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Received: 1 March 2023 | Accepted: 30 August 2023

DOI: 10.1111/een.13280

ORIGINAL ARTICLE

Ecological Entomology

Exploring spatial and temporal patterns of viral infection across populations of the Melissa blue butterfly

Kelli J. McKeegan[1,2] | Nadya D. Muchoney[1,2] | Mike B. Teglas[1,3] | Matthew L. Forister[1,2] | Angela M. Smilanich[1,2]

[1]Program in Ecology, Evolution, and Conservation Biology, University of Nevada, Reno, Nevada, USA

[2]Department of Biology, University of Nevada, Reno, Nevada, USA

[3]Department of Agriculture, Veterinary and Rangeland Sciences, University of Nevada, Reno, Nevada, USA

Correspondence
Kelli J. McKeegan, Program in Ecology, Evolution, and Conservation Biology, University of Nevada, 1664 North Virginia Street, Reno, NV 89557, USA.
Email: kelli.mckeegan@nevada.unr.edu

Funding information
Graduate Student Association, University of Nevada; National Science Foundation, Grant/Award Numbers: DEB-1929522, DEB-2114793; Nevada IDeA Network of Biomedical Research Excellence; Core Service, Grant/Award Number: GM-103440

Associate Editor: Julia Ferrari

Abstract

1. Identifying patterns of pathogen infection in natural systems is crucial to understanding mechanisms of host–pathogen interactions. In this study, we explored how Junonia coenia densovirus (JcDV) infection varies over space and time in populations of the Melissa blue butterfly (*Lycaeides melissa*: Lycaenidae) using two different host plants. Collections of *L. melissa* adults from multiple populations and years, along with host plant tissue and community samples of arthropods found on host plants, were screened to determine JcDV prevalence and load. Additionally, we sampled at multiple time points within a single *L. melissa* flight season to investigate intra-annual variation in infection patterns.
2. We found population-specific variation in viral prevalence of *L. melissa* across collection years, with historical samples potentially having higher viral prevalence than contemporary samples, although host plant diet was not informative for these patterns. Patterns of infection across multiple generations within a flight season showed that late-season samples had a higher proportion of JcDV-positive individuals, suggesting an accumulation of virus over the season. Sequence data from a segment of the JcDV capsid gene showed a lack of viral genetic diversity between *L. melissa* collected from different localities, and little to no viral particles were found in the surrounding environment.
3. Our discovery of temporal variation in infection suggests that multiple sampling efforts must be made when describing pathogen prevalence in multivoltine hosts. Our findings represent an important first step towards further exploration of the ecological factors mediating disease prevalence and host-specific variability of infection in wild insect populations.

KEYWORDS
densovirus, host–pathogen interactions, *Lycaeides melissa*, spatial ecology, temporal patterns

INTRODUCTION

All major pathogenic taxa (fungi, viruses, bacteria and nematodes) cause disease in insects, and identifying patterns of infection in natural systems is crucial to improving our general understanding of host–pathogen dynamics (Fuxa & Tanada, 1987; Hajek & Shapiro-Ilan, 2018). Most of the research on these entomopathogens is conducted in the context of biological pest control in agricultural systems (Inceoglu et al., 2001). However, it is also necessary to study host–pathogen interactions in the field to understand potential impacts of biocontrol agents on non-target or beneficial species, uncover important mediators of infection or host population

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regulation that cannot be detected in laboratory settings, and identify consequences of emerging disease (Hajek & Shapiro-Ilan, 2018). Exploring the spatial and temporal patterns of infection, especially in non-model systems, has the potential to improve our understanding of pathogen host range, transmission, and other ecological and evolutionary phenomena (Campos-Herrera & Lacey, 2018; Dwyer & Elkington, 1995; Myers & Cory, 2016).

Insect-specific viruses in the well-studied family *Baculoviridae* are known for their host specificity and pathogenic effects causing high mortality in insect hosts (Clarke & Clem, 2003; Nouri et al., 2018; Sosa-Gomez et al., 2020). Baculoviruses have been the foundation for understanding how viruses interact with the host immune system (Sparks et al., 2008), how biotic and abiotic variables influence viral persistence and interactions within an insect community (Cory et al., 2000; Fuxa, 2004; Hajek & Shapiro-Ilan, 2018; Peng et al., 1999), and how viruses vary genetically both within and across host populations (Harrison et al., 2013; Thézé et al., 2014; Williams et al., 2017). However, given the enormous diversity of viruses, insect host–virus interactions have many unexplored avenues (Suttle, 2013; Williams, 2018), and there is limited knowledge of infection patterns outside of a few well-studied systems (e.g., Nucleopolyhedroviruses in Lepidoptera; Myers & Cory, 2016). Here, we investigate patterns of infection in natural populations caused by a densovirus that infects Lepidoptera within the family Lycaenidae.

Densovirinae is a subfamily of *Parvoviridae*: small, non-occluded, single-stranded DNA viruses that have well-conserved genomes but are paradoxically quite diverse (Bergoin & Tijssen, 2000; Fédiére, 2000). Parvoviruses are relatively resilient to environmental stressors such as UV and heat exposure, with previous studies showing some non-occluded pathogens remain active many days longer than enveloped viruses when challenged with acidification and storage time (Clifford & Watson, 2008; Vinnerås et al., 2012). The persistence of densoviruses, which infect invertebrates, outside of the host is not as well known, but there is evidence that some DVs are environmentally stable (Carlson et al., 2006; Johnson & Rasgon, 2018). Many DVs have been extensively researched for their applicability as biocontrol agents (e.g., those infecting insect pests; Carlson et al., 2006; Jiang et al., 2007; Johnson & Rasgon, 2018), while others impact economically beneficial species such as commercially produced arthropods (e.g., crickets and shrimp; Liu et al., 2011; Rai et al., 2012), and more are emerging or still being described (Jackson et al., 2020). Densoviruses vary widely in virulence and host range across taxa (Gupta et al., 2015; Johnson & Rasgon, 2018), with some showing a mutualistic relationship with their host (Xu et al., 2014). Studies have also shown variation in DV effects within host species, explained by host sex (Perrin et al., 2020; Xu et al., 2014), diet (Muchoney et al., 2022; Smilanich et al., 2018) and genomic diversity of the virus (El-Margawy et al., 2003; Federici & Maddox, 1996; Rai et al., 2012). Our study focused on a host–densovirus relationship, specifically working with natural populations of the widespread Melissa blue butterfly, *Lycaeides melissa* (Lepidoptera: Lycaenidae), and the Junonia coenia densovirus (*Protoambidensovirus lepidopteran1*).

