University of Nevada, Reno

Tritrophic Consequences of Host Range Expansion: The Impacts of Exotic Host Plants on Infection and Immunity in Native Insect Herbivores

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Ecology, Evolution, and Conservation Biology

by

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THE GRADUATE SCHOOL

We recommend that the dissertation
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Abstract

Species introductions are a pervasive aspect of global change. Exotic plants, in particular, are present in nearly all terrestrial environments and have been incorporated into the diets of many native herbivores, giving rise to novel multitrophic interactions. These recent examples of host range evolution provide naturally occurring experiments through which to investigate the complex ecological factors that facilitate, or constrain, herbivore persistence within novel niches. Though adoption of exotic plants into the diets of native insect herbivores is common, their use is often associated with negative effects on herbivore growth and performance, relative to native host plants. However, herbivore fitness on different host plants is context-dependent and shaped by a multitude of factors beyond suitability for development, including interactions with diverse natural enemies. Consideration of herbivore performance within a tritrophic framework, including attack by and defense against these enemies, may be essential for understanding the outcomes of dietary expansion for native herbivores. In particular, entomopathogens represent critical agents of mortality for insect herbivores, yet their ecological impacts and importance in mediating diet breadth evolution remain poorly understood in many natural systems.

In this dissertation, I combined approaches from the fields of eco-immunology, chemical ecology, and disease ecology to investigate the consequences of exotic host plant use for immune performance, chemical defense (i.e., phytochemical sequestration), and vulnerability to a viral pathogen in two North American herbivores: *Euphydryas phaeton,* the Baltimore checkerspot (Lepidoptera: Nymphalidae), and *Anartia jatrophae,* the white peacock (Lepidoptera: Nymphalidae). These herbivores provide compelling

systems in which to compare the tritrophic outcomes of host range expansion, as they: (1) recently incorporated the same exotic plant, *Plantago lanceolata* (Plantaginaceae), into their diets, (2) exhibit reduced growth on the exotic plant, relative to native host plants, (3) are infected by the same entomopathogen, Junonia coenia densovirus, across wild populations, and (4) differ in their degree of dietary specialization and relationships with plant secondary chemistry, which can impact immunity and susceptibility to pathogens.

Employing a combination of field-based surveys and manipulative laboratory experiments, I found that the outcomes of dietary expansion for herbivore infection and immunity differed across the two focal species. In *E. phaeton,* use of the exotic plant was associated with suppression of multiple immune parameters, differential sequestration of defensive phytochemicals (iridoid glycosides), and higher viral burdens during certain stages of development, representing potential costs of host range expansion. However, *E. phaeton*'s ability to survive densovirus infection was not reduced on the exotic host plant, suggesting that additional factors (e.g., phytochemical sequestration) may contribute to defense against this pathogen even when immunity is compromised. In contrast, use of the exotic plant dramatically increased resistance to viral infection in *A. jatrophae*, likely through suppression of replication, though immune performance did not vary based on host plant use. Together, this research demonstrates that, in certain systems, exotic host plants may represent equally suitable or even superior resources for supporting herbivore development, relative to native host plants, when the impacts of pathogen infection are considered. Moving forward, evaluation of the role of host plant use in mediating defense against infectious diseases across wild populations may provide a deeper understanding of the complex ecological factors shaping host range evolution in herbivorous species.

Dedication

To my grandparents: Carole, Millie, and Bear.

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Introduction

The existence of exotic species within ecosystems is a near-ubiquitous aspect of the modern world. Exotic species, which can also be referred to as non-native or non-indigenous species, may be broadly defined as those that arrive at a region to which they are not indigenous, often with the intentional or unintentional assistance of humans, and subsequently establish a persistent population (Simberloff, 2013). These species may interact with native ecosystems in a wide variety of manners, including as predators, competitors, parasites, mutualists, and hosts for native organisms, and can bring about substantial ecological and evolutionary change within existing communities (Carroll and Fox, 2007; Mack et al., 2000; Strauss et al., 2006). The establishment of exotic plants, in particular, has been pervasive (van Kleunen et al., 2015) and has given rise to novel trophic and multitrophic interactions following the incorporation of these plants into the diets of native herbivores (Bezemer et al., 2014; Singer et al., 1993; Sunny et al., 2015).

In insect herbivores, incorporation of exotic host plants appears to be common; for example, 34% of butterfly species in California use introduced plants for either larval feeding or oviposition (Graves and Shapiro, 2003). In many cases, exotic plants represent inferior resources for larval growth and development, relative to native or ancestral host plants (Bowers et al., 1992; Forister et al., 2009; Yoon and Read, 2016), and in certain cases, they can create population sinks (Keeler and Chew, 2008; Schlaepfer et al., 2005). In other systems, exotic plants may provide suitable niches for native herbivores, and interactions between the two can be characterized as beneficial or facilitative (Rodriguez,

2006). For example, exploitation of exotic plants can sustain herbivore populations when native host plants are scarce or absent (Shapiro, 2002) or facilitate expansion of herbivore geographic range, population size, or breeding season (Brown et al., 2017; Graves and Shapiro, 2003). As the adoption of exotic plants into the diets of native herbivores may be expected to increase in frequency as native plants decline or are displaced (Tallamy et al., 2021), evaluating the consequences of these dietary expansions for herbivore ecology and evolution represents an important aspect of understanding the impacts of introduced species on native ecosystems. Furthermore, these recent examples of host range evolution provide natural experiments through which to investigate the complex ecological factors that facilitate, or constrain, herbivore persistence on novel plants (Forister et al., 2020).

The outcomes of novel associations between native herbivores and exotic plants fundamentally depend upon: (1) recognition and acceptance of the plant by larvae and egg-laying adults (Janz and Nylin, 1997) and (2) the extent to which the plant supports development (Forister and Wilson, 2013). It follows that research on the consequences of exotic host plant use has focused on documenting differences in oviposition preference and performance (i.e., development rate, body weight, and survival) on native and exotic plants (e.g., Bowers et al., 1992; Davis and Cipollini, 2014; Forister et al., 2009; Fortuna et al., 2013). However, herbivore fitness on novel plants is context-dependent and shaped by a broad range of factors beyond physiological suitability for development, including interactions with natural enemies (Price et al., 1980). Evaluation of performance within a tritrophic framework, including attack by and defense against enemies, may therefore be essential for understanding the outcomes of dietary expansion for native herbivores.

The ability of host plant use to modulate interactions between herbivores and their natural enemies, with potential outcomes for herbivore fitness and persistence on exotic host plant species, is the primary focus of this dissertation. Interactions with predators, parasitoids, and pathogens can result in high mortality in insect herbivores (Hawkins et al., 1997), and may therefore constitute important mediators of host range evolution (Bernays and Graham, 1988; Singer and Stireman, 2005). Importantly, even if an exotic plant supports relatively poor herbivore development, the "enemy-free space" hypothesis suggests that its use may be advantageous if it reduces or eliminates vulnerability to natural enemies (Jeffries and Lawton, 1984). While the importance of incorporating interactions with the third trophic level into the study of herbivore diet breadth evolution has been recognized (Lill et al., 2002; Singer and Stireman, 2005), relatively few studies have investigated the role of natural enemies in shaping herbivore fitness on exotic plants, in particular (Fortuna et al., 2013). Fewer still have focused on interactions with pathogens, as opposed to predators or parasitoids, representing a critical knowledge gap.

My dissertation research combined approaches from the fields of ecological immunology (Schulenburg et al., 2009), disease ecology (Campos-Herrera and Lacey, 2018), invertebrate pathology, and chemical ecology (Dyer et al., 2018) to gain insight into the multifaceted outcomes of host range expansion for interactions between native insect herbivores and their natural enemies. Specifically, I investigated the consequences of exotic host plant use for immunity, chemical defense, and susceptibility to a naturally occurring viral pathogen in two North American herbivores: *Euphydryas phaeton,* the Baltimore checkerspot (Lepidoptera: Nymphalidae), and *Anartia jatrophae,* the white peacock (Lepidoptera: Nymphalidae). These herbivores provide compelling systems in

which to compare the tritrophic outcomes of dietary expansion, as these species: (1) recently incorporated the same exotic plant, *Plantago lanceolata* (Plantaginaceae)*,* into their diets, (2) exhibit reduced growth on the exotic plant, relative to native host plants, (3) are infected by the same entomopathogen, Junonia coenia densovirus (JcDV), across wild populations, and (4) differ in their degree of dietary specialization and relationships with plant secondary chemistry, which has the potential to influence herbivore immunity (Smilanich and Muchoney, 2022) and resistance to pathogens (Cory and Hoover, 2006).

My dissertation is divided into four chapters, two of which employed field-based approaches and two of which primarily consisted of controlled laboratory experiments. For my first chapter, I characterized variation in immunity, chemical defense, and viral burdens across wild populations of *E. phaeton,* aiming to determine: (1) how use of an exotic plant impacts physiological defenses against the third trophic level, and (2) how physiological defenses themselves (i.e., immunity and phytochemical sequestration) are interrelated in wild herbivores. This study also represented the first record of infection with the focal virus (JcDV) in *E. phaeton* and the first investigation of this pathogen's occurrence across wild populations of any host. This chapter was recently published in the journal *Ecology and Evolution* (Muchoney et al., 2022). My second chapter, also a field-based experiment, documented variation in JcDV prevalence and infection loads across the course of the life cycle in wild populations of *E. phaeton*, aiming to elucidate the role of host plant use in mediating infection during different stages of development.

For my third chapter, I performed a manipulative experiment that evaluated the effects of exotic host plant use on immune function, development, and susceptibility to viral infection in the second focal herbivore, *A. jatrophae*. This chapter also includes the

results of a field survey conducted in wild populations of *A. jatrophae,* representing the first record of JcDV infection in this host species, along with an evaluation of the genetic similarity between JcDV isolated from wild butterflies and the laboratory-propagated isolate that has been employed in many experimental studies of this pathogen (including this dissertation). Finally, my fourth chapter consisted of two laboratory experiments, one focusing on *A. jatrophae* and one focusing on *E. phaeton,* which characterized the effects of exotic host plant use on resistance to JcDV infection across a range of infectious doses. Altogether, these studies provide novel insight into the role of host plant use in mediating relationships between insect herbivores and their entomopathogens and demonstrate the tritrophic context-dependence of herbivore performance on exotic host plant species.

In addition to the research presented in this dissertation, engagement in outreach, training, and mentorship has been a priority for me throughout my time at UNR. Within the Smilanich Lab, I have mentored 16 undergraduate students and led training sessions on immune assays and pathogen screening for 12 graduate students from UNR and other universities. I have also served as an Outreach Coordinator for the Ecology, Evolution, and Conservation Biology program for six years and, in this role, have organized events that connect researchers at UNR with the broader community, including Science Days hosted for Big Brothers Big Sisters of Northern Nevada and Open House events at the UNR Museum of Natural History. I have also been a member of the Board of Directors of Nevada Bugs and Butterflies, a local education and conservation nonprofit, since 2019 and have contributed to several educational initiatives, including a science activity packet for families engaged in distance learning during the Covid-19 pandemic and two Summer Nature Blitzes using the platform iNaturalist. In addition, I have led lessons for school

groups at UNR's Museum of Natural History, presented insect-focused programs for the South Valleys Library STEAM Program, and volunteered at local events including the Desert Research Institute's Girls' Day of STEM and the Western Nevada Science & Engineering Fair. These activities have been among my most rewarding experiences as a graduate student, allowing me to engage the community in my research, hone my skills in scientific communication, and foster enthusiasm about science in young learners.

In conclusion, my dissertation research has afforded me the opportunity to gain valuable experience in field-based ecological research, laboratory-based manipulative experiments, and molecular techniques for detecting and quantifying pathogens, as well as science education and mentorship. I look forward to continuing to apply these skills toward further understanding the complex relationships between herbivores, host plants, and entomopathogens during a postdoctoral research position with Dr. Angela Smilanich.

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Chapter One

Use of an exotic host plant shifts immunity, chemical defense, and viral burden in wild populations of a specialist insect herbivore

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ABSTRACT

Defense against natural enemies constitutes an important driver of herbivore host range evolution in the wild. Populations of the Baltimore checkerspot butterfly, *Euphydryas phaeton* (Nymphalidae), have recently incorporated an exotic plant, *Plantago lanceolata* (Plantaginaceae), into their dietary range. To understand the tritrophic consequences of utilizing this exotic host plant, we examined immune performance, chemical defense, and interactions with a natural entomopathogen (Junonia coenia densovirus, *Parvoviridae*) across wild populations of this specialist herbivore. We measured three immune parameters, sequestration of defensive iridoid glycosides (IGs), and viral infection load in field-collected caterpillars using either *P. lanceolata* or a native plant, *Chelone glabra* (Plantaginaceae). We found that larvae using the exotic plant exhibited reduced immunocompetence, compositional differences in IG sequestration, and higher in situ viral burdens compared to those using the native plant. On both host plants, high IG sequestration was associated with reduced hemocyte concentration in the larval hemolymph, providing the first evidence of incompatibility between sequestered chemical defenses and the immune response (i.e., the "vulnerable host" hypothesis) from a field-based study. However, despite this negative relationship between IG sequestration and cellular immunity, caterpillars with greater sequestration harbored lower viral loads. While survival of virus-infected individuals decreased with increasing viral burden, it ultimately did not differ between the exotic and native plants. These results provide evidence that (1) phytochemical sequestration may contribute to defense against pathogens even when immunity is compromised, and (2) herbivore

persistence on exotic plant species may be facilitated by sequestration and its role in defense against natural enemies.

1 | INTRODUCTION

The introduction of exotic species into ecosystems has profound impacts on the ecology and evolution of native organisms (Bezemer et al., 2014; Singer et al., 1993; Strauss et al., 2006; Sunny et al., 2015). In particular, the arrival of exotic plant species may create opportunities for the evolution of herbivore host range if these plants are incorporated into the diets of native herbivores (Bowers et al., 1992; Carroll and Fox, 2007; Forister et al., 2009; Singer et al., 1993). Incorporation of exotic host plant species is a common phenomenon in insect herbivores; for example, Graves and Shapiro (2003) reported that 34% of butterfly species in California utilize introduced plant taxa for either larval feeding or oviposition. Paradoxically, use of exotic host plants is overwhelmingly associated with negative effects on herbivore performance relative to native host plants in Lepidoptera (Yoon and Read, 2016), as indicated by metrics including development rate, weight, feeding efficiency, and survival (e.g., Bowers et al., 1992; Forister et al., 2009; Fortuna et al., 2013; Keeler and Chew, 2008). Therefore, the incorporation of exotic host plants into the diets of native herbivores frequently presents the question of which ecological benefits, if any, facilitate herbivore persistence on these species.

While optimization of herbivore performance, as measured by bitrophic indices focusing solely on the herbivore-plant interaction, often fails to explain patterns of host plant use (Forister et al., 2009; Mason et al., 2011), persistence on novel host plants involves an array of ecological, behavioral, and physiological factors beyond suitability for development (Forister and Wilson, 2013; Mason, 2016). In particular, herbivore interactions with natural enemies, including predators (Grosman et al., 2017; Murphy, 2004), parasitoids (Fortuna et al., 2013; Harvey and Fortuna, 2012), and pathogens (Cory

and Hoover, 2006; Shikano, 2017) can differ substantially between native and exotic host plant species. As these interactions comprise a major source of mortality for insect herbivores (Hawkins et al., 1997), exploitation of enemy-free or enemy-reduced space may constitute an important driver of host range evolution (Bernays and Graham, 1988; Jeffries and Lawton, 1984; Singer and Stireman, 2005). Consideration of herbivore performance within a tritrophic framework, including attack by and defense against natural enemies, may therefore be essential for understanding the ecological mediators of persistence on exotic host plants (Fortuna et al., 2013; Harvey et al., 2010; Singer and Stireman, 2005).

In recent years, eco-immunological research has highlighted the immune response as a critical physiological link between herbivore diet and interactions with natural enemies. Such studies have revealed substantial diet-mediated variation in herbivore immune function (Singer et al., 2014), with repercussions for resistance against diverse natural enemies (reviewed in Smilanich and Muchoney, 2022). For example, several studies have documented positive effects of dietary protein content on caterpillar immune function, which have been linked to increased survival following bacterial or viral challenge in experimental settings (e.g., Cotter et al., 2019; Lee et al., 2006). In the wild, immunological variation may be driven by differences in plant quality (Diamond and Kingsolver, 2011; Klemola et al., 2008), macronutrient composition (Cotter et al., 2019; Lee et al., 2006), and/or secondary chemistry (Bukovinszky et al., 2009; Haviola et al., 2007; Smilanich et al., 2009a; Trowbridge et al., 2016). As the immune response provides insects with effective defenses against parasitoids (Carton et al., 2008; Smilanich et al., 2009b) and pathogens (Rantala and Roff, 2007; Washburn et al., 1996),

host plant mediated variation in herbivore immunity may contribute to the ecological costs, or benefits, of host range expansion.

A second physiological link between herbivore diet and interactions with enemies is phytochemical sequestration. Some herbivores possess the ability to sequester toxic plant compounds and employ them in defense against natural enemies (Bowers, 1993; Nishida, 2002; Opitz and Müller, 2009). Use of exotic host plants, which may differ from native host plants in both composition and concentrations of secondary metabolites, can impact chemical defense in sequestering species, with ramifications for predator deterrence (Bowers, 1980; Knerl and Bowers, 2013) and fitness of internal parasites (Barbosa et al., 1991; De Roode et al., 2008; Ode, 2006; Singer et al., 2009). A less-explored question is whether sequestration also indirectly affects herbivore-natural enemy interactions through modulation of the immune response. Diets containing high concentrations of certain secondary metabolites can suppress lepidopteran immune responses (Lampert and Bowers, 2015; Richards et al., 2012; Smilanich et al., 2009a), putatively rendering caterpillars more vulnerable to pathogens and parasitoids (but see Barthel et al., 2016; Garvey et al., 2021; Laurentz et al., 2012 for examples of positive immunological effects of secondary metabolites). This “vulnerable host” hypothesis (Smilanich et al., 2009a) has rarely been investigated, and typically only in laboratory settings, but offers potential for insight into the role of host plants in mediating physiological defenses against different types of natural enemies. In particular, characterizing the impacts of sequestration on herbivore immunity may reveal tradeoffs between investment in chemical and immunological forms of defense.

In this study, we examined the consequences of host range expansion by a native insect herbivore onto an exotic plant by measuring a suite of defenses employed by herbivores against natural enemies. We asked: can host plant mediated effects on insect defense help explain the paradox of herbivore persistence on exotic plants that confer relatively poor bitrophic performance? To address this question, we focused on a specialist lepidopteran herbivore, the Baltimore checkerspot (*Euphydryas phaeton* Drury, Nymphalidae), and an entomopathogen that occurs naturally in *E. phaeton* populations, Junonia coenia densovirus (*Parvoviridae*). *Euphydryas phaeton* recently incorporated the non-native *Plantago lanceolata* L. into its host range (Stamp, 1979) and exhibits reduced performance on this exotic plant, compared to its primary native host plant, *Chelone glabra* L. (Bowers et al., 1992). Importantly, these two plant species differ in their composition of iridoid glycosides (Bowers et al., 1992; Duff et al., 1965), which are secondary metabolites that are sequestered by *E. phaeton* caterpillars (Bowers and Puttick, 1986) and have been shown to negatively impact the immune response of another specialist nymphalid butterfly species (Richards et al., 2012; Smilanich et al., 2009a). We combined approaches from the fields of eco-immunology and chemical ecology to characterize the multifaceted effects of host plant use on herbivore defenses, focusing on the interacting roles of the immune response and phytochemical sequestration. We specifically addressed three questions: (1) Does use of an exotic host plant impact herbivore immunocompetence and/or sequestration? (2) Are higher levels of sequestration associated with reduced immunocompetence? (3) Do host plant effects on immunocompetence and/or sequestration affect interactions with a natural pathogen? By evaluating variation in physiological defenses and viral infection across naturally

occurring, wild herbivore populations, we provide insight into the tritrophic outcomes of host range expansion.

