

A citizen science project reveals contrasting latitudinal gradients of wing deformity and parasite infection of monarch butterflies in New Zealand

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Abstract. 1. Host–parasite interactions represent complex relationships among species, often with considerable spatial variation. We examined latitudinal variation in the prevalence of monarch butterfly (*Danaus plexippus*) infection by the protozoan parasite *Ophryocystis elektroscirrha* via a citizen science project in New Zealand. Parasitism by *O. elektroscirrha* can result in incomplete development causing mortality, or adult butterflies with deformed wings and reduced fitness.

2. We established the existence of counter-latitudinal clines of wing deformities and parasite prevalence in monarchs, which in New Zealand are non-migratory. The prevalence of wing deformities increased with increasing latitude, whereas the prevalence of parasite infections decreased with increasing latitude. No parasitism was observed in our highest latitudinal location, while all butterfly samples were infected in the warmest and lowest latitudinal site. The prevalence of parasitism or wing deformity did not vary with the butterfly sex.

3. Cold temperatures appear to independently affect the parasite and host butterfly development, with lower temperatures limiting parasite development and inducing wing deformities in butterflies. Other protozoan parasites display similar latitudinal clines that are thought to be temperature related.

4. Environmental factors appear to independently influence insect populations through different mechanistic pathways. The high prevalence of parasite infections may affect butterfly fitness in warmer regions, but the cooler conditions at higher latitudes manifest as a major fitness cost, deformed wings. Monarch butterflies in New Zealand and around the world may perform better where it is not too hot (and optimal for these parasites), but not cold (where the pupae experience developmental issues).

Key words. *Danaus plexippus*, deformity, *Ophryocystis elektroscirrha*, parasitism, temperature.

Introduction

Host–parasite interactions represent common albeit complex relationships among species (Musgrave *et al.*, 2019). Parasite richness, prevalence, and the outcome of the host–parasite interactions vary significantly between geographical regions (Dunn *et al.*, 2010). For many plants and animals, species richness and abundance peak in the tropics and decrease towards the poles. Spatial variation in parasitism is well documented in a variety of taxa including trees (Albrechtsen *et al.*, 2010), insects

(Webberley *et al.*, 2006; Corby-Harris & Promislow, 2008), crustacean (Wolinska *et al.*, 2011), fish (Rohde, 1993, 2002; Timi, 2003), birds (Calvete *et al.*, 2003; Loiseau *et al.*, 2010), and mammals (Cumming, 2000; Nunn *et al.*, 2005). For marine fish, for example, the diversity of monogenean parasites increases with decreasing latitude or increasing water temperature (Rohde, 1993; Rohde & Heap, 1998). Explanations for these patterns include the energy hypothesis and the evolutionary time hypothesis (reviewed by Rohde, 1999; Poulin, 2007). These processes may act in synergy.

Monarch butterflies (*Danaus plexippus*) are an iconic and popular species, which are parasitised by the neogregarine protozoan *Ophryocystis elektroscirrha* in their native and introduced

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ranges (McLaughlin & Myers, 1970; Leong *et al.*, 1997). This parasite is a specialist on *Danaus* spp. (Gao *et al.*, 2020). It can harm butterflies by negatively affecting several life-history parameters including pre-adult survival, adult body mass, mating ability, fecundity, flight ability, and adult lifespan (de Roode *et al.*, 2019). Heavy infections appear to impair normal development leading to crippling wing deformities (Bradley & Altizer, 2005; de Roode *et al.*, 2008a). Parasitism by *O. elektroscirra* has been hypothesised to be a contributing factor to monarch butterfly declines (Davis & Rendon-Salinas, 2010; Thogmartin *et al.*, 2017). It is also thought to have contributed to the evolution of monarch migratory behaviour. The 'migratory escape' hypothesis predicts that migrating butterflies benefit from escaping pathogen accumulation in contaminated environments (Bartel *et al.*, 2011; Flockhart *et al.*, 2018; de Roode *et al.*, 2019). The 'migratory culling' hypothesis predicts the selective removal of infected butterflies occurs during their migration (Bradley & Altizer, 2005; Bartel *et al.*, 2011; de Roode *et al.*, 2019). Debate continues as to how parasitism by *O. elektroscirra* has contributed to the evolution of migratory behaviour in these butterflies.

