** (likely residents)
Proportion recovered	0.08	0.40
Total captured and released	39	102
Site 1: Houston (site 1)	1	10
Site 2: Houston (site 2)	10	35
Site 3: Galveston	28	57
Total natal origins determined	11	12
Migrants	11	3
Residents	0	9
Model results		
Site fidelity	0.74 ± 0.14	1.0 (assumed)
Apparent survival	0.43 ± 0.08	0.57 ± 0.04
Capture probability	0.38 ± 0.05	

CHAPTER 6

CONCLUSIONS

Previous studies indicated that long-distance migrations can reduce parasite burdens for wild hosts. In these cases, migration can act to limit host exposure in parasite-contaminated habitats or cull infected individuals from the population [1]. Building on this foundation, my dissertation research investigated consequences for infectious disease patterns when migrations unravel due to human activities. We found that sedentary monarchs breeding year-round on exotic milkweed experienced significantly higher infection risk compared to migratory butterflies. This pattern was striking in two distinct monarch populations, in the southern U.S. (Ch. 2) and in California (Ch. 3). These studies are among the first to show that the loss of host migratory behaviors can lead to greater parasite infection prevalence. The loss of migration could enhance both direct transmission (because infected hosts that forego migration could be more likely to survive) and environmental transmission (because host populations using the same habitat year-round can continuously acquire parasites from the environment). Our mathematical model for local transmission of protozoan parasites among monarchs showed that environmental transmission and parasite longevity in the environment was critical for the parasite to sustain itself locally even during a typical breeding season during the summer (Ch. 4). Finally, we found that migratory monarchs share habitat with resident monarchs during the fall and spring migrations, with implications for parasite transmission (Ch. 5).

This research also uncovered questions for future work. One area for further investigation is to identify other factors that influence patterns in infection prevalence. My dissertation

research showed that host migratory behavior at a large geographic scale (Ch. 2 and 3) and parasite environmental persistence and shedding rate at a local scale (Ch. 4) were important drivers of infection dynamics. However, field studies in the monarch-OE system (in this dissertation and in previous studies) demonstrate site-to-site heterogeneity in infection prevalence, for which we cannot identify explanatory factors. Future studies could examine how site-level variables such as host larval density and habitat patchiness affect infection patterns. Influential site-level drivers of infection as well as a measurement of parasite decay rate in the environment (which our experiment in Ch. 4 was not able to quantify) could inform a mathematical model similar to the one we have constructed and better predict prevalence levels observed in the wild.

A second outstanding area for future work is to quantify the net impact of year-round resident sites on migratory monarchs. This would require first estimating the geographic extent and density of locations with year-round exotic milkweed and resident monarchs. Overlaying the geographic distribution of year-round milkweed sites with the range for migratory monarchs' re-colonization into the southern U.S. in the spring could be useful for understanding to the threat of infection for migrants' offspring. Future work could then incorporate year-round milkweed locations into a spatially explicit mathematical model for monarchs' annual cycle in North America, capturing the movement, re-colonization, and population growth of monarchs through their multiple generations each year. This would help conservationists better assess the effect of these exotic milkweed gardens on the larger migratory monarch population, which our work here was not able to measure. Our work showed that year-round monarch breeding in tropical milkweed gardens harbor high levels of a debilitating parasite, with demonstrated opportunities for transmission to migrants and their progeny.

This dissertation research raises concerns about infectious disease responses in other recently established sedentary populations. Numerous migratory bird and mammal populations have become more sedentary in recent decades. These changes are described in the literature for several dozen species, many of which formed in the 1980s or later around supplemental resources (e.g., wheat fields, garbage dumps) or were associated with mild winters [3–6]. Not all sedentary populations will experience high levels of infection, as we observed in monarchs. A study in European blackbirds, for instance, demonstrated lower tick and avian malaria prevalence in urbanized sedentary birds relative to rural birds [7]. A handful of other studies, however, have shown similar infection outcomes as we observed here: Sedentary populations of elk, salmon, and fruit bats (formed through supplemental feeding or aquaculture) experienced higher levels of parasitism from *Brucella*, sea lice, and Hendra virus, respectively, relative to more mobile populations [8–10]. While field studies comparing sedentary and migratory populations have been rare, my dissertation work and the existing literature emphasize the need to investigate whether increased disease burdens due to the break-down of migration are common for resident populations.

Sedentary populations that do experience high levels of parasitism could affect migratory animals that encounter them. Interactions between residents and migrants are common across taxa, but historically have been difficult to study [11]. Understanding parasite transmission between residents and migrants could inform conservation efforts and help identify risks to threatened species. In my dissertation research, I investigated the degree to which resident monarch populations influence behaviors and transmit pathogens to migratory monarchs (Ch. 5). This study showed that migratory monarchs shared habitat with infected sedentary monarchs during their fall and spring journeys through Texas (Ch. 5). In addition, a subtle (though

statistically significant) pattern suggested that sedentary sites with tropical milkweed may attract reproductive and infected migrants, or may induce reproductive development in migrants, during a time when most monarchs prioritize energy allocation toward migration and overwintering. In the spring, some migrants and residents also shared milkweed breeding habitat, which could facilitate resident-to-migrant parasite transmission.

Our findings inform management recommendations for monarch habitat. Taken as a whole, our results show that tropical milkweed gardens in the southern coastal U.S. enable monarchs to breed continuously and that these non-migratory behaviors lead to declines in monarch health. Further, our findings suggest that tropical milkweed host plants in the U.S. and Canada do not significantly contribute to the larger migratory monarch population. There were two lines of evidence for this: *A. curassavica* plants produced few of the migrants (~1% or less) in our study from Mexico in the winter or Texas in the fall, based on results from cardenolide quantification (Ch. 5). This suggests that tropical milkweed is not producing a large number of migratory butterflies that arrive in Mexico, and that the vast majority of migrants come from native species of milkweed. Year-round tropical milkweed could also enhance infection prevalence for offspring of migrants in the spring. This depends on the extent to which migrants oviposit in tropical milkweed gardens along the Gulf coast (as we directly observed). Migrants that use these habitats for ovipositing would introduce first-generation spring monarchs into high-risk habitats [12]. Infected migrants from these locations would then transport parasites northward.

Altogether, these findings highlight the need for native milkweed species with phenology that more closely synchronizes with the monarch migratory cycle. Seasonal milkweeds that die back in the winter will not support the year-round monarch breeding behaviors that lead to high

infection risk. However, gardeners particularly in the South often cannot find native milkweeds at commercial nurseries, most of which do not stock milkweed or exclusively provide exotic varieties. For areas where tropical milkweed gardens are already established but cannot yet be replaced with native species, we recommend cutting back the plants each month during Oct.-Feb. to discourage monarch winter-breeding. For future milkweed planting efforts in the southern U.S., we recommend avoiding tropical milkweed. These management steps, coordinated at a regional scale, could reduce parasite burdens for monarchs in the southern U.S.

Our findings provide support that the loss of migration enhanced pathogen transmission in monarchs. Similar processes could be occurring in other newly sedentary populations. Conservation priorities for imperiled species should focus not only on restoring population sizes but protecting population health. For many species, this will require preserving the migratory behaviors that reduce parasite infection risk. Limiting supplemental resources – particularly year-round resources – could promote this aim.