2 | METHODS

2.1 Caterpillars, host plants, and virus

Euphydryas phaeton (Nymphalidae)*,* the Baltimore checkerspot butterfly, is a univoltine North American herbivore that specializes on host plants containing iridoid glycosides (Bowers, 1980). Iridoid glycosides (hereafter, IGs) are monoterpenoid plant secondary metabolites that can be toxic and/or deterrent to generalist or non-adapted specialist herbivores (Bowers and Puttick, 1988). *Euphydryas phaeton* caterpillars sequester IGs from host plants and retain them through the adult stage (Bowers and Puttick, 1986), rendering both larvae and butterflies unpalatable to predators (Bowers, 1980). Sequestration of IGs has been found to suppress larval immune responses in two specialist lepidopteran species (Lampert and Bowers, 2015; Richards et al., 2012; Smilanich et al., 2009a; but see Laurentz et al., 2012); however, the effects of IG sequestration on herbivore interactions with entomopathogens remain unknown.

The primary host plant for *E. phaeton* in the northeastern U.S. is *Chelone glabra* (Plantaginaceae), white turtlehead, a native, long-lived, perennial herb (Pennell, 1935). *Chelone glabra* primarily contains the IG catalpol, and may also contain smaller amounts of aucubin (Bowers et al., 1992), which is the chemical precursor of catalpol (Damtoft et al., 1983). Over the past 40 years, researchers have documented an expansion of *E. phaeton*'s host range to include a non-native plant, *Plantago lanceolata* (Plantaginaceae), narrowleaf plantain (Bowers et al., 1992; Stamp, 1979). This short-lived, perennial herb

was introduced to North America during the 19th century (Cavers et al., 1980). *Plantago lanceolata* also contains IGs (Bowers and Stamp, 1992), consisting of mainly aucubin with smaller amounts of catalpol (Duff et al., 1965; Fajer, 1989). Where these two host plant species co-occur, *E. phaeton* may utilize both plants (Bowers and Richardson, 2013), while some populations use *P. lanceolata* or *C. glabra* exclusively (Bowers et al., 1992; Stamp, 1979). Despite this host range expansion, *E. phaeton* prefers the native *C. glabra* over *P. lanceolata* for both larval feeding and oviposition (Bowers et al., 1992). This is likely driven in part by costs associated with the exotic host plant, including reduced performance (Bowers et al., 1992) and increased palatability to predators (Bowers, 1980). However, *E. phaeton* population growth rates can be higher on *P. lanceolata* (Brown et al., 2017), suggesting that use of this exotic plant may entail both costs and benefits.

Junonia coenia densovirus (hereafter, JcDV) is a nonenveloped, single-stranded DNA virus in the family *Parvoviridae* (*Densovirinae: Lepidopteran protoambidensovirus 1*). Though first identified in *Junonia coenia* (Nymphalidae), JcDV is capable of infecting Lepidoptera in the Bombycidae, Erebidae, Noctuidae, and Nymphalidae in a laboratory setting (Mutuel et al., 2010; Resnik and Smilanich, 2020; Rivers and Longworth, 1968). Larvae become infected by JcDV through ingestion of contaminated food, after which viral particles cross the midgut and replicate in tracheae, hemocytes, visceral muscle, and epidermis (Mutuel et al., 2010; Wang et al., 2013). Infection may result in hypoxia, molting and metamorphosis failure, and death; however, pathogenesis is dose-dependent and does not always result in mortality (Mutuel et al., 2010; Smilanich et al., 2018). Despite its potentially broad host range, little is known of JcDV in the wild; this study

represents the first record of JcDV infection in the focal herbivore species, *E. phaeton,* and the first investigation of its occurrence in wild populations of any host species.

2.2 Experiment overview

To compare caterpillar immune responses, IG sequestration, and JcDV prevalence and infection loads across populations using the native and exotic host plant, we sampled caterpillars from wild *E. phaeton* populations in May 2016 and 2017. Caterpillars were brought to the University of Nevada, Reno, where they underwent a series of immune assays. In 2016, larvae were freeze-killed following immune assessment to evaluate in situ relationships between host plant use, immune performance, IG sequestration, and viral infection. In 2017, caterpillars were not freeze-killed following immune assessment, but reared out to ascertain the effects of host plant use, larval immune responses, and viral infection on survival. In both years, postmortem insects were screened for JcDV, and viral loads of infected individuals were quantified.

2.3 Population sampling

Euphydryas phaeton caterpillars were collected from populations located throughout the northeastern U.S. (Figure 1a). In 2016, we sampled caterpillars from eight sites: three sites using *C. glabra*, three sites using *P. lanceolata*, and two sites using both plants. In 2017, caterpillars were again collected from eight sites: two sites using *C. glabra,* three sites using *P. lanceolata*, and three sites using both. We did not collect caterpillars at three previously sampled sites in 2017 due to relatively small population sizes, and three additional sites were visited and sampled in 2017 only (Figure 1a). At each site, post-diapause (fifth or sixth instar) larvae were collected and placed alive into individual, sterile culture tubes (USA Scientific, Ocala, FL, USA) with foliage from

nearby host plants (2016: *n* = 390; 2017: *n* = 229; see Appendix: Table S1 for *n* at each site). At sites where both host plants were utilized, plant species occurred in discrete spatial patches and larvae were collected based on observed host plant use at the time of collection.

2.4 Caterpillar rearing

Field-collected caterpillars were reared in an incubator at the University of Nevada, Reno using a 16-hour photoperiod (day temperature: 25°C, night temperature: 20°C) and fed daily with foliage corresponding to their host plant use at the time of collection. *Plantago lanceolata* leaves were collected from the wild in Reno, NV, while *C. glabra* leaves were collected from sampling sites in Montpelier, VT and stored in a refrigerator. Leaf surfaces were sterilized prior to feeding by soaking in 5% bleach solution for 10 min and rinsing thoroughly. Caterpillars were reared in individual 2 oz plastic cups and checked daily to monitor development and mortality. Sterile technique was used between handling of each individual, which entailed soaking instruments in a 30% solution of bleach, followed by a 70% solution of ethanol.

Upon reaching the sixth instar and a threshold mass of 0.18 g, caterpillars underwent immune assays (below). Individuals that died prior to reaching this stage (*n* = 148 across both years) were not included in immunological assessments. Following immune assays, caterpillars were either frozen (2016) or returned to the incubator to complete development (2017). Pupated individuals were weighed and transferred to 32 oz plastic containers with mesh lids for eclosion, and butterflies were maintained on a diet of 10% honey water and monitored daily for mortality.

2.5 Caterpillar immune assays

To evaluate the immunocompetence of caterpillars utilizing different host plants, we measured a combination of humoral and cellular immune parameters, including phenoloxidase (hereafter, PO) enzymatic activity, hemocyte concentrations, and melanization. In insects, PO initiates the melanization response, which involves deposition of melanin on the surface of foreign invaders and generation of cytotoxic compounds that contribute to parasite killing (González-Santoyo and Córdoba-Aguilar, 2012). The PO cascade and its reactive products have been shown to contribute to antiviral defense in certain insect systems (Shelby and Popham, 2006; Zhao et al., 2011; but see Saejeng et al., 2010; Shikano et al., 2010). Differentiated hemocytes (including granulocytes, oenocytoids, and plasmatocytes) are important mediators of phagocytosis, melanization, and encapsulation (Lavine and Strand, 2002) and can suppress viral infection in Lepidoptera through encapsulation of infected tissues (McNeil et al., 2010; Washburn et al., 1996) and elimination of virus from the hemolymph (Trudeau et al., 2001).

To measure standing PO activity, hemolymph was extracted from caterpillars (n = 454) and a spectrophotometric assay of enzymatic activity was immediately performed following the protocol of Smilanich et al. (2018). Hemolymph was extracted by piercing the cuticle of the A6 segment using a fine needle sterilized with 70% ethanol. Using a pipette, 10 µl of hemolymph was added to 500 µl of cold phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO, USA) and vortexed. To provide a substrate for the reaction, 200 µl of l-Dopa solution (4.0 mM; Sigma-Aldrich) was added to 100 µl of each PBS-bound hemolymph sample in a 96-well microplate (Bio-Rad, Hercules, CA, USA). Colorimetric measurements were recorded using an iMark Microplate Absorbance

Reader (Bio-Rad), which measured absorbance at 490 nm every 30 s for 45 min. PO activity was calculated as the slope (OD_{490}/min) over the entire 45-minute period, during which time the enzymatic reaction remained in the linear phase. PO activity measurements with values less than zero ($n = 2$) were excluded from analyses.

To estimate hemocyte concentrations, an additional sample of hemolymph (4-10 μl) was extracted from each caterpillar ($n = 446$) and mixed with twice the volume of cold anticoagulant, which was prepared by mixing 0.684 g of EDTA, 0.346 g of citric acid, and 180 ml of PBS and adjusting the pH to 7.4 before each use (Triggs and Knell, 2012). Hemolymph mixtures were refrigerated and examined within 24 h of extraction by pipetting a 10 μl aliquot of each sample into a Neubauer Bright-Line hemocytometer (Sigma-Aldrich). Cells falling within the central grid were counted using a compound microscope at 400x magnification, and hemocytes were distinguished as granulocytes, oenocytoids, or plasmatocytes based on morphology when possible (Ribeiro and Brehélin, 2006). Cells that were not differentiable as these hemocyte types were included in total hemocyte counts. Hemolymph samples with fewer than two cells visible on the entire grid ($n = 9$) were excluded from analyses due to potential sampling error. Total and differential hemocyte concentrations (cells/ml) were calculated by multiplying each hemocyte count (cells/100 nl) by a factor of 30,000 to account for sample dilution (2:1) and convert units.

To assess the melanization response, we simulated immune challenge using abiotic implants, which can provide effective estimates of insect resistance to pathogens (Rantala and Roff, 2007) and parasitoids (Smilanich et al., 2009b). Monofilament implants were made from abraded nylon fishing line (0.2 mm diameter) cut into 2 mm

lengths and knotted at one end to facilitate removal (Rantala and Roff, 2007). Implants were sterilized with 70% ethanol and inserted into the larval hemocoel via the abdominal wound created during hemolymph extraction. Caterpillars (n = 391) were allowed to react to implants for 24 h, during which time they were maintained without food in order to clear the gut prior to IG quantification (see below). In 2016, implants were stored in 70% ethanol following removal; due to degradation of encapsulating material, measurements from a subset of individuals (n = 30) were excluded from analyses. In 2017, implants were stored dry and frozen, which effectively prevented sample degradation. Implants were photographed at 3.2x magnification using a dissecting microscope mounted with a digital camera (Carl Zeiss Discovery V.8, AxioCam Software, Oberkochen, Baden-Wurttenburg, Germany). Using the "quick selection" tool in Adobe Photoshop CC 2018 (Adobe Systems Inc., San Jose, California, USA), a mean grey value (MGV) was generated for each photographed implant. MGV is a numerical measure of greyness ranging from 0 to 255, where 0 = pure grey and 255 = pure white. For ease of interpretation, MGVs were transformed into a percentage of melanization [1 – (MGV/maximum MGV)]*100 prior to analysis (Smilanich et al., 2009a).

2.6 Iridoid glycoside quantification in caterpillars

To evaluate the effects of host plant use on chemical defense, caterpillars were freeze-killed and homogenized following implant removal in 2016. Tissues remaining following removal of a small sample for viral screening (see below) were used to quantify IG sequestration (n = 276). Larvae that died prior to immune assessment (n = 85) were excluded from analysis, as they were not starved prior to freezing. Aucubin and catalpol concentrations of caterpillar tissues were quantified using gas chromatography

following the methods of Bowers and Stamp (1992). Samples were weighed and extracted overnight in 5 ml methanol, then filtered, and the extract was evaporated. An internal standard, phenyl β-D-glucopyranoside (0.500 mg), was added to each sample, which was then partitioned between water and ether to remove lipids. The ether fraction was discarded and the water fraction, containing IGs and sugars, was evaporated and extracted in 1 ml methanol overnight. A 100 µl aliquot was removed, evaporated, and then derivatized with 100 µl of Tri-Sil Z (Sigma-Aldrich). Aliquots were injected into an Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector (FID) and Agilent DB-1 column (Bowers and Collinge, 1992; Fajer et al., 1992; Gardner and Stermitz, 1988) calibrated with aucubin and catalpol standards (HP ChemStation software, v. A.03.34). All IG concentrations were corrected for sample mass and are presented as percent dry weight (mg IGs/mg dry weight), with total IG concentrations representing the sum of aucubin and catalpol concentrations in each sample. A subset of caterpillars with zero values for total IG sequestration (n = 17) were excluded from analyses as potential sample degradation from over-homogenization may have led to undetectable amounts of IGs.

2.7 Viral screening of caterpillars

To detect and quantify JcDV infection in *E. phaeton,* DNA was extracted from an aliquot of homogenized tissue from each freeze-killed caterpillar in 2016 (n = 389) and each caterpillar, pupa, or butterfly that died in the laboratory in 2017 (n = 198). For the subset of individuals that were reared to the adult stage, wings were removed prior to homogenization, whereas whole larvae and pupae were used. Total DNA was extracted from each tissue sample (mass: $\bar{x}$ =16.06 ± 0.25 mg) using Qiagen DNeasy 96 Blood and

Tissue Kits (Qiagen, Hilden, North Rhine-Westphalia, Germany), following the Protocol for Purification of Total DNA from Animal Tissues.

Extracted DNA was screened for JcDV using quantitative PCR, with primers specific to the VP4 capsid protein gene of JcDV (Wang et al., 2013) and arthropod 28S rDNA primers as an internal control (Nice et al., 2009). DNA samples were screened in duplicate for both VP4 and 28S using iTaq Universal SYBR Green Supermix (Bio-Rad) at a total reaction volume of 10 µl. Reactions were run on a Bio-Rad CFX96 Optics Module with C1000 Thermal Cycler following the protocol of Smilanich *et al.* (2018) for VP4, with an initial denaturing step at 95°C for 5 min, followed by 45 cycles of: 95°C for 10 s, 60°C for 15 s, 72°C for 15 s. A modified protocol was used for 28S, with an initial step at 95°C for 5 min, followed by 45 cycles of: 95°C for 10 s, 57°C for 15 s, 72°C for 15 s. A melt curve from 65°C to 95°C was performed following each reaction to verify amplification of a single product. Relative viral loads were calculated as $2^{-\Delta C_t}$ (Schmittgen and Livak, 2008), representing the abundance of the JcDV VP4 gene relative to the abundance of the internal control [ΔC_t = mean C_t (threshold cycle) for VP4 – mean C_t for 28S] and log-transformed.

2.8 Statistical analyses

All statistical analyses were performed in R version 4.0.4 (R Core Team, 2021). Linear mixed-effects models (LMMs) were fitted with the ‘nlme’ package (Pinheiro et al., 2020) using REML, and generalized linear mixed-effects models (GLMMs) were fitted with ‘lme4’ (Bates et al., 2015). Fixed effects structures of LMMs and GLMMs were selected using an information theoretic (IT) approach (Burnham and Anderson, 2002): for each analysis, a set of candidate models that included focal predictor variables,

along with combinations of potentially influential covariates and two-way interactions, was specified. Akaike information criteria corrected for small sample sizes (AICc) were compared among candidate model sets using the 'MuMIn' package (Bartoń, 2020), and the model with the best fit (i.e., lowest AICc value) was selected. In cases where a simpler or equally simple candidate model received a similarly high level of AICc support to the best-fit model (ΔAICc < 2), the results of all suitable models are presented, and the models with higher AICc values are referred to as "alternative model structures." Results of the best-fit model(s) for each analysis are presented below; for details on candidate models and parameters relevant to model selection, see Tables S2-6. In addition to IT-specified fixed effects, random intercepts for sampling sites were included in all models. All LMM residuals were inspected for normality and homoscedasticity; if heteroscedasticity was detected, the varIdent or varExp structures were applied to accommodate variable spread of residuals (Zuur et al., 2009; Tables S2-6). Marginal R^2 values were calculated using Nakagawa's method with the 'performance' package (Lüdecke et al., 2020) and post-hoc examinations of estimated marginal means were performed using the 'emmeans' package (Lenth 2021). For all analyses, statistical significance was assessed using an alpha level of 0.05.

2.8.1 JcDV occurrence in E. phaeton populations

The probabilities of detecting JcDV in *E. phaeton* individuals using the native or exotic host plant species were examined using GLMMs with a binomial distribution and logit link-function. Host plant species was included as a fixed effect, with JcDV presence (Y/N) in freeze-killed caterpillars (2016), or lab-deceased caterpillars, pupae, and adults (2017), as response variables. For JcDV-positive individuals, log-transformed viral loads

were then compared using LMMs with host plant species and life stage at the time of death (for 2017 only) as fixed effects.

2.8.2 Host plant effects on immunocompetence and sequestration

Host plant effects on caterpillar immune responses were evaluated separately for each immune parameter using LMMs with host plant, year, and larval weight as fixed effects and PO activity, melanization, and granulocyte, oenocytoid, plasmatocyte, and total hemocyte concentrations as responses. In addition, the interaction between host plant species and year was included in the best-fit models for PO activity and oenocytoids, and the effects of JcDV infection (Y/N) and its interaction with host plant were included in the best-fit model for plasmatocytes. These predictors were not retained in the other models, as their inclusion did not improve model fit (Table S3). All hemocyte concentrations were cube-root transformed, and melanization scores were squared, to improve normality of residuals. The effects of host plant species and significant two-way interactions are summarized in Figure 2; see Table S7 for full models.

Caterpillar sequestration of aucubin, catalpol, and total IGs was compared across host plant species using separate LMMs including the covariate of larval weight. All IG concentrations were cube-root transformed to improve normality of residuals. Relationships between IG sequestration and larval immunity were assessed using separate LMMs including the total concentration of sequestered IGs, the composition of sequestered IGs (quantified as the proportion of aucubin out of total IGs), host plant, and influential two-way interactions (see Table S5) as fixed effects and PO, melanization, and total hemocytes as responses. Follow-up LMMs evaluated the effects of total IG concentration on each type of hemocytes (granulocytes, oenocytoids, and plasmatocytes).

2.8.3 Effects of immunity and sequestration on viral infection

The effect of sequestration on viral burden was investigated using an LMM with total IG sequestration and host plant species as fixed effects and larval JcDV load as the response. As granulocytes were the primary hemocyte type impacted by sequestration (see Results), we then performed an LMM with total IG sequestration, granulocyte concentration, and the interaction between IG sequestration and granulocytes as fixed effects, and JcDV load as the response. To probe this interaction, Johnson-Neyman intervals for significance of the conditional effect of granulocytes on JcDV load were calculated using the 'interactions' package (Long, 2019).

Survivorship of JcDV-infected individuals in 2017 was assessed using two GLMMs with survival to the adult stage (Y/N) as the binomial response variable. The first model evaluated load-dependent effects on survival, with JcDV load and host plant as fixed effects, while the second examined the effects of larval immune responses (along with larval weight) on survival.

3 | RESULTS

3.1 Occurrence of JcDV in *E. phaeton* populations

JcDV was detected in *E. phaeton* individuals originating from all sites, at overall frequencies of 12% in 2016 and 25% in 2017 (see Figure 1b-c for site-level frequencies). In 2016, viral frequency did not differ significantly between caterpillars utilizing the two host plant species, though the odds of viral detection were slightly higher in individuals using the exotic *P. lanceolata* than those using the native *C. glabra* [odds ratio (OR) = 1.50, 95% confidence interval (CI) = (0.79-2.85), p = 0.2]. However, caterpillars using *P.*

lanceolata harbored significantly higher JcDV loads than those using *C. glabra* (Figure 1b) ($\beta = 0.81 \pm 0.31$, $t = 2.6$, $df = 34$, $p = 0.01$; marginal $R^2 = 0.14$, $n = 43$), with 138-fold greater untransformed loads.

In 2017, when individuals were reared out to adulthood or death before viral screening, neither JcDV frequency [OR = 0.64, 95% CI = (0.32-1.38), $p = 0.2$] nor postmortem loads ($\beta = -0.61 \pm 0.38$, $t = -1.6$, $df = 36$, $p = 0.1$) differed based on host plant (Figure 1c). However, viral loads were higher in deceased pupae ($\beta = 1.57 \pm 0.42$, $t = 3.8$, $df = 36$, $p < 0.001$) and larvae ($\beta = 2.19 \pm 0.63$, $t = 3.5$, $df = 36$, $p = 0.001$) than in butterflies (marginal $R^2 = 0.35$, $n = 47$).