Monarch butterflies have expanded their distribution to at least 74 countries around the world (Nail *et al.*, 2019). They are the only resident *Danaus* species and host to *O. elektroscirra* in New Zealand, although *D. chrysippus* and *D. hamatus* are infrequently blown to the country from Australia (Clarke & Zalucki, 2004; Zalucki & Clarke, 2004; Gibbs, 2013). In New Zealand, monarch butterflies occupy a latitudinal range of -34.5 to -46.0°S (Gibbs, 1980), which encompasses a wide array of temperatures and growing conditions.

Temperature seems the most critical factor affecting Lepidoptera dynamics (Li *et al.*, 2017; Thogmartin *et al.*, 2017). Protozoan parasites in other species have previously been observed to vary with latitude owing to their sensitivity to factors such as temperature, pH, rainfall, and host diversity and abundance (Zilberstein & Shapira, 1994; Harvell *et al.*, 2002). In addition, temperature-dependent effects on parasite abundance and virulence have been widely observed (Dawson *et al.*, 2005; Schar-sack *et al.*, 2016; Musgrave *et al.*, 2019).

The New Zealand monarch populations offer a simplification of the tritrophic system present in their home range in North America. Serial founder effects have resulted in monarch populations displaying low genetic diversity (Pierce *et al.*, 2014). The limited diversity of these butterflies likely also limits the number of parasite genotypes, which have been shown to have varying levels of virulence in North America (de Roode *et al.*, 2007). There are at least 33 different milkweed plants in North America that can alter the parasite–host relationship (de Roode *et al.*, 2008b; Nail *et al.*, 2019), but only four milkweed species occur in New Zealand. Two species of *Gomphocarpus* (*G. fruticosus* and *G. physocarpus*) were introduced from South Africa and two *Asclepias* species (*A. curassavica* and *A. incarnata*) were introduced from North America. With the exception of *A. incarnata*, which loses its leaves over winter, the other three milkweed species are perennial in New Zealand, with *A. incarnata* being very uncommon (Gibbs, 2013). These shrubs are usually grown in gardens but they can also propagate naturally (Gibbs, 2013). Additionally, monarch butterfly populations in

New Zealand are non-migratory with one study finding <3% of individuals fly more than 20 km (Wise, 1980).

One previous study found that *O. elektroscirra* parasitism occurs worldwide in nearly every monarch population examined (Altizer & de Roode, 2015). That survey included data from New Zealand, and showed that *O. elektroscirra* was present, at approximately 50% infection prevalence, albeit a sample size of just six individuals. In our study, we sampled monarch butterflies via a citizen science project. Previous studies have similarly used citizen scientists to sample and help understand this host–parasite relationship (Bartel *et al.*, 2011; Satterfield *et al.*, 2015, 2016) and many other aspects of monarch biology (Ries & Oberhauser, 2015).

The objectives of our study were to determine the prevalence of *O. elektroscirra* infections in monarch butterflies across New Zealand, and test whether wing deformities are correlated with parasitism rates. Our initial prediction was that higher parasite loads will have a higher fitness cost that can be phenotypically observed as crumpled or deformed wings. We also asked if the monarch–*O. elektroscirra* interaction varied significantly by latitude, a proxy for air temperature.