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APPENDIX A

CHAPTER 2 SUPPLEMNTARY INFORMATION

To accompany: Loss of migratory behaviour increases infection risk for a butterfly host (Satterfield, Maerz, and Altizer)

I. Historical occurrences of tropical milkweed and monarch winter-breeding

(a) Herbaria and record searches

We conducted an herbaria search and reviewed historical documents to better understand when the planting of tropical milkweed and monarch winter-breeding became more common in the southern U.S. Through the Index Herbariorium [1] and other web resources, we identified 175 distinct herbaria in the U.S. for which records were accessible online (in addition to the University of Georgia Herbarium), relative to an estimated 800 total herbaria nationwide [2]. We searched for records of *Asclepias curassavica* in the continental U.S. without date restrictions and eliminated duplicate specimen records. We performed a similar search for a native species, butterfly milkweed (*Asclepias tuberosa)*, to observe if any trends in tropical milkweed records were simply attributable to greater collector interest or awareness about milkweeds. Further, to survey distribution and gardening practices concerning tropical milkweed, we searched the Biodiversity Heritage Library (www.biodiversitylibrary.org) for occurrences of "*Asclepias curassavica*" in English-language documents from the U.S. Finally, we surveyed natural history literature on monarchs to understand when winter-breeding was previously documented in the southern U.S.

(b) Results

Eleven historical documents from the Biodiversity Heritage Library indicate that tropical milkweed was being planted in American gardens and hot-houses in the 19th century, as early as 1806 [3]. Articles from historical gardening journals and manuals discuss how to plant tropical milkweed (e.g., [4], dated 1898) and describe *A. curassavica* as “often cultivated for ornament in our Southern States" ([5], dated 1901), “worthy of a place in our gardens,” (see [6] from 1890), and a "choice flowering annual adapted for sowing on a hot-bed" ([7] from 1841). An additional eight records note the distribution of tropical milkweed. By the dawn of the 20th century, tropical milkweed was described as occurring in the southern U.S. with a limited distribution (e.g., see [8] from 1897, [9] from 1912). In 1954, Woodson [10] noted its occurrence as “occasional ruderals” in southern California, Florida, Louisiana, and Texas.

The herbaria search showed that records of tropical milkweed have become more common in the southern U.S. in recent years. The search yielded 101 records of tropical milkweed; most records (n=72) were from Florida. Of these, records from south Florida (south of Sarasota, n=43) tended to have earlier collection dates (mean date=1978, range = 1936-2011) than those from north Florida (n=29; mean date = 1995, range=1960-2012). Other records came from Arizona (n=1), California (n=13 with mean date = 1986, range = 1909-2011), Connecticut (n=2), Louisiana (n=4), Mississippi (n=1), Missouri (n=2), South Carolina (n=5), and Utah (n=1). Examining temporal changes in these records across the southern half of the U.S. (n=96, including AZ, CA, LA, MS, SC, and FL), we found that few records existed before 1940 and a modest increase in records occurred over the last 50 years. Notably, a rise in records occurred in the 1960s and 1970s, followed by a sharper rise in the 2000s (Figure S1*a*). A similar temporal

trend is observed when excluding south Florida, which was likely the first place in the U.S. to be colonized by tropical milkweed. The herbaria search of the native butterfly milkweed (*A. tuberosa*) yielded 349 records, primarily from South Carolina, Florida, and Alabama. As with tropical milkweed, records of butterfly milkweed were scarce before 1940 and increased in the 1960s. However, record frequency for native butterfly milkweed appears fairly constant in recent decades, with little change in number of specimens per decade since 1970 (Figure S1*b*).

A survey of historical scientific literature indicates that monarchs have used tropical milkweed during the regular breeding season for as long as it has been available, but that breeding behaviours during winter were not observed until later. For example, two early records note monarch caterpillars feeding on tropical milkweed during the spring and summer. The first mention of *A. curassavica* in the U.S. occurred in a Georgia natural history book published in 1797 [11]. Abbot illustrates a monarch caterpillar ("Papilio Archippus") feeding on tropical milkweed in April, presumably in 1797 or before. Another report describes monarch larvae on *A. curassavica* in Missouri in August 1868 [12]. The first mention of winter-breeding in the southern U.S. appeared in 1937 [13], when monarch eggs and larvae were observed in January on *A. curassavica* in Orlando, FL. Four additional records in Texas and Jacksonville, FL note monarch larvae during the winter of 1957-1958 [14]. To our knowledge, no additional records of winter-breeding in the southern U.S (excluding south Florida) appear in the literature until 2010, when 95 sightings of winter-breeding monarchs (larvae, pupae or ovipositing females) were recorded in the southern U.S. between 2002-2010 above 27ºN [15].

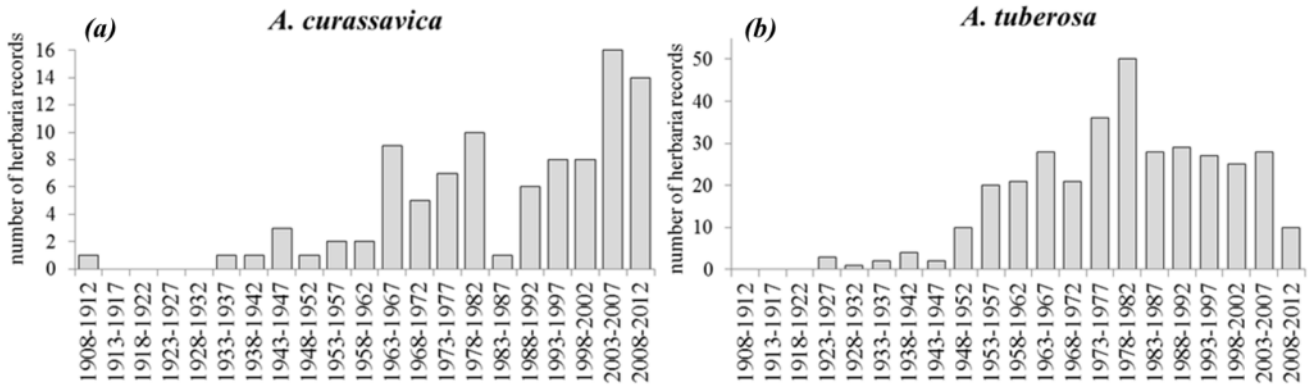

Figure S1. Number of herbaria records documented in the southern U.S. between 1908 and 2012, for (a) tropical milkweed, *A. curassavica*, and (b) butterfly milkweed, *A. tuberosa*, based on a search of 176 herbaria. A simple analysis of covariance examining records over time showed significant differences for *A. curassavica* and *A. tuberosa* records.

(c) Conclusions

These searches suggest that, at least to a limited extent, tropical milkweed has long been a part of North American gardens, and monarchs utilized it during the typical breeding season. During the past two decades, modest increases in *A. curassavica* herbaria records and widespread observations of winter-breeding activity on tropical milkweed have occurred. It is important to distinguish sightings of *winter-breeding* monarchs (eggs, larvae, or pupae during Dec-Feb) from sightings of *adult* monarchs found during the winter. Small sub-populations of adult monarchs have been found in the southern U.S. flying or roosting (but not breeding) during the winter months as early as 1875 [16]. These adults overwinter especially along the Florida Gulf coast and to a lesser degree along the southern Atlantic coast. We examined parasite samples from these "coastal overwintering" populations, discussed in the main text.