3.2 Does use of an exotic host plant impact immunocompetence?

Use of the exotic plant, *P. lanceolata,* was associated with a significant reduction in the melanization response of *E. phaeton* caterpillars, compared to *C. glabra* (Figure 2a) ($\beta = -190 \pm 86$, $t = -2.3$, $df = 377$, $p = 0.03$). PO activity was also reduced in larvae using *P. lanceolata* in 2016 but did not differ significantly based on host plant in 2017 (Figure 2b) (host plant x year interaction: $\beta = 13.2 \pm 5.7$, $t = 2.3$, $df = 439$, $p = 0.02$). An alternative model structure that excluded the interaction between host plant and year showed no significant effect of host plant species on PO activity ($\beta = -4.0 \pm 3.3$, $t = -1.2$, $df = 440$, $p = 0.2$) but had lower support (Table S7).

Total hemocyte concentrations did not vary based on host plant (Figure 2c) ($\beta = -3.3 \pm 5.5$, $t = -0.6$, $df = 432$, $p = 0.6$); however, interesting patterns emerged when investigating different types of hemocytes. Specifically, using *P. lanceolata* had a negative impact on plasmatocytes in uninfected caterpillars, but the opposite pattern was observed when caterpillars were infected with JcDV (Figure 2f) (host plant x JcDV

interaction: $\beta = 32 \pm 14$, $t = 2.3$, $df = 277$, $p = 0.03$), suggesting that JcDV infection mediated host plant effects on plasmatocyte concentrations. An alternative model structure that excluded the effect of JcDV infection showed no significant effect of host plant species on plasmatocytes ($\beta = -10.0 \pm 6.8$, $t = -1.5$, $df = 279$, $p = 0.1$) but had lower support (Table S7). Granulocyte concentrations did not differ based on host plant (Figure 2d) ($\beta = -3.0 \pm 5.6$, $t = -0.53$, $df = 292$, $p = 0.6$), while oenocytoid concentrations were marginally lower on *P. lanceolata* than *C. glabra* in 2017 but not in 2016 (Figure 2e) (host plant x year interaction: $\beta = -14.1 \pm 6.0$, $t = -2.4$, $df = 307$, $p = 0.02$). The effect of JcDV infection was not retained in the models for melanization, PO activity, total hemocytes, granulocytes, or oenocytoids, as its inclusion did not improve model fit (Table S3).

3.3 Does use of an exotic host plant impact sequestration?

Euphydryas phaeton caterpillars exhibited distinct patterns of IG sequestration when feeding on *C. glabra* and *P. lanceolata*, mirroring the typical IG profiles of their respective host plant species (Bowers et al., 1992; Bowers and Stamp, 1992; Duff et al., 1965; Fajer, 1989). While larvae consuming *C. glabra* primarily sequestered catalpol, with little to no aucubin, larvae consuming *P. lanceolata* sequestered a more even mixture of catalpol and aucubin (Figure 3a). Overall, the total concentration of IGs sequestered by larvae did not differ between the two host plants ($\beta = 0.039 \pm 0.076$, $t = 0.51$, $df = 266$, $p = 0.6$; marginal $R^2 = 0.09$; $n = 276$). However, caterpillars using *P. lanceolata* sequestered over seven times more aucubin than those using *C. glabra* ($\beta = 0.469 \pm 0.044$, $t = 11$, $df = 249$, $p < 0.001$; marginal $R^2 = 0.73$, $n = 260$), while

caterpillars using *C. glabra* sequestered 42% more catalpol than those using *P. lanceolata* (β = -0.152 ± 0.074, t = -2.1, df = 258, p = 0.04; marginal R^2 = 0.09, n = 268).

3.4 Is higher sequestration associated with reduced immunocompetence?

There was a significant negative relationship between the total concentration of IGs sequestered by *E. phaeton* larvae and total hemocyte concentration in the hemolymph (Figure 3b) (β = -13.0 ± 5.4, t = -2.4, df = 238, p = 0.02). This pattern was evident in individuals utilizing both *C. glabra* and *P. lanceolata* and was consistent across alternative model structures that varied in their inclusion of two-way interaction terms (Table S8). In addition, there was no significant relationship between the composition of sequestered IGs (proportion of aucubin) and total hemocytes (β = 47 ± 25, t = 1.9, df = 238, p = 0.06). Follow-up analysis of the relationships between sequestration and different types of hemocytes revealed that this pattern was mediated by significant negative associations between IG concentration and granulocytes (β = -18.4 ± 6.2, t = -3.0, df = 138, p = 0.004) and IG concentration and plasmatocytes (β = -14.6 ± 5.8, t = -2.5, df = 146, p = 0.01) (Table S9).

The concentration of IGs sequestered by caterpillars was not significantly associated with PO activity (β = -3.4 ± 2.5, t = -1.4, df = 241, p = 0.2) or melanization (β = 67 ± 99, t = 0.68, df = 206, p = 0.5); however, compositional variation in IG sequestration did exhibit correlations with these parameters. There was a significant negative relationship between the proportion of aucubin sequestered by caterpillars (aucubin/total IGs) and PO activity on both plants (Figure 3c) (β = -26.3 ± 9.4, t = -2.8, df = 241, p = 0.006). A similar pattern was documented for melanization: sequestration of a greater proportion of aucubin was associated with a significant reduction in the

melanization response of caterpillars utilizing *P. lanceolata,* but not *C. glabra* (Figure 3d) (IG composition x host plant interaction: β = -1203 ± 460, t = -2.6, df = 206, p = 0.009). This pattern was consistent in an alternative model structure (see Table S8 for full models).

3.5 Do host plant effects on sequestration and/or immunocompetence affect interactions with a pathogen?

Increased IG sequestration was associated with reduced JcDV load in *E. phaeton* larvae using both *P. lanceolata* and *C. glabra* (Figure 4) (β = -0.46 ± 0.22, t = -2.1, df = 24, p = 0.04). Though the interaction between host plant and IG sequestration was not significant (β = -0.41 ± 0.47, t = -0.89, df = 23, p = 0.4), the magnitude of this relationship was greater for caterpillars using *P. lanceolata* (slope: -0.59 ± 0.26 for *P. lanceolata*; slope: -0.18 ± 0.38 for *C. glabra*). Since granulocyte concentrations were negatively associated with sequestration (see above), we examined the interacting influences of sequestration and granulocytes on viral load in the subset of larvae for which all three parameters were quantified. Interestingly, JcDV load was negatively associated with both total IG sequestration (β = -4.7 ± 1.1, t = -4.4, df = 6, p = 0.005) and granulocyte concentration (β = -0.0411 ± 0.0090, t = -4.6, df = 6, p = 0.004). Moreover, there was a significant interaction between sequestration and granulocyte concentration (β = 0.0214 ± 0.0064, t = 3.3, df = 6, p = 0.02; marginal R^2 = 0.67, n = 16). This interaction indicates that when larvae sequestered IGs at low levels, granulocytes had a significant negative effect on viral load; however, this effect attenuated as caterpillars sequestered higher IG concentrations. The threshold or tipping point at which IG sequestration began to erode the putative immunological effect of granulocytes on JcDV

(Johnson-Neyman interval: transformed IG sequestration value < 1.5) corresponded to an untransformed value of 3.1% sequestered IGs by dry weight. Above this value, granulocyte concentrations no longer exhibited a significant negative relationship with JcDV burden.

Survival of JcDV-infected individuals was not host plant specific: 46% of infected individuals reared on *C. glabra* (n = 43) versus 45% of those reared on *P. lanceolata* (n = 47) died before reaching the adult stage. However, JcDV exhibited a load-dependent effect on survival: insects with higher viral loads had a lower probability of surviving to the adult stage (Figure 5) [OR = 0.32, 95% CI = (0.14-0.50), p = 0.002]. Notably, the strength of larval immune responses did not significantly impact survival of infected individuals (Table S10).

4 | DISCUSSION

This study provides evidence that host range expansion mediates a multifaceted shift in herbivore physiological defenses against natural enemies. Use of the exotic host plant, *P. lanceolata,* was associated with: (1) suppression of multiple immune parameters (Figure 2), which may influence vulnerability to a broad range of pathogens and parasites, and (2) differential composition of sequestered IGs (Figure 3a), which may compromise the efficacy of chemical defense against predators (Bowers, 1980) and impact the strength of immunological defenses against pathogens and parasites (Figure 3c-d). Additionally, in situ JcDV infection loads were higher on *P. lanceolata* (Figure 1b), suggesting that use of the exotic plant may entail greater exposure or vulnerability to this pathogen during larval development. Despite these defensive differences, larvae

using both *P. lanceolata* and *C. glabra* exhibited reduced JcDV burdens when sequestering high concentrations of IGs (Figure 4), and survival of JcDV-infected individuals was ultimately similar on the two plants (Figure 5). These results indicate that use of an exotic plant is characterized by reduced immunocompetence but comparable levels of chemical protection against a virus, potentially supporting sustainable populations of *E. phaeton* on this exotic plant.

Enhancement of immunity does not appear to be promoting *E. phaeton* persistence on the exotic plant, as field-collected caterpillars exhibited reductions in multiple immune responses when consuming *P. lanceolata*. The suite of immune parameters suppressed in caterpillars using the exotic plant, including melanization (Figure 2a), plasmatocyte concentration (Figure 2f), and PO activity (in 2016; Figure 2b), provides defense against eukaryotic parasites (Carton et al., 2008; Richman and Kafatos, 1996) and can also contribute to antiviral (McNeil et al., 2010; Trudeau et al., 2001; Washburn et al., 1996) and antimicrobial (Lavine and Strand, 2002; Rantala and Roff, 2007) immunity. In particular, decreased melanization can be an effective predictor of successful parasitism by dipteran and hymenopteran parasitoids in Lepidoptera (Smilanich et al., 2009b; but see Klemola et al., 2008), indicating that caterpillars with weaker melanization responses may be more vulnerable to parasitism in the wild. Reduced plasmatocyte densities may additionally impair critical cell-mediated defenses against microbial pathogens, including phagocytosis and expression of antimicrobial peptides (Strand, 2008). Overall, suppression of humoral and cellular components of the immune response represents a substantial putative cost of exotic host plant use for *E. phaeton* larvae; however, the extent to which these patterns are consistent across time

warrants consideration. Variation in host plant effects across the two sampling years was evident for two out of six measured immune parameters (PO activity, Figure 2b; and oenocytoid densities, Figure 2e), underscoring the likelihood that aspects of larval history beyond host plant identity, including both abiotic and biotic factors, impact patterns of immunocompetence in wild settings.

Host plant use may additionally impact herbivore defense against natural enemies through effects of phytochemical sequestration on the immune response. We documented a negative relationship between IG sequestration and hemocyte concentrations in *E. phaeton* caterpillars (Figure 3b), indicating that sequestration of plant secondary metabolites may suppress components of the cellular immune response (specifically, granulocytes and plasmatocytes; Table S8). This pattern could be a product of direct cytotoxicity of sequestered IGs (Smilanich et al., 2009a) or indirect effects arising from the competing energetic demands of sequestration and immunity. These findings provide the first support for the "vulnerable host" hypothesis from a field-based study, as our results indicate that *E. phaeton* larvae that sequester high concentrations of IGs may be more immunologically vulnerable to parasitism and/or infectious disease in the wild (Lampert and Bowers, 2015; Smilanich et al., 2009a).

Although the total concentration of IGs sequestered by *E. phaeton* did not differ based on host plant species, caterpillars consuming the exotic *P. lanceolata* sequestered a relatively even mixture of aucubin and catalpol, while those consuming *C. glabra* sequestered primarily catalpol (Figure 3a). This compositional difference in IG sequestration may have repercussions for the strength of immune responses: both PO activity (Figure 3c) and melanization (Figure 3d) decreased as caterpillars sequestered a

greater proportion of aucubin, relative to catalpol. These findings are consistent with previous research documenting negative synergistic effects of aucubin and catalpol sequestration on melanization in another specialist caterpillar (Richards et al., 2012). Though additional research will be necessary to determine the role of synergy in mediating these patterns in *E. phaeton*, these results indicate that the distinct IG mixture sequestered by larvae using *P. lanceolata* may contribute to immunosuppression on this plant (Figure 2), highlighting a potential tritrophic outcome of compositional phytochemical variation in this system.

Although individuals that sequestered high levels of IGs were more immunologically vulnerable, our results indicate that sequestered phytochemicals may provide herbivores with an additional form of defense against viral infection (Figure 4). We observed a negative relationship between IG sequestration and JcDV load in *E. phaeton* caterpillars using both the exotic and native host plants (Figure 4). This apparently protective effect of IG sequestration was surprising, given the negative relationship between IG sequestration and hemocyte concentrations also documented in this study (Figure 3b), and suggests that these compounds may attenuate viral infection through mechanisms external to the immune responses measured in this study. Antiviral properties of IGs are well-documented in mammalian systems (e.g., Tundis et al., 2008); thus, sequestration of high concentrations of these compounds could directly interfere with JcDV infectivity or replication within the host. Similar protective effects of toxic phytochemicals against pathogens have been reported in several insect systems (Cory and Hoover, 2006; De Roode et al., 2008; Smilanich et al., 2018). Our data expand upon this research by directly associating variation in sequestration of plant secondary metabolites

with defense against an entomopathogen, a relationship which has rarely been explicitly assessed.

Together, these results highlight an important caveat to the “vulnerable host” hypothesis: the immune response is not the only form of defense against enemies, and host vulnerability may be influenced by both direct and indirect effects (mediated by the immune response) of sequestered compounds on parasites and pathogens. Although not tested here, the potential effects of IG sequestration may give rise to a tradeoff between chemical and immunological forms of defense against the virus, as high levels of sequestration were associated with both attenuation of infection (Figure 4) and suppression of cellular immune components (Figure 3b). In particular, JcDV loads were negatively associated with granulocyte concentrations, indicating that these cells may play a role in suppressing infection. However, the negative relationship between granulocytes and viral load was only evident in larvae sequestering low concentrations of IGs, further suggesting an incompatibility between sequestration and hemocytic immunity.

This immunological cost of sequestration may be expected to give rise to distinct defensive syndromes across herbivore populations experiencing differential pressure from natural enemies. Intermediate sequestration phenotypes may be favored when chemical and hemocytic defenses are both critical for survival; this may be expected in *E. phaeton* populations where both JcDV infection (Figure 1a-b) and attack by other natural enemies, including parasitoids and microbial pathogens, are common. Alternatively, chemical defense may be favored over hemocytic immunity in cases where: (1) sequestration provides comparable protection against enemies, but incurs a lesser

energetic cost, or (2) sequestration provides defense against a broader suite of enemies, including predators, parasitoids, and pathogens. In such cases, we may predict a disinvestment in immunity (see Tan et al., 2019). Variation in selective pressures from multiple types of natural enemies across *E. phaeton* populations using either *C. glabra* or *P. lanceolata*, and its role in driving tradeoffs in defensive strategies, warrants further study.

Our understanding of the ecological factors facilitating herbivore persistence on exotic host plants may be improved through field-based investigations within a tritrophic framework. Though the immunological disadvantages of using *P. lanceolata* may limit *E. phaeton*'s ability to persist on this exotic plant, the putatively protective role of IG sequestration against JcDV infection was consistent between host plants. This comparable level of chemical defense may facilitate continued use of the exotic plant, particularly in populations where JcDV pressure is high and mounting an immune response is costly. Importantly, the likelihood of surviving JcDV infection did not differ between individuals reared on the native and exotic host plants (Figure 5), suggesting that chemical defense effectively compensated for immunosuppression on the exotic plant where this pathogen was concerned. These findings provide insight into the paradox of exotic host plant use (Yoon and Read, 2016) in *E. phaeton,* indicating that *P. lanceolata* may represent an equally suitable host plant, relative to the native *C. glabra*, within certain tritrophic contexts. While these defensive traits represent a subset of many dimensions of herbivore fitness that can differ on native and exotic host plants (Forister et al., 2020; Nylin et al., 2018), they provide novel insight into the interacting roles of immune defense and phytochemical sequestration in mediating tritrophic interactions in

the wild. Moving forward, consideration of herbivore physiological defenses against higher trophic levels may provide opportunities for more comprehensive evaluation of the ecological costs and benefits of host range expansion, with applications to understanding the evolution of herbivore diet breadth.

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AUTHOR CONTRIBUTIONS

AMS, PAM, and MDB conceived the study and all authors contributed to its design. NDM, PAM, and ALC collected samples, NDM performed immune assays, viral screening, and insect rearing, and ALC and MDB performed chemical analyses. NDM analyzed data and wrote the manuscript, and all authors contributed to revisions.

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FIGURE LEGENDS

Figure 1 (A) Locations of sites in Connecticut (CT1), Massachusetts (MA1–4), Rhode Island (RI1), and Vermont (VT1–5) from which *Euphydryas phaeton* caterpillars were sampled. At each site, either the native host plant (*Chelone glabra*), exotic host plant (*Plantago lanceolata*), or both host plants were utilized. Caterpillars were collected in 2016 and 2017 unless otherwise indicated. (B-C) Junonia coenia densovirus loads of freeze-killed larvae in 2016 (B) and lab-deceased larvae, pupae, and adults in 2017 (C) across sites. Points represent mean viral load ± SE, with site-level JcDV frequency (% infected individuals) above each point. Based on linear mixed-effects models with an alpha level of 0.05, in situ JcDV loads were significantly higher in larvae using *P. lanceolata* (B), but postmortem loads did not differ significantly based on host plant (C).

Figure 2 Effects of host plant species on immune responses of *Euphydryas phaeton* caterpillars, including implant melanization score (A), phenoloxidase activity (B), and concentrations of total hemocytes (C), granulocytes (D), oenocytoids (E), and plasmatocytes (F) in the hemolymph. Points represent estimated marginal means (EMMs) ± SE based on linear mixed-effects models, which included host plant (*Chelone glabra* or *Plantago lanceolata*) and year (2016 or 2017) as fixed effects, larval weight as a covariate, and random intercepts for sites (see Table S7 for full models). EMMs were averaged across larval weights (all models) and years (with the exceptions of B and E, which included significant interactions between host plant and year). The effects of JcDV and its interaction with host plant species were included in only the plasmatocyte model (F). Lettering sets (a-b and a_1-b_1) indicate significant differences between EMMs based

on pairwise contrasts, evaluated using an alpha level of 0.05. Use of the exotic *P. lanceolata* was associated with significant reductions in melanization (A), PO activity (in 2016 only; B), and plasmatocyte concentrations (in uninfected individuals only; F).

Figure 3 (A) Iridoid glycoside sequestration of *Euphydryas phaeton* caterpillars utilizing either *Chelone glabra* or *Plantago lanceolata* across sites. Bars represent mean total IG concentrations (% dry weight) ± SE, delineated by proportions of aucubin and catalpol (n = 7-36 per bar). (B) Relationship between IG sequestration and total hemocyte concentration in *E. phaeton* larvae (untransformed values). Caterpillars that sequestered higher concentrations of IGs exhibited significantly lower hemocyte densities, regardless of host plant species (LMM: marginal $R^2 = 0.04$, $n = 250$). (C) Relationship between the proportion of aucubin sequestered by larvae (aucubin/total IGs) and phenoloxidase activity. Caterpillars that sequestered a higher proportion of aucubin exhibited significantly lower PO activity on both host plants (LMM: marginal $R^2 = 0.06$, $n = 252$). (D) Relationship between the proportion of aucubin sequestered by larvae and implant melanization. Larvae that sequestered higher proportions of aucubin exhibited significantly lower melanization on the exotic plant, *P. lanceolata,* but not the native *C. glabra* (LMM: marginal $R^2 = 0.06$, $n = 219$). Significance was assessed using an alpha level of 0.05.

Figure 4 Relationship between iridoid glycoside sequestration and Junonia coenia densovirus load of *Euphydryas phaeton* caterpillars. Based on a linear mixed-effects model with an alpha level of 0.05, JcDV-infected caterpillars that sequestered higher total

concentrations of IGs exhibited significantly lower viral infection loads when utilizing both the native plant, *Chelone glabra,* and the exotic plant, *Plantago lanceolata* (marginal $R^2 = 0.26$, $n = 34$). Though the negative effect of IG sequestration on viral burden did not differ significantly based on host plant species, separate slopes are provided for individuals utilizing *C. glabra* and *P. lanceolata* for illustrative purposes.