Materials and methods

We sampled butterflies during the austral autumn of 2020. Autumn is when high numbers of *O. elektroscirra* spores occur (Bartel *et al.*, 2011) and coincided with the observation of a high number of wing deformities and monarch pupae having difficulties emerging from their chrysalis (Fig. 1a,b). We developed a website and press release asking for samples from New Zealand citizens (Young, 2020). Citizens were asked to sample any adult monarch butterflies present in their gardens. Sampling *O. elektroscirra* can be harmlessly and easily achieved by placing clear sticky tape (e.g. Scotch Tape) on the adult butterfly abdomen, to which a combination of butterfly scales and distinctive parasite spores stick (Altizer *et al.*, 2000; Davis *et al.*, 2004) (Fig. 1c,d). The spores can only be seen under >50x magnification. Citizens were asked to mail the tape with information on the wing condition (normal or deformed), butterfly sex, sampling location, and whether the samples were taken from wild or reared butterflies to the university. Many submitters also emailed us photographs of the butterflies showing the wing deformities. Not all submitted samples contained information on butterfly sex, wing deformity, or rearing information, so our sample size is different in different analyses. Our request coincided with the lockdown period for New Zealand during the COVID-19 virus pandemic and we had a total of 97 individuals/families, aged between 4 and 86 submit samples. Sampling took place between 15 April 2020 and 27 July 2020.

Following previous research (Altizer *et al.*, 2000; de Roode *et al.*, 2009; Flockhart *et al.*, 2018), we quantified spore counts into four categories based on the transmission biology of the parasite: 0 = no spores present, 1 = 1–99 spores, i.e., mild infection likely due to contamination as adults via mating or other activity (see de Roode *et al.*, 2007, 2009; Majewska *et al.*, 2019), 2 = 100–499 spores, medium infection and 3 = >500 spores, heavy to very heavy infection (Table S1). Thus,

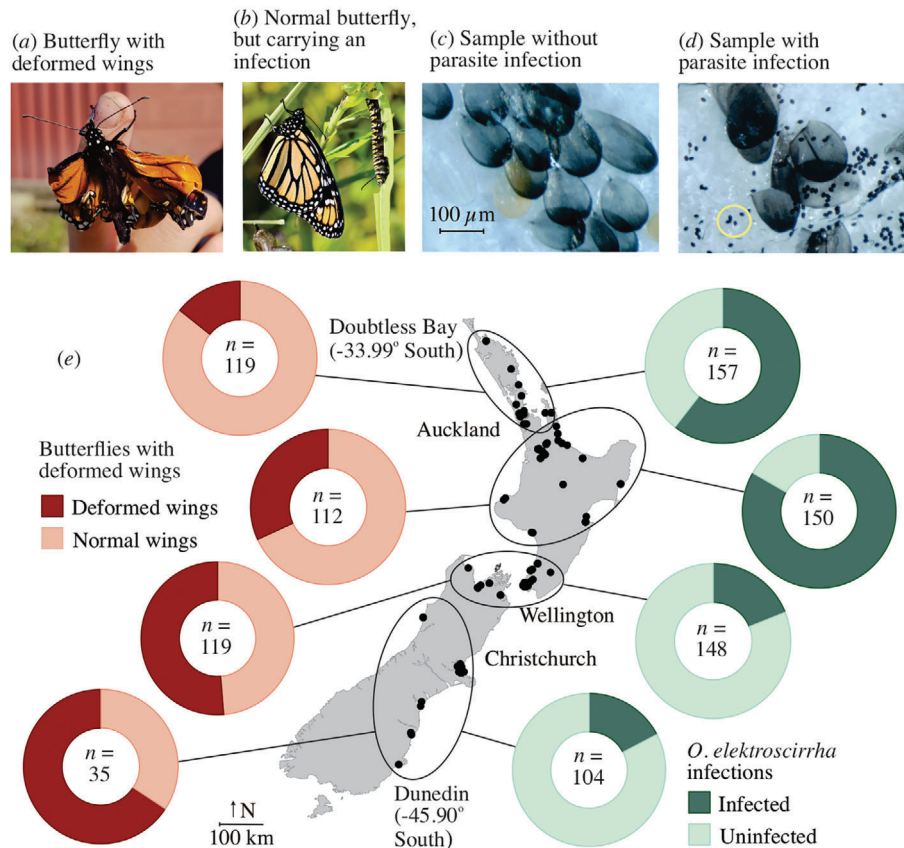


Fig. 1. (a) A butterfly with crumpled wings raised by 6-year-old Nicolas of Rotorua (photograph by Nicky King); (b) this adult butterfly has normal wings, but observed to be heavily infected by the protozoan parasite *Ophryocystis elektroscirrha*; (c) an example of samples showing butterfly scales but no spores, and (d) butterfly scales along with *O. elektroscirrha* spores (circled in yellow); (e) a map of New Zealand with pie charts showing deformed wing or infection prevalence. n = sample size; some people did not indicate the wing status of their butterfly samples (photographs b–d by Phil Lester). [Colour figure can be viewed at wileyonlinelibrary.com.]