The Junonia coenia densovirus (JcDV; first isolated from *Junonia coenia* in the region of Berkeley, California by Rivers & Longworth, 1972) has a broad host range within Lepidoptera and has been studied in several families (e.g., Nymphalidae, Bombycidae, Noctuidae, Erebidae; Gupta et al., 2015; Mutuel et al., 2010; Muchoney et al., 2022; Smilanich et al., 2018). JcDV is contracted during the larval stage by consumption of contaminated plant matter, after which viral particles cross the larval midgut by transcytosis and replicate in the epidermis and tracheal tissue (Kemmerer & Bonning, 2020; Mutuel et al., 2010). In vivo studies of JcDV in *Spodoptera frugiperda* found that infection and pathogenesis of this virus are dependent on both dose and time, or host life stage (Mutuel et al., 2010). Individuals infected with high loads ($LD_{50} = 5.0 \times 10^9$ viral genomes) of JcDV as early-instar larvae experience structural defects in the affected tissues, disrupting important functions such as respiration and moulting. Diseased hosts may become oxygen-depleted and "stuck" between moults, often leading to death before pupation or adult emergence (Mutuel et al., 2010). Data on transmission strategies are not yet reported for JcDV, although recent data with white peacocks (*Anartia jatrophae*: Nymphalidae) show that infected individuals that make it to the adult stage have survived infection, but still test positive for the virus (Muchoney et al., 2023). Vertical transmission from mother to offspring has been observed in several other insect-densovirus systems (Altinli et al., 2019; Kittayapong et al., 1999). Additionally, while other densoviruses have been described in the field (Kittayapong et al., 1999; Xu et al., 2014), much of JcDV research to-date has taken place predominantly in a laboratory setting (but see Muchoney et al., 2022).

The Melissa blue (*Lycaeides melissa*) is a non-migratory, sexually dimorphic butterfly in the family Lycaenidae that is found in discrete patches based on host plant distribution across western North America, with limited dispersal between populations (Forister et al., 2020; Gompert et al., 2014). Melissa blues utilise members of the Fabaceae family (in our region, the western Great Basin, these are most commonly *Astragalus canadensis* or *Lupinus argenteus*) as host plants for larval diet and oviposition but have recently (~200ya) expanded to include alfalfa, *Medicago sativa*, often in roadside or weedy environments (Chaturvedi et al., 2018). There is evidence that this novel host plant is nutritionally inferior to native host plants, resulting in lower mass and reduced diversity of gut microbiota of larvae utilising *M. sativa* (Forister et al., 2013; Yoon et al., 2019). Melissa blue populations are typically multivoltine, undergoing three to four overlapping generations throughout the flight season and overwintering as eggs. This suite of life history traits makes *L. melissa* unique from other lepidopteran families in which JcDV has been studied (Noctuidae, Mutuel et al., 2010; Nymphalidae, Smilanich et al., 2018; Nymphalidae, Muchoney et al., 2022, 2023). While *L. melissa* has been shown to be a viable JcDV host in laboratory settings (Yoon, 2021), the host–pathogen relationship has yet to be described for any densovirus infecting natural populations of *L. melissa*, nor have patterns of JcDV infection been described for any butterfly species in the family Lycaenidae.

Our overarching goal was to describe the spatial and temporal patterns of JcDV infection in wild Melissa blue populations. With prior knowledge that many densoviruses are generalists and *L. melissa* is a viable host, we first hypothesized that JcDV would be present in

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natural populations of the butterfly, which would represent a new host record for the virus. Moreover, because feral alfalfa (*M. sativa*) is nutritionally inferior and there is evidence of dietary effects on herbivore immune response to JcDV (Muchoney et al., 2022; Yoon et al., 2019), we predicted that Melissa blue populations utilising the novel host plant would be less robust and thus more susceptible to pathogens compared with populations using native host plant. Specifically, we predicted that populations utilising feral alfalfa would have higher JcDV prevalence (percentage of infected individuals in the sampled population) and average viral load (i.e., viral burden) compared with the native host populations.

The second goal in this study was to understand how viral infection patterns change over time in natural populations of host insects. We hypothesized that since *L. melissa* is multivoltine and exists in genetically discrete populations, we would find intra- and interannual fluctuations in JcDV infection. To investigate this, we compared viral infection among historical samples and contemporary collections, as well as repeated samples from a subset of populations made within the same flying season. Thirdly, we investigated the community presence of JcDV by screening rinses of *L. melissa* host plants, as well as ants (Formicidae) and treehoppers (Membracidae) found on these plants. Given that Melissa blue caterpillars and treehoppers are ant-tended and coexist (Forister et al., 2020), we hypothesized JcDV would be present in, or on the surface of, these other community members, possibly informing us of potential routes for horizontal viral transmission.

The study of emerging diseases in natural systems warrants pathogen genomic sequence comparisons, not only to confirm the identity of the pathogen but to potentially understand sources of variation in different host species and populations. Although other densoviruses are highly genetically conserved (Tijssen & Bergoin, 1995), we suspected diversity might be found in the viral capsid gene (Roos et al., 2007; Song et al., 2016). Therefore, we also compared JcDV capsid gene fragments among wild *L. melissa* localities to explore the possibility that variation in infection prevalence is explained by viral genetic variation between discrete host populations. This study represents a first step towards understanding this particular host–pathogen system and identifying influential variables.

MATERIALS AND METHODS

Sampling for host plant effects on JcDV infection in wild Melissa blues

To evaluate the historical prevalence of Junonia coenia densovirus (JcDV) in wild Melissa blue populations, we screened DNA extracted from butterflies collected in previous years (2001–2015; see Gompert et al., 2014, for sampling protocol). Briefly, the collections consisted of 226 *L. melissa* adults from 19 locations in western North America (Figure 1), mostly collected during single sampling events, with a few populations having two or three collection dates over a span of a few years (Table 1). Of the 19 populations surveyed, 11 utilised native host plants (in the genera *Astragalus*, *Glycyrrhiza* and *Lupinus*), 7 utilised roadside or naturalised alfalfa (*Medicago sativa*) as a novel host, and one population was found on both native and novel host plants (Table 1). Individuals that were screened for JcDV from the historical collection were chosen based upon availability and amount of DNA and with the goal of representing populations that use either native host plants or the novel host, alfalfa, over a broad geographic range (Figure 1). Due to the amount of time that had elapsed since collection, some DNA failed to amplify in initial screenings, and the sample sizes varied among populations and specific years of collection (Table 1). qPCR screening of these historical samples was conducted in 2018, and viral gene sequencing was conducted on a subset of these samples in 2019 (see below).

