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Thermal tolerance and environmental persistence of a protozoan parasite in monarch butterflies

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ABSTRACT

Many parasites have external transmission stages that persist in the environment prior to infecting a new host. Understanding how long these stages can persist, and how abiotic conditions such as temperature affect parasite persistence, is important for predicting infection dynamics and parasite responses to future environmental change. In this study, we explored environmental persistence and thermal tolerance of a debilitating protozoan parasite that infects monarch butterflies. Parasite transmission occurs when dormant spores, shed by adult butterflies onto host plants and other surfaces, are later consumed by caterpillars. We exposed parasite spores to a gradient of ecologically-relevant temperatures for 2, 35, or 93 weeks. We tested spore viability by feeding controlled spore doses to susceptible monarch larvae, and examined relationships between temperature, time, and resulting infection metrics. We also examined whether distinct parasite genotypes derived from replicate migratory and resident monarch populations differed in their thermal tolerance. Finally, we examined evidence for a trade-off between short-term within-host replication and long-term persistence ability. Parasite viability decreased in response to warmer temperatures over moderate-to-long time scales. Individual parasite genotypes showed high heterogeneity in viability, but differences did not cluster by migratory vs. resident monarch populations. We found no support for a negative relationship between environmental persistence and within-host replication, as might be expected if parasites invest in short-term reproduction at the cost of longer-term survival. Findings here indicate that dormant spores can survive for many months under cooler conditions, and that heat dramatically shortens the window of transmission for this widespread and virulent butterfly parasite.

1. Introduction

Many parasites produce transmission stages that are exposed to environmental stressors prior to encountering a susceptible host. These transmission stages often persist for days or weeks (e.g. chytrid fungus infecting amphibians (Johnson and Speare 2003, Kolby et al., 2015)), and in some cases, can remain viable for a year or longer (e.g. anthrax that infects mammals (Sinclair et al., 2008), nucleopolyhedroviruses (NPVs) that infect Lepidoptera (Thompson et al., 1981)). Environmental transmission is particularly common among diverse entomopathogens used for biological control of insect pests, where transmission stages remain dormant in soil or other substrates until they encounter a new host (Vega and Kaya 2012). Among beneficial insects like bees, parasites can be transmitted between hosts via environmental substrates such as flowers, and even strains with limited persistence (surviving only a few hours) can spread effectively both within and between species (Graystock et al., 2015).

Many factors, including ultraviolet radiation, precipitation, and humidity can influence the ability of insect pathogens to persist (i.e. retain viability; we use persistence and viability interchangeably hereafter) in the environment (Benz 1987, Ignoffo 1992, Fernandes et al., 2015). Temperature in particular determines the persistence time and infectiousness of many insect parasites (Table 1) (Benz 1987, Thomas and Blanford 2003). For instance, spores of muscardine fungi (biological

Abbreviations: GLMM, generalized linear mixed model; LMM, linear mixed model; NA, North America; NPV, nucleopolyhedrovirus; OE, *Ophryocystis elektroscirrha*.

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Table 1
Examples of environmental tolerance of insect parasites, based on work testing responses to temperature, time, and outdoor exposure on microbial persistence. Rows are colored by parasite type (fungus: green; protozoan: blue; virus: yellow). (See below-mentioned references for further information.)

Environmental factor	Host	Parasite	Parasite type	Response measure	Main finding	Citation
Temperature	Tiger striped hissing cockroach (*Princisia vanwaerebecki*) & discoid cockroach (*Blaberus discoidalis*)	*Blabericola migrator* & *Blabericola cubensis*	Protozoan	Ability of spore to release sporozoites	Temperature significantly reduced viability at 27°C, 35°C and 40°C	Kolman et al. (2015)
Time	Gypsy moth (*Lymantria dispar*)	NPV	Virus	Infection	Proportion of infected hosts declined with time. The best estimate of viral persistence was 2.56 days	Fuller et al. (2012)
	Pine sawfly (*Neodiprion sertifer*)	NPV	Virus	Infection	Virus persisted for at least 2 years on pine foliage	Kaupp (1983)
	Douglas fir tussock moth (*Orgyia pseudotsugata*)	NPV	Virus	Infection	Nearly one third of virus in the organic soil layer remained active 11 years after an epizootic	Thompson et al. (1981)
Outdoor exposure	Large white (*Pieris brassicae*)	Granulovirus	Virus	Infection	The virus remained infective after 16 weeks of weathering	David and Gardiner (1966)
Temperature and time	Pales weevil (*Hylobius pales*)	*Beauveria bassiana* & *Metarrhizium anisopliae*	Fungus	Germination, sporulation	Spore viability responded nonlinearly to temperature, peaking between 25-30°C. Spores were viable for longer when stored at 8°C than at 21°C	Walstad et al. (1970)
	Whitefly (*Bemisia tabaci*)	*Cordyceps javanica*	Fungus	Germination	Spore survivorship was inversely proportional to duration of heat shock exposure (45°C)	Mascarin et al. (2018)
	Tenebrionid insects	10 species of Cephaline gregarines	Protozoan	Ability of spore to release sporozoites	Spore viability declined with temperature at and above 40°C. At 35°C and below, spores were viable for a year after exposure	Patil et al. (1983)
	Glabrous cabinet beetle (*Trogoderma glabrum*)	*Mattesia trogodermae*	Protozoan	Infection	Optimal spore longevity was at -19°C, with 66% infection after 1 year. Exposure to 73°C and above for just 30 minutes was lethal to spores	Nara et al. (1981)
	Greater wax moth (*Galleria mellonella*)	Invertebrate iridescent virus 6	Virus	Infection; ID_{50}	Non-significant increase in rate of viral inactivation at high temperatures. After 50 days, infectivity fell by an order of magnitude.	Marina et al. (2000)
Outdoor exposure and time	Monarch butterfly (*Danaus plexippus*)	*Ophryocystis elektroscirrha*	Protozoan	Infection; spore load	Spores maintained infectivity for at least 16 days in environment, though resulting infection load was lower after exposure. Modeling indicated spores must persist for more than 6 weeks for prevalence to reach levels observed in wild populations.	Satterfield et al. (2017)
	Corn earworm (*Helicoverpa zea*) & tobacco budworm (*Heliothis virescens*)	NPV	Virus	Host mortality	Most viral activity was lost by 1-2 days after application on leaf surfaces in the field	Bullock (1967)