we considered butterflies to be infected with *O. elektroscirrha* if their spore counts were categorised as either 2 or 3 in the above rationale. We used mixed-effects binary logistic regression to model the presence or absence of wing deformity, or parasite presence or absence, using the package lme4 (Bates *et al.*, 2015) in R (R Core Team, 2020) with ggplot2 (Wickham, 2016). lme4 uses maximum likelihood to fit models. The random effect in the models was the submitting individual/family. The fixed effects were latitude, and the sex of the butterfly (male or female). Our initial models included an additional fixed effect on whether the butterfly cocoon was known to have completed development in an artificial environment (e.g. a glasshouse, porch) or it developed in a wild setting. Akaike information criterion (AIC) values were used to compare different models. For the final model, we calculated the proportion of variance explained by both fixed and random factors (the conditional R^2 value) using the R package MuMIn (Bartoń, 2020).

Latitudinal gradients are correlated with variation in temperature. We explored this relationship using temperature data from the National Climate Database (CliFlo) of New Zealand's National Institute of Water and Atmospheric Research. We downloaded the average air temperature for the months of

March, April, and May 2020 (Anonymous, 2020), which encompassed the developmental period in the New Zealand autumn for our butterfly samples. Temperature stations were typically located within 10 km of the georeferenced sample collection.

We first used a linear regression analysis to show the relationship between latitude and average temperature, which showed a highly significant, positive relationship ($P < 0.001$) with an adjusted R^2 of 0.787. The relationships between the likelihood of wing deformity and parasite infection and temperature mirrored that with the analyses on latitude. The fit of the relationships, as assessed by AIC values, was better for the prevalence of parasites when using latitude as an independent variable rather than temperature (529.25 vs. 539.88, respectively). For wing deformities, the latitude and temperature gave similar AIC values (408.02 and 407.91, respectively). For consistency between analyses, we used latitude as the explanatory variable rather than temperature.

To confirm the identity of the parasite via molecular methods, we extracted DNA from parasite spores collected from eight monarch butterflies in sticky tape from three sampling locations (Auckland, Levin, and Christchurch). We selected samples

with the highest parasite infections (Table S1). Under the microscope, we separated the parasite spores from the sticky tape (making sure not to include butterfly wing scales) with small tweezers and needles and placed them in a microcentrifuge tube with 1 ml of isopropanol. The majority of spores were stuck to small pieces of tape, so they were left in isopropanol for 3 days. We then proceeded to DNA extraction using the Quick-DNA MiniPrep Kit (Zymo Research, USA). Spores were mechanically homogenised in a Precellys Evolution homogeniser (Bertin Instruments, France) in 500 µl of genomic lysis buffer for two cycles of 9900 rpm each. Samples were incubated in a heat block at 56°C for 2 h. The remaining of the protocol followed the kit's manufacturer instructions. Following DNA extraction, we amplified a 558 bp fragment of 18S rRNA using the primers designed by Gao *et al.* (2020). Reaction volume was 50 µl containing 10 ng DNA, 1x MyTaq Mix (Bioline, Australia), 5 pmol of each primer (Thermo Fisher, New Zealand), Bovine Serum Albumin (Sigma Aldrich, New Zealand), and ultra-pure, distilled water (Invitrogen, New Zealand). The PCR product was examined by agarose gel electrophoresis and purified with rSap combined with Exo 1 (New England Biolabs, USA). Sequencing was performed on an ABI 3130x1 Genetic Analyzer (Applied Biosystems, USA) at Massey Genome Service (Palmerston North, New Zealand). We aligned the forward and reverse gene sequences using the default alignment algorithm implemented in the software Geneious v. 10.2.6 (<http://www.geneious.com>). The resulting sequence was submitted to GenBank (GenBank accession number: MT742950).