To obtain a snapshot of contemporary viral presence in *L. melissa* populations and quantify community JcDV presence, 516 adults were collected over the 2020 *L. melissa* flight season (late May through early September). We were unable to access the site or locate adults at seven of the locations sampled in the past, but nine sample sites were added for the 2020 survey. Twelve populations overlapped between those surveyed prior to 2016 and those visited in 2020 (Table 1). Of the 21 total distinct *L. melissa* populations surveyed for contemporary measures of JcDV presence, 9 utilised native host plants, 8 utilised alfalfa, and 3 populations utilised both native and novel host plant types (Table 1). Collections of adult butterflies were typically made midday as temperatures were highest and wind speed was lowest, and all specimens were promptly freeze-killed and stored in individual glassine envelopes at −20°C until viral analysis.

Sampling for temporal variation of JcDV infection over the Melissa blue flying season

To measure intra-annual viral infection, *L. melissa* adults were collected multiple times during the 2020 season from a subset ($N = 4$) of the contemporary populations (BWP, WAL, VUH, VEC; Table 1). Sampling intensity was influenced by densities observed at each site: VUH was the only location with enough adults to be sampled in late May; VUH, WAL and BWP were sampled in the third week of June; all four sites were sampled in early July; and finally, VUH and VEC were sampled in mid-August and the first week of September. BWP experienced a fire after the second collection event, which prevented that site from being sampled further throughout the season. At the WAL site, adults could not be found for a late-season collection, unlike the two Verdi populations (VUH and VEC), where *L. melissa* is typically more abundant.

Sampling for environmental JcDV presence in communities including Melissa blues

At 14 of the sites sampled in 2020, tissue samples from *L. melissa* host plants were obtained. Because ants are known to tend *L. melissa* larvae at many of these locations, ants (Formicidae: mostly of the genus *Formica*) and ant-tended treehoppers (Membracidae: *Campylenchia*) found on each plant were also collected (Scholl et al., 2014). Plants were selected systematically to represent the entire locality, with an emphasis on maximising the density of arthropods collected.

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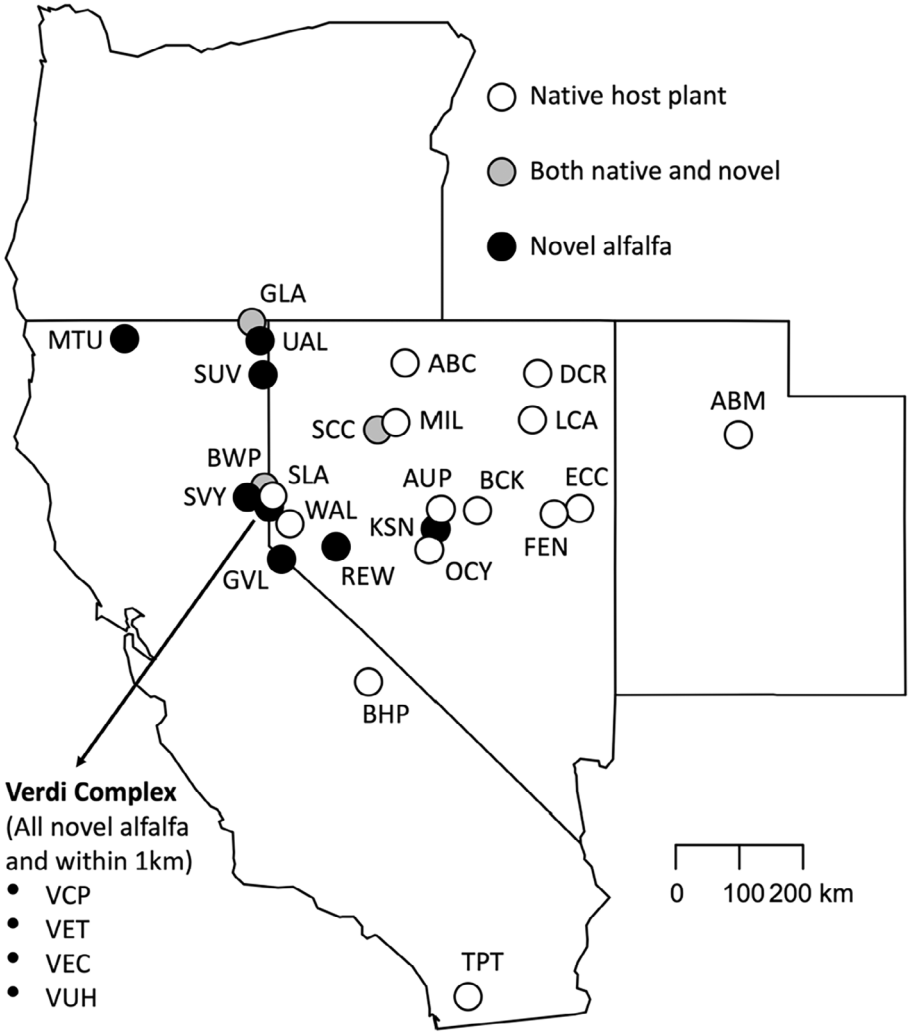

FIGURE 1 Map of *Lycaeides melissa* collection sites in the western United States of California, Oregon, Nevada and Utah. Black points represent populations that utilise novel alfalfa (*Medicago sativa*), white points represent native host plant sites and grey points represent populations that utilise both native host plants and novel alfalfa. Coordinates for localities in Table S1.

Tissue was collected using sterile technique from 10 plants at each site by covering the plant part with a Whirl-Pak (Nasco) and pulling to detach the tissue. To account for potential variation in surface viral presence due to factors such as UV exposure or moisture, each plant sample consisted of a combination of three stems closer to the soil, terminal plant tissue from each stem including leaves, and three to four flower clusters (if present). Ants and treehoppers were collected whenever present on the plant being sampled using forceps (rinsed in 10% bleach solution between uses), then placed in sterile 2-mL microcentrifuge tubes ($N = 148$ samples). All samples (*L. melissa* larvae and adults included) were promptly placed in −20°C freezers upon return from the field.

To detect JcDV on sample surfaces, ultrapure water (1 mL for plants, 500 μL for arthropods) was added to plant, ant and treehopper Whirl-Pak bags and tubes in the laboratory. Samples were shaken gently for 30 s to cover all surfaces. An aliquot of rinse water was taken from each container (500 μL for plants, 300 μL for arthropods) and pipetted into new microcentrifuge tubes, which were then refrozen until used as template for qPCR (protocol below).

qPCR protocol for viral detection

JcDV was assayed in sample DNA and surface rinses using quantitative PCR. DNA from adult *L. melissa* thoraxes, and whole-specimen DNA from a subset of 25 ant and 15 treehopper samples, was extracted using DNeasy Blood and Tissue Kits (QIAGEN). Using iTaq universal SYBR Green supermix (Bio-Rad) and primers specific to the viral capsid gene (VP4, Wang et al., 2013), reactions were run in triplicate with a Bio-Rad CFX96 Optics Module and C1000 thermocycler. Primers specific to an arthropod 28 s mitochondrial gene were used as an internal control in a separate PCR to verify the quality of extracted DNA samples (Nice et al., 2009). All qPCR reactions (for both the VP4 gene and 28 s gene) were performed according to the protocols described by Muchoney et al. (2022). Each qPCR run had

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TABLE 1 Overview of sampling effort among *Lycaeides melissa* localities and years (historical = 2001–2015; contemporary = 2020), with host plant type (native; alfalfa).