Figure 5 Estimated survival probabilities of *Euphydryas phaeton* based on Junonia coenia densovirus load. Based on a generalized linear mixed-effects models with an alpha level of 0.05, higher JcDV infection loads corresponded to a significantly lower probability of surviving to the adult stage, regardless of larval host plant species (GLMM: marginal $R^2 = 0.47$, $n = 47$). Arrows indicate mean postmortem viral loads of field-collected insects in 2017 (reared out and deceased in the laboratory as larvae, pupae, or adults), which did not differ significantly between individuals reared on the native host plant, *Chelone glabra,* and the exotic host plant, *Plantago lanceolata*.

FIGURES

Figure 1

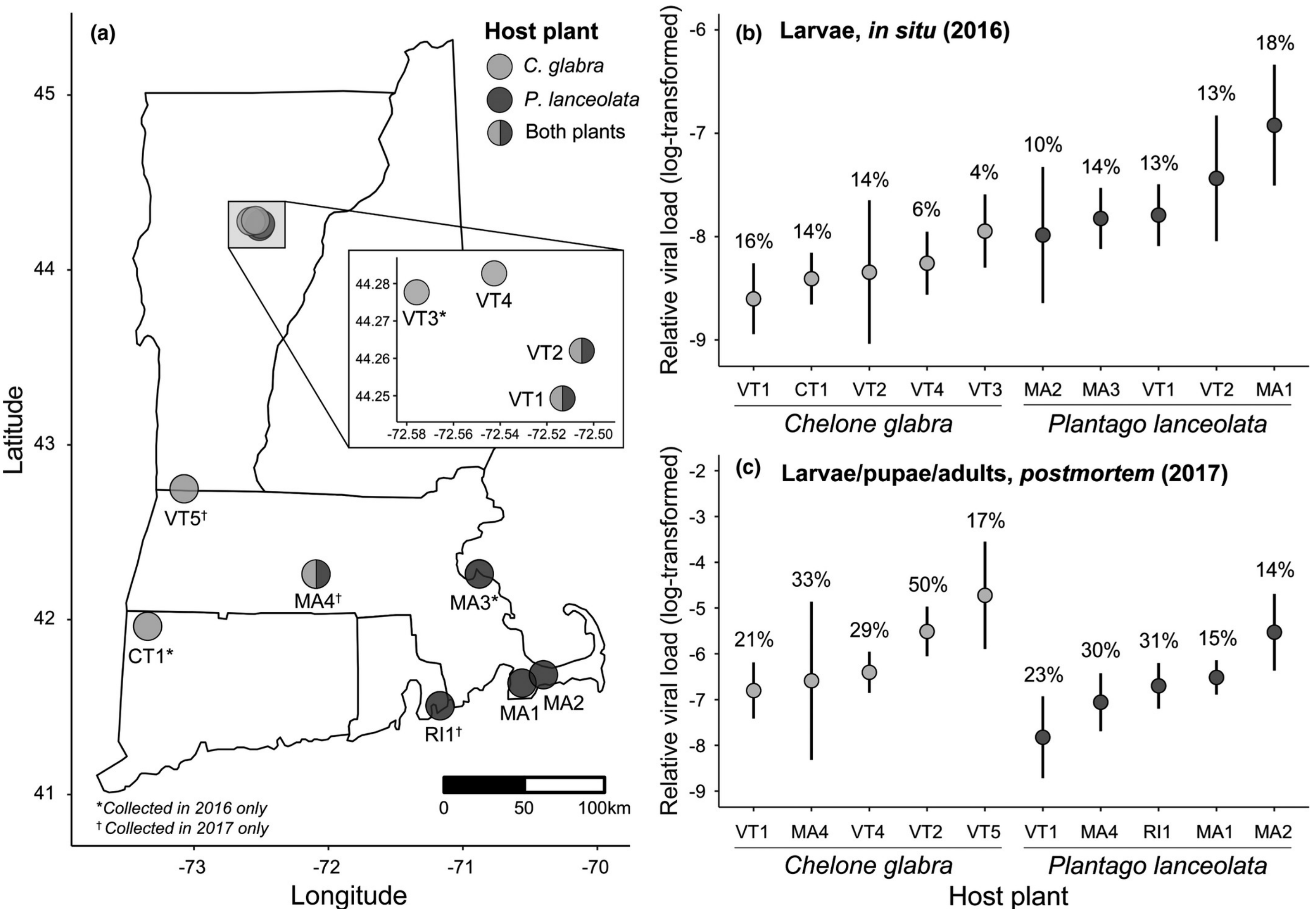


Figure 2

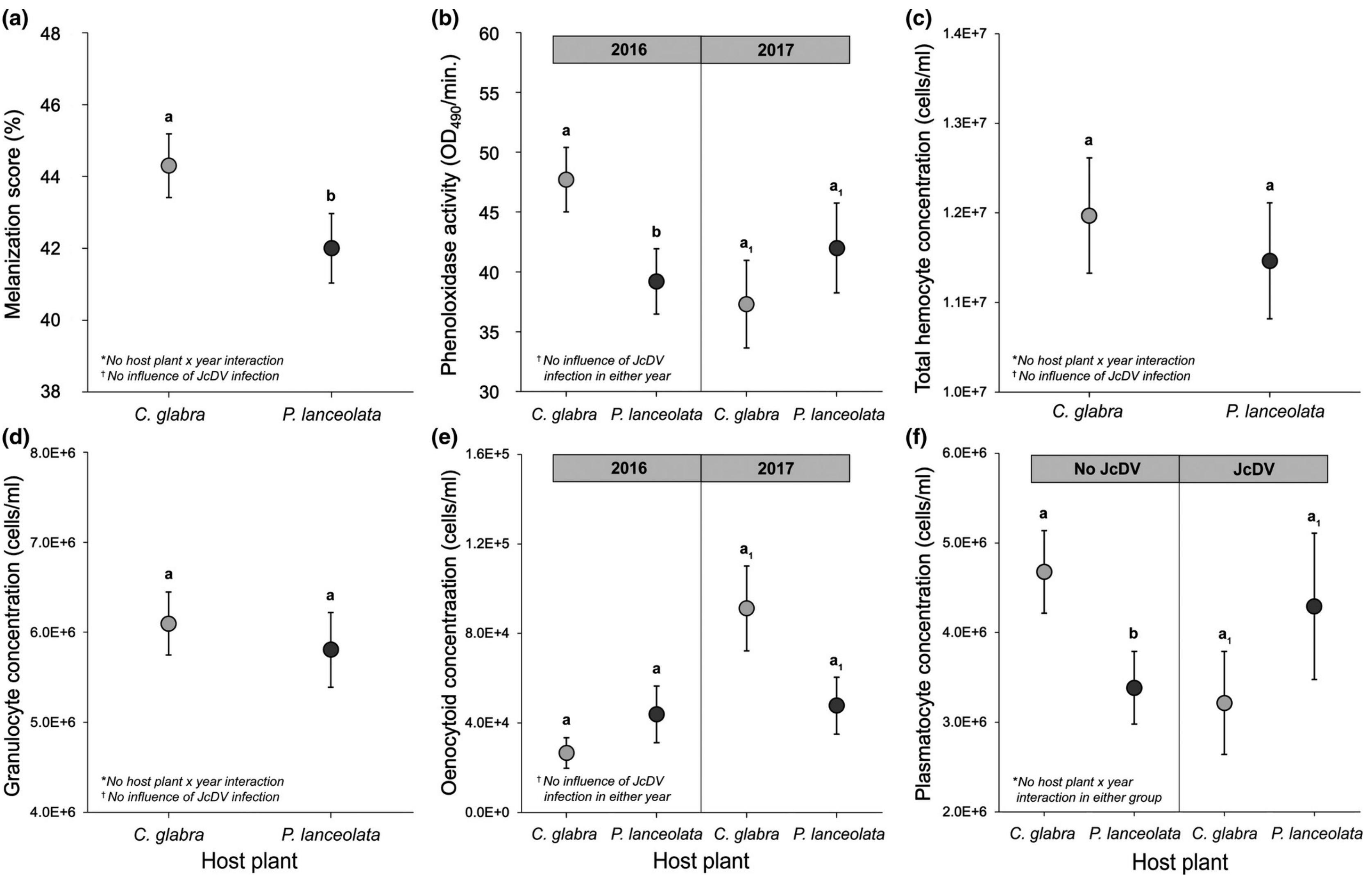


Figure 3

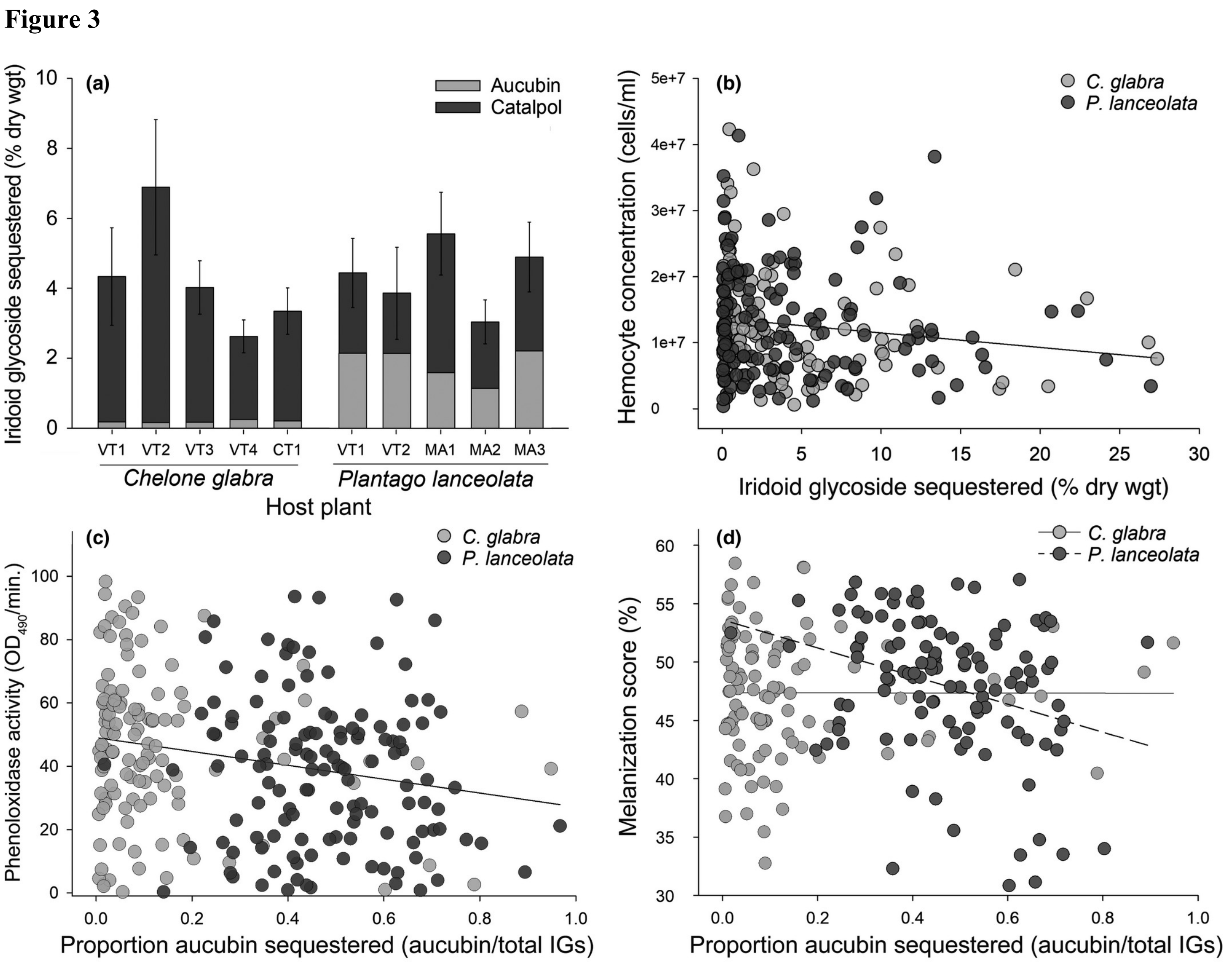


Figure 4

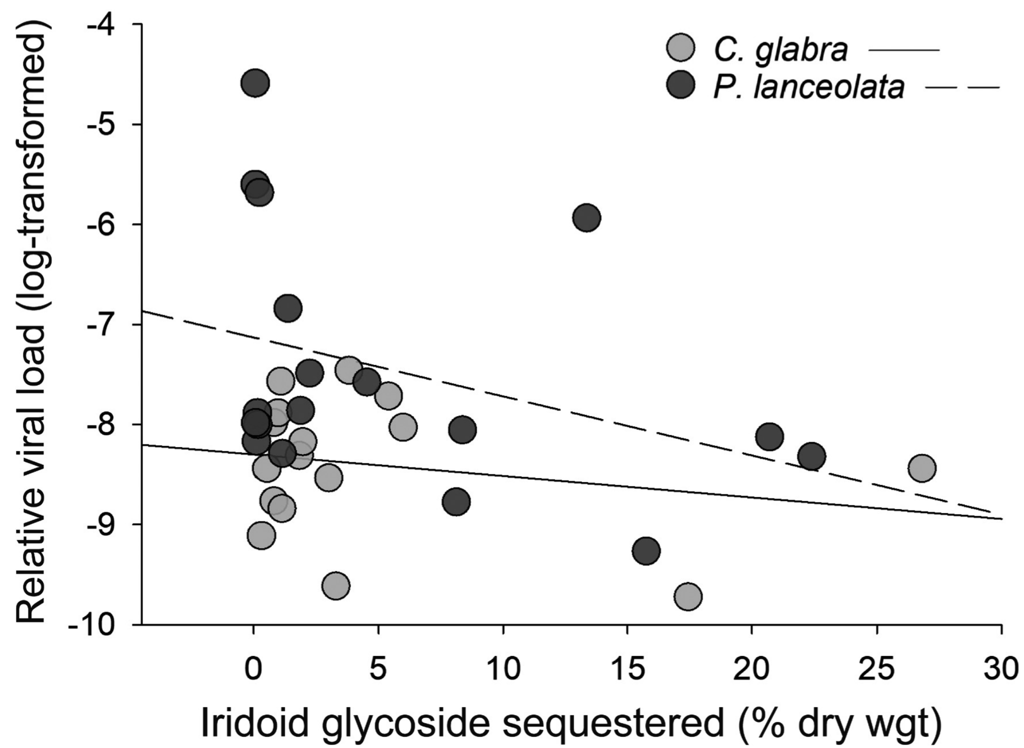
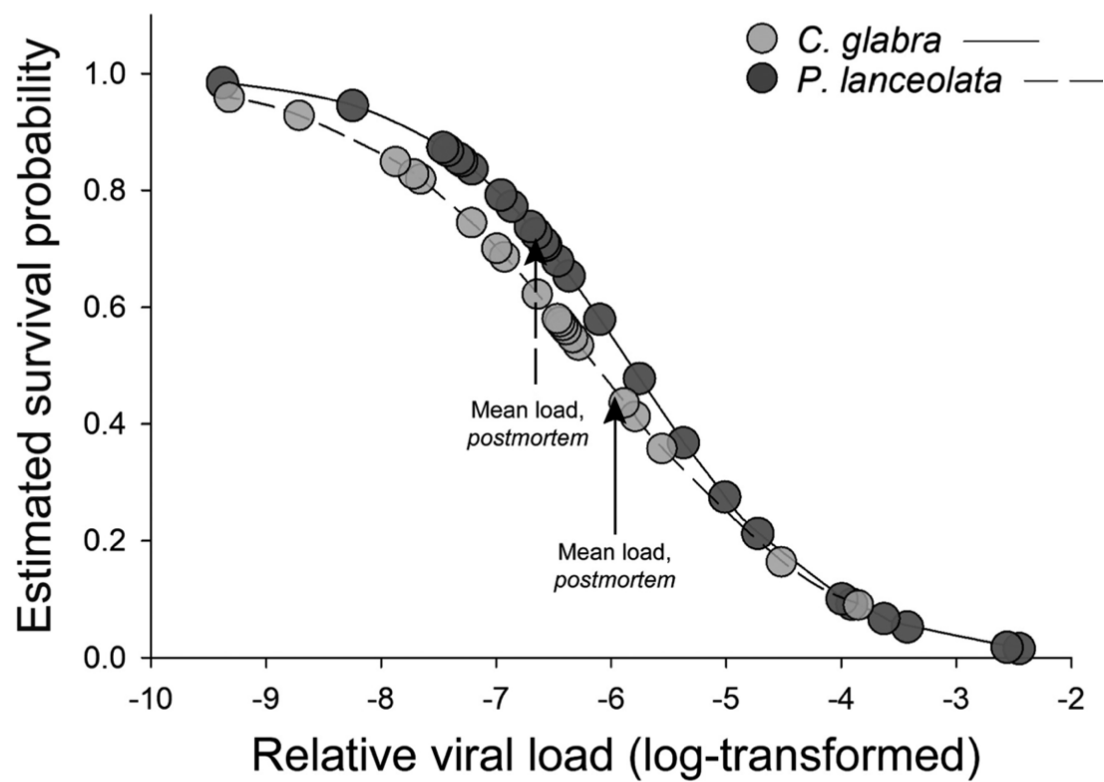


Figure 5



APPENDIX

Table S1 Sampling details for *Euphydryas phaeton* caterpillars collected from the wild in May 2016 and 2017. Field sites were located throughout the northeastern U.S. in Connecticut (CT), Massachusetts (MA), Rhode Island (RI), and Vermont (VT), spanning a range of approximately 330 km. At each site, post-diapause *E. phaeton* caterpillars utilized either the native host plant, *Chelone glabra,* the exotic host plant, *Plantago lanceolata,* or both host plants (MA4, VT1, VT2). Sample sizes of caterpillars collected from each site and host plant species (*n*) are provided for each year (2016 or 2017).

Site ID	Host plant species	Larvae 2016 (*n*)	Larvae 2017 (*n*)	Latitude	Longitude
CT1	*Chelone glabra*	49	Not collected*	41.961877	-73.344344
MA1	*Plantago lanceolata*	50	30	41.636994	-70.559876
MA2	*Plantago lanceolata*	50	30	41.684528	-70.400126
MA3	*Plantago lanceolata*	50	Not collected*	42.260065	-70.877680
MA4	*Chelone glabra*	Not visited	9	42.259651	-72.092595
MA4	*Plantago lanceolata*	Not visited	12	42.259651	-72.092595
RI1	*Plantago lanceolata*	Not visited	30	41.507292	-71.168968
VT1	*Chelone glabra*	25	22	44.249298	-72.513361
VT1	*Plantago lanceolata*	25	18	44.249298	-72.513361
VT2	*Chelone glabra*	25	29	44.262020	-72.505049
VT2	*Plantago lanceolata*	16	Not collected*	44.262020	-72.505049
VT3	*Chelone glabra*	50	Not collected*	44.277640	-72.575906
VT4	*Chelone glabra*	50	30	44.282757	-72.542557
VT5	*Chelone glabra*	Not visited	19	42.747740	-73.073560

** Caterpillars not collected due to relatively small population sizes.*

Table S2 Details of model selection for linear mixed-effects models (LMMs) and generalized linear mixed-effects models (GLMMs) presented in the Results section: "Occurrence of JcDV in *E. phaeton* populations." For each analysis, a set of candidate models was specified, and the degrees of freedom (*df*), log-likelihood (*logLik*), Akaike's information criterion corrected for small sample sizes (*AICc*), and Akaike weight (*weight*) of each model was calculated. Fixed effects structures were selected using an information theoretic (IT) approach: the model with the best fit (i.e., the lowest AICc value; indicated with shading) was reported in the Results. All models included random intercepts for sampling sites. Host plant = host plant species (*Chelone glabra* or *Plantago lanceolata*); weight = larval body weight at the time of immune assessment; JcDV load = log-normalized JcDV load relative to an internal control gene; deceased = lab-deceased larvae, pupae, and adults; stage = life stage (larva, pupa, or adult) at the time of death.