Results

We received 564 samples of adult butterflies spanning the distribution of monarch butterflies in New Zealand (Fig. 1e). Out of these, five samples were excluded as there were no scales in the sticky tape, thus our effective sample size was 559. For samples where the butterfly sex was recorded ($n = 510$), 53.1% were female and 46.9% were male. Out of the 559 samples, 58.9% ($n = 329$) were infected with *O. elektroscirra* spores, with 38.4% of specimens being recorded as having deformed wings (134 butterflies with deformed wings out of 385 butterflies with wing status reported; Fig. S1). No parasitism was observed in our highest latitudinal location of Dunedin, while all butterfly samples were infected in the warmest and lowest latitudinal site of Doubtless Bay (Fig. 1e). Our 18S rRNA sequence confirmed that the parasite present in New Zealand is indeed *O. elektroscirra*. Our sample matched the sequences of *O. elektroscirra* extracted from monarch butterflies from Costa Rica and the Netherlands in the GenBank database with a similarity of 100%.

Contrasting patterns of wing deformity and parasitism infection were observed. The rate of wing deformities increased with increasing latitude (Figs 1e and 2). Our logistic regression model predicted <20% specimens would have wing deformities in the lowest latitude or warmest field sites. In the highest latitudinal sites, the prevalence of wing deformities was predicted to increase to >80%. In contrast, parasitism by *O. elektroscirra* was shown to be lowest in the higher latitudinal localities

sampled (<10%), though increased to >90% in the lowest latitudinal sites (Fig. 2). These counter-cline results were contrary to our expectations, in which we expected wing deformities to be correlated with parasitism rates. The average temperature in the austral autumn months (when samples were collected, from April until the end of May) declines along this latitudinal range from 17.0°C in Auckland, to 14.7°C in Wellington, to 13.5°C in Dunedin (Anonymous, 2020). Latitude in New Zealand is highly correlated with temperature.

Our mixed-effects logistic regression model examined how the presence or absence of parasitism was related to latitude, butterfly sex, and wing deformity. Our initial model included the categorisation of whether the butterfly cocoon was known to have completed development in an artificial environment (e.g. a glasshouse) or it developed in a wild setting. Because the rearing method was not significant in the analyses for either parasitism or wing deformities ($P \geq 0.655$), we excluded it from the final logistic regression models. These final models incorporated the individual submitter or family as a random factor and included only the main effects of latitude and butterfly sex. For the presence or absence of spores, latitude was statistically significant with a positive slope ($z = 4.933$; $\beta = 0.805 \pm 0.161$; $P < 0.001$). The conditional R^2 value indicated that 75.7% of the variation in the presence or absence of spores was explained by this model. For wing deformity, latitude was also statistically significant, but with the opposing effect of a negative slope ($z = -2.965$; $\beta = -0.357 \pm 0.120$; $P = 0.003$). The conditional R^2 value indicated that 55.5% of the variation in the presence or absence of wing deformity was explained by this model. The prevalence of parasitism or wing deformity did not significantly vary with the butterfly sex ($P \geq 0.068$).

We obtained the average air temperature for each sampling location for the autumn period. Substituting the average air temperature for latitude gave very similar results in our mixed-effects logistic regression models. For the presence or absence of spores, temperature was statistically significant with a positive slope ($z = 4.418$; $\beta = 1.525 \pm 0.345$; $P < 0.001$). For wing deformity, temperature was also statistically significant with the opposing effect of a negative slope ($z = -2.928$; $\beta = -0.682 \pm 0.233$; $P = 0.003$). Again, either the prevalence of parasitism or wing deformity did not vary significantly with the sex of the butterfly ($P \geq 0.079$).