Population	Code	Host	Historical N (year)	Contemporary (2020) N
Bishop	BHP	Native	14 (2011)	14
Deeth Charleston Rd.	DCR	Native	15 (2011)	2
East Creek Cg.	ECC	Native	21 (2014)	9
Gardnerville	GVL	Alfalfa	3 (2001), 11 (2004), 1 (2007)	11
Lamoille Canyon	LCA	Native	1 (2010), 7(2011)	12
Montague	MTU	Alfalfa	8 (2007)	52
Ophir City	OCY	Native	5 (2012)	13
Star Creek Canyon	SCC	Both	10 (2012)	13
Surprise Valley	SUV	Alfalfa	12 (2011)	64
Sierra Valley	SVY	Alfalfa	9 (2001), 6 (2002)	75
Upper Alkali Lake	UAL	Alfalfa	11 (2012)	15
Little Washoe Lake	WAL	Native	7 (2011), 2 (2012)	12
Abel Creek	ABC	Native	15 (2012)	–
Albion Meadow	ABM	Native	21 (2012)	–
Mill Creek	MIL	Native	11 (2015)	–
Red Earth Way	REW	Alfalfa	9 (2011)	–
Silver Lake	SLA	Native	10 (2012)	–
Trout Pond Trailhead	TPT	Native	10 (2010)	–
Verdi Crystal Peak	VCP	Alfalfa	3 (2011), 4 (2012)	–
Austin Summit	AUP	Native	–	1
Big Creek Cg.	BCK	Native	–	3
Beckwourth Pass	BWP	Both	–	50
Fifty East NV	FEN	Native	–	4
Goose Lake	GLA	Both	–	25
Kingston Canyon	KSN	Alfalfa	–	10
Verdi Classic	VEC	Alfalfa	–	61
Verdi New Tracks	VET	Alfalfa	–	13
Verdi Under Highway	VUH	Alfalfa	–	65

Note: The number of individuals collected at each site is represented by N. Sites also represented geographically on a map in Figure 1.

two wells of a positive control (with 10^8 JcDV genome units/μL water used as template) and two negative, or no-template, controls using only water.

Viral loads reported here and used in the analyses were determined using a standard curve to calculate absolute number of JcDV genome copies. Collaborators in the M. Ogliastro Lab (University of Montpellier) provided us with viral stock of known concentration, which then underwent a series of seven 1:10 dilutions (1.0×10^9 through 1.0×10^3). We screened each dilution using the same JcDV primers and qPCR protocol described above, plotting the average cycle threshold (Ct) values of individual replicates. The regression equation obtained from the slope of the plotted Ct values of known JcDV concentration ($y = -0.2413x + 11.601$; $R^2 = 0.99$) was then used to algebraically determine JcDV quantity in each sample (viral genomes, vg) from the Ct values of the VP4 gene. All measures of viral prevalence, or frequency, are represented as a proportion of JcDV-positive individuals out of the total number of individuals collected in each group.

Viral sequence comparisons

To explore whether patterns of viral infection were associated with variation in the viral genome, we compared sections of the JcDV capsid genes (VP1-4; length: 445 bp) in a subset of virus-positive individuals in 10 historical populations from which we had remaining DNA after qPCR analysis. Due to low overall viral DNA concentration in wild-caught hosts, genes were amplified using a nested PCR design. For the first round of nested PCR on the JcDV viral capsid (VP4) gene, the external primer sequences were obtained from collaborators at the University of Montpellier, France (Ogliastro Lab) and were as follows: 5′-ACGCTCCACATAACTCGCAA-3′ (forward) and 5′-GGT

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GCACTAGTAGCAGTGGG-3′ (reverse). The total reaction volume for each well was 25 μL, which consisted of Promega GoTaq® Flexi DNA polymerase (0.125 μL per well) and buffer (5 μL), DNA nucleotides (0.5 μL) and primers (1 μL each forward/reverse, concentration: 10 μM), magnesium chloride (3 μL), and 5 μL of template DNA. Reactions were performed using a Bio-Rad T100 Thermal Cycler using the following parameters: 95°C for 2 min; followed by 35 cycles of 95°C for 30 s, 57°C for 30 s and 72°C for 50 s; then a final extension step at 72°C for 5 min.

The recipe, concentrations and parameters for the second round of PCR, using the products of the first round as the template, were the same as the first round except for a 60°C annealing step (instead of 57°C) and 2 μL magnesium chloride (instead of 3 μL). The internal primer sequences were as follows: 5′-TCCTAGTTCTTCCGGAGCAA-3′ (forward) and 5′-TGATCTATCAATACCCCATCCAAGT-3′ (reverse). Products were then visualised on a 1% agarose gel using ethidium bromide. Samples showing clear bands on the gel ($N = 31$) were processed using the QIAquick PCR Purification Kit (QIAGEN) and submitted to the Nevada Genomics Center for Sanger sequencing.

Statistical analyses

All statistical analyses were performed in R (v4.2.1) with general utilisation of the "dplyr" package (v1.0.10, Wickham, Chang, et al., 2022) to manipulate datasets. The R package "lubridate" (v1.8.0, Spinu et al., 2021) was used to calculate ordinal day from collection dates, and the packages "ggplot2" (v3.3.6, Wickham, François, et al., 2022) and "ggmap" (v3.0.0, Kahle et al., 2019) were used to construct the collection site map figure. Data figures were made in SigmaPlot (v14, Systat Software, Inc).

A generalised linear mixed-effects model (GLMM, binomial distribution, Table 2a) and a linear mixed-effects model (LMM, Gaussian distribution, Table 2c) were performed to analyse the effects of host plant, sex and collection year on viral prevalence and viral load, respectively. Estimated coefficients of prevalence analyses were exponentiated to be on an odds-scale for the purpose of data reporting. Site was included as both a random effect and fixed effect in different versions of the model to quantify population-level differences, and we implemented models with the "lme4" package and used the "car" package to calculate Wald X^2 statistics (v1.1.30, Bates et al., 2022; v3.1-2, Fox & Weisberg, 2019). Because of an imbalance in sample sizes between collections made in 2020 ($N = 516$) and those made in historical years (2001–2015, $N = 226$), we conducted an additional analysis to investigate the effect of sampling year on viral prevalence (Table 2b). We initially constructed a separate linear model from just the historical data (excluding the recent year), then combined a random subset of individuals ($N = 25$) from 2020 with the historical dataset for 1000 iterations of the linear model to analyse the effects of samples from 2020 on the overall proportion of viral infection. Finally, to analyse temporal variation of viral prevalence over the season, a generalised linear model (GLM, binomial, Table 2d) was used, fitting both simple and quadratic predictors for collection date (thus allowing for the possibility of non-linear increase during the season).