Model Selection: ***Occurrence of JcDV in E. phaeton populations***

Response variable	*Year*	*Type*	*n*	*Variance structure*	*Fixed effects structure*	*df*	*logLik*	*AICc*	*weight*
Caterpillar JcDV (presence/absence)	2016	GLMM	389	None	*Model 1.1:* Host plant	3	-112.4	230.9	0.62
					Model 1.2: Host plant + weight	4	-112.4	232.9	0.23
					Model 1.3: Host plant + weight + host plant*weight	5	-111.8	233.8	0.15
Caterpillar JcDV load	2016	LMM	43	None	*Model 2.1:* Host plant	4	-51.2	111.7	0.73
					Model 2.2: Host plant + weight	5	-51.1	114.2	0.21
					Model 2.3: Host plant + weight + host plant*weight	6	-51.0	116.8	0.06
Deceased JcDV (presence/absence)	2017	GLMM	196	None	*Model 3:* Host plant	*No model selection performed*			
Deceased JcDV load	2017	LMM	47	None	*Model 4.1:* Host plant	4	-86.4	181.7	0.00
					Model 4.2: Host plant + life stage	6	-77.0	168.1	0.79
					Model 4.3: Host plant + life stage + host plant*life stage	8	-75.5	170.7	0.21

Table S3 Details of model selection for linear mixed-effects models (LMMs) reported in the Results section: "Does use of an exotic host plant impact immunocompetence?" For each analysis, a set of candidate models was specified, and the degrees of freedom (*df*), log-likelihood (*logLik*), Akaike's information criterion corrected for small sample sizes (*AICc*), and Akaike weight (*weight*) of each model was calculated. Fixed effects structures were selected using an information theoretic (IT) approach: the model with the best fit (i.e., the lowest AICc value; indicated with shading) was reported in the Results. For PO activity and plasmatocytes (model sets 5 and 9), the results of two models were reported, as a simpler model structure that excluded certain fixed effects was found to receive a similarly high level of AICc support to the best-fit model (ΔAICc < 2). All models included random intercepts for sampling sites, and variance structures (varIdent or varExp) were applied to a subset of LMMs to account for heterogeneity of variance across levels of fixed effects. Host plant = host plant species (*Chelone glabra* or *Plantago lanceolata*); year = sampling year (2016 or 2017); weight = larval body weight at the time of immune assessment; JcDV = presence/absence of Junonia coenia densovirus.

Model Selection: ***Does use of an exotic host plant impact immunocompetence?***

Response variable	*Year*	*Type*	*n*	*Variance structure*	*Fixed effects structure*	*df*	*logLik*	*AICc*	*weight*
PO activity	Both	LMM	454	varIdent (year)	*Model 5.1:* Host plant + year + weight	7	-1991.2	3996.7	0.17
					Model 5.2: Host plant + year + weight + JcDV	8	-1991.2	3998.7	0.06
					Model 5.3: Host plant + year + weight + JcDV+ host plant*JcDV	9	-1990.8	3999.9	0.03
					Model 5.4: Host plant + year + weight + host plant*year	8	-1989.3	3994.9	0.43
					Model 5.5: Host plant + year + weight + JcDV+ host plant*year	9	-1989.2	3996.9	0.15
					Model 5.6: Host plant + year + weight + JcDV+ host plant*year + host plant*JcDV	10	-1988.3	3997.0	0.15
Melanization	Both	LMM	391	varExp (weight)	*Model 6.1:* Host plant + year + weight	7	-2830.7	5675.7	0.33
					Model 6.2: Host plant + year + weight + JcDV	8	-2829.8	5676.1	0.28
					Model 6.3: Host plant + year + weight + JcDV+ host plant*JcDV	9	-2829.8	5678.1	0.10
					Model 6.4: Host plant + year + weight + host plant*year	8	-2830.6	5677.5	0.13
					Model 6.5: Host plant + year + weight + JcDV+ host plant*year	9	-2829.6	5677.8	0.12
					Model 6.6: Host plant + year + weight + JcDV+ host plant*year + host plant*JcDV	10	-2829.6	5679.9	0.04
Granulocytes	Both	LMM	306	None	*Model 7.1:* Host plant + year + weight	6	-1488.2	2988.6	0.45
					Model 7.2: Host plant + year + weight + JcDV	7	-1488.1	2990.7	0.16
					Model 7.3: Host plant + year + weight + JcDV+ host plant*JcDV	8	-1487.7	2991.9	0.09
					Model 7.4: Host plant + year + weight + host plant*year	7	-1487.9	2990.3	0.20
					Model 7.5: Host plant + year + weight + JcDV+ host plant*year	8	-1487.9	2992.4	0.07
					Model 7.6: Host plant + year + weight + JcDV+ host plant*year + host plant*JcDV	9	-1487.6	2993.8	0.03

Model Selection: ***Does use of an exotic host plant impact immunocompetence? Cont.***

Response variable	*Year*	*Type*	*n*	*Variance structure*	*Fixed effects structure*	*df*	*logLik*	*AICc*	*weight*
Oenocytoids	Both	LMM	322	None	*Model 8.1:* Host plant + year + weight	6	-1391.4	2795.1	0.08
					Model 8.2: Host plant + year + weight + JcDV	7	-1391.2	2796.9	0.03
					Model 8.3: Host plant + year + weight + JcDV+ host plant*JcDV	8	-1390.5	2797.5	0.02
					Model 8.4: Host plant + year + weight + host plant*year	7	-1388.8	2792.0	0.37
					Model 8.5: Host plant + year + weight + JcDV+ host plant*year	8	-1388.7	2793.9	0.15
					Model 8.6: Host plant + year + weight + JcDV+ host plant*year + host plant*JcDV	9	-1386.8	2792.2	0.34
Plasmatocytes	Both	LMM	293	None	*Model 9.1:* Host plant + year + weight	6	-1545.6	3103.4	0.18
					Model 9.2: Host plant + year + weight + JcDV	7	-1545.2	3104.8	0.09
					Model 9.3: Host plant + year + weight + JcDV+ host plant*JcDV	8	-1542.7	3101.9	0.38
					Model 9.4: Host plant + year + weight + host plant*year	7	-1544.8	3104.0	0.13
					Model 9.5: Host plant + year + weight + JcDV+ host plant*year	8	-1544.5	3105.5	0.06
					Model 9.6: Host plant + year + weight + JcDV+ host plant*year + host plant*JcDV	9	-1542.5	3103.6	0.16
Total hemocytes	Both	LMM	446	varExp (weight)	*Model 10.1:* Host plant + year + weight	7	-2254.5	4523.2	0.44
					Model 10.2: Host plant + year + weight + JcDV	8	-2254.4	4525.2	0.16
					Model 10.3: Host plant + year + weight + JcDV+ host plant*JcDV	9	-2253.9	4526.3	0.10
					Model 10.4: Host plant + year + weight + host plant*year	8	-2254.3	4525.0	0.16
					Model 10.5: Host plant + year + weight + JcDV+ host plant*year	9	-2254.3	4527.0	0.07
					Model 10.6: Host plant + year + weight + JcDV+ host plant*year + host plant*JcDV	10	-2253.6	4527.7	0.05

Table S4 Details of model selection for linear mixed-effects models (LMMs) reported in the Results section: "Does use of an exotic host plant impact sequestration?" For each analysis, a set of candidate models was specified, and the degrees of freedom (*df*), log-likelihood (*logLik*), Akaike's information criterion corrected for small sample sizes (*AICc*), and Akaike weight (*weight*) of each model was calculated. Fixed effects structures were selected using an information theoretic (IT) approach: the model with the best fit (i.e., the lowest AICc value; indicated with shading) was reported in the Results. All models included random intercepts for sampling sites, and variance structures (varIdent or varExp) were applied to a subset of LMMs to account for heterogeneity of variance across levels of fixed effects. IG concentration = total concentration of iridoid glycosides sequestered by caterpillars; host plant = host plant species (*Chelone glabra* or *Plantago lanceolata*); weight = larval body weight at the time of immune assessment.

Model Selection: ***Does use of an exotic host plant impact sequestration?***

Response variable	*Year*	*Type*	*n*	*Variance structure*	*Fixed effects structure*	*df*	*logLik*	*AICc*	*weight*
IG concentration	2016	LMM	276	None	*Model 11.1:* Host plant	4	-273.2	554.5	0.00
					Model 11.2: Host plant + weight	5	-260.7	532.0	0.71
					Model 11.3: Host plant + weight + host plant*weight	6	-260.9	533.7	0.29
Aucubin concentration	2016	LMM	260	varIdent (host plant)	*Model 12.1:* Host plant	5	-45.2	100.7	0.00
					Model 12.2: Host plant + weight	6	-39.6	91.6	0.02
					Model 12.3: Host plant + weight + host plant*weight	7	-34.8	84.0	0.98
Catalpol concentration	2016	LMM	268	None	*Model 13.1:* Host plant	4	-250.8	509.8	0.00
					Model 13.2: Host plant + weight	5	-241.3	492.8	0.50
					Model 13.3: Host plant + weight + host plant*weight	6	-240.3	492.9	0.50

Table S5 Details of model selection for linear mixed-effects models (LMMs) reported in the Results section: "Is higher sequestration associated with reduced immunocompetence?" For each analysis, a set of candidate models was specified, and the degrees of freedom (*df*), log-likelihood (*logLik*), Akaike's information criterion corrected for small sample sizes (*AICc*), and Akaike weight (*weight*) of each model was calculated. Fixed effects structures were selected using an information theoretic (IT) approach: the model with the best fit (i.e., the lowest AICc value; indicated with shading) was reported in the Results. For melanization and total hemocytes (model sets 15 and 16), the results of 2-3 models were reported, as model structures that included an equal or lesser number of fixed effects were found to receive a similarly high level of AICc support to the best-fit model (ΔAICc < 2). All models included random intercepts for sampling sites, and the varIdent variance structure was applied to one set of LMMs (model set 16) to account for heterogeneity of variance. Host plant = host plant species (*Chelone glabra* or *Plantago lanceolata*); IG concentration = total concentration of iridoid glycosides sequestered by caterpillars; IG composition = proportion of aucubin sequestered out of total IGs.

Model Selection: ***Is higher sequestration associated with reduced immunocompetence?***

Response variable	*Year*	*Type*	*n*	*Variance structure*	*Fixed effects structure*	*df*	*logLik*	*AICc*	*weight*
PO activity	2016	LMM	252	None	*Model 14.1:* Host plant + IG concentration + IG composition	6	-1147.0	2306.3	0.53
					Model 14.2: Host plant + IG concentration + IG composition + host plant*IG concentration	7	-1146.9	2308.3	0.19
					Model 14.3: Host plant + IG concentration + IG composition + host plant*IG composition	7	-1146.9	2308.2	0.20
					Model 14.4: Host plant + IG concentration + IG composition + host plant*IG concentration + host plant*IG composition	8	-1146.8	2310.2	0.08
Melanization	2016	LMM	219	None	*Model 15.1:* Host plant + IG concentration + IG composition	6	-1675.1	3362.6	0.10
					Model 15.2: Host plant + IG concentration + IG composition + host plant*IG concentration	7	-1675.0	3364.6	0.04
					Model 15.3: Host plant + IG concentration + IG composition + host plant*IG composition	7	-1672.6	3359.7	0.41
					Model 15.4: Host plant + IG concentration + IG composition + host plant*IG concentration + host plant*IG composition	8	-1671.4	3359.5	0.45
Total hemocytes	2016	LMM	250	varIdent (host plant)	*Model 16.1:* Host plant + IG concentration + IG composition	7	-1329.0	2672.5	0.26
					Model 16.2: Host plant + IG concentration + IG composition + host plant*IG concentration	8	-1327.8	2672.3	0.28
					Model 16.3: Host plant + IG concentration + IG composition + host plant*IG composition	8	-1327.8	2672.1	0.30
					Model 16.4: Host plant + IG concentration + IG composition + host plant*IG concentration + host plant*IG composition	9	-1327.3	2673.3	0.16
Granulocytes	2016	LMM	148	None	*Model 17.1:* Host plant + IG concentration	5	-773.4	1557.2	0.67
					Model 17.2: Host plant + IG concentration + host plant*IG concentration	6	-773.0	1558.6	0.33
Oenocyoids	2016	LMM	165	None	*Model 18.1:* Host plant + IG concentration	5	-766.8	1544.1	0.60
					Model 18.2: Host plant + IG concentration + host plant*IG concentration	6	-766.2	1544.9	0.40
Plasmatocytes	2016	LMM	156	None	*Model 19.1:* Host plant + IG concentration	5	-814.8	1640.0	0.74
					Model 19.2: Host plant + IG concentration + host plant*IG concentration	6	-814.7	1642.0	0.26

Table S6 Details of model selection for linear mixed-effects models (LMMs) reported in the Results section: "Do host plant effects on sequestration and/or immunocompetence affect interactions with a pathogen?" For each analysis, a set of candidate models was specified, and the degrees of freedom (*df*), log-likelihood (*logLik*), Akaike's information criterion corrected for small sample sizes (*AICc*), and Akaike weight (*weight*) of each model was calculated. Fixed effects structures were selected using an information theoretic (IT) approach: the model with the best fit (i.e., the lowest AICc value; indicated with shading) was reported in the Results. All models included random intercepts for sampling sites. JcDV load = log-normalized JcDV load relative to an internal control gene; IG concentration = total concentration of iridoid glycosides sequestered by caterpillars; host plant = host plant species (*Chelone glabra* or *Plantago lanceolata*); weight = larval body weight at the time of immune assessment.

Model Selection: ***Do host plant effects on sequestration and/or immunocompetence affect interactions with a pathogen?***

Response variable	*Year*	*Type*	*n*	*Variance structure*	*Fixed effects structure*	*df*	*logLik*	*AICc*	*weight*
Caterpillar JcDV load	2016	LMM	34	None	*Model 20.1:* IG concentration + host plant	5	-45.3	102.7	0.60
					Model 20.2: IG concentration + host plant + IG concentration*host plant	6	-44.9	104.8	0.21
					Model 20.3: IG concentration + host plant + weight	6	-45.3	105.6	0.14
					Model 20.4: IG concentration + host plant + IG concentration*host plant + weight	7	-44.8	108.0	0.04
Caterpillar JcDV load	2016	LMM	16	None	*Model 21.1:* IG concentration + granulocytes	5	-16.4	48.8	0.06
					Model 21.2: IG concentration + granulocytes + IG concentration*granulocytes	6	-11.2	43.6	0.82
					Model 21.3: IG concentration + granulocytes + host plant	6	-15.0	51.4	0.02
					Model 21.4: IG concentration + granulocytes + host plant + IG concentration*granulocytes	7	-9.9	47.8	0.10
Survival to adult stage (Y/N)	2017	GLMM	47	None	*Model 22.1:* JcDV load + host plant	4	-23.2	55.4	0.77
					Model 22.2: JcDV load + host plant + JcDV load*host plant	5	-23.2	57.8	0.23
Survival to adult stage (Y/N)	2017	GLMM	35	None	*Model 23.1:* PO activity + melanization + total hemocytes	5	-21.6	55.2	0.06
					Model 23.2: PO activity + melanization + total hemocytes + host plant	6	-20.4	55.8	0.04
					Model 23.3: PO activity + melanization + total hemocytes + weight	6	-17.6	50.2	0.70
					Model 23.4: PO activity + melanization + total hemocytes + host plant + weight	7	-17.3	52.8	0.19

Table S7 Effects of host plant species on immune responses of wild-collected *Euphydryas phaeton* caterpillars, including phenoloxidase (PO) activity, melanization, and concentrations of total hemocytes, granulocytes, oenocytoids, and plasmatocytes. The effects of host plant species (*Chelone glabra* or *Plantago lanceolata*), sampling year (2016 or 2017), and larval body weight at the time of immune assessment were evaluated using linear mixed-effects models including random intercepts for sites. In addition, the interaction between host plant species and year was included in the AICc- best models for PO activity and oenocytoids, and the effects of JcDV infection (Y/N) and its interaction with host plant were included in the AICc- best model for plasmatocytes. For PO activity and plasmatocytes, alternative model structures that excluded these additional fixed effects are also reported, as they received a similarly high level of AICc support to the best-fit model. These predictors were not retained in the models for melanization, total hemocytes, or granulocytes, as their inclusion did not improve model fit (Table S3).

	PO Activity (AICc-best)				**PO Activity** (ΔAICc = 1.81)				**Melanization**				**Total Hemocytes**			
Predictor	*Estimate* ± *SE*	*t*	*df*	*p*	*Estimate* ± *SE*	*t*	*df*	*p*	*Estimate* ± *SE*	*t*	*df*	*p*	*Estimate* ± *SE*	*t*	*df*	*p*
Host plant	-8.47 ± 3.61	-2.34	439	**0.020**	-4.05 ± 3.34	-1.21	440	0.226	-194.17 ± 86.42	-2.25	377	**0.025**	-3.26 ± 5.54	-0.59	432	0.557
Year	-10.43 ± 4.08	-2.56	439	**0.011**	-3.72 ± 3.00	-1.24	440	0.215	-833.90 ± 62.83	-13.27	377	**<0.001**	2.84 ± 5.72	0.50	432	0.620
Body weight	0.10 ± 0.03	3.61	439	**<0.001**	0.09 ± 0.03	3.40	440	**<0.001**	1.86 ± 0.52	3.59	377	**<0.001**	0.09 ± 0.06	1.45	432	0.147
JcDV infection																
Host plant x year	13.22 ± 5.66	2.34	439	**0.020**												
Host plant x JcDV																
Marginal R^2	0.05				0.03				0.33				0.01			
n	454				454				391				446			

	Granulocytes				**Oenocytoids**				**Plasmatocytes** (AICc-best)				**Plasmatocytes** (ΔAICc = 1.57)			
Predictor	*Estimate* ± *SE*	*t*	*df*	*p*	*Estimate* ± *SE*	*t*	*df*	*p*	*Estimate* ± *SE*	*t*	*df*	*p*	*Estimate* ± *SE*	*t*	*df*	*p*
Host plant	-2.96 ± 5.57	-0.53	292	0.596	5.41 ± 4.15	1.31	307	0.193	-17.08 ± 7.55	-2.26	277	**0.025**	-10.03 ± 6.80	-1.48	279	0.141
Year	-20.98 ± 6.47	-3.24	292	**0.001**	15.15 ± 4.14	3.66	307	**<0.001**	38.24 ± 7.07	5.41	277	**<0.001**	35.79 ± 6.89	5.19	279	**<0.001**
Body weight	-0.03 ± 0.08	-0.34	292	0.734	0.03 ± 0.04	0.86	307	0.393	0.23 ± 0.08	2.74	277	**0.007**	0.24 ± 0.09	2.84	279	**0.005**
JcDV infection									-19.60 ± 9.34	-2.10	277	**0.037**				
Host plant x year					-14.15 ± 5.99	-2.36	307	**0.019**								
Host plant x JcDV									31.98 ± 14.17	2.26	277	**0.025**				
Marginal R^2	0.06				0.06				0.22				0.23			
n	306				322				293				293			

Table S8 Effects of iridoid glycoside sequestration on immune responses of *Euphydryas phaeton* caterpillars. The effects of IG concentration (total concentration of sequestered IGs), IG composition (proportion of aucubin sequestered out of total IGs), host plant species (*Chelone glabra* or *Plantago lanceolata*), and influential two-way interactions on phenoloxidase (PO) activity, melanization score, and total hemocyte concentrations were evaluated using linear mixed-effects models including random intercepts for sites. The results of two models are reported for melanization, and the results of three models are reported for total hemocytes, as alternative model structures that varied in their inclusion of two-way interactions terms (host plant species x IG concentration; host plant species x IG composition) were found to receive a similarly high levels of AICc support to the best-fit model for these analyses. These interaction terms were not retained in the model for PO activity, as their inclusion did not improve model fit (Table S5).