Discussion

We observed a distinct gradient in butterfly parasitism rates by *O. elektroscirra* in New Zealand. Infection prevalence was substantially higher in the warmer environments to the north and lowest in the coolest, southernmost sites of New Zealand. High infections of this protozoan parasite can have substantial fitness costs for monarch butterflies, manifesting as a reduced mating ability, fecundity, flight ability, and adult lifespan (de Roode *et al.*, 2019). Our results suggest that the high prevalence of parasite infections may affect butterfly fitness in warmer regions, but the low *O. elektroscirra* prevalence in higher latitudes means that any detrimental effects from this parasite must be substantially reduced in these cooler environmental conditions.

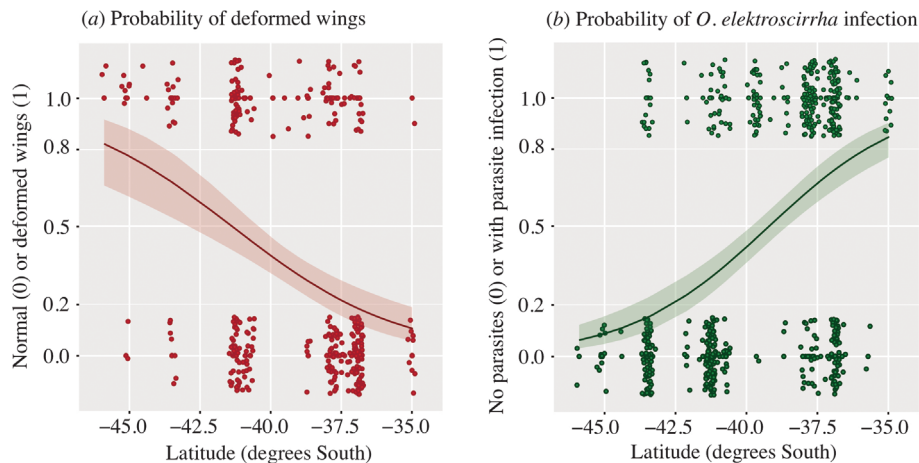


Fig. 2. Logistic regression analyses of (a) butterflies displaying normal or deformed wings, and (b) the presence or absence of *O. elektroscirra* spores. All values were either 0 or 1, but are displayed here using the 'jitter' function of ggplot2 in order to provide an indication of sample sizes. The fitted curves are shown with 99% confidence intervals. [Colour figure can be viewed at wileyonlinelibrary.com].

Butterflies at higher latitudes with cooler conditions, however, have a major fitness cost expressed as wing deformity. We hypothesise that monarch butterfly populations may perform better at intermediate latitudes and temperatures.

A latitudinal gradient in parasitism is similarly observed in populations of monarch butterflies in North America, where three distinct populations occur. A population in southeastern USA where the majority of butterflies are non-migratory and breed year-round; a population in the western coast of North America with some individuals migrating short distances to overwinter in California, where seasonal and year-round breeding sites can also be found (Satterfield *et al.*, 2016); and a population in eastern North America that migrates from as far north as Canada to Central Mexico (Altizer *et al.*, 2000). The population migrating from the highest latitudes has the lowest rate of parasitism by *O. elektroscirra* (reviewed by de Roode *et al.*, 2019). The year-round breeding, non-migratory populations found in the warmer areas of southern Texas, Louisiana, Mississippi, Alabama, Georgia, and Florida together with the non-migratory southern California populations have the highest parasitism rate (Satterfield *et al.*, 2016; de Roode *et al.*, 2019). In addition, these non-migratory populations of monarchs have also been described as susceptible to chilling injury and unable to withstand freezing (Troyer *et al.*, 1996). The lower development threshold for monarch larvae is 11–12°C (Zalucki, 1982); larvae are freeze-intolerant, whereas butterfly eggs are chill-intolerant, with third instars being most sensitive to extreme cold and heat exposure (Nail *et al.*, 2015). The increase in wing deformities with increasing latitude in our study seems likely to be related to cold temperatures. Our results from the comparatively simple tritrophic system with non-migratory butterflies in New Zealand suggest that limited cold tolerance may be a driver of parasitism rates, wing deformities, and butterfly migratory behaviour. However, given the potential for inbreeding in New Zealand monarchs due to the founder effects experienced (Pierce *et al.*, 2014), we cannot discount the possibility that a low level of genetic diversity could be affecting the development of wings through

altering mutation frequencies in the wingless gene, bacterial infections, and/or the absence of the endosymbiont *Wolbachia* (Pierzynowska *et al.*, 2019). Future research studies that analyse the known causes of wing malformations in butterflies in New Zealand monarchs are indeed warranted.