Sequences obtained from the Nevada Genomics Center were entered into NCBI's BLAST. All sequence results had at least 98% similarity to JcDV, indicating that they did not belong to other similar densoviruses. Each sequence was aligned with the JcDV reference genome (GenBank: KC883978; Pham et al., 2013) for identity confirmation. FASTA files obtained from Sanger sequencing were trimmed and aligned in UGENE (v33, Unipro, 2012), and further compared using Clustal Omega (v1.2.2, Sievers & Higgins, 2018). Because JcDV is an ssDNA virus, certain analyses formulated for haplotypes can also be applied to measure sequence similarity. Therefore, a sequence alignment (Figure S2) and haplotype network (Figure S3) were constructed to visualise viral sequence differences between host populations using the "msa" (v1.26.0, Bodenhofer et al., 2015) and "pegas" packages (v0.12, Paradis et al., 2019), respectively, for R.

RESULTS

Effects of host plant diet, host sex and collection period on JcDV infection

Contemporary samples (those collected in 2020) had a significantly lower proportion of JcDV-positive individuals (53 of 516 adults, or 10.3%) compared with samples collected in 2001–2015 (79 of 226 adults, or 34.9%), ($\beta = 0.155 \pm 1.35$, $p < 0.001$, Table 2a). Estimated coefficients (β) for all models predicting viral presence or absence (including this one) are on an odds-scale. Within samples from historical collections made prior to 2016, JcDV was detected in at least one individual in 17 of the 19 locations, and viral prevalence was unevenly distributed across sites (Figures 2 and S1). JcDV was detected in at least one adult in 14 of the 21 *L. melissa* populations from which the contemporary samples were collected in 2020 (Figures 2 and S1), but there were several populations with 0 or 1 JcDV-positive individuals, and only a few populations with viral prevalence higher than 20% (Figure 3).

Initially, we detected a significant, negative trend in JcDV infection based on specific collection year (odds-scale β for year effect $= 0.89 \pm 1.02$, $p < 0.001$, Table 2a), but this was driven by the lower prevalence of virus in contemporary samples and the much larger sampling effort for those individuals from 2020. After statistically resampling to account for differences in historical and contemporary sample sizes, the effect of collection year on JcDV infection prevalence approaches zero (Table 2b). JcDV infection prevalence actually increased slightly with increasing year between 2001 and 2015 (Figure 3b; $\beta = 1.58 \pm 1.27$, $p = 0.06$, Table 2b). When site was treated as a fixed effect, it explained a large amount of variation in viral prevalence showing strong population-specific effects ($X^2_{[27]} = 91.5$, $p < 0.001$; compared with the effect of year: $X^2_{[1]} = 36.2$, $p < 0.001$).

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TABLE 2 Statistical models analyzing variation in viral prevalence and load.

a. (Linear mixed model) JcDV prevalence in *Lycaeides melissa* population as explained by host plant type, sex, and collection time (Contemporary = 2020). Coefficients on logit-scale in table, but exponentiated for results text. Estimates are with reference to novel alfalfa host plant, female, and historical sampling period (2001–2015) Values on the left include estimates for each factor level; values on the right include Wald X^2 and associated statistics.

Fixed effects	Estimate (±SE)	*Z*-value	*p*-value	Wald X^2	DF	*p*-value
Intercept	−0.71 (0.41)	−1.75	0.08			
Host:Both	−0.48 (0.69)	−0.69	0.49	0.65	2	0.72
Host: Native	−0.30 (0.48)	−0.62	0.53			
Sex: Male	0.46 (0.24)	1.90	0.06	3.60	1	0.06
Contemporary	−1.86 (0.30)	−6.53	3.9e−10***	39.16	1	3.9e−10***

b. Viral prevalence as explained by collection year and day of the year, accounting for bias in sampling effort by combining historical data with a random subset of contemporary (2020) data for 1000 iterations of a linear model. The first columns with estimates and *p*-values refer to the model with all data; the historical only estimate refers to the model excluding 2020; the subset of 25 estimate refers to the repeated resampling.

Fixed effects	Estimate (±SE)	*p*-value	Estimate (±SE) historical only	Estimate (±SE) subset of 25
Intercept	263 (38.2)	5.02e−12***	−98.9 (84.7)	42.0 (61.8)
Year collected	−0.13 (0.02)	2.83e−12***	0.05 (0.04)	−0.02 (0.03)
Day of year	−0.02 (0.48)	7.12e−4***	3.21e−3 (6.97e−3)	8.69e−4 (6.40e−3)

c. (Generalised linear mixed model) Viral load as explained by host plant type, sex, and collection year (contemporary = 2020). Estimates are with reference to novel alfalfa host plant, female, and historical sampling period (2001–2015). Values on the left include estimates for each factor level; values on the right include Wald X^2 and associated statistics.

Fixed effects	Estimate (±SE)	*t*-value	Wald X^2	DF	*p*-value
Intercept	2.89 (0.11)	26.7			
Host:Both	−0.11 (0.18)	−0.62	1.11	2	0.57
Host: Native	−0.10 (0.11)	−0.98			
Sex: Male	0.04 (0.10)	0.36	0.13	1	0.72
Contemporary	−0.27 (0.11)	−2.46	6.06	1	0.01***

d. (Generalised linear model) Variation in viral prevalence over the course of *L. melissa* flying season (late May–early September) for four populations (BWP, VUH, VEC, WAL). Estimates are with reference to population BWP (R^2 = 0.36); values on the left include estimates for each factor level; values on the right include Wald X^2 and associated statistics.

Fixed effects	Estimate (±SE)	*Z*-value	*p*-value	Wald X^2	DF	*p*-value
Intercept	25.05 (13.84)	1.81	0.07			
Day of year	−0.31 (0.13)	−2.40	0.02*	5.01	1	0.03*
(Day of year)^2	8.7e−4 (3.1e−4)	2.78	0.01**	6.90	1	0.01**
Population: VEC	0.63 (0.86)	0.73	0.47			
Population: VUH	−0.52 (1.04)	−0.50	0.62	Population: 14.2	3	2.7e−3***
Population: WAL	2.70 (0.93)	2.89	3.9e−3***			

Note: Asterisks indicate significant effects with alpha cut-off of 0.05.
*$p \le 0.05$; **$p \le 0.01$; ***p 0.001.