control agents of the pales weevil, *Hylobius pales*) require temperatures between 10 and 35 °C to germinate, with germination peaking between 25 and 30 °C (Walstad et al., 1970). Granulovirus can persist for many months in stored foods, but viral persistence time and infectivity against the pest moth *Plodia interpunctella* drops off at temperatures of 32 °C and higher (Vail et al., 1991). Across diverse insect pathogens including viruses, fungi and protozoa, some are especially tolerant of thermal variation, whereas others are more sensitive (summarized in Table 1). In particular, some pathogens have thick proteinaceous coatings and heat shock proteins that protect against extreme temperatures, drought, and ultraviolet light (Xavier and Khachatourians 1996, Caraco and Wang 2008). For instance, occlusion bodies (protective protein lattices) of NPVs containing virions are released into the environment following host death. These occlusion bodies can protect virions against environmental stressors until susceptible larvae consume contaminated foliage (Caraco and Wang 2008, Myers and Cory 2016).

Understanding the duration and determinants of environmental persistence is important for predicting the dynamics, evolution, and population-level impacts of parasites on both beneficial and pest insects. Mathematical models show that persistence time outside the host can determine temporal infection dynamics (Anderson and May 1981, Briggs and Godfray 1995). For example, a model of NPV infection in gypsy moths showed that viruses that decayed quickly in the environment caused shorter epidemics (Fuller et al., 2012) and were less likely to induce host population cycles (Vezina and Peterman 1985). If investment in environmental persistence is resource-intensive, then parasites with long-lived external stages might produce fewer propagules (Caraco and Wang 2008, Rafaluk-Mohr 2019). For instance, there is evidence for a trade-off between free-living survival and replication rate in phages that infect *Escherichia coli* (De Paepe and Taddei 2006). Alternatively, some work predicts that higher virulence can evolve in parasites with persistent infective stages because they can "sit and wait" in the environment, incurring little cost from killing their hosts (Kamo and Boots 2004, Caraco and Wang 2008, Cressler et al., 2016, Rafaluk-Mohr 2019). Past research in insect systems has explored parasite virulence-transmission trade-offs (Myers and Rothman 1995, de Roode et al., 2007, de Roode et al., 2008b, Chapuis et al., 2012), but there remains a crucial need to understand the consequences of environmental persistence for insect-pathogen dynamics and evolution more broadly.

In this study, we explored thermal tolerance in a debilitating protozoan, *Ophryocystis elektroscirrha* (OE), infecting monarch butterflies (*Danaus plexippus*). Environmental transmission is critically important for infection in this system (Satterfield et al., 2017, Majewska et al., 2019). Infections develop internally in monarch larvae and pupae, and adult monarchs emerge covered with millions of dormant protozoan spores on the outsides of their bodies (McLaughlin and Myers 1970, de Roode et al., 2007). Transmission occurs when infected butterflies shed infectious spores onto eggs or milkweed leaves, and spores are later consumed by monarch larvae (Altizer et al., 2004, de Roode et al., 2009). Infection with OE can reduce monarch survival to adulthood, adult lifespan, reproductive success, and flight ability (Bradley and Altizer 2005, de Roode et al., 2007). This protozoan has been detected in all monarch populations examined to date, and infection prevalence varies by location, season, and year (Altizer et al., 2000, Pierce et al., 2014, Altizer and de Roode 2015).

Previous work suggests that environmental persistence of OE spores determines monarch-OE infection dynamics. Results of a stage-structured model indicated that spores must persist more than 6 weeks in the environment for simulated infection prevalence to match observed prevalence in wild populations (Satterfield et al., 2017). Further modeling work reported that a longer duration of milkweed contamination could increase late-season parasite prevalence and decrease monarch population size (Majewska et al., 2019). Importantly, OE spores have a thick, amber-colored wall that appears to offer protection from environmental stressors (McLaughlin and Myers 1970). Although experimental work has demonstrated that OE spores can remain infectious for at least 16 days outdoors (Satterfield et al., 2017), longer-term viability remains untested. Recent work provides evidence for heritable variation in OE spore size and color, which could determine variation in environmental persistence (Sander et al., 2013).

We experimentally tested how temperature affects parasite persistence outside of the host, using a range of temperatures experienced throughout the monarchs' migratory and non-migratory range, and testing across short, moderate, and long time periods that map onto the monarchs' annual migratory cycle. We summarize the following predictions in Table 2.

We predicted that spore viability, as measured by infectivity and within-host replication, would decrease with increasing spore age (**1a**), and that spores from migratory populations would retain viability over longer timescales (**1b**). North America migratory monarchs spend approximately 8 months in a non-reproductive state during phases of migration and overwintering (Urquhart and Urquhart 1978, Nagano et al., 1993). In contrast, adults that emerge during summer breeding phases, and those from resident (non-migratory) populations, live only 2–4 weeks. This variation in migratory and reproductive behavior could produce different selection pressures on spore longevity. In particular, dormant spores on infected adult butterflies in the overwintering generation must retain viability over the entire migration period to infect new generations of larvae in the spring. Parasites from tropical and subtropical areas with resident monarch populations (e.g. Hawaii, Florida) (Altizer et al., 2000, Satterfield et al., 2015) are transmitted over shorter timescales, and might therefore show more rapid loss of viability.

In terms of temperature, we predicted that spore persistence would decline with increasing temperature (**2a**). Because resident monarchs inhabit consistently warm environments, we predicted that their parasites would better tolerate warmer temperatures, whereas spores

Table 2
Experimental predictions of how temperature, time, and parasite genotype would affect viability of OE spores.