	PO Activity				**Melanization** (AICc-best)				**Melanization** (ΔAICc = 0.12)			
Predictor	*Estimate* ± *SE*	*t*	*df*	*p*	*Estimate* ± *SE*	*t*	*df*	*p*	*Estimate* ± *SE*	*t*	*df*	*p*
IG concentration	-3.41 ± 2.50	-1.36	241	0.174	67.10 ± 98.90	0.68	206	0.498	-53.07 ± 58.57	-0.91	207	0.366
IG composition	-26.29 ± 9.42	-2.79	241	**0.006**	-59.52 ± 308.24	-0.19	206	0.847	-246.68 ± 283.36	-0.87	207	0.385
Host plant	1.66 ± 5.16	0.32	241	0.748	875.15 ± 298.83	2.93	206	**0.004**	528.21 ± 193.72	2.73	207	**0.007**
Host plant x IG concentration					-183.98 ± 122.05	-1.51	206	0.133				
Host plant x IG composition					-1202.92 ± 457.71	-2.63	206	**0.009**	-898.38 ± 412.90	-2.18	207	**0.031**
Marginal R^2	0.06				0.06				0.06			
n	252				219				219			

	Total Hemocytes (AICc-best)				**Total Hemocytes** (ΔAICc = 0.16)				**Total Hemocytes** (ΔAICc = 0.34)			
Predictor	*Estimate* ± *SE*	*t*	*df*	*p*	*Estimate* ± *SE*	*t*	*df*	*p*	*Estimate* ± *SE*	*t*	*df*	*p*
IG concentration	-12.95 ± 5.44	-2.38	238	**0.018**	-21.56 ± 7.98	-2.70	238	**0.007**	-12.80 ± 5.46	-2.35	239	**0.020**
IG composition	46.71 ± 25.02	1.87	238	0.063	19.86 ± 20.20	0.98	238	0.326	23.91 ± 20.07	1.19	239	0.235
Host plant	10.79 ± 16.29	0.66	238	0.508	-28.23 ± 16.87	-1.67	238	0.096	-8.42 ± 10.53	-0.80	239	0.425
Host plant x IG concentration					15.12 ± 10.08	1.50	238	0.135				
Host plant x IG composition	-56.16 ± 37.34	-1.50	238	0.134								
Marginal R^2	0.06				0.05				0.05			
n	250				250				250			

Table S9 Effects of iridoid glycoside sequestration on differentiated hemocytes of *Euphydryas phaeton* caterpillars. The effects of IG concentration (total concentration of sequestered IGs) and host plant species (*Chelone glabra* or *Plantago lanceolata*) on concentrations of granulocytes, oenocytoids, and plasmatocytes in the hemolymph were evaluated using linear mixed-effects models including random intercepts for sites.

	Granulocytes			
Predictor	*Estimate ± SE*	*t*	*df*	*p*
IG concentration	-18.37 ± 6.22	-2.95	138	**0.004**
Host plant	9.96 ± 8.24	1.21	138	0.229
Marginal R^2	0.06			
n	148			
	Oenocytoids			
Predictor	*Estimate ± SE*	*t*	*df*	*p*
IG concentration	-2.32 ± 3.10	-0.75	155	0.454
Host plant	5.15 ± 4.83	1.07	155	0.288
Marginal R^2	0.01			
n	165			
	Plasmatocytes			
Predictor	*Estimate ± SE*	*t*	*df*	*p*
IG concentration	-14.60 ± 5.84	-2.50	146	**0.014**
Host plant	-15.33 ± 9.49	-1.61	146	0.109
Marginal R^2	0.08			
n	156			

Table S10 Effects of larval immune responses on survival of *Euphydryas phaeton* individuals infected with Junonia coenia densovirus. A binomial generalized linear mixed-effects model was used to evaluate the effects of standing phenoloxidase (PO) activity, implant melanization score, and total hemocyte concentration, along with the covariate of larval body weight at the time of immune assessment, on survival of infected individuals to the adult stage (Y/N). The strength of measured immune responses did not significantly impact the probability of surviving infection to reach the adult stage.

	Survivorship			
Predictor	*Odds Ratio*	*95% CI*	*z*	*p*
PO activity	0.974	0.000-1.007	-1.48	0.139
Melanization	0.999	0.998-1.001	-0.94	0.347
Total hemocytes	0.988	0.970-1.003	-1.45	0.147
Body weight	1.039	1.011-1.079	2.36	**0.018**
Marginal R^2	0.39			
n	35			

Chapter Two

Host plant and developmental stage impact prevalence and load of a viral entomopathogen, Junonia coenia densovirus, in wild butterflies

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ABSTRACT

Pathogens represent critical agents of mortality for insect herbivores, yet their ecological impacts remain poorly understood in many natural systems. Characterizing variation in pathogen pressure across herbivore populations using native or exotic host plant species may provide insight into the tritrophic outcomes of host range evolution. The Baltimore checkerspot butterfly, *Euphydryas phaeton* (Nymphalidae), recently incorporated an exotic plant, *Plantago lanceolata* (Plantaginaceae), into its host range. We investigated the consequences of this dietary expansion for interactions between *E. phaeton* and a naturally occurring entomopathogen, Junonia coenia densovirus (JcDV), across the course of herbivore development, which includes an obligate overwintering diapause during the larval stage. We quantified viral prevalence and loads in populations using either *P. lanceolata* or a native plant, *Chelone glabra* (Plantaginaceae) during three stages of the life cycle: post-diapause, pre-diapause, and diapause. We found that viral prevalence was higher in post-diapause caterpillars, pupae, and adults, compared to early-instar caterpillars in the pre-diapause and diapause stages. However, early-instar larvae that were infected with JcDV harbored higher viral loads, compared to late-instar larvae, pupae, and butterflies. In addition, viral loads were substantially higher in post-diapause herbivores utilizing the exotic plant, *P. lanceolata,* compared to *C. glabra,* while host plant effects were minimal during earlier developmental stages. Together, these results demonstrate that viral prevalence and burdens can vary considerably across the life cycle of an insect host and suggest that host plant mediated effects on herbivore susceptibility to infection may be most evident or consequential during later stages of development.

1 | INTRODUCTION

Insects are attacked by diverse assemblages of natural enemies in the wild, from predators and parasitoids to microbial pathogens (Anderson and May, 1981; Greeney et al., 2012; Hawkins et al., 1997). These natural enemies can exert powerful top-down control on insect populations, potentially suppressing outbreaks of forest defoliators and agricultural pests (Dwyer et al., 2004; Mason et al., 1983; Parry et al., 1997), but can also threaten species by contributing to population declines and local extinction (Cameron et al., 2011; Richards et al., 1999; Washburn and Cornell, 1981). Although advances in molecular technologies have facilitated the detection and study of infectious diseases in invertebrate hosts (Campos-Herrera and Lacey, 2018), relatively little is known of insect-pathogen interactions outside of forest and agricultural pest systems (Cory and Hoover, 2006) and commercially significant species (Cox-Foster et al., 2007; Eilenberg and Jensen, 2018). In particular, interactions between insects and pathogenic viruses remain critically understudied in many natural systems (Williams, 2018), creating substantial gaps in our understanding of the disease ecology of wild insects. Characterizing patterns of pathogen occurrence across wild insect populations, and elucidating the ecological mediators of susceptibility to infection, represent critical steps toward understanding the roles of entomopathogens in shaping the ecology and evolution of their insect hosts.

In insect herbivores, a primary determinant of both exposure and susceptibility to pathogens is host plant use (Cory and Hoover, 2006; Shikano, 2017). As most viral and bacterial entomopathogens must be ingested to initiate infection, host plants are often directly implicated in the infection process, providing opportunities for direct interactions between plants and pathogens (e.g., on the phylloplane or in the midgut lumen), along

with indirect interactions mediated by herbivore body condition or immune responses (Diamond and Kingsolver, 2011; Shikano et al., 2010; Yoon et al., 2019). For example, secondary metabolites in host plants can interfere with the establishment of baculovirus infection in the caterpillar midgut (Felton & Duffey 1990), while diets rich in protein can enhance herbivore survival following viral or bacterial challenge by offsetting the nutritional requirements of resisting infection (Lee et al., 2006; Povey et al., 2009). It follows that susceptibility to pathogen infection can vary substantially across herbivores consuming different host plant species, which may be reflected through differences in mortality rate, speed-of-kill, and/or pathogen yield (i.e., production of progeny) (Ali et al., 1998; Duffey et al., 1995; Kouassi et al., 2001; Raymond et al., 2002). Furthermore, the likelihood of pathogen encounter may differ according to host plant use; for instance, the habitat or microclimate in which a plant occurs, or the architecture of the plant itself, can impact pathogen persistence on the phylloplane (Raymond et al., 2005), thereby influencing rates of encounter between herbivores and pathogens. Thus, characterizing host plant mediated variation in both exposure and vulnerability to pathogens may offer considerable insight into patterns of disease risk across wild herbivore populations.

In addition to host plant mediated effects, exposure and susceptibility to pathogen infection may exhibit substantial variation across the life cycle of an insect host. For holometabolous insects such as Lepidoptera, wherein different developmental stages vary markedly in feeding habits, mobility, and a variety of other behavioral and physiological traits, this variation may be particularly pronounced (Zalucki et al., 2002). For example, phytophagous larvae may encounter different types of pathogens than adults that have siphoning mouthparts, and as the majority of feeding occurs during the larval stage, they

may also experience a higher overall risk of infection. Vulnerability to infection may additionally change across the course of larval development (McNeil et al., 2010; Teakle et al., 1986). In many cases, early-instar larvae are more susceptible to pathogen infection than late-instar larvae: for example, the viral dose required to kill 50% of larvae (LD_{50}) can increase by orders of magnitude over the course of larval development in Lepidoptera infected by baculoviruses and a densovirus (Briese DT, 1986; Mutuel et al., 2010). This phenomenon, called "developmental resistance," may be explained by the increasing surface area-to-volume ratio of the gut (Hochberg, 1991), changes in the physiology of the peritrophic membrane or midgut epithelium (Levy et al., 2011), and/or the maturation of immune responses (McNeil et al., 2010; Urbański et al., 2014; Wago and Ichikawa, 1979) as larvae grow. However, high resistance in late-instar larvae may be coupled with greater risk of encountering and ingesting pathogens, due to higher mobility and feeding rates during this stage (Dwyer, 1991). Given the great potential for variation in herbivore-pathogen dynamics across host ontogeny, the ecological impact of a given pathogen on wild insect populations may be expected to exhibit temporal fluctuations in many cases.

In this study, we characterized the influences of host plant use and herbivore ontogeny on interactions between a native insect herbivore, *Euphydryas phaeton* Drury (Lepidoptera: Nymphalidae) and a naturally occurring viral pathogen, Junonia coenia densovirus (JcDV). *Euphydryas phaeton,* the Baltimore checkerspot, is a univoltine butterfly that occurs throughout eastern North America. In the northeastern United States, adults fly from late June to July; during this period, females typically lay two to three egg masses consisting of 100-600 eggs each (Bowers, 1978). Caterpillars hatch, construct a feeding web on their host plant, and develop gregariously with their natal clusters until

reaching the fourth instar, when they cease feeding and enter obligate diapause in August or September (Figure 1). These half-grown larvae overwinter in the leaf litter in groups of 10 to 100 individuals, remaining close to the host plant upon which they initially built their feeding web, and reemerge in early- to mid-May (Bowers, 1978). At this point, the caterpillars resume feeding and complete development through the sixth instar, typically pupating in June. Due to the occurrence of this overwintering diapause during the larval stage, a single “growing season” for *E. phaeton* consists of late-instar development of the prior year’s larvae during the early summer, followed by early-instar development of the next generation of larvae during the late summer, providing a useful study system for investigating variation in pathogen pressure across the course of larval development.

Euphydryas phaeton caterpillars specialize on host plants containing iridoid glycosides (IGs): monoterpenoid plant secondary metabolites that can be toxic and/or deterrent to non-adapted herbivores (Bowers and Puttick, 1988). Caterpillars sequester IGs from their host plants and retain them through the adult stage as a form of chemical defense (Bowers, 1980; Bowers and Puttick, 1986). Historically, the primary host plant for *E. phaeton* in the northeastern U.S. was *Chelone glabra* (hereafter, *Chelone*), a native, long-lived, perennial herb. Recently, however, *E. phaeton* expanded its host range to include an exotic plant, *Plantago lanceolata* (hereafter, *Plantago*) (Bowers et al., 1992; Stamp, 1979), a short-lived, perennial herb introduced from Europe during the 19th century (Cavers et al., 1980). Though certain populations persist on *Plantago* exclusively (Stamp, 1979), *E. phaeton* prefers the native *Chelone* for both feeding and oviposition (Bowers et al., 1992), and there are several costs associated with using the exotic plant,

including increased palatability to predators (Bowers, 1980), reduced larval growth rate (Bowers et al., 1992), and inferior immune performance (Muchoney et al., 2022).

Recent research revealed that *E. phaeton* caterpillars are naturally infected by a viral pathogen, Junonia coenia densovirus (hereafter, JcDV), across wild populations in the northeastern U.S. (Muchoney et al., 2022). JcDV is a nonenveloped, single-stranded DNA virus in the family *Parvoviridae* (*Densovirinae: Lepidopteran protoambidensovirus 1*) capable of infecting Lepidoptera in multiple families (Mutuel et al., 2010; Resnik and Smilanich, 2020; Rivers and Longworth, 1968). Infection with JcDV occurs via an oral route: viral particles are ingested on contaminated foliage and subsequently cross the midgut to replicate in tracheae, hemocytes, visceral muscles, and epidermis (Mutuel et al., 2010; Wang et al., 2013). Infection often results in hypoxia and disruptions to molting and metamorphosis (Mutuel et al., 2010; Smilanich et al., 2018). In *E. phaeton,* mortality of JcDV-infected individuals was found to vary depending on viral load, with high loads corresponding to increased mortality in immature stages (Muchoney et al., 2022).

Our previous study revealed that wild *E. phaeton* caterpillars harbored higher JcDV infection loads when utilizing the exotic plant, *Plantago,* compared to the native plant, *Chelone,* during the final larval instar (Muchoney et al., 2022). This pattern may be indicative of higher susceptibility to JcDV infection in caterpillars consuming *Plantago* or greater exposure to JcDV on *Plantago*, either of which would represent an additional, tritrophic cost of using this exotic plant for *E. phaeton* (see Bowers, 1980; Bowers et al., 1992). Further insight into the outcomes of higher viral burdens for herbivore fitness and persistence on the exotic plant may be gained by investigating: (1) the relationship

between host plant use and JcDV infection during additional stages of development, and (2) sublethal effects of viral infection on individuals that survive to the adult stage.

Here, we examined variation in herbivore-JcDV interactions across the host life cycle in wild populations of *E. phaeton*, aiming to determine the role of host plant use in mediating viral prevalence and loads throughout different stages of development. We specifically addressed three questions: (1) How do viral prevalence and infection loads vary across herbivore life stages? (2) How does host plant use influence viral prevalence and infection loads during each life stage? (3) What are the outcomes of viral infection and host plant use for survival and adult longevity? By evaluating temporal variation in herbivore-pathogen interactions across wild populations, we provide insight into the role of host plant use in mediating herbivore susceptibility to infectious disease and the potential influence of a pathogenic densovirus on the survival of herbivores utilizing either a native or exotic host plant species.

2 | METHODS

2.1 Experiment overview

To evaluate variation in JcDV prevalence and infection loads across the course of herbivore development in populations using the native or exotic host plant, we collected *E. phaeton* caterpillars from the wild in 2019. Sampling occurred at three time points throughout the life cycle: post-diapause larvae (late-instar, generation 1) were sampled in early summer, pre-diapause larvae (early-instar, generation 2) of the next generation were sampled in late summer, and diapausing caterpillars (fourth instar, generation 2) were overwintered in the laboratory and sampled the following spring (Figure 1; see below for

details). Caterpillars were collected from four populations in the northeastern U.S. (see Appendix: Table S1). Two of these populations (located in Vermont; VT1-2) utilized the native host plant, *Chelone,* while two populations (located in Massachusetts; MA1-2) utilized the exotic *Plantago*. In addition, caterpillars were collected from a fifth site in upstate New York (NY1), which used the native *Chelone,* during the post-diapause stage; however, pre-diapause sampling did not occur at this site due to small population size. As such, caterpillars from the NY1 population were included in analyses of post-diapause survival and longevity but were excluded from analyses across all three sampling stages.

2.2 Post-diapause sampling

Post-diapause caterpillars were collected from the wild during the sixth instar between May 30 and June 7, 2019. Caterpillars (n = 184; see Table S1 for n at each site) were sampled throughout the occupied patch of host plants at each site and placed alive into individual, sterile culture tubes (USA Scientific, Ocala, FL, USA) along with foliage from nearby host plants. Caterpillars were brought to the University of Nevada, Reno to complete development, where they were reared in incubators using a 16-hour photoperiod (day temperature: 25°C, night temperature: 20°C) and fed ad libitum with foliage corresponding to the host plant species utilized at their site of origin. *Plantago* leaves were collected from the wild in Reno, NV, while *Chelone* leaves were collected from a sampling site in Montpelier, VT and stored in a refrigerator. Leaf surfaces were sterilized prior to feeding by soaking in 5% bleach solution for 10 min and rinsing thoroughly. Caterpillars were reared in individual 2 oz plastic cups until pupation and checked daily to monitor development and mortality. Sterile technique was used between handling of each individual, which entailed soaking instruments in a 30% solution of bleach,

followed by a 70% solution of ethanol. Pupated individuals were weighed and transferred to 32 oz plastic containers with mesh lids for eclosion, and butterflies were maintained on a diet of 10% honey water. Following death in the larval (n = 30), pupal (n = 5), or adult (n = 149) stage, all individuals were promptly frozen for viral screening.

2.3 Pre-diapause sampling

Pre-diapause caterpillars were collected from the wild between August 16 and 19, 2019. At each site, either whole or partial clusters of caterpillars that shared a feeding web were sampled throughout the occupied patch of host plants, with the exception of one site in Vermont (VT2) where caterpillars had not yet hatched and egg masses were collected as an alternative (see Table S1 for details). Clusters were placed together into plastic containers with foliage from nearby host plants and brought to the University of Colorado, Boulder for the remainder of pre-diapause development. During this time, clusters were reared in incubators using a 12-hour photoperiod at 25°C and fed ad libitum with sterilized *Chelone* or *Plantago* foliage collected from their site of origin. Laboratory clusters ranged in size from 10 to 262 larvae. Upon reaching the fourth instar, when diapause began, five caterpillars were sampled from each cluster (34 clusters, n = 170) and freeze-killed for viral screening.

2.4 Overwintering and diapause sampling

Remaining caterpillars were overwintered in their original, wild-collected clusters at the University of Colorado, Boulder. Following molting to the fourth instar and the cessation of feeding, clusters were cooled in an incubator for 30 days (day temperature: 10°C, night temperature: 5°C) and then transferred to a cold room and kept at a constant temperature of 4°C until April 2020. Over the winter, clusters were not fed but were

monitored weekly for mortality, removal of dead larvae, and dampening of the substrate to prevent desiccation. In mid-April, five larvae were sampled from each successfully overwintered cluster (23 clusters, n = 115) and freeze-killed for viral screening.

2.5 Viral screening and quantification

To detect JcDV infection in *E. phaeton*, DNA was extracted from a tissue sample from each insect using Qiagen DNeasy 96 Blood and Tissue Kits (Qiagen, Hilden, North Rhine-Westphalia, Germany) following the Protocol for Purification of Total DNA from Animal Tissues. For post-diapause individuals that died during the larval stage, DNA was extracted from an aliquot of homogenized tissue (mass: $\bar{x} = 20.0 \pm 0.4$ mg), whereas whole pupae and dissected abdomens were used for those that died during the pupal and adult stages, respectively ($\bar{x} = 66.6 \pm 3.5$ mg). For pre-diapause and overwintered larvae, which were smaller in size, DNA was extracted from whole insects ($\bar{x} = 5.3 \pm 0.1$ mg).

Extracted DNA was screened for JcDV using quantitative PCR, with primers specific to the VP4 capsid protein gene of JcDV (Wang et al., 2013) and arthropod 28S rDNA primers (Nice et al., 2009) as an internal control. DNA samples were screened in duplicate for both VP4 and 28S using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) at a volume of 10 µl. Reactions were run on a Bio-Rad CFX96 Optics Module with C1000 Thermal Cycler following the protocols of Muchoney *et al.* (2022). Viral loads were calculated as $2^{-\Delta Ct}$ (Schmittgen and Livak, 2008), representing the abundance of the JcDV VP4 gene relative to the abundance of the internal control gene [ΔC_t = mean C_t (threshold cycle) for VP4 – mean C_t for 28S], and log-transformed.