II. Additional tables and figures

Table S1. Number of monarch field samples and sampling locations by year and source population. Site locations varied over the two years of the study, with some sites sampled both years and other sites sampled during only one year. Summer 2011 through winter 2012 is considered Year 1; summer 2012 through winter 2013 is Year 2. Winter-breeding samples included those collected by investigators at the University of Georgia (N=51 and N=45 for years 1 and 2, respectively) and by volunteer citizen scientists (N = 352 and 219 for years 1 and 2). Samples were assigned to sub-regions nested within each source for the purpose of the statistical analysis (linear mixed model) described in section III below. Our sub-regions were based on NOAA U.S. climate regions [17], however, we note exceptions: (i) a single site in Kansas was grouped into the Ohio Valley, (ii) sites in Manitoba, Canada were grouped into the Upper Midwest, (iii) sites in Southern Ontario comprise an additional sub-region, and (iv) sites in the southeast are separated into coastal and non-coastal sub-regions. U.S. states are abbreviated.

source population	sub-regions	states/colonies	milkweed habitat	time periods	total samples	total sites
summer-breeding	Ohio Valley Northeast Southeast Southern Ontario Upper Midwest	OH, IL, IN, TN, KS CT, NH, NJ, NY, PA NC, VA Ontario, Canada IA, MI, MN, WI; Manitoba, Canada	primarily native milkweeds including *A. syriaca*, *A. incarnata*, *A. tuberosa*	year 1 (June 1-Oct. 1, 2011)	1276	54
				year 2 (June 1-Oct. 1, 2012)	1290	52
Mexico overwintering	Michoacán	Sierra Chincua and Cerro Pelón colonies	no milkweed	year 1 (March 5, 2012)	835	1
				year 2 (Feb. 12-15, 2013)	1555	2
winter-breeding	South central Coastal southeast	LA, TX FL, GA, SC	tropical milkweed (*A. curassavica*)	year 1 (Dec. 1-March 1, 2011-2012)	403	23
				year 2 (Dec. 1-March 1, 2012-2013)	264	18
coastal overwintering	Coastal southeast	FL, SC	no milkweed	year 1 (Dec. 1-March 1, 2011-2012)	169	3
				year 2 (Dec. 1-March 1, 2012-2013)	85	3

Table S2. Origins of *OE* isolates in virulence experiment (location, date, sex, and population).

isolate	city/colony	state	date collected	sex	source population
1	Atlanta	GA	10/14/2011	M	Summer-breeding
6	Sylvester	GA	10/24/2011	M	Summer-breeding
7	Hazelton	IA	8/25/2011	M	Summer-breeding
15	Lawrence	KS	9/12/2011	M	Summer-breeding
20	Dugald	Manitoba, Canada	7/23/2011	F	Summer-breeding
21	Pinconning	MI	9/13/2011	M	Summer-breeding
31	Gilbert	MN	8/25/2011	F	Summer-breeding
36	Durham	NC	9/28/2011	F	Summer-breeding
42	Athens	GA	9/12/2011	F	Summer-breeding
46	Katonah	NY	9/5/2011	F	Summer-breeding
47	Willoughby Hills	OH	7/8/2011	F	Summer-breeding
61	Millersburg	PA	9/21/2011	F	Summer-breeding
64	Newtown	PA	10/4/2011	F	Summer-breeding
66	Phoenixville	PA	9/14/2011	M	Summer-breeding
69	Oakridge	TN	8/1/2011	M	Summer-breeding
74	Arlington	TX	10/8/2011	M	Summer-breeding
78	Wisconsin Rapids	WI	6/12/2011	M	Summer-breeding
2	Cerro Pelón	Michoacán, MX	3/5/2012	M	Mexico overwintering
12	Cerro Pelón	Michoacán, MX	3/5/2012	F	Mexico overwintering
13	Cerro Pelón	Michoacán, MX	3/5/2012	M	Mexico overwintering
14	Cerro Pelón	Michoacán, MX	3/5/2012	M	Mexico overwintering
19	Cerro Pelón	Michoacán, MX	3/5/2012	M	Mexico overwintering
23	Cerro Pelón	Michoacán, MX	3/5/2012	M	Mexico overwintering
24	Cerro Pelón	Michoacán, MX	3/5/2012	F	Mexico overwintering
26	Cerro Pelón	Michoacán, MX	3/5/2012	M	Mexico overwintering
34	Cerro Pelón	Michoacán, MX	3/5/2012	F	Mexico overwintering
38	Cerro Pelón	Michoacán, MX	3/5/2012	M	Mexico overwintering
45	Cerro Pelón	Michoacán, MX	3/5/2012	F	Mexico overwintering
48	Cerro Pelón	Michoacán, MX	3/5/2012	F	Mexico overwintering
51	Cerro Pelón	Michoacán, MX	3/5/2012	F	Mexico overwintering
54	Cerro Pelón	Michoacán, MX	3/5/2012	M	Mexico overwintering
55	Cerro Pelón	Michoacán, MX	3/5/2012	F	Mexico overwintering
62	Cerro Pelón	Michoacán, MX	3/5/2012	F	Mexico overwintering
68	Cerro Pelón	Michoacán, MX	3/5/2012	F	Mexico overwintering
72	Cerro Pelón	Michoacán, MX	3/5/2012	F	Mexico overwintering
73	Cerro Pelón	Michoacán, MX	3/5/2012	M	Mexico overwintering
77	Cerro Pelón	Michoacán, MX	3/5/2012	F	Mexico overwintering
4	Savannah	GA	1/5/2012	M	Winter-breeding
8	San Antonio	TX	2/10/2012	M	Winter-breeding
9	Galveston	TX	2/15/2012	M	Winter-breeding
17	Hitchcock	TX	1/5/2012	F	Winter-breeding
25	Kenner	LA	2/19/2012	M	Winter-breeding
32	Useppa Island	FL	2/21/2012	M	Winter-breeding
33	Austin	TX	12/19/2011	F	Winter-breeding
35	Seabrook	TX	2/2/2012	F	Winter-breeding
37	Lakeland	FL	1/22/2012	F	Winter-breeding
41	New Orleans	LA	1/25/2012	F	Winter-breeding
43	Melbourne	FL	2/12/2012	M	Winter-breeding
49	Houston	TX	12/28/2011	F	Winter-breeding
52	League City	TX	1/26/2012	F	Winter-breeding
53	Port St. Joe	FL	12/4/2011	F	Winter-breeding
56	League City	TX	2/14/2012	M	Winter-breeding
58	Orlando	FL	1/7/2012	F	Winter-breeding
63	Hitchcock	TX	12/21/2011	F	Winter-breeding
65	Houston	TX	12/30/2011	M	Winter-breeding
70	Seabrook	TX	12/25/2011	F	Winter-breeding
75	New Orleans	LA	12/15/2011	M	Winter-breeding