Experimental factor	Prediction	Reasoning
Time	**1a.** Older spores are less viable (as measured by infectivity and within-host replication)	Spores will physically degrade over time, or sporozoites will lose activity over time
	1b. Spores from migratory populations retain viability over longer timescales	Spores of migratory monarchs must retain viability over the entire migration period (~8 months), while parasites of resident monarchs are transmitted over shorter timescales (2–4 weeks), and might therefore show more rapid loss of viability
Temperature	**2a.** Spore persistence declines with increasing temperature	High temperatures will inhibit sporozoite survival and activity
	2b. Parasites of resident monarchs better tolerate warmer temperatures, whereas spores of migratory monarchs tolerate a wider range of temperatures	Resident monarchs inhabit consistently warm environments, while migratory monarchs experience a broader range of temperatures (i.e. cooler overwintering sites and warmer breeding sites)
Parasite genotype	**3a.** There is a negative correlation between short-term within-host replication and long-term persistence ability	If investment in environmental persistence is resource-intensive, then parasites with long-lived external stages might produce fewer propagules
	3b. There is a positive correlation between short-term within-host replication and long-term persistence ability	More environmentally persistent strains could afford to be more virulent (and exploit their hosts via faster replication), as their transmission depends less on host longevity and mobility

derived from migratory monarchs might better tolerate a wider range of temperatures (Davis et al., 2005) (**2b**). In addition to testing effects of temperature and source population on spore persistence, we examined the influence of parasite genotype within each source population.

Finally, we examined evidence for a trade-off between virulence and environmental persistence in OE genotypes. We expected to find a negative correlation between short-term within-host replication and long-term persistence ability (**3a**). Alternatively, finding a positive correlation would support the "sit and wait" hypothesis, which posits that more environmentally persistent strains can afford to be more virulent (and exploit their hosts via faster replication), as their transmission depends less on host longevity and mobility (**3b**).

2. Methods and materials

2.1. Host and parasite sources

Wild-caught infected monarchs from four source populations were used to create 20 parasite clonal isolates in our laboratory. Source populations are denoted here as Eastern NA (eastern North American long-distance migratory population), Western NA (western North American short-distance migratory population), Florida (south Florida resident population), and Hawaii (resident monarchs from Hawaiian archipelago). Five clonal parasite lineages per source population were generated prior to the experiment by infecting monarch caterpillars with single haploid parasite spores, and using their offspring as the sources for this experiment (de Roode et al., 2007). These same parasite clones were used previously in different experiments (de Roode et al., 2007, de Roode and Altizer 2010, Sander et al., 2013), where they infected monarchs and showed variation in spore loads, virulence, and spore morphology. Collection sites and dates of wild infected monarchs used to generate the 20 parasite clones are provided in Table A.1.

Monarchs used to propagate clones were derived from the Eastern NA population. Briefly, we inoculated the parasite-free grand-progeny of uninfected wild-captured adults with 10–20 spores per early instar larvae. Approximately 5 monarchs were inoculated with each clone and reared to the adult stage. Methods for inoculation and rearing followed previously described protocols (de Roode et al., 2007, de Roode et al., 2008a, de Roode et al., 2008b). Following adult eclosion, cotton swabs were rubbed on the abdomens of infected monarchs (to pick up several