Regardless of whether wild *L. melissa* populations utilised a native host plant, alfalfa as a novel host, or a combination of native and novel host plants, there was no effect of host plant type on viral prevalence ($X^2 = 0.65$, $p = 0.72$, Table 2a). In an analysis of sex as another potential driver of variation in viral effects, females had only slightly higher viral prevalence compared with males in collections from prior to 2016 ($X^2 = 2.28$, $p = 0.13$), but significantly lower viral prevalence than males in contemporary samples ($X^2 = 13.3$, $p < 0.001$). When all collection years were considered together in our model, males were predicted to have higher JcDV prevalence than females ($\beta = 1.58 \pm 1.27$, $p = 0.06$, Table 2a).

Average viral load of the populations that contained infected individuals—represented by the exponential value of 10^n viral genomic units—ranged from 1.819 to 3.299, with samples made in 2020 having lower loads than historical samples ($\beta = -0.27 \pm 0.11$, $p < 0.05$; Table 2c). This is a relatively low viral load, which agrees with viral concentration of other adults in previous descriptions of dose- and life-stage-dependency of JcDV effects (Muchoney et al., 2022; Mutuel et al., 2010; although these studies were conducted in larger-bodied Lepidoptera). There was no effect of host plant type on viral load ($X^2 = 1.11$, $p = 0.58$) or sex on viral load ($X^2 = 0.13$, $p = 0.72$; Table 2c).

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Temporal variation of JcDV infection over Melissa blue flying season

For three out of the four populations sampled repeatedly, there was a greater proportion of individuals that were JcDV positive in collections of *L. melissa* adults made later in the year (Figure 4; $\beta = 1.00 \pm 1.00$, $p < 0.05$, Table 2d), indicating there was temporal variation in viral prevalence across the flying season in 2020. Because *L. melissa* is multivoltine, this means that later generations of flying adults had higher viral prevalence. The one locality that showed decreased JcDV infection in its final collection was BWP (Figure 4), which burned before a later season collection could be made. For the late-season (early September) collections at VUH and VEC, the percentage of samples positive for JcDV was much higher (64.3% and 88.9%, respectively) than the across-season average frequencies for these populations (15.7% and 21.0%, respectively, $p < 0.005$, $R^2 = 0.36$; Table 2d). Overall, for this portion of the analysis but also across the entire dataset, population-level viral frequency increased as ordinal day (date at which collections were made) increased ($\beta = 1.02 \pm 1.01$, $p < 0.001$, Table 2b; Table 2d), meaning that collections made later in the season had a higher percentage of infected individuals.

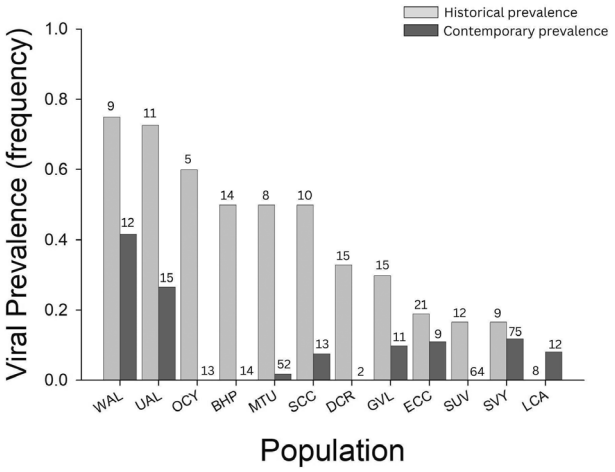

FIGURE 2 JcDV infection prevalence (proportion of sample JcDV positive) across *L. melissa* populations visited for both historical (prior to 2016) and contemporary (2020) sampling. Numbers above bars represent sample sizes for each collection event.

Community presence of JcDV

Of the 317 plant surface rinses screened, none of them had detectable JcDV levels and only one ant surface rinse (from MTU locality) out of 148 total ant and treehopper sample rinses was positive for JcDV (i.e., the rinse sample fluoresced at the amplification temperature and had the same melt temperature as the positive JcDV control). The subset of community arthropods whose DNA was extracted consisted of 25 ant samples and 15 treehopper samples. One DNA sample from ants (site: VUH) and one treehopper DNA sample (site: VEC) came back positive for JcDV.

Viral sequence comparisons

BLAST results indicated that all viral capsid gene (VP1-4) sequence segments (length = 445 base pairs) obtained from wild *L. melissa* ($N = 31$) had over a 98% sequence similar in identity to the Junonia coenia densovirus Oxford reference genome (GenBank: KC883978; Pham et al., 2013). Other lepidopteran densoviruses were returned by that search at notable percentages (some up to 80% similarity) but

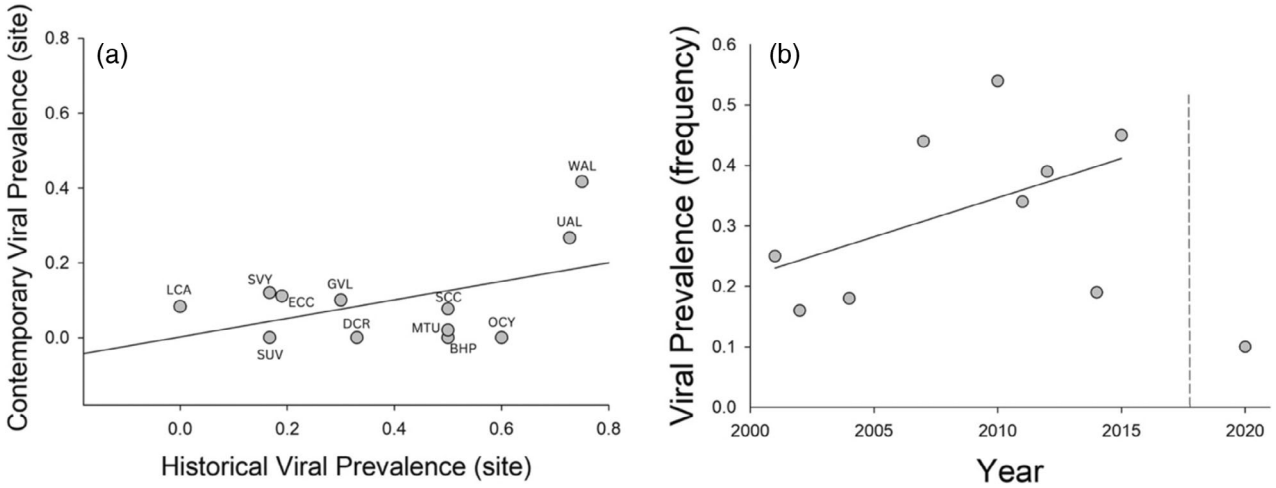

FIGURE 3 Scatterplots of (a) historical (2001–2015) and contemporary (2020) viral prevalence for the 12 populations sampled at both collection points; and (b) overall viral prevalence (infection frequency) for each year, showing a gradual increasing trend of infection with collection year, with the exception of 2020 samples, which exhibit a year of particularly low viral frequency.