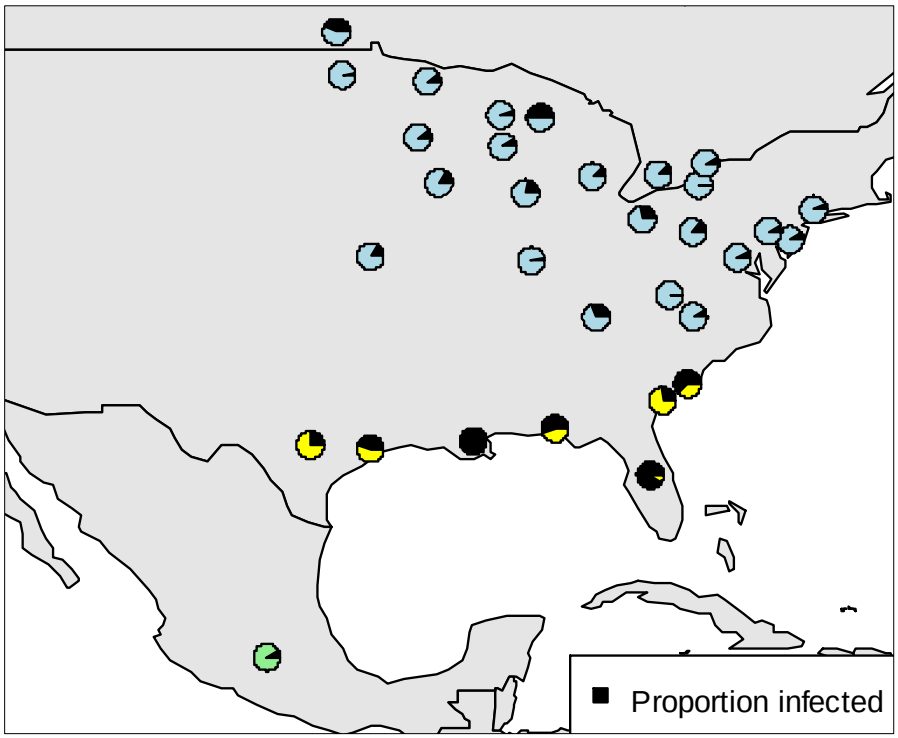

Figure S2. Proportion of monarchs heavily infected with *OE* during 2011-2013, aggregated at the state or sub-state level. Pie charts indicate prevalence (black shading) at winter-breeding sites (yellow), summer-breeding sites (blue), and Mexico overwintering sites (green). Coastal overwintering sites are not shown. Each pie chart represents multiple sites grouped by proximity for easier visual display. In the U.S., sample size per pie chart ranges from 8 to 380 samples.

Table S3. Model-averaged parameter estimates for generalized linear mixed model for infection status among all monarchs. For each predictor variable, the table shows relative importance Σ (sum of AIC weights for all top models that included the predictor), regression coefficient b, standard errors, z-values and p-values. Model-averaged parameters are based on top models for which ΔAIC<10. Component models are in table S4.

predictor	**relative importance, Σ**	**level**	**regression coefficient, b**	**standard error**	**z value**	**p value**
source (*reference*: winter-breeding sites)	1.00	Mexico overwintering	-3.32	1.17	2.85	0.0044**
		coastal overwintering	-1.68	0.40	4.18	<0.0001***
		summer-breeding	-3.54	0.44	8.10	<<0.0001***
collection stage (*reference*: larva)	1.00	adult	-0.79	0.21	3.80	0.0001***
year (*reference*: year 1)	0.96	year 2	0.44	0.15	2.91	0.0036**
sex (*reference*: female)	0.69	male	0.22	0.12	1.91	0.0566 NS

Table S4. Component models for model-averaged generalized linear mixed model for infectious status among monarchs. For each component model, the table shows factors included, log-likelihood value, ΔAICc relative to full model, AICc weight, log-likelihood, deviance, marginal R^2 (variance explained by fixed factors) and conditional R^2 (variance explained by both fixed and random factors). For all models, site was treated as a random factor nested within source.

	fixed factors				**Δ AICc**	**AICc weight**	**log-likelihood**	**deviance**	**marginal R^2**	**conditional R^2**
	source	**collection stage**	**year**	**sex**						
model 16	yes	yes	yes	yes	0.00	0.662	-2082.3	4164.5	0.128	0.377
model 14	yes	yes	yes		1.61	0.296	-2084.1	4168.1	0.126	0.375
model 8	yes	yes		yes	6.32	0.028	-2086.4	4172.8	0.121	0.382
model 6	yes	yes			8.14	0.011	-2088.3	4176.7	0.120	0.380

III. Analysis of infection prevalence with a linear mixed model

(a) Methods

We used a linear mixed model in package *nlme* in R v.3.0.3 to examine effects of source, year, and a source-year interaction on infection prevalence. We assigned each site to one of eight sub-regions based on climate (as detailed in table S1). Sub-region was treated as a random effect nested within source. The purpose of aggregating sites was to reduce spatial correlation among prevalence data and determine whether source remained an important explanatory factor. Prevalence per site per year was arcsine-square-root-transformed to normalize variance, and sites with fewer than 8 samples were excluded from this analysis. Because coastal overwintering sites overlap spatially with some winter-breeding sites, coastal overwintering sites are excluded from this analysis to allow sub-region to be fully nested within source (i.e., coastal southeast sub-region nested within only the winter-breeding source population).

(b) Results and conclusions

Large differences in prevalence attributed to source were significant in the linear mixed model ($F_{2,5}$=18.50, p=0.005). Infection prevalence was several times higher among non-migratory (winter-breeding) monarchs sampled in the southern U.S. compared to migratory monarchs sampled at Mexico overwintering sites (t_5=3.12, p=0.03) or in the summer-breeding range (t_5=6.10, p=0.0017). Prevalence estimates for migratory monarchs from summer-breeding sites compared to Mexico overwintering sites were not statistically different. Infection prevalence during the second year of the study was marginally higher than in the first year in the linear mixed model ($F_{1,83}$=4.12, p=0.046).

Results from this linear mixed model, in which sites were aggregated in sub-regions, are congruent with those found using individual-level infection status data (reported in the main

text), indicating that significant differences in prevalence persist among sources even when spatial correlation among sites is removed.

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APPENDIX B

CHAPTER 3 SUPPLEMENTARY INFORMATION

To accompany: Migratory monarchs wintering in California experience low infection risk compared to monarchs breeding year-round on non-native milkweed *(Satterfield, Villablanca, Maerz, and Altizer)*

Table S1. Parameter estimates for generalized linear mixed model for infection status among all monarchs. For each predictor variable, the table shows regression coefficient b, standard errors, z-values and p-values.

predictor	**level**	**regression coefficient, b**	**standard error**	**z value**	**p value**
source (*reference*: overwintering sites)	year-round breeding sites	4.41	0.84	5.24	<<0.0001***
year (*reference*: year 1, 2013-2014)	year 2 (2014-2015)	0.57	0.30	1.90	0.057, NS
	year 3 (2015-2016)	1.12	0.32	3.48	0.0005***
sex (*reference*: females)	males	0.20	0.12	1.67	0.95, NS
Source*year interaction (*reference*: female)	year-round breeding sites: year 2	-0.28	0.37	-0.75	0.46, NS
	year-round breeding sites: year 3	-1.24	0.51	-2.43	0.015*

APPENDIX C

CHAPTER 4 SUPPLEMENTARY INFORMATION

To accompany: Environmental Persistence Influences Infection Dynamics for a Butterfly Pathogen (Satterfield, Altizer, Williams, and Hall 2016)