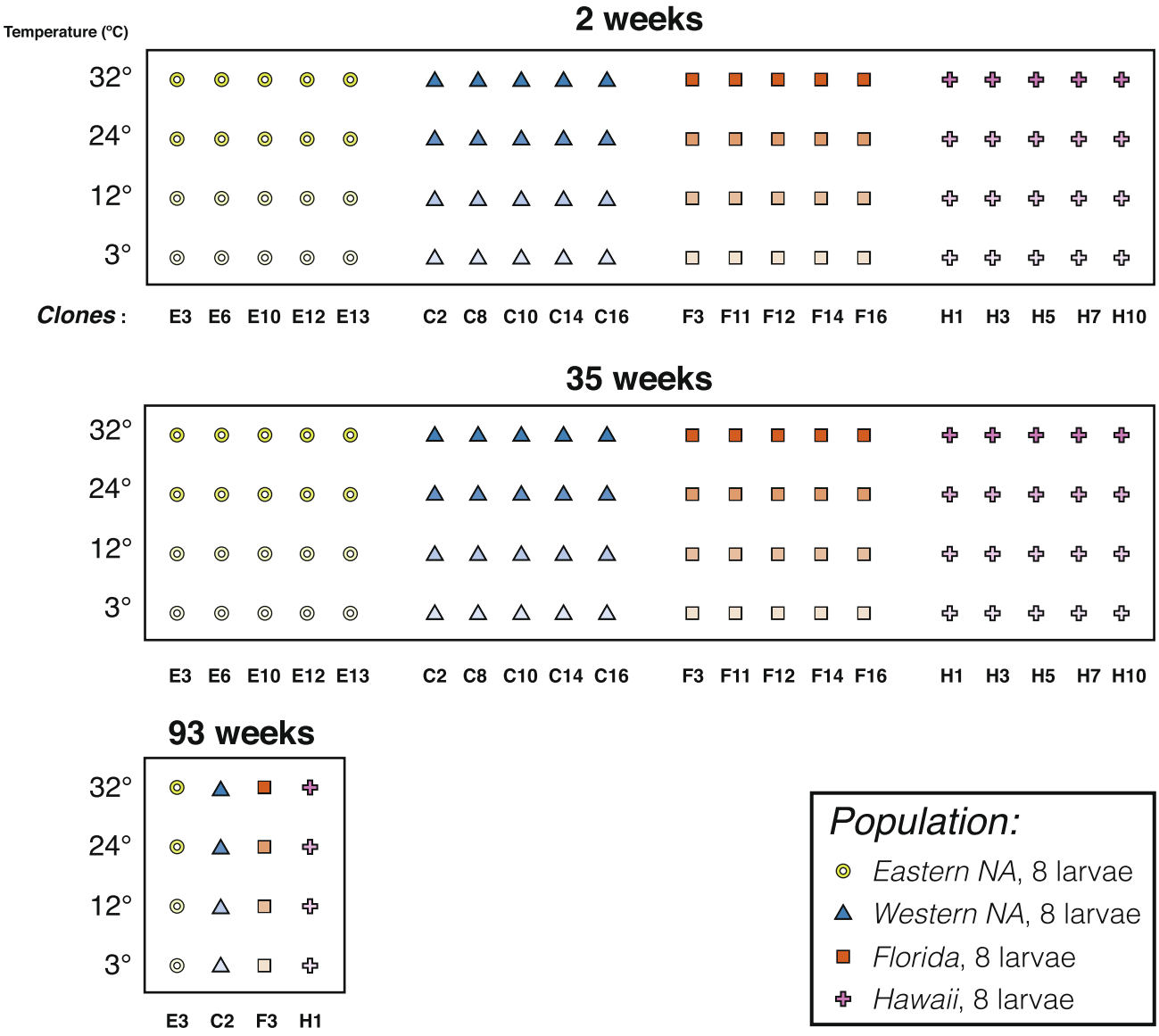

Fig. 1. Experimental design showing temperature and duration of exposure across 20 parasite clones from four source populations. For each clone, eight larvae were inoculated at each duration and temperature. All 20 clones were tested from the 2- and 35-week exposure durations; only four clones were tested from the 93-week duration owing to size constraints of the final experiment.

thousand spores per swab), and placed singly into 5 cm^2 glassine envelopes. Two envelopes per clone were placed into one of four temperature treatments as described below.

Monarchs used to test spore viability following spore thermal exposures were the laboratory-reared, parasite-free grand-progeny of 20 wild spring migratory monarchs caught in San Antonio, TX and Athens, GA (USA) in April 2009. We obtained eggs from 6 non-inbred host genetic lineages, where a lineage included a mix of full- and half-siblings.

2.2. Experimental thermal and time treatments

Spores were held in incubators at one of four temperatures (3 °C, 12 °C, 24 °C, or 32 °C) for a short (2 week), moderate (35 week), or long (93 week) time period (Fig. 1). Experimental temperatures were chosen to represent a range of temperatures to which spores are exposed in nature (overwintering sites: 0–15 °C; breeding sites in summer: 14–35 °C) (Brower et al., 2004, Weiss 2005). Data loggers (iButtons) were placed together with parasite samples to record temperature every 4 h for the duration of each treatment. The 2- and 35-week time periods were chosen based on durations that parasites on resident and migratory butterflies, respectively, must remain viable on their hosts to infect a new generation of larvae, and the 93-week interval was chosen to test whether spores can persist over multiple years. Spores from all 20 clones were held for 2 and 35 weeks; only spores from four clones (E3, *C*2, F3, H1) were held for 93 weeks (Fig. 1).

2.3. Host inoculation

We inoculated a target of 8 caterpillars per temperature–time-clone combination ($n = 1408$, Fig. 1). Owing to the deaths of a small number of larvae during the inoculation phase, actual numbers of inoculated larvae sometimes deviated by 1–2 from the target. To control for host effects on spore load (de Roode and Altizer 2010), treatments were randomized over the 6 host lineages. Second instar larvae were fed 1 cm^2 squares of milkweed to which 10 OE spores were manually applied; spores were counted visually using a stereo microscope at 60× magnification. Although the minimum infectious dose is 1 spore (de Roode et al., 2007), 10 spores were used to increase the likelihood of infection. Control larvae ($n = 100$) consumed milkweed squares with no spores.

Infected larvae were reared in a walk-in room with exposure to natural ambient light and temperatures at 26–28 °C. Larvae and pupae were reared singly in 0.47L containers using cuttings of greenhouse-raised *Asclepias incarnata* (de Roode et al., 2007, de Roode et al., 2008a, de Roode and Altizer 2010). Upon adult emergence, monarchs were transferred to individual glassine envelopes, held in a 12 °C incubator and checked daily to record the date of host death. Due to the space-intensive nature of the experimental set-up, inoculations and rearing were performed in two consecutive blocks (Block 1: $n = 742$; Block 2: $n = 763$) staggered by 2 weeks (pupation is completed in less than 2 weeks).

2.4. Metrics of infection and virulence

Monarch infection was measured in two ways: first, we recorded whether or not monarchs were infected (0/1) using a non-destructive method. Clear tape was pressed onto abdomens of adults, and parasite spores were counted using a stereo microscope at 50X magnification. Samples with >100 spores were considered to be heavily infected, following past work (Altizer et al., 2000, Bartel et al., 2011). Second, we examined the spore load of infected adult butterflies, as this measure is strongly positively correlated with the level of within-host replication (de Roode et al., 2007) and transmission of parasite spores (de Roode et al., 2009). Following host death, parasite spore load was determined by vortexing monarch bodies and counting spores using a hemocytometer at 100× magnification (de Roode et al., 2007). Spore loads were $\log_{10}$-transformed prior to analysis to normalize the error variance.

2.5. Statistical analyses

We tested how temperature, duration (i.e. time period), source population (Eastern NA, Western NA, Florida, or Hawaii), and population migratory status (migratory or resident) influenced metrics of OE viability. Our primary metric of parasite survival was host infection status as a binary variable, to indicate whether a viable infection could result from a given inoculation. We tested quantitative spore load as a secondary metric of spore viability, to reflect the effective number of viable spores ingested by a monarch, in combination with the rate of within-host replication. Owing to slight temperature fluctuations in the incubators (Fig. A.1), we calculated median temperature values for each duration separately, and treated temperature as a continuous, rather than categorical, variable in the model. Duration was treated as a categorical variable. Statistical analyses were performed in the R statistical environment v. 3.6.1 (R Core Team 2019).