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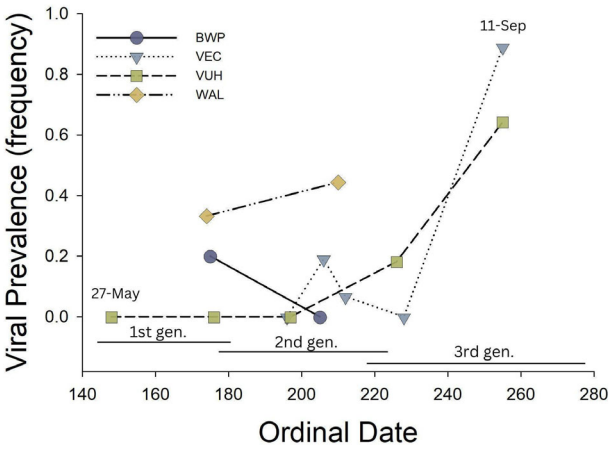

FIGURE 4 Between-generation JcDV infection prevalence for four *L. melissa* sites (BWP, VEC, VUH and WAL) collected multiple times during the 2020 flying season. Adult generation times approximated due to overlap and phenological variation between populations.

given the small genomes and overall similarity of densoviruses (Bergoin & Tijssen, 2000); this was not enough to suggest co-infection or infection by a virus that was not JcDV. There was a total of five JcDV haplotype variants defined by single nucleotide polymorphisms (SNPs) across the 10 *L. melissa* populations compared (Figure S2). Three haplotype variants were identified in single individuals, and the other two haplotype variants were shared by multiple individuals from either the same or different collection sites (Figure S3). While the largest number of sequences from wild individuals were of the same haplotype as the Junonia coenia densovirus Oxford reference genome (haplotype A), none of the host localities had only a single haplotype, so there was no apparent pattern of segregating sites based on *L. melissa* locality.

DISCUSSION

This study provides the first record of JcDV infection in wild populations of *L. melissa* and represents a description of spatial and temporal variation in the interaction between JcDV and butterflies in the family Lycaenidae. Perhaps the most compelling findings involve heterogeneity in viral prevalence (percentage of individuals infected with the virus) across populations (Figures 2 and 3b), within a season (Figure 4), and between historical and contemporary samples (Figure 3). Because we did not find evidence of a strong effect of host plant species on JcDV prevalence or load in Melissa blue adults, our hypothesis that populations utilising the novel host plant (alfalfa) would experience more frequent or severe infections in nature was not supported. The effect of sex on viral frequency suggests that males might be more susceptible to JcDV infection, which is interesting to note as *L. melissa* males are the smaller sex and exhibit patrolling behaviour, but this effect was not consistent across historical and contemporary samples.

The lack of detectable JcDV on the surfaces of host plants and co-occurring arthropods conflicts with our predictions regarding viral occurrence at the community level. These findings are unique compared with other JcDV–lepidopteran interactions where the host plant was found to be an important predictor of infection status (Muchoney et al., 2022; Smilanich et al., 2018) and JcDV was detected on host plant material (Muchoney et al. unpublished data). However, the relatively small sample size of 10 plants at each site could explain the apparent absence of JcDV in the surrounding environment. Previous evidence of JcDV and other densoviruses being environmentally stable and persisting on plant surfaces (Muchoney et al. unpublished data; Carlson et al., 2006) would encourage screening more plant samples for finer resolution before saying the virus is totally absent in the environment. Because of the temporal variation in viral prevalence we found, it would be useful to sample plants several times throughout the flying season and to test specifically flowers, as adult *L. melissa* are believed to have incorporated exotic alfalfa at least somewhat due to female preference to oviposit and nectar at its flowers (Forister et al., 2013).

Another compelling finding was the increase in JcDV prevalence documented throughout the flying season. Vertical transmission in this multivoltine host could account for accumulation of viral infection over the course of the season, allowing JcDV to persist and transfer across overlapping generations in a population (Fuxa, 2004). This would be contingent upon infected individuals being reproductively viable, of course, and we did not test female reproductive capability in this study. However, a prior study found that adult white peacocks (*Anartia jatrophae*) harbouring JcDV are still able to reproduce (Muchoney et al., 2023), and additionally, the virus was detected on the surface of eggs oviposited by infected painted lady butterflies (*Vanessa cardui*: Nymphalidae) (Smilanich et al., 2022 unpublished data). Because of this possibility and evidence of reproductive fitness increasing with infection in another densovirus system (Xu et al., 2014), it is conceivable that JcDV-infected *L. melissa* adults may pass the virus to their offspring in later generations if they survive to adulthood (Fuxa, 2004). Additionally, temporal accumulation of the proportion of infected individuals could have implications in management systems that depend on phenology, such as integrated pest management (IPM) (Crimmins et al., 2020). If, for instance, an agricultural pest species is multivoltine, it may be erroneous to assume that infection status would remain static and pathogen prevalence should be monitored throughout the growing season. Continuation of repeated surveys in focal populations, followed by comparisons between parental and offspring infection status, would be critical for determining the importance of vertical transmission in this system.

Our findings support the general idea that patterns of JcDV in *L. melissa* are location-specific and population effects are fairly consistent over time. Although there were differences in viral infection between collections made in earlier years and samples from 2020, with 2020 appearing to be a low-prevalence year, some populations had low JcDV prevalence historically and little to no viral infection in contemporary samples. Likewise, populations with high historical JcDV prevalence tended to be the populations where JcDV was

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detected in contemporary samples, but those trends did not hold for all populations (Figure 3a). In addition, we found that *L. melissa* populations along the eastern slope of the Sierra Nevada mountains (see UAL, VCP, VEC, VUH and WAL; Figure 1) had higher viral prevalence, which was consistent in both earlier and later years (see UAL and WAL, Figures 2 and 3a). This pattern across the landscape suggests that although *L. melissa* tend to have strong population structure with relatively little dispersal among locations (Gompert et al., 2014), JcDV transmission may have been facilitated by colonisation history and gene flow among these locations (Chaturvedi et al., 2018). Indeed, Chaturvedi et al. (2018) found that populations along the eastern slope of the Sierra Nevada Mountains were more similar to each other genetically relative to populations in other parts of the range, suggesting a common lineage in this area. Alternatively, JcDV prevalence could be maintained by other site-dependent factors such as interactions with endosymbionts (e.g., *Wolbachia*, Altinli et al., 2020; Shastry et al., 2022), or abiotic factors (Carlson et al., 2006).