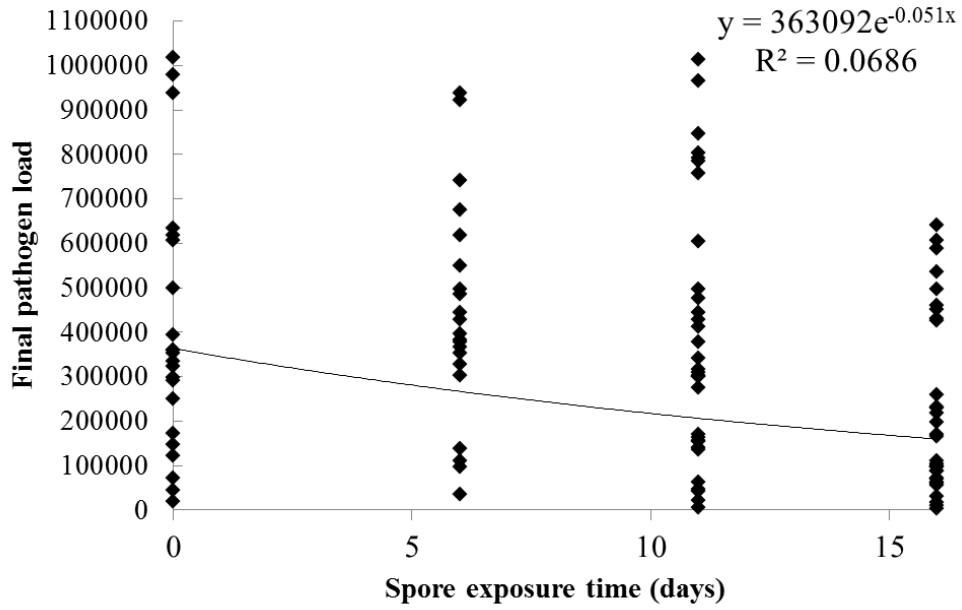
S1 Pathogen information

Origin of pathogen isolates

We chose three pathogen isolates for our environmental persistence experiment. Isolates E3, E13, and E10 were selected to represent low, moderate, and high levels of virulence, respectively, as documented in a previous experiment [1]. Virulence was measured as the inverse of the lifespan of infected adult monarchs; shorter lifespan indicated higher virulence. Isolates were originally collected from wild monarchs in eastern North America, with isolate E3 collected from Cape May, NJ; isolate E13 from Sweet Briar, VA; and isolate E10 from St. Paul, MN. Isolates have been passed through live monarchs to propagate spores multiple times since collection. Isolates were stored at 12°C until we exposed spores to outdoor conditions in our experiment. Prior work suggested that spores can remain viable for long periods at 12°C. The high rate of infection in our experiment even within the control treatment (not exposed to outdoor conditions) indicated the spores used in our study were viable [S. Altizer, unpublished].

Experimental Results: Pathogen load

We measured pathogen load of infected butterflies at the end of our experiment as a proxy of *infection severity.* Lower spore loads indicate less severe infections. Among infected monarchs, total pathogen load declined with greater exposure time to the environment.



S1 Fig 1. Exponential decay curve fit to pathogen load data over time.

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S2 Model parameterization

We represent the number of monarchs in pre-adult stages (egg, larvae, and pupae) in model compartments S_L (susceptible) and I_L (infected). Monarchs in the adult stage are represented in the S_A (uninfected) and I_A (infected) compartments. Because monarchs are among the most well studied invertebrates in the natural world, we can derive most parameter values for our model from the published literature. We use the degree-day model (described in [1]) to calculate development time for wild monarchs experiencing typical temperatures in the summer-breeding range: At 25.6°C (an average low daily temperature for June in Pennsylvania), breeding monarchs develop from eggs to eclosion in 26 days. Thus, we assume larval development in our transmission model occurs at a rate of g=1/26.

Previous work using outdoor cages showed that uninfected adult female monarchs live 24 days on average and infected adult females live 20 days on average [2]. Based on this study, our model assumes adult monarch mortality occurs at μ_A=1/24 for uninfected individuals and μ_I=1/20 for infected individuals.

We assume larvae (S_L and I_L) are produced at per-capita fecundity rate b. Past work showed that females produce an average of 715 eggs over their lifespan [3]. Assuming a 24-day adult lifespan and multiplying by ½ to only consider females in the population, we represent fecundity as b=15 eggs per day.

We represent larval consumption rate c as the total number of *Asclepias incarnata* milkweed leaves consumed per monarch. As larvae eat approximately 35 leaves over the 26-day time span from egg to eclosion [Satterfield, personal observations], we use c=1.35 leaves/day in the model.

We estimate the probability for infected monarchs successfully eclosing and reproducing, p_E, using experimental data from infected monarchs in captivity [4]: Monarchs with an average pathogen load of $10^{5.75}$ spores had a 0.9 probability of eclosing and a 0.8 probability of mating, yielding p_E=0.72.

Per capita larval mortality includes both density-dependent and density-independent components. In the absence of density dependence, the density-independent mortality rate μ_0 is related to the probability of surviving the 26-day period from egg to eclosion, *s*, such that

$$s = e^{-\mu 0 * 26}$$

Rearranging the equation to solve for density-independent mortality gives:

$$\mu_0 = -\frac{\ln s}{26}$$

Survival from egg to pupation has previously been reported as 0.12 [5] and is consistent with other estimates of survival [6]. Assuming s=0.12 yields μ_0=0.08 for density-independent larval mortality rate. To estimate density-dependent mortality μ_1, we first describe host dynamics in the absence of disease using a system of differential equations for number of larvae (N_L) and number of adults (N_A):

$$\frac{\mathrm{d}N_L}{dt} = bN_A - \left(\mu_0 + \mu_1 \frac{N_L}{M}\right) N_L - gN_L$$

$$\frac{\mathrm{d}N_A}{dt} = gN_L - \mu_A N_A$$

We can then solve the system of equations for μ_1 at equilibrium, when dN_L/dt and dN_A/dt are equal to 0:

$$\mu_1 = \left(\frac{M}{N_L}\right)\left(\frac{bg}{\mu_A} - \mu_0 - g\right)$$

We assume equilibrium larval density is 0.25 larvae/plant, based on the upper range of densities documented during the summer-breeding season in wild milkweed patches in the Midwest [6]. This places larval density per milkweed leaf at 0.01, assuming *A. incarnata* plants have an average of 25 leaves each [Satterfield, personal observation], making M/N_L=100 in the expression above. We use this and the parameter values previously described to obtain density-dependent larval mortality rate $\mu_{1=}$1372.

We incorporate pathogen environmental stages into the model by representing the number of pathogen-exposed milkweed leaves as *W*. Transmission of OE pathogens occurs when infected adult monarch deposit spores onto milkweed leaves and larvae consume the spores before they become inviable. However, little is known about the deposition and decay of OE pathogens on milkweed leaves in natural settings. Thus, we vary spore shedding rate λ ($1<\lambda<300$ leaves/day/infected adult) and pathogen environmental persistence ($1<\frac{1}{\mu_W}<80$ days) in the model and observe effects on infection prevalence and adult abundance. Our experimental findings suggested spores commonly persist at least 16 days and thus have the potential to persist for longer periods. Spore shedding rate λ could reasonably occur at a rate between 1 and 300 leaves/day/infected monarch, based on observations that breeding monarchs can visit up to 70 milkweed stalks per hour and remain active for several hours per day [A. Majewska, personal communication].