In addition to relative viral loads, the absolute quantity of JcDV in each insect was estimated using a standard curve. A stock solution of JcDV was serially diluted and

used to generate a standard curve over seven orders of magnitude (1.0 x 10^3 to 1.0 x 10^9 viral genomes), which was used to calculate the amount of JcDV in each sample of DNA based on C_t values for the VP4 gene. These values were then scaled to account for the proportion of total body mass that was used for DNA extraction to extrapolate whole-body virus quantity, which was expressed as viral genomes (vg) and log-transformed.

2.6 Statistical analyses

All statistical analyses were performed in R version 4.1.0 (R Core Team, 2021). The likelihood of detecting JcDV across all *E. phaeton* individuals was examined using two logistic regression models: the first assessed the effects of host plant species (*Chelone* or *Plantago*), collection stage (post-diapause, pre-diapause, or diapause), and their interactions on the presence/absence of JcDV infection, while the second assessed the effects of sampling site, collection stage, and their interactions on the same binomial response. Within the subset of individuals that were found to be infected with JcDV, viral burdens were investigated using two multiple regression models: the first assessed the effects of host plant species, collection stage, and their interactions on relative viral load (the abundance of JcDV relative to insect DNA), while the second assessed the effects of site, collection stage, and their interactions. In both cases, the second model was fitted in order to examine patterns of variation across sampling sites, regardless of host plant use. A third multiple regression model evaluated the effects of host plant species, collection stage, and their interactions on the absolute quantity of JcDV in each insect. Post-hoc pairwise comparisons were performed using the ‘emmeans’ package (Lenth, 2021).

Patterns of viral occurrence were also investigated at the cluster level for larvae sampled during the pre-diapause and diapause stages. The effect of viral prevalence (%

infected individuals per cluster) on the mean load of infected individuals in that cluster was evaluated using a linear mixed-effects model (LMM), which included sampling site as a fixed effect and random intercepts for clusters. An additional LMM assessed the effect of cluster size (*n* larvae) on viral load, and mixed-effects logistic regression was used to evaluate the effect of cluster size on the presence/absence of viral infection. Both models included sampling site and collection stage as fixed effects and random intercepts for clusters. All linear mixed-effects models were fitted with ‘lme4’ (Bates et al., 2015), and *p*-values were generated using the ‘lmerTest’ package (Kuznetsova et al., 2017).

Caterpillars collected during the post-diapause stage were reared until death to assess the effects of JcDV infection and host plant use on survival, adult longevity, and adult body mass. Survivorship was assessed using two logistic regression models with survival to the adult stage (Y/N) as the binomial response. The first evaluated the effects of host plant species and JcDV infection status (infected/uninfected) on survival across all individuals, while the second examined the effect of viral load on survival in JcDV-infected individuals. Postmortem viral loads of post-diapause individuals were further probed using a multiple regression model that included host plant and life stage at death (larva, pupa, or adult) as predictors and tissue sample mass as a covariate (to account for variation across life stages; see Section 2.2). Adult longevity was evaluated using two regression models: the first assessed the effects of host plant species and JcDV infection on longevity (*n* days between eclosion and death), while the second examined the effect of viral load in JcDV-infected adults. Adult body mass was assessed using a multiple regression model that included host plant, JcDV infection status, sex (to account for dimorphism in body size), and the interaction between host plant and sex as predictors.

3 | RESULTS

3.1 Viral prevalence across life stages

JcDV infection was detected in 30% of *E. phaeton* individuals that were collected from the wild during the post-diapause stage (May-June 2019), 11% of individuals that were collected from the wild during the pre-diapause stage (August 2019), and 16% of diapausing caterpillars that were overwintered in the laboratory (April 2020). Overall, viral prevalence did not vary consistently based on host plant species (Figure 2a; Table 1) or across sampling sites (Figure 2b; Table S3). The likelihood of infection was slightly higher on the native *Chelone* during both post-diapause [odds ratio (OR) = 0.71 ± 0.24, $z = -1.0$, $p = 0.3$] and pre-diapause (OR = 0.40 ± 0.22, $z = -1.7$, $p = 0.09$), but slightly higher on the exotic *Plantago* during diapause (OR = 1.89 ± 0.99, $z = 1.2$, $p = 0.2$), which was mirrored at the site level (Figure 2b). On both plants, there was a substantial decrease in JcDV prevalence between the post-diapause stage (generation 1) and the pre-diapause stage (generation 2; Table 1). Across overwintering, patterns varied based on host plant use: larvae reared on *Chelone* showed little change in viral frequency (OR = 0.78 ± 0.39, $z = -0.5$, $p = 0.6$), while those reared on *Plantago* exhibited an increase in frequency between pre-diapause and diapause (OR = 3.75 ± 2.14, $z = 2.3$, $p = 0.02$).

3.2 Viral loads across life stages

Within the subset of individuals that were found to be infected with JcDV, viral loads were higher in those utilizing *Plantago,* compared to *Chelone,* during the post-diapause stage (Figure 3a), exhibiting 15-fold greater loads (Table 1). In contrast, infection loads were similar on the two plants during both pre-diapause and diapause

(Table 1). By developmental stage, viral loads were higher in individuals that were collected during pre-diapause and diapause than those collected during post-diapause (Table 1), indicating that these smaller, less mature insects harbored a greater proportion of viral DNA relative to their own DNA. When the total amount of virus in each insect was estimated, accounting for differences in body mass across developmental stages, viral quantity was found to be similar across all stages (Table S2; Figure S1). Together, these results indicate that smaller-bodied pre-diapause and diapausing larvae harbored a similar quantity of virus to larger-bodied post-diapause individuals, due to the higher concentration of virus in their tissues. When examined at the site level, relationships between viral load and life stage varied across sampling sites (Figure 3b; Table S3).

3.3 Viral occurrence in clusters (pre-diapause and diapause)

At the cluster level, there was a positive relationship between the frequency of viral occurrence (% infected larvae out of a subsample of five) and the mean viral load of infected individuals within that cluster (Figure 4) ($\beta = 0.15 \pm 0.07$, $t = 2.2$, $p = 0.05$). On average, caterpillars sampled from clusters with a higher prevalence of JcDV infection ($n = 2$ infected larvae) exhibited 70-fold higher loads than those originating from clusters with a lower prevalence ($n = 1$ infected larva). No clusters contained more than two infected larvae out of the five that were screened at each stage. Cluster-level prevalence and load during pre-diapause were not strongly correlated with prevalence and load in the same cluster following overwintering (prevalence: Pearson's $R = 0.02$, $p = 0.9$; load: Pearson's $R = -0.65$, $p = 0.2$). Though laboratory clusters varied considerably in size, cluster size did not influence the likelihood of JcDV infection [OR = 1.00, 95% CI = (1.00-1.01), $z = 1.0$, $p = 0.3$] or infection loads ($\beta = -0.02 \pm 0.01$, $t = -1.3$, $p = 0.2$).

3.4 Survival and adult longevity (post-diapause)

Use of *Plantago* had a strong negative effect on probability of survival to the adult stage in both JcDV-infected and uninfected individuals (Figure 5a) [OR = 0.24, 95% CI = (0.08-0.65), $z = -2.7$, $p = 0.006$). JcDV infection had an additive, though weaker, negative effect on survival on both host plants [OR = 0.47, 95% CI = (0.21-1.03), $z = -1.9$, $p = 0.06$]. In JcDV-infected individuals, postmortem viral loads were higher on *Plantago* than *Chelone,* regardless of the life stage at which the insect died (Figure 5b) ($\beta = 1.19 \pm 0.36$, t = 3.3, $df = 54$, $p = 0.002$), and there was a negative overall relationship between viral load and survival (Figure S2) [OR = 0.63, 95% CI = (0.39-0.96), $z = -2.1$, $p = 0.04$].

In individuals that survived to the adult stage, JcDV infection occurred in a greater proportion of females (40%) than males (9%) ($X^2 = 13.8$, $df = 1$, $p = 0.0002$), though infection loads were similar between the sexes ($\beta = -0.32 \pm 0.68$, $t = -0.5$, $df = 40$, $p = 0.6$). Longevity during the adult stage did not vary based on larval host plant species ($\beta = 0.63 \pm 0.75$, $t = 0.8$, $df = 120$, $p = 0.4$) or JcDV infection status ($\beta = -0.77 \pm 0.79$, $t = -1.0$, $df = 120$, $p = 0.3$), nor was it influenced by viral load in JcDV-infected individuals ($\beta = -0.49 \pm 0.48$, $t = -1.0$, $df = 34$, $p = 0.3$). However, total body mass was reduced in female butterflies that were reared on *Plantago,* relative to *Chelone* ($\beta = -35.51 \pm 8.01$, $t = -4.4$, $df = 136$, $p < 0.0001$), but did not differ based upon host plant species in male butterflies ($\beta = -8.52 \pm 10.86$, $t = -0.8$, $df = 136$, $p = 0.4$) or based upon JcDV infection status in either sex ($\beta = -2.70 \pm 7.09$, $t = -0.4$, $df = 136$, $p = 0.7$).

4 | DISCUSSION

This study demonstrates that both prevalence and load of a naturally occurring entomopathogen exhibit substantial variation across the life cycle of its herbivorous insect host. Sampling during more mature developmental stages revealed relatively high viral prevalence (Figure 2) coupled with low infection loads (Figure 3), while sampling during earlier developmental stages revealed the opposite pattern of low viral prevalence paired with high infection loads. In addition, a strong effect of host plant on JcDV load was evident solely during the post-diapause stage, when viral burdens were substantially higher in individuals using the exotic plant, *Plantago* (Figure 3a; Figure 5b). As higher viral burdens during post-diapause were associated with an increased likelihood of pre-adult mortality (Figure S2), this pathogen may be expected to impact the fitness of individuals using *Plantago* more so than those using the native *Chelone*. Together, these findings suggest that: (1) developmental stage may be a more important determinant of viral prevalence than host plant use in this particular system, (2) host plant mediated effects on susceptibility to infection may be most pronounced during later stages of development, and (3) overall vulnerability to JcDV infection may be greater on the exotic host plant, *Plantago,* representing a putative cost of host range expansion for *E. phaeton.*

We found viral prevalence to be relatively consistent across sampling sites and host plant species (Figure 2), contributing to growing evidence that JcDV is a widespread and persistent pathogen in *E. phaeton* populations, and that neither host plant provides a refuge from encounters with this natural enemy. Our previous research within this system reported similar patterns: viral prevalence did not vary based on host plant use during the post-diapause stage, and 25% of field-collected individuals reared until death harbored infection (compared to 30% in the present study; Muchoney et al., 2022). Although JcDV

infection was present at all sites and life stages, there was a considerable decrease in infection frequency between the post-diapause and pre-diapause stages (Figure 2). This pattern may be reflective of different feeding habits: late-instar caterpillars are larger, more mobile, and consume a greater volume of foliage relative to early instars, providing increased opportunity for exposure to, and ingestion of, viral particles on contaminated host plants (i.e., horizontal transmission). This premise has been illustrated in caterpillar-nucleopolyhedrovirus systems; for example, late-instar *Orgyia pseudotsugata* caterpillars (Lymantriidae) were both more infectious and more likely to become infected than early instars due to higher mobility and feeding rates (Dwyer, 1991). As pathogen prevalence and viability can also exhibit temporal variation driven by abiotic factors, including temperature, UV radiation, humidity, and precipitation (Ment et al., 2018), it is also possible that JcDV occurrence in the environment (e.g., on host plant surfaces) decreases over the course of the summer growing season; this possibility warrants further study.

Variation in viral loads may provide insight into the susceptibility of different developmental stages to JcDV infection in the wild. We found that relative viral burdens were higher in early-instar caterpillars, compared to late-instar larvae, pupae, and adults (Figure 3). Similar patterns have been documented in *Spodoptera exigua* (Noctuidae) harboring nucleopolyhedrovirus infection (Graham et al., 2015; Murillo et al., 2011), suggesting that the ability to suppress viral infection increases over the course of host development. One physiological factor that may mediate this pattern is developmental variation in immunity (Gillespie et al., 1997). The functionality of cell-mediated immune responses typically increases as insects age (Urbański et al., 2014; Wago and Ichikawa, 1979), due in part to proliferation and differentiation of hemocytes. For example, Carper

et al. (2019) found that hemocyte densities increased across larval instars in the specialist caterpillar *Junonia coenia* (Nymphalidae). It is therefore possible that the relatively low viral burdens observed in post-diapause individuals are indicative of a more developed immune response and greater capacity to suppress infection (see Muchoney et al., 2022), while the opposite is true of early instars. In addition, the concentration of iridoid glycosides sequestered by *E. phaeton* was found to be negatively correlated with JcDV loads during the sixth instar (Muchoney et al., 2022), indicating that IG sequestration may contribute to defense against JcDV. As lower levels of IGs are sequestered by pre-diapause (third-instar) larvae, compared to post-diapause (sixth instar) larvae (Carper et al., 2021), variation in IG-mediated chemical defense may provide additional explanatory power into the pattern of more severe infections observed in less mature caterpillars.

Host plant mediated effects on interactions between *E. phaeton* and JcDV were primarily evident during the post-diapause stage, when both relative viral burdens and whole-body viral quantity were higher on *Plantago* compared to *Chelone* (Figure 3a; Figure S1). This pattern corroborates the findings of our previous study (Muchoney et al., 2022) and suggests that JcDV-infected individuals may experience an increased risk of death prior to reaching the adult stage when feeding on *Plantago* due to load-dependent effects on mortality (Figure S2). Importantly, by dying with higher viral burdens (Figure 5b), these insects also exhibit higher "yield" and are expected to release a greater number of viral particles into their environment via their cadavers, increasing the likelihood of horizontal transmission to conspecifics (Anderson and May, 1981). Reduced immune responses in caterpillars using *Plantago,* compared to *Chelone* (Muchoney et al., 2022) or reduced growth rates on this host plant (Bowers et al., 1992) may contribute to this

pattern, though the role of specific immune defenses in suppressing JcDV infection remains somewhat unclear (Resnik and Smilanich, 2020). Although further study will be necessary to elucidate the mechanism(s) through which using *Plantago* corresponds to higher JcDV loads*,* the outcomes of this pattern are likely consequential for *E. phaeton* fitness and therefore represent a potential cost of incorporating this exotic species.

The ability of herbivore populations to persist on exotic host plants is shaped by both bitrophic factors (e.g., the physiological suitability of the host plant for supporting herbivore development) and tritrophic interactions (Bernays and Graham, 1988; Forister and Wilson, 2013; Singer and Stireman, 2005). In considering the "bitrophic" suitability of *Plantago* as a host plant for *E. phaeton,* we found evidence of lower post-diapause survival (Figure 5a) and reduced female body mass on *Plantago*, compared to *Chelone,* though adult longevity was similar on the two plants. These findings corroborate the results of a study by Brown et al. (2017), which found that the relative suitability of the two plants varied depending upon the life stage and demographic parameter in question, with higher post-diapause survival and female mass on *Chelone* but higher pre-diapause nest size, overwintering survival, and overall population growth rates on *Plantago*. Thus, although *Chelone* may represent a superior host plant during post-diapause development (Bowers et al., 1992), *Plantago* may be superior during pre-diapause and diapause. Our study contributes a novel, tritrophic perspective to these patterns, illustrating that host plant effects on viral infection, much like host plant effects on demographic parameters, vary across the herbivore life cycle, with a negative consequence of using *Plantago* (higher viral loads) evident only during later stages of development. Characterizing such variation in herbivore-pathogen dynamics not only improves our understanding of the

ecological impact of understudied entomopathogens in natural systems, but also provides insight into the tritrophic factors shaping herbivore performance on different host plants.

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AUTHOR CONTRIBUTIONS

The study was conceived and designed by NDM, ALC, MDB, and AMS. NDM, ALC, and MDB collected samples, NDM reared post-diapause insects, ALC reared pre-diapause and diapausing insects, and NDM and DGL performed viral screening. NDM analyzed the data and wrote the manuscript, and all authors contributed to revisions.

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TABLES

Table 1 Prevalence and loads of Junonia coenia densovirus in *Euphydryas phaeton,* based on larval host plant species (*Chelone glabra* or *Plantago lanceolata*) and life stage at the time of collection (post-diapause/generation 1, pre-diapause/generation 2, or diapause/generation 2; see Figure 1). The probability of detecting JcDV ("viral prevalence") was assessed using logistic regression, with host plant species, collection stage, and their interaction as predictors and presence/absence of JcDV as the binomial response. Viral loads (log-transformed, relative to an internal control gene) were compared across the same predictors using multiple regression. The intercepts of both models represented individuals utilizing the native host plant, *Chelone,* during the post-diapause stage. Thus, odds ratios less than one represent lower odds of viral detection, and estimates less than zero represent lower viral loads, compared to this reference group.

	Viral prevalence			**Viral load**		
Predictors	*Odds Ratio [95% CI]*	*z*	*p*	*Estimate ± SE*	*t*	*p*
Host plant (*Plantago*)	0.71 [0.36–1.39]	-0.99	0.320	1.17 ± 0.50	2.32	**0.023**
Stage (*pre-diapause*)	0.33 [0.15–0.68]	-2.93	**0.003**	4.04 ± 0.58	6.92	**<0.001**
Stage (*diapause)*	0.26 [0.10–0.61]	-2.92	**0.004**	3.82 ± 0.74	5.17	**<0.001**
Host plant (*Plantago*) x stage (*pre-diapause*)	0.55 [0.15–1.91]	-0.91	0.363	-1.72 ± 1.05	-1.64	0.105
Host plant (*Plantago*) x stage (*diapause*)	2.66 [0.79–9.37]	1.56	0.118	-0.32 ± 1.00	-0.32	0.752
n	450			85		
R^2	0.06			0.55		

FIGURE LEGENDS

Figure 1 Summary of the life cycle of *Euphydryas phaeton* (outlined in black) and the sampling design employed in this study (outlined in orange). Letters A-C indicate the three developmental stages at which screening of *E. phaeton* individuals for Junonia coenia densovirus occurred, and dashed arrows indicate portions of the life cycle that occurred in the laboratory. Post-diapause (sixth instar) larvae were collected from wild populations in May/June 2019 and reared out in the laboratory until death in the larval, pupal, or adult stage before undergoing viral screening (A; n = 184). Clusters of pre-diapause individuals (early-instar larvae or eggs) from the next generation were collected from the wild in August 2019. These individuals completed pre-diapause development in the laboratory before a subset of larvae from each cluster underwent viral screening (B; n = 170). All remaining larvae entered diapause and were overwintered in the laboratory in their original clusters. Following overwintering, an additional subset of individuals from each cluster underwent viral screening before emerging from diapause (C; n = 115).

Figure 2 Prevalence of Junonia coenia densovirus across the *Euphydryas phaeton* life cycle based upon host plant use (A) and sampling site (B). Points represent % infected individuals at each collection stage (post-diapause/generation 1, pre-diapause/generation 2, or diapause/generation 2; see Figure 1). At each site, *E. phaeton* caterpillars utilized either the native host plant, *Chelone glabra* (VT1-2), or the exotic host plant, *Plantago lanceolata* (MA1-2). On both host plants and across all sites, there was a decrease in infection frequency between the post-diapause stage and the pre-diapause stage of the

next generation. Across overwintering, patterns varied based upon host plant use: caterpillars reared on *Chelone* showed little change in prevalence, while those reared on *Plantago* exhibited an increase in viral frequency between pre-diapause and diapause.

Figure 3 Junonia coenia densovirus loads of infected individuals across the *Euphydryas phaeton* life cycle based upon host plant use (A) and sampling site (B). Points represent mean load (log-transformed, relative to an internal control gene) ± SE at each collection stage (post-diapause/generation 1, pre-diapause/generation 2, or diapause/generation 2; see Figure 1). At each site, *E. phaeton* caterpillars utilized either the native host plant, *Chelone glabra* (VT1-2), or the exotic host plant, *Plantago lanceolata* (MA1-2). Viral loads were higher during the pre-diapause and diapause stages, compared to the post-diapause stage. In addition, infection loads were 15-fold higher in individuals utilizing the exotic *Plantago,* compared to the native *Chelone,* during the post-diapause stage, but did not differ based on host plant use during the pre-diapause and diapause stages.