Our comparisons of the genetic sequences of the viral capsid genes revealed only a few SNPs, and all were singletons that did not show any spatial pattern based on collection site (i.e., *L. melissa* locality). This confirms that the JcDV genome is highly conserved like other members of the *Parvoviridae* family (Tijssen & Bergoin, 1995). On the other hand, our findings are in contrast with other studies of densoviruses that show high genetic diversity among host localities in regions of the capsid gene (Song et al., 2016), which is responsible for packaging and delivering the viral genome (Roos et al., 2007). Although whole-genome comparisons could be conducted in the future to further support this, it appears that genetic variation in JcDV is not dependent on *L. melissa* locality, at least not at the spatial scale encompassed by our study.

One caveat worth noting is that JcDV screening of *L. melissa* for this study occurred entirely in adults, for the logistical reason that the small, cryptic larvae are difficult to find in the field. This presents a challenge in drawing definitive conclusions about the occurrence of JcDV in host populations since the pathogenic effects of the virus primarily occur in the larval and pupal stages (Muchoney et al., 2022; Mutuel et al., 2010). Individuals that were most susceptible to JcDV and succumbed to the virus in earlier life stages were not part of our sampling. Thus, it is likely that the JcDV-negative adults we caught and screened represent those individuals that were either uninfected or able to successfully eliminate viral infection. Furthermore, we can speculate that JcDV-positive adults were not able to resist infection but either contracted the disease in a later larval stage just before pupation, or perhaps tolerated it through development (Roy & Kirchner, 2000). Because we know that larval infection causes increased pre-metamorphosis mortality from previous laboratory studies with other host species (Muchoney et al., 2023; Mutuel et al., 2010), the observation of live-caught adults that are JcDV positive suggests that sub-lethal infections could occur in this system. This might explain the persistence of JcDV at low prevalence and loads in certain populations, as covert or sub-lethal infection can still be horizontally transmitted or pass throughout generations by vertical transmission (Elderd, 2018). Future studies should investigate the infection patterns of JcDV in individual *L. melissa* throughout the larval and adult stages to uncover whether larvae effectively reduce viral burden or remain actively infected and able to transmit the virus through to adulthood, which will bolster our understanding of the persistence of this virus in natural host systems.

Although further investigation is needed to understand the mechanisms behind later host generations having higher viral prevalence and we cannot eliminate the importance of horizontal transmission in this system, we speculate that vertical transmission occurs and is an important route for transmission. Importantly, we found that a single "snapshot" of a host population may produce different results depending on when sampling was conducted, and therefore is not representative of overall pathogen presence. When studying pathogens in multivoltine hosts, multiple collection efforts should be made throughout the season. Field surveys should be continued to describe long-term patterns, and future studies should explore the physiological response (i.e., immunity) of the caterpillars to the virus to better understand causes of variation in viral load and frequency.

AUTHOR CONTRIBUTIONS

Kelli J. McKeegan: Conceptualization; investigation; funding acquisition; writing – original draft; methodology; validation; visualization; writing – review and editing; formal analysis; project administration; data curation; resources. **Nadya D. Muchoney:** Conceptualization; investigation; funding acquisition; writing – review and editing; visualization; methodology; formal analysis; data curation; resources. **Mike B. Teglas:** Resources; supervision; software; methodology; writing – review and editing; project administration; funding acquisition. **Matthew L. Forister:** Conceptualization; funding acquisition; writing – review and editing; methodology; formal analysis; project administration; data curation; resources. **Angela M. Smilanich:** Conceptualization; funding acquisition; writing – review and editing; writing – original draft; resources; supervision; data curation; software; formal analysis; project administration; visualization.

ACKNOWLEDGEMENTS

Conclusions expressed in this article are those of the authors and do not reflect the views of the UNR GSA, NIH or NSF. Funding for this work was supported by a National Science Foundation grant (DEB-1929522 to AMS, MLF and MBT) and the University of Nevada, Reno Graduate Student Association Research, Travel, and Materials award. MLF thanks the National Science Foundation (DEB-2114793). Funding for Sanger sequencing and bioinformatics training was supported by the Core Service Award (GM-103440) from the IDeA Network of Biomedical Research Excellence, a National Institute of Health Program. We would like to thank Zachariah Gompert for sending historical sample DNA, and Mylene Ogliastro for supplying JcDV stock and VP primer sequences. Special thanks to Zachariah Gompert, Denali Lowder, Samantha DiFulvio, Joshua Turner, Richard Tillett, Paul Hartley, Craig Osborne, Zachary Marion, Trevor Faske, Eliza Grames and other members of our greater research group for assistance in the field, laboratory or analyses.

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CONFLICT OF INTEREST STATEMENT

All authors of this manuscript confirm that there are no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

Data supporting the results presented in this manuscript are publicly available on Figshare Repository (10.6084/m9.figshare.24116013, and DNA sequences have been deposited in the NCBI GenBank database (accession numbers OR540207-OR540237).

ORCID

Kelli J. McKeegan https://orcid.org/0000-0002-8982-3200
Nadya D. Muchoney https://orcid.org/0000-0001-6780-5848

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Data S1. Table S1. Population codes, host plant type, and geographic coordinates (decimal degrees) of *Lycaeides melissa* sampling localities. Increased accuracy in populations of the Verdi complex (VCP, VEC, VET, VUH) due to proximity to one another.

Figure S1. Viral prevalence (proportion of sample JcDV positive) across all *L. melissa* collection sites ($N = 28$), with light gray bars representing historical (prior to 2016) samples, and dark gray bars representing contemporary (2020) samples.

Figure S2. Multiple alignment comparing sequences for the Junonia coenia densovirus (JcDV) viral capsid protein (VP1-4) genes isolated from wild *Lycaeides melissa* butterflies ($N = 31$) collected from 10 locations to the published genome for this pathogen (KC883978; Pham et al., 2013). Polymorphism was evident at only four sites out of the 445 nucleotide-long amplicons, indicating 98%–100% sequence identity across isolates. The "M" present in the KC883978 reference sequence (location 9) refers to the occurrence of adenine (A) or cytosine (C).

Figure S3. Haplotype network of JcDV capsid gene (VP1-4) sequences isolated from ten *L. melissa* populations. Size of circles represents the number of individuals with each haplotype variant, and each hatch mark represents one nucleotide of separation.

How to cite this article: McKeegan, K.J., Muchoney, N.D., Teglas, M.B., Forister, M.L. & Smilanich, A.M. (2024) Exploring spatial and temporal patterns of viral infection across populations of the Melissa blue butterfly. *Ecological Entomology*, 49(1), 54–66. Available from: https://doi.org/10.1111/een.13280

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