We first modeled infection status (0/1) in adult monarchs ($n = 1050$) with a penalized quasi-likelihood generalized linear mixed model (GLMM; binomial distribution, logit link) using the *MASS* package (Venables and Ripley 2002). Penalized quasi-likelihood was chosen to correct for overdispersion. We included temperature, time period, population migratory status, and their two-way interactions as predictor variables. Monarch sex and experimental block were included as fixed factors, and parasite clone was included as a random effect. Population migratory status was used instead of parasite source population because when source population was included, a combination of predictors perfectly separated a subset of monarchs by infection status (i.e. perfect separation). Marginal and conditional pseudo R^2 values were calculated as measures of fit using the *MuMIn* package (Barton 2014).

To next examine spore load, we fit a linear mixed model (LMM) using the *lme4* package (Bates et al., 2015). We analyzed data for infected monarchs from the 2- and 35-week durations only ($n = 722$) because too few positive cases were available for the 93-week duration. All fixed effects were the same as above, except that source population was used instead of population migratory status. As before, parasite clone was included as a random effect. Because graphical exploration of the data indicated a possible three-way interaction between temperature, duration, and source population, we fit a second LMM that included this interaction. We compared models using a likelihood ratio test with the 'anova' function of the *stats* package (R Core Team 2019). P-value estimation for the models was done using Kenward-Roger approximated denominator degrees of freedom calculated with the *pbkrtest* package (Halekoh and Højsgaard 2014).

We followed up with a more focused analysis to test whether spores derived from resident populations had *greater* viability at *higher* temperatures, but *reduced* viability at *lower* temperatures, compared to spores derived from migratory populations. We excluded the 2-week duration from these analyses given that temperature did not seem to affect viability over this short timescale (see Results). We used one-tailed *t*-tests to compare the proportion of infected monarchs and spore load caused by spores with migratory versus resident origins for each temperature treatment, and corrected for multiple comparisons by applying a Benjamini-Hochberg adjustment to P values.

Lastly, we examined whether the environmental persistence of OE shows evidence for a genetic trade-off against within-host replication, and whether this relationship depends on population migratory status. For each clone, we calculated 1) the proportion of infected monarchs in the 35-week duration from the two middle temperature treatments as a measure of long-term persistence ability, and 2) the average spore load in the 2-week duration as a measure of maximum within-host replication. We used a linear model to model the proportion of infected monarchs as a function of spore load, migratory status, and their two-way interaction.

3. Results

Of 1408 larvae inoculated with OE spores, 3 died during the inoculation phase. Survival to the adult stage was similar for inoculated (74.7%, 1050/1405) and control (74.0%, 74/100) monarchs. Survival was higher for monarchs in Block 2 (86%, 611/713) compared to Block 1 (63%, 439/692). Of the inoculated monarchs that successfully eclosed, 71.5% (751/1050) were infected with OE (Table A.2). No control butterflies showed signs of infection with OE.

3.1. Effects of temperature, time, and parasite origin on spore viability

Over 95% of monarchs inoculated with 2-week-old spores became infected, with similarly high infection rates across the range of experimental temperatures. In contrast, warmer temperatures reduced the infectivity of spores held for 35 and 93 weeks (Fig. 2). A penalized quasi-likelihood GLMM showed that this interaction between temperature and time was a significant predictor of OE infection status in adult monarchs (Table 3). In other words, infection ability dropped off steeply with temperature for spores held for moderate and long time periods, but not spores held for the shortest time. Additionally, there was a significant main effect of time: inoculation with spores held for 93 weeks was less likely to result in infection ($\beta \pm SE = -3.86 \pm 1.70$, $P = 0.023$; Table 3). Spores held at the upper limits of time and temperature (93 weeks at 24 °C and 32 °C) caused no infections (Table A.2). Fewer monarchs inoculated in Block 2 were infected relative to Block 1 (Table 3; Block 1 = 75.4%; Block 2 = 67.2%). Neither population migratory status nor host sex were significant predictors of OE infection. The model's fixed effects explained 70% of the variance in infection probability (marginal $R^2 = 0.70$). Including the random effect of parasite clone explained 2% additional variance (conditional $R^2 = 0.72$).

Spore loads of infected monarchs ranged over 4 orders of magnitude ($10^{3.5}$ – $10^{7.3}$; median spore load: $10^{6.0}$). Spore load decreased with increasing temperature, and was lower for spores held for longer durations (Fig. 3, Table 4), but these changes were weaker than those observed for infection status. For example, average spore load for spores held for 2 weeks was $10^{6.0}$, for 35 weeks was $10^{5.8}$, and for 93 weeks was $10^{5.5}$. Spores from different parasite clones differed in the average spore load they caused (Fig. 4), but average spore loads were similar across the four source populations.

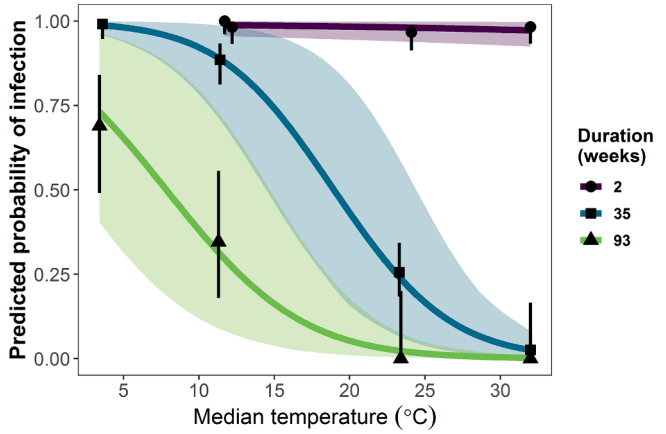

Fig. 2. Average marginal predicted probabilities of OE infection are plotted over the range of experimental temperatures for the three exposure durations: 2 weeks (purple/circles), 35 weeks (blue/squares), and 93 weeks (green/triangles). Shaded regions show the range in which 95% of the predicted probabilities fell. Points represent the proportion of monarchs infected with OE after experimental inoculation; error bars represent 95% confidence intervals around those proportions. Spores held for 2 and 35 weeks were derived from all 20 clone lines, whereas spores held for 93 weeks were derived from 4 clone lines (E3, C2, F3, H1). A malfunction in the lowest temperature treatment in the 2-week duration (see Fig. A.1) caused the median temperature over this time period to be 12.2 °C rather than 3 °C as intended. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Results of a penalized quasi-likelihood GLMM (binomial distribution, logit link) to examine effects of temperature, duration, migratory status of the source population, and other design variables on the likelihood of OE infection (n = 1050). P values < 0.05 are in bold.