We set initial conditions in the model to represent a milkweed patch early in the breeding season – when no larvae are present (S_L=0 and I_L=0), when newly sprouted milkweed leaves are abundant in the patch (M=25000 leaves assuming a patch of 1000 plants with 25 leaves/plant), and when no leaves have been exposed to pathogens (W_0=0). We initially allow 18 uninfected adult monarchs (S_A=18 at T=0) and 2 infected adult monarchs (I_A=2 at T=0) to colonize the

milkweed patch. This assumes an initial infection prevalence of 10%, as observed in samples from wild monarchs collected by citizen scientists in the *Monarch Health* program (www.monarchpathogens.org). Specifically, *Monarch Health* samples from 1142 wild monarchs in the summer-breeding range collected early in the breeding season (April-July) in 2011-2014 indicate that early-season prevalence ranges from 5% to 20%, with an average of 12% across years. To use whole numbers of monarchs, we assumed 10% of monarchs were initially infected in our model.

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APPENDIX D

CHAPTER 5 SUPPLEMENTARY INFORMATION

To accompany: Migratory monarchs that share stopover habitat with sedentary conspecifics show greater reproductive activity and infection risk (Satterfield et al.)

I. Collection methods

In Fall 2014, adult monarchs were collected at three year-round breeding locations with tropical milkweed and at four stopover locations without year-round milkweed or monarchs. Stopover sites were sampled during the peak migration period (when the highest number of monarchs are observed for an area), which occurs earlier in the central flyway and several weeks later in the coastal flyway. Year-round breeding locations were sampled before, during, and after the peak migration in the coastal flyway. Additional site information is reported in Table S1. All collected monarchs were stored in an ice chest until processing (within 6 hours typically), when each individuals were tested for parasites, sexed, and measured for morphometric data. We limited parasite contamination during capture by wearing disposable gloves (changed multiple times per day), storing monarchs in individual glassine envelopes, and using a separate net between stopover sites and year-round breeding sites. During processing, we changed gloves after sampling every 5 individuals and wiped gloves with a cloth after every individual. Collected monarchs were then assessed for reproduction (Section III) and kept for chemical analyses.

Table S1. Collection dates and location information by site for collected monarchs in Fall 2014.

Site type	Site ID	Location	Description	Flyway	N	Phase in migration for monarch collection	Dates of monarch collection (2014)
Stopover	1	Fort Worth, TX (32.74º,-97.36º)	Multi-acre garden	central	80	During peak	Oct. 7-8
	2	Fair Oaks Ranch, TX (29.73º,-98.65º)	Small garden (<1 acre)	central	28	During peak	Oct. 17-20
	3	Dallas, TX (32.78º,-96.76º)	Multi-acre garden, with seasonal *A. curassavica*, A. *vertillicata*	central	50	During peak	Oct. 9-12
	4	Port Lavaca, TX (28.66º,-96.58º)	Causeway with wild flowers & few *A. oenotheroides*	coastal	42	During peak	Oct. 31-Nov. 2
Year-round breeding	5	Houston, TX (29.72º,-95.39º)	Medium garden, with a large patch of year-round *A. curassavica*	coastal	32	Before peak During peak After peak	Oct. 16 Oct. 31-Nov. 1 Dec. 4
	6	Houston, TX (29.82º,-95.54º)	Medium garden, with a large patch of year-round *A. curassavica*	coastal	52	Before peak During peak After peak	Oct. 17-23 Oct. 28-Nov. 7 Nov. 21-Dec. 5
	7	Galveston, TX (29.28 º, -94.85º)	Multi-acre garden with a large patch of year-round *A. curassavica*	coastal	61	Before peak During peak After peak	Oct. 14-22 Oct. 29-Nov. 3 Nov. 23

In Spring 2015, monarch adults and pre-adults (eggs, larvae, and pupae) were captured at three year-round breeding locations and two stopover locations during the spring migration, when monarchs are more spatially and temporally dispersed as they migrate northward. Monarchs were processed for infection and morphometric data but not for reproductive status during the spring, which is part of the traditional monarch breeding season. Natal origins were determined with chemical analyses. Additional information is in Table S3. We note that very few monarchs were sampled at Site 9.

Table S2. Collection dates, sample sizes, and location by site for spring 2015.

Site type	**Site ID**	**Location**	**Description**	**N adults**	**N pre-adults** (that survived to adulthood)	**Dates of monarch collection (2015)**
Stopover	8	Cheapside, TX (29.25, -97.42)	Old field, with wild flowers and *A. asperula*	12	10	April 6-10
	9	Cuero, TX	Roadside with wild flowers and *A. viridis*	0	3	April 7-11
Year-round breeding	5	Houston, TX (29.72º,-95.39º)	Medium garden, with a large patch of year-round *A. curassavica*	2	8	April 3
	6	Houston, TX (29.82º,-95.54º)	Medium garden, with a large patch of year-round *A. curassavica*	19	10	April 1-9
	7	Galveston, TX (29.28 º, -94.85º)	Multi-acre garden with a large patch of year-round *A. curassavica*	19	9	April 2-9

II. Capture-mark-recapture analyses

We conducted a capture-mark-recapture study at three year-round breeding sites during Fall 2014. Our main aim was to compare site fidelity and duration of stopover of migrant monarchs (or presumed migrants) relative to residents, rather than to precisely measure population size or vital rates. We also measured changes in monarch mass over time. During Oct. 14-Nov. 2, we sampled each site at least once within 4-day periods, such that each site was visited five times. During Nov. 3 –Dec. 5, we focused primarily on two sites (sites 6 and 7, with higher monarch abundance) and visited these locations four additional times at less regular intervals. Monarchs captured for the first time were processed for infection status, morphometric measurements, sex, and mass and marked with an adhesive tag with a unique code. Monarchs were stored temporary in a cool and typically processed within 3 hours after capture. Monarchs that were recaptured were re-weighed and assessed for wing condition. A subset of recaptured monarchs (with at least 5 days between captures) was collected for chemical analysis.

We tagged and released a total of 141 monarchs and recovered 44 individuals. Infected individuals were recaptured through the study period. Only 3 uninfected butterflies (of 39 tagged) were recaptured. (Table S3). For recaptured monarchs (most of which were infected), mass declined over time between initial and final capture dates (Figure S1). This could indicate that monarchs lose mass as they age, possibly as a result of lipid depletion or desiccation.