In North American monarch populations, migration has been considered likely to mediate interactions between the monarch host and the *O. elektroscirra* parasite (Altizer *et al.*, 1999). The parasite–host interaction was suggested as a major force in driving seasonal migration behaviour (Altizer *et al.*, 2000; Bradley & Altizer, 2005; Bartel *et al.*, 2011; Flockhart *et al.*, 2018; de Roode *et al.*, 2019). The use of the same butterfly habitats for long periods allows for the accumulation of the parasite infectious stages over time; migration helps the butterflies escape from the contaminated habitats. This 'migratory escape' of the parasites might play a role in the evolution of migration (Altizer & de Roode, 2015). 'Migratory culling' or the selective removal of weak, infected butterflies during their energetically costly migration could also drive the host–parasite patterns seen in North America (Bradley & Altizer, 2005; Bartel *et al.*, 2011). Migratory culling or migratory escape could well be evolutionary drivers of the monarch's migration behaviour in North America. A benefit of the migration would be to avoid chilling injuries resulting in the wing deformities that we observed in the New Zealand populations, which are non-migratory (Wise, 1980). The apparent intolerance of cold temperatures by the parasite that we observed may also contribute to the *O. elektroscirra* prevalence patterns in North America and other countries. Laboratory studies rearing butterflies at different temperatures, with and without parasites, will be needed to control for the effects of migration and further understand the role of these parasites and the environment on monarch fitness.

Laboratory studies with monarch butterflies and their parasite may also enable researchers to control for other effects that could have contributed to our results. Monarch butterfly distribution is certainly influenced by the availability of host plants (Zalucki & Rochester, 1999). Monarch butterfly density may have varied

across our study region, which could also have influenced the abundance of the parasite. The effects of butterfly density on susceptibility to *O. elektroscirra* have been quantified in the laboratory, with the probability of infection increasing with increasing larval density. Monarchs reared at intermediate and high densities suffered the greatest negative effects of parasitism (Lindsey *et al.*, 2009). Variation in relative humidity, particularly dry stress as seen in Australia, might have also played a role in the outcome of the host–parasite relationship (Altizer *et al.*, 1999).

Monarch butterfly populations have been considered to be threatened, due to a variety of factors including climate change that may result in the freezing of butterfly populations (Nail *et al.*, 2019; Pennisi, 2021). Other researchers have examined the role of climatic factors, habitat loss, disease, and agricultural insecticide use, finding that breeding season temperature was an especially important determinant of annual variation in abundance (Thogmartin *et al.*, 2017). Our results suggest that disease and climate might interact in a latitude-specific fashion. Conservation approaches may need to be regionally tailored to account for interacting factors, such as parasite–climate interactions. Finally, these results further highlight the benefits and immense value of the “citizen army for science” in understanding monarch butterfly biology and behaviour (Ries & Oberhauser, 2015). Citizen science has had a long history in deciphering the migration of these butterflies (Urquhart, 1976). Our study supports the conclusion that opportunistically-collected citizen science data can transform our understanding of species distributions, phenology, and diversity gradients for these butterflies and many other species (Soroye *et al.*, 2018).

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The authors have no conflict of interest to declare.

Author contributions

P.J.L. conceived and coordinated the study. Both authors analysed the samples and wrote the manuscript. M.B. conducted the genetics lab work and categorised the spore counts.

Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1: The complete dataset used in our analysis. Data on sample location, parasitism, butterfly sex and wing deformities.

Fig. S1: Graph showing the spore samples that fell into each of the four categories for spore counts.

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