Variable		β	SE	t value	P
Temperature		−0.073	0.056	−1.29	0.20
Duration	2 weeks	Reference	–	–	–
	35 weeks	4.8e-05	1.50	3.2e-05	1
	93 weeks	−3.86	1.70	−2.28	**0.02**
Migratory status	Migratory	Reference	–	–	–
	Resident	−1.66	1.41	−1.17	0.26
Sex	Female	Reference	–	–	–
	Male	0.11	0.32	0.35	0.73
Block	1	Reference	–	–	–
	2	−1.51	0.35	−4.35	**0.00**
Duration: Temperature	2 weeks: Temperature	Reference	–	–	–
	35 weeks: Temperature	−0.26	0.056	−4.64	**0.00**
	93 weeks: Temperature	−0.22	0.085	−2.52	**0.01**
Duration: Migratory status	2 weeks:Resident	Reference	–	–	–
	35 weeks: Resident	0.76	0.96	0.80	0.43
	93 weeks: Resident	0.91	1.41	0.65	0.52
Temperature: Migratory status	Temperature: Migratory	Reference	–	–	–
	Temperature: Resident	0.045	0.045	0.99	0.32

A likelihood ratio test indicated that including a three-way interaction between duration, temperature, and source population significantly improved spore load model fit ($\chi^2 = 19.7$, df = 3, $P = 0.0002$) compared to the model including only two-way interactions between those predictors (Table 4). In particular, associations between spore load, temperature, and duration depended on source population (Fig. 3). For example, spores derived from the Western NA population appeared to better tolerate higher temperatures, as indicated by a shallower slope in the predicted fit between spore load and temperature in the 35-week condition (Fig. 3). Although spores derived from the Florida population caused lower spore loads when held at warmer temperatures, it is important to note that only spores from this population and no others retained viability at the highest temperature (Fig. 3). Monarchs in Block 2 had significantly lower spore loads than those in Block 1 (Table 4). The model's fixed effects explained 33% of the variance in spore load (marginal $R^2 = 0.33$). Including the random effect of clone explained 5% additional variance (conditional $R^2 = 0.38$).

Based on one-tailed t-tests, spores derived from migratory versus resident monarch populations did not differ significantly in 1) their ability to infect monarch larvae or 2) the spore load they caused, at any of the four temperature treatments (Table A.3).

3.2. Association between parasite persistence and within-host replication

In a linear model, neither 2-week spore load ($P = 0.72$), migratory status ($P = 0.20$), nor their 2-way interaction ($P = 0.20$) were significant predictors of the proportion of infected monarchs caused by 35-week-old spores (Table A.4). Plotting the proportion of infected monarchs (35-week duration, two middle temperatures) versus average spore load (2-week duration) for each of the 20 clones, and predicted fits from the model, suggested a slight negative relationship (tradeoff) between persistence and virulence for clones from resident populations, and an

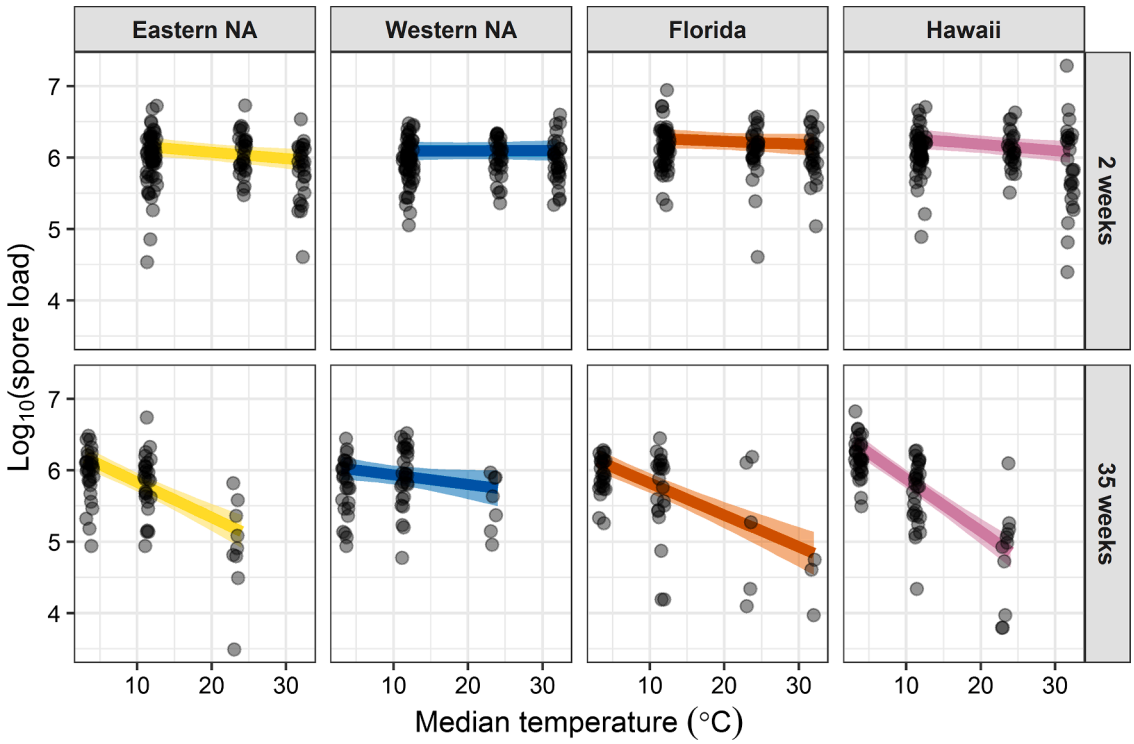

Fig. 3. A visualization of fixed effects of a linear mixed model predicting spore load over the range of experimental temperatures, for the 2-week duration (top row) and 35-week duration (bottom row). 95% confidence intervals are shaded. Jittered points represent log-transformed spore loads. Predictions are displayed at the reference levels of non-focal predictors (i.e. sex = female, block = 1).

even weaker positive relationship for clones from migratory populations (Fig. 5).