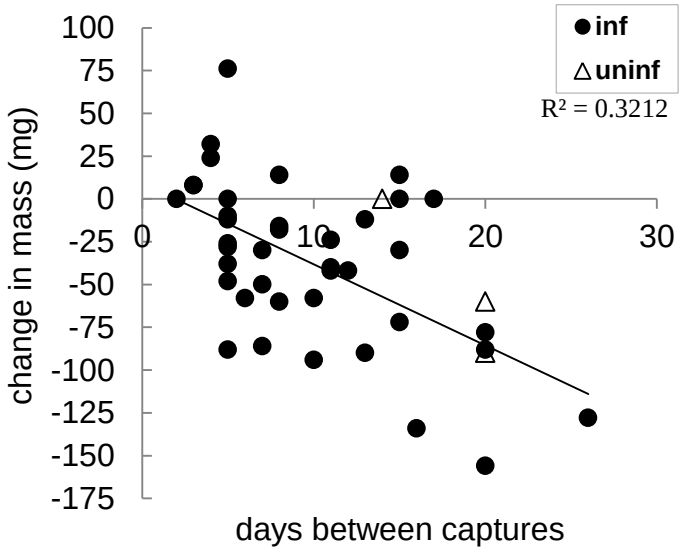

Figure S1. Changes in mass for infected and uninfected recaptured monarchs. Most monarchs lost mass between initial and later capture dates, with greater losses occurring as time elapsed.

Table S3. Time between first and last captures for uninfected and infected monarchs in the capture-mark-recapture study at three year-round breeding sites in Fall 2014. Uninfected monarchs are likely migrants whereas infected monarchs are likely residents or southern monarchs, based on known prevalence.

Time between first and last capture at original site	**Uninfected count** (likely migrants)	**Infected count** (likely residents)
not recaptured	36	61
2 days	0	1
3 days	0	2
4 days	0	2
5 days	0	9
6 days	0	1
7 days	0	4
8 days	0	4
10 days	0	2
11 days	0	3
12 days	0	1
13 days	0	2
14 days	1	0
15 days	0	4
16 days	0	1
17 days	0	1
20 days	2	3
26 days	0	1
Total tagged	**39**	**102**

We used Cormack-Jolly-Seber (CJS) models on capture-mark-recapture data to estimate capture rate and apparent survival, a function of fidelity and true survival. We used Bayesian hierarchical inference with Gibbs sampling (known as BUGS models) in the program *jags* through R to fit CJS models. We estimated group-specific rates for uninfected and infected monarchs separately, as a proxy for comparing migrants (91% of which are not infected) and residents (95% of which are parasitized). This was necessary as chemical confirmation of migratory status was not possible for most released monarchs. We evaluated (using DIC) three

models, with Model 1 assuming group-specific survival and site fidelity, Model 2 assuming group-specific fidelity but constant survival, and Model 3 assuming group-specific apparent survival. All models assumed constant capture rates. Model 3 was the best supported, as evaluated by DIC. We made two additional assumptions to estimate group-specific site fidelity. First, we assumed site fidelity was 1.0 for residents, an assumption that allowed us to calculate true survival for residents. Second, because we also assumed that true survival of migrants was equal to survival of residents, we were able to estimate site fidelity for migrants. Models were fit using 3 Markov-chain Monte Carlo (MCMC) chains with 10,000 iterations, with the first 5000 iterations removed. Model results are reported in the main text (Table 1). Note that due to small samples from site 5, we used samples only from sites 6 and 7 in CJS models.

III. Reproductive assessments in mating cages

We assessed male reproductive status based on mating activity in cages (0.5 m^2). Monarchs collected in the early to mid-fall were assessed in outdoor mating cages set up as close to the original collection sites as possible to mimic natural conditions. Wild males collected in Fort Worth and Dallas were housed in cages in Dallas (site 3), and males from Houston and Galveston were placed in cages in Houston and Katy (at other locations). Due to logistical limitations, monarchs from Fair Oaks Ranch and Port Lavaca had to be placed in cages in Houston. Temperatures became too cold in the late fall for outdoor cages to accurately show reproductive status, thus monarchs collected in the late fall (after Nov. 22, all from year-round breeding sites) were caged 8 days after capture at the University of Georgia in incubators set to temperatures and day length for Houston during the dates Oct. 31-Nov. 11. These conditions

allowed for temperatures that were high enough to for monarch flight but represented natural conditions as closely as was tenable.

Males collected before Nov. 22 were placed in outdoor mating cages within 1 to 4 days after capture. Outdoor mating cages were set up in the same way, secured onto a table with a tarp providing shade for half of the cage. We provided 20% honey water, refreshed daily ad libitum, and placed monarchs on sponges daily to encourage eating. Tropical milkweed did not occur in the areas in which we housed mating cages to assess reproduction. Cages were set up with 27 to 38 individuals per cage, with females (most of which were lab-reared) co-housed with wild males at an approximately 1:1 ratio. A small number of wild females (N=32) were used in some mating cages; the reproductive status of cage females (which requires dissection) was not assessed. Any mating pairs were recorded every morning and evening, frequently enough to observe all matings. Capture monarchs in outdoor cages at different locations (in Dallas, Katy, and Houston) experienced different temperatures and weather, much as they would in the wild. We maintained cages for such that each cage had 7 days of temperatures that reached >21.1 C (70F), thus total number of days per cage ranged from 8 to 10 days depending on weather.

IV. Stable isotope procedures and analyses

We measured stable hydrogen (δ^2H) isotope composition from wing tissue in collected monarchs from the fall to trace natal origins. Hydrogen isotope values are related to latitude, with more depleted (negative) values occurring farther north. An isoscape for monarchs in North America was previously constructed, and shows that monarchs with natal origins south of Dallas, TX should typically have hydrogen values more positive than -100‰. However, because several of our sites were irrigated with groundwater that could alter plant δ^2H based on precipitation, we

validated expected monarch δ^2H values by processing 25 of the wild monarch larvae/pupae (known to be residents) from year-round breeding sites collected in Spring 2015 (Figure S2). We also measured, in a previous study, stable isotope composition for 96 known migrants collected at two overwintering colonies in Mexico 2013.