4. Discussion

Our results show that external transmission stages of the parasite *O. elektroscirrha* can persist for many months outside of the host when exposed to cool temperatures. Importantly, warmer temperatures sharply reduced the viability of parasite transmission stages, and the magnitude of this effect increased over time. In contrast to the high infection rates from spores held for 35–93 weeks at cool temperatures, relatively few monarchs became infected when exposed to spores held for long durations at the highest temperatures. We observed wide heterogeneity in viability between parasite clones, but little to no population-level differences in infection rates. We found no support for a tradeoff between short-term within-host replication and long-term persistence ability.

In agreement with **Prediction 2a**, both infection probability and spore load decreased unidirectionally with increasing temperature. Our finding that spore viability remained high at cool temperatures approaching (but above) freezing, and declined at warmer temperatures, aligns with previous work demonstrating that external stages of insect parasites lose viability with increasing temperature (Table 1). Spores were resilient to all experimental temperatures for a short time (2 weeks), consistent with past findings that OE spores remain highly infectious for at least 16 days in outdoor conditions (Satterfield et al., 2017). We also found that infectivity and replication declined with increasing time, supporting **Prediction 1a**. Because the lemon-shaped, amber spores of OE remained similar in appearance across all of the experimental treatments, the lower infection rates and spore loads likely resulted from the inability of sporozoites to emerge from the spore wall, penetrate the gut, and/or replicate internally early in the infection process (McLaughlin and Myers 1970).

Parasites derived from migratory versus resident populations showed similar patterns of persistence across the experimental treatments, counter to **Predictions 1b** and **2b**. Thus, even though parasites from migratory populations must persist longer in the wild between cycles of transmission (when monarchs are migrating and overwintering), and are likely exposed to a broader range of temperatures (from near-freezing to extreme heat) throughout the monarch's annual cycle, spores derived from migratory populations did not show greater viability across temperatures or over time. It may be that temperature conditions experienced by migratory and resident populations are not different enough to lead to selection for different temperature optimums in OE. Alternatively, it is possible that selection acts on parasite tolerance of fluctuating temperatures, as opposed to the constant temperature exposures in our experiment.

Findings here provide some support for differences among source populations (Eastern NA, Western NA, Florida, Hawaii) and parasite clones. Only spores derived from the resident Florida population successfully infected larvae after being held at the hottest experimental temperature for 35 weeks, as might be expected if these parasites are predictably exposed to heat in the wild. Moreover, parasites derived from the Western NA population better tolerated a range of temperatures, as indicated by a shallow slope in the predicted fit between spore load and temperature in the 35-week condition (Fig. 3). It might be that the elevational variation west of the continental divide produces a sharper gradient of temperatures across a narrower geographic range, thus selecting for greater thermal tolerance. Within source populations, parasite clones varied in their infection probabilities and spore loads, consistent with past findings of high variability in infectivity, spore load, and morphology among OE clones (de Roode et al., 2008b, de Roode and Altizer 2010, Sander et al., 2013, Sternberg et al., 2013).

Our statistical analyses showed no evidence of a tradeoff between environmental persistence and within-host parasite replication, counter to **Prediction 3a**, as might be expected if the production of long-lived spores requires greater resource investment on a per-spore basis. Other life history trade-offs have been documented in parasitic

Table 4
Results of a LMM to examine effects of temperature, time, parasite source population, and control variables on $\log_{10}$-transformed spore load of infected butterflies ($n = 722$). P values were calculated using Kenward-Roger approximated degrees of freedom calculated with the *pbkrtest* package. P values < 0.05 are in bold.

Variable		β	SE	t value	P
Temperature		−8.8e-3	0.004	−2.26	**0.03**
Duration	2 weeks	Reference	–	–	–
	35 weeks	0.077	0.11	0.67	0.50
Source population	Eastern NA	Reference	–	–	–
	Western NA	−0.17	0.13	−1.25	0.21
	Florida	0.066	0.14	0.48	0.63
	Hawaii	0.11	0.14	0.80	0.42
Sex	Female	Reference	–	–	–
	Male	−0.031	0.027	−1.16	0.25
Block	1	Reference	–	–	–
	2	−0.20	0.027	−7.22	**<1e-4**
Duration: Temperature	2 weeks: Temperature	Reference	–	–	–
	35 weeks: Temperature	−0.041	0.008	−5.26	**<1e-4**
Duration: Source population	2 weeks:Eastern NA	Reference	–	–	–
	35 weeks:Western NA	−0.091	0.16	−0.56	0.58
	35 weeks:Florida	−0.13	0.16	−0.78	0.44
	35 weeks:Hawaii	0.17	0.16	1.04	0.30
Temperature: Source population	Temperature: Eastern NA	Reference	–	–	–
	Temperature: Western NA	9.3e-3	5.4e-3	1.73	0.09
	Temperature: Florida	4.4e-3	5.6e-3	0.79	0.43
	Temperature: Hawaii	5.6e-6	5.6e-3	0.001	1
Duration: Temperature: Source population	2 weeks: Temperature: Eastern NA	Reference	–	–	–
	35 weeks: Temperature: Western NA	0.027	0.014	2.34	**0.02**
	35 weeks: Temperature: Florida	2.1e-4	0.011	0.02	0.98
	35 weeks: Temperature: Hawaii	−0.024	0.011	−2.21	**0.03**