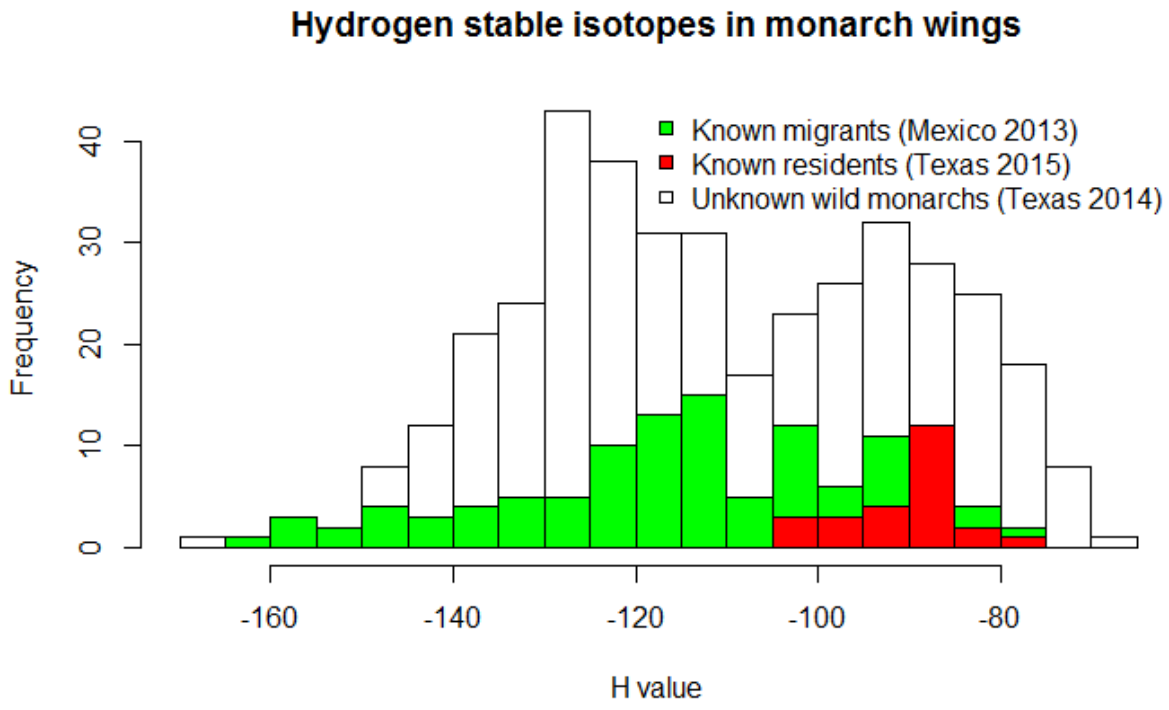

Figure S2. Histogram of hydrogen isotope values for known residents and migrants and for unknown wild monarchs collected in Fall 2014 in Texas.

V. Cardenolide analyses

Cardenolide composition in monarch wings has been informative in past studies to observe milkweed plant species of natal origin using chromatography. Here, we developed a method using ultra-high-pressure high-performance liquid chromatography (HPLC) to determine whether wild monarchs originated from *A. curassavica* or another (non-*A. curassavica*) milkweed.

To prepare samples, we pulverized wing tissue (right forewings), dissolved cardenolides into methanol followed by evaporation, and re-suspended the resulting film with a known internal standard (digitoxin), a toxic cardenolide that does not appear in monarchs. We then filtered this product and used HPLC to separate and quantify distinct cardenolide toxins across a solvent gradient. Following this procedure, we grouped cardenolide fingerprints for monarchs into distinct groups by combining information from the HPLC into a non-metric multidimensional scaling (NMDS) analysis using a permutation multiple ANOVA (MANOVA). Included in the NMDS analysis was total cardenolide concentration (across all cardenolide peaks in the chromatogram), cardenolide non-polarity (a measure of toxicity, with non-polarity being more toxic, and measured as retention time on the solvent membrane of each cardenolide peak), and cardenolide diversity (measurement of peaks using a Shannon-Weiner index).

We considered wild Texas butterflies to have originated from *A. curassavica* if its NMDS coordinates occurred within a specifically defined polygon, previously constructed around a tight cluster of lab-raised and field monarchs known to be from *A. curassavica* (Figure S3b). We considered a butterfly to be from a non-*A. curassavica* species if its coordinates fell outside of this space, as validated by additional lab-raised and field monarchs from 11 native milkweed species. To test the accuracy of this method, we blind-tested 36 *A. curassavica* monarchs and

placed their cardenolide information into the NMDS space. Of these known butterflies, 97% were correctly grouped as from tropical milkweed. To test non-*A. curassavica* monarchs, we blind-tested 86 *A. syriaca* butterflies and 99% were correctly grouped as non-*A. curassavica* . In total, we used 133 wild and lab-reared monarchs from tropical milkweed to define the known-*A. curassavica* NMDS space. We tested a total of 214 non-*A. curassavica* monarchs; all occurred outside the tropical milkweed cluster (Figure S3a).

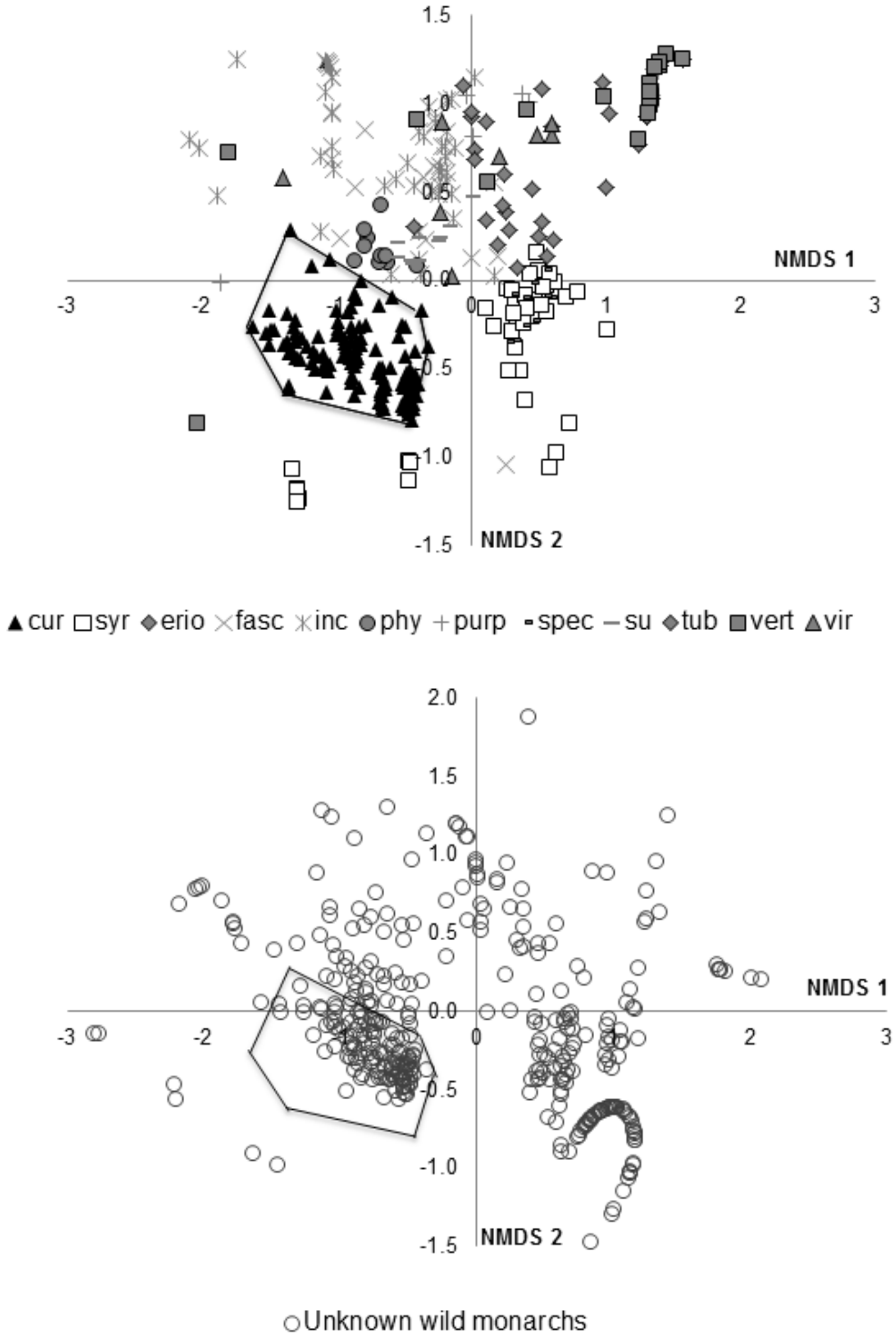

Figure S3. Cardenolide composition from monarch wing tissue, represented with NMDS coordinates, for known-origin monarchs (top panel) and for wild monarchs (bottom panel).