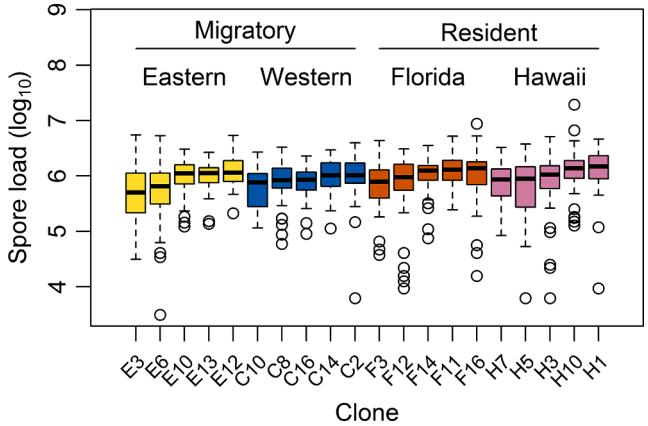

Fig. 4. Log_{10} spore loads caused by the 20 parasite clones used in the experiment. Spore loads were calculated over all experimental temperatures and durations from all infected monarchs. Boxplots are arranged by increasing median spore load within source population.

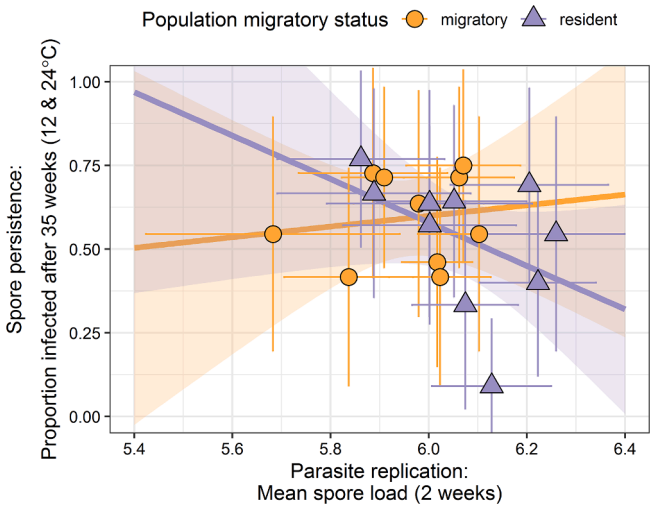

Fig. 5. Relationship between parasite replication (as measured by spore load caused by inoculation with spores held for 2 weeks) and long-term persistence (as measured by the proportion of infected hosts following inoculation with spores held for 35 weeks at intermediate temperatures). Values were calculated separately for the 20 parasite clones. Colors represent population migratory status (migratory: orange circles, resident: purple triangles). Predicted fit lines and confidence intervals (shaded) are from a linear model. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

organisms, including between traits affecting transmission versus virulence (Paterson and Barber 2007, de Roode et al., 2008b, Nørgaard et al., 2020 preprint). Visual inspection of among-clone differences suggested a slight negative relationship for spores from resident populations, and a weaker positive relationship for parasites from migratory populations. This suggests that parasites might be able to maintain both high replication and long-term environmental persistence simultaneously. Further studies, using larger numbers of clones, will be needed to confirm these findings, and to test for other parasite traits that might predict variation in environmental persistence.

Findings here point towards several avenues for future research. First, under natural conditions, parasite transmission stages contend with multiple stressors simultaneously (e.g. rain, sunlight, temperature fluctuations). Field experiments could explore whether parasites decay at a faster rate under more natural conditions, and how parasites respond to pulsed stressors such as short-duration temperature extremes. Selection experiments could test whether tolerance of environmental stressors (such as high temperature) selects for more virulent parasite strains that exploit their hosts, as has been suggested by some empirical and modeling work (Bonhoeffer et al., 1996, Rafaluk-Mohr 2019). The cellular mechanisms underlying temperature- and age-induced declines in parasite viability could also be explored. A study on *Cryptosporidium parvum,* another Apicomplexan parasite, found that carbohydrate energy reserves were depleted as storage time and temperature increased, and that these reserves predicted infectivity (Fayer et al., 1998). In addition, the effect of temperature on the within-host dynamics of infection in developing larvae and pupae is important to investigate, especially because thermal sensitivity profiles can differ for insect hosts and their parasites (Thomas and Blanford 2003).

Exploring how parasite transmission in monarchs might change with climate warming could help predict the parasite's future impact on monarchs. The eastern North American monarch population has experienced declines since the mid-1990s (Brower et al., 2012, Thogmartin et al., 2017), and modeling work suggested that parasite infection can cause substantially lower monarch population size (Altizer and Oberhauser 1999, Bradley and Altizer 2005, Bartel et al., 2011, Majewska et al., 2019). Given our finding that higher temperatures limit spore

viability, it seems plausible that infection prevalence might decrease, rather than increase, with climate change. Because other host and parasite traits (e.g. within-host replication of OE, host contact rates) will also respond to temperature variation, integrated modeling and experimental work, as has been done in other systems (Molnár et al., 2013, Gehman et al., 2018), is needed to better predict future responses of insects and their parasites to contemporary climate change.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The data underlying this publication have been submitted to Mendeley Data and will be available at https://nam03.safelinks.protection.outlook.com/?url=http%3A%2F%2Fdx.doi.org%2F10.17632%2F398kdjh6x5&data=04%7C01%7CC.Corrections%40elsevier.com%7Cf6018ef9f483439696b208d8d2f584d8%7C9274ee3f94254109a27f9fb15c10675d%7C0%7C0%7C637491299796397069%7CUnknown%7CTWFpbGZsb3d8eyJWIjoiMC4wLjAwMDAiLCJQIjoiV2luMzIiLCJBTiI6Ik1haWwiLCJXVCI6Mn0%3D%7C3000&sdata=URipoJieFo4LGqRWw6HoYQBxYto8kq%2FTR%2BXinHzd%2FuE%3D&reserved=0.Data.

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