Figure 4 Relationship between Junonia coenia densovirus prevalence and mean viral loads in *Euphydryas phaeton* clusters during the pre-diapause and diapause stages. Five larvae were subsampled from each cluster at each collection stage, and no subsample contained more than two infected individuals. Points represent mean viral load (log-transformed, relative to an internal control gene) of each cluster at each collection stage (n = 24), and SE values are provided for clusters with n infected individuals > 1. There was a positive relationship between JcDV prevalence in each cluster (n infected larvae out of five) and the mean viral load of infected individuals within that cluster.

Figure 5 (A) Survival of wild-collected *Euphydryas phaeton* individuals based on host plant species and infection with Junonia coenia densovirus. Points represent frequencies of survival to the adult stage in individuals utilizing either the native host plant, *Chelone glabra* (*n* = 105) or the exotic host plant, *Plantago lanceolata* (*n* = 79). Survival was lower on *Plantago* than *Chelone* in both JcDV-infected and uninfected individuals. (B) JcDV loads of *E. phaeton* based on host plant species and life stage at the time of death. Points represent estimated marginal means (EMMs) ± SE for viral load (log-transformed, relative to an internal control gene) based on a multiple regression model, which included host plant and life stage as predictors and sample mass as a covariate, in order to account for variation in the mass of the tissue sample that was screened for JcDV across different life stages (see Methods). Presented EMMs are averaged across tissue sample masses. Postmortem viral burdens were higher in individuals reared on *Plantago* compared to *Chelone,* regardless of life stage at the time of death. In addition, viral loads of deceased larvae (*n* = 13) were higher than those of pupae (*n* = 4) and butterflies (*n* = 42).

FIGURES

Figure 1

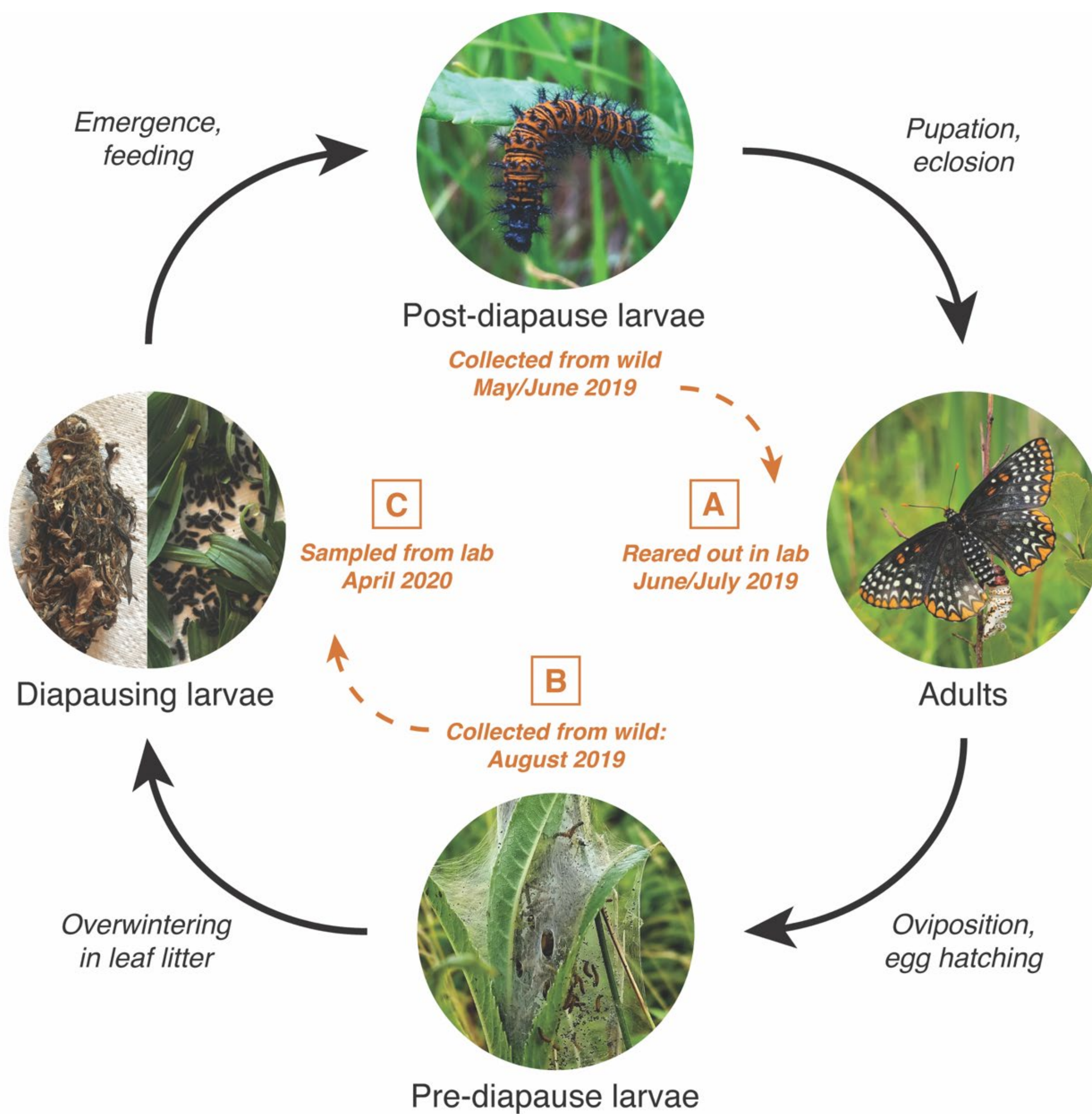


Figure 2

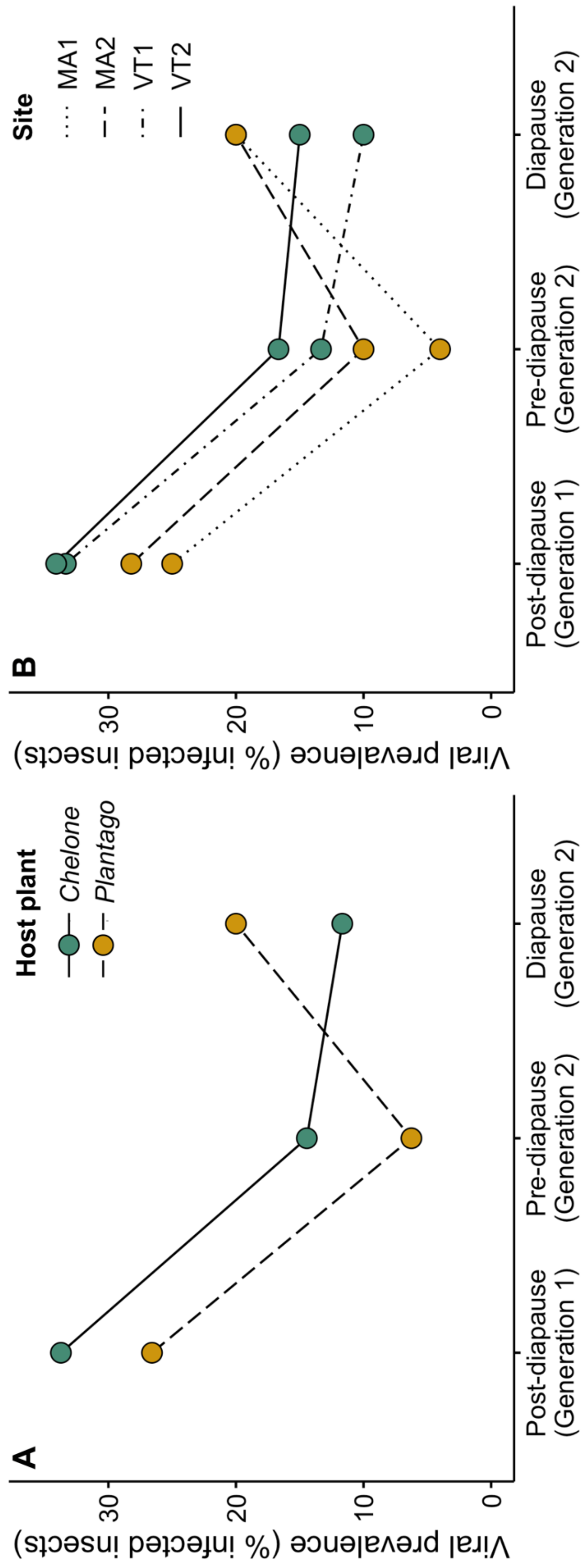


Figure 3

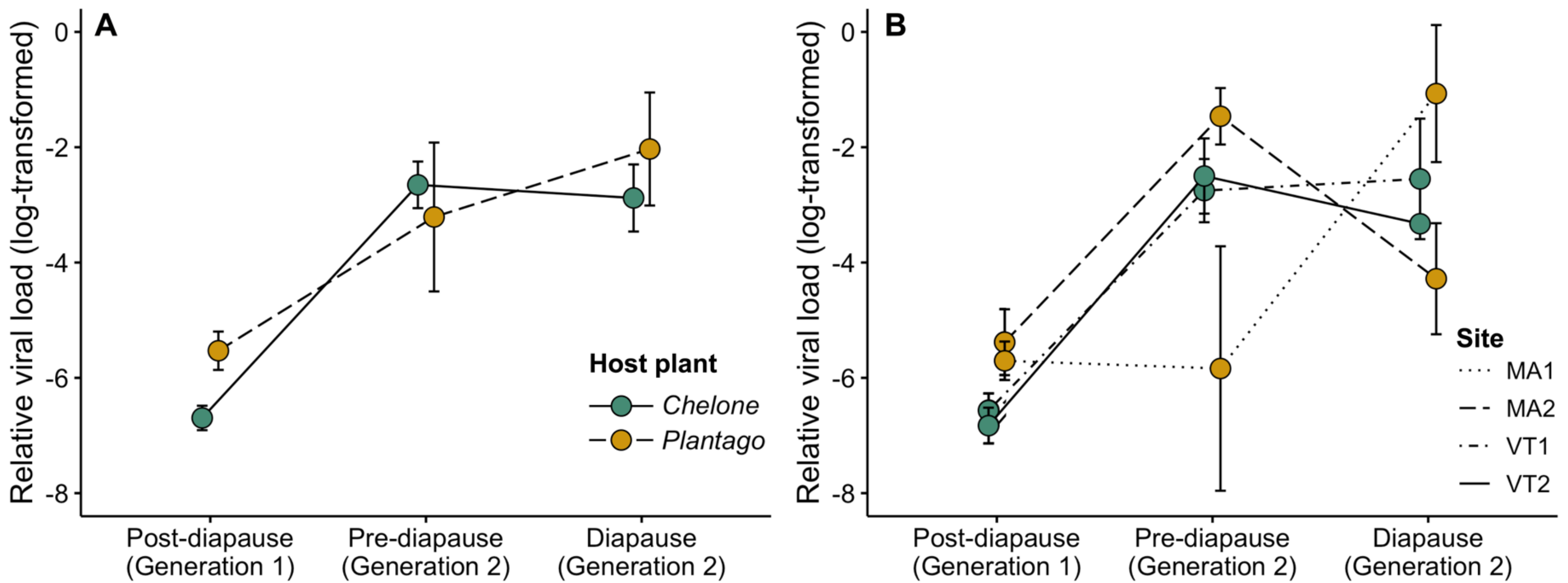


Figure 4

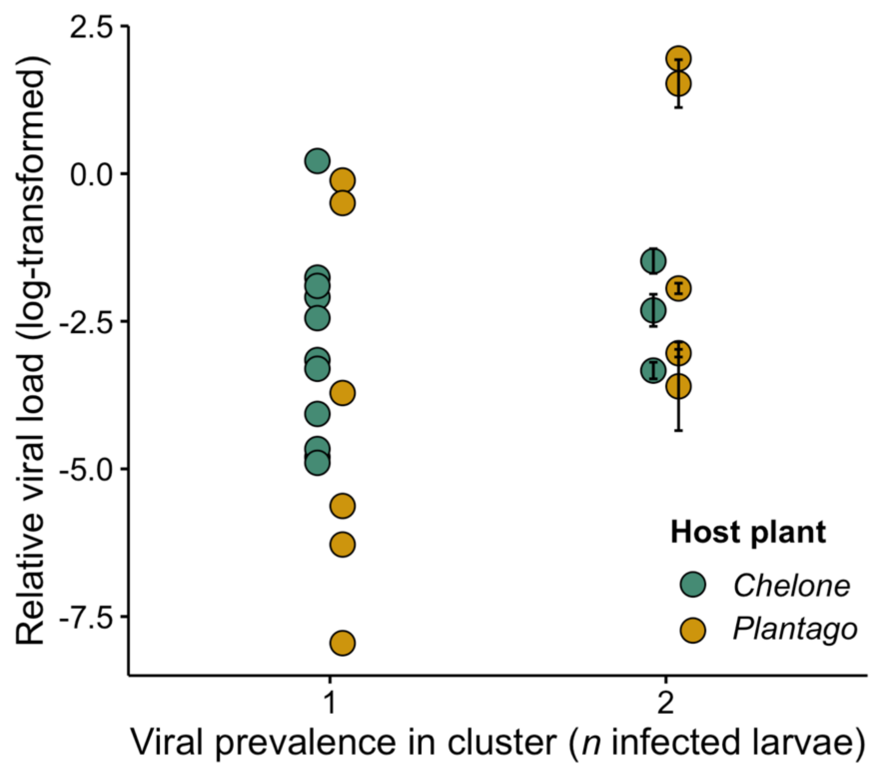
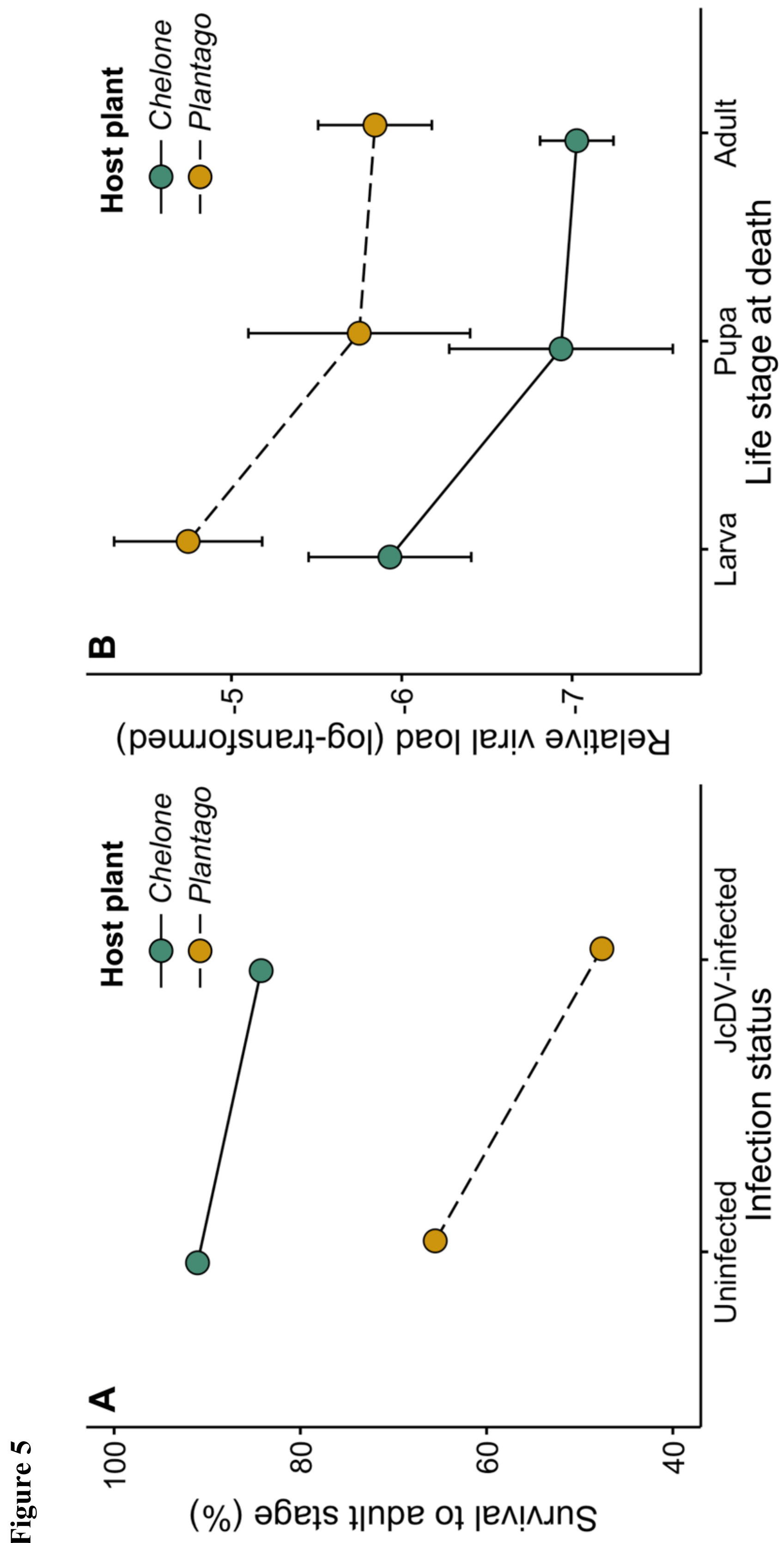


Figure 5



APPENDIX

Table S1 Sampling details for *Euphydryas phaeton* caterpillars collected from the wild in 2019. Field sites were located throughout the northeastern U.S. in Massachusetts (MA1-2), New York (NY1), and Vermont (VT1-2). At each site, *E. phaeton* caterpillars utilized either the native host plant, *Chelone glabra,* or the exotic host plant, *Plantago lanceolata.* Sample sizes of individuals that were screened for Junonia coenia densovirus infection (*n*) are provided for each site and collection stage. Post-diapause caterpillars were collected from the wild in May/June 2019 and clusters of pre-diapause caterpillars from the subsequent generation were collected from the wild in August 2019. These clusters completed pre-diapause development in the laboratory before a subset of larvae underwent viral screening ("pre-diapause caterpillars"). Remaining caterpillars were overwintered in the lab, after which an additional subset of larvae from each cluster underwent viral screening before emerging from diapause ("diapause caterpillars").

Site	Host plant	Post-diapause larvae (*n*)	Pre-diapause clusters (*n*)	Pre-diapause larvae (*n*)	Diapause larvae (*n*)	Latitude	Longitude
MA1	*Plantago*	40	10 (whole)	50	40	41.636994	-70.559876
MA2	*Plantago*	39	6 (3 whole/ 3 partial)	30	15	41.684528	-70.400126
NY1	*Chelone*	19	Not coll.*	Not coll.*	Not coll.*	42.5313871	-74.1590844
VT1	*Chelone*	42	12 (whole)	60	40	44.262020	-72.505049
VT2	*Chelone*	44	6 (egg masses)	30	20	44.282757	-72.542557

* *Pre-diapause clusters not collected due to small population size.*

Table S2 Whole-body quantities of Junonia coenia densovirus in *Euphydryas phaeton,* based on larval host plant species (*Chelone glabra* or *Plantago lanceolata*) and life stage at the time of collection (post-diapause/generation 1, pre-diapause/generation 2, or diapause/generation 2; see Figure 1). Viral quantities were estimated using a standard curve and adjusted for variation in body mass across stages. Whole-body quantities of JcDV were log-transformed and compared across host plant species, collection stages, and their interactions using multiple regression. The intercept of this model represented individuals utilizing the native plant, *Chelone,* during the post-diapause stage. Thus, estimates less than zero represent lower viral loads compared to this reference group.

	Whole-body viral quantity		
Predictors	*Estimate ± SE*	*t*	*p*
Host plant (*Plantago*)	0.41 ± 0.17	2.43	**0.017**
Stage (*pre-diapause*)	-0.06 ± 0.20	-0.30	0.766
Stage (*diapause)*	-0.23 ± 0.25	-0.91	0.365
Host plant (*Plantago*) x stage (*pre-diapause*)	-0.85 ± 0.35	-2.42	**0.018**
Host plant (*Plantago*) x stage (*diapause*)	-0.22 ± 0.34	-0.65	0.520
n	85		
R^2	0.16		

Table S3 Prevalence and loads of Junonia coenia densovirus in *Euphydryas phaeton,* based on sampling site (MA1, MA2, VT1, or VT2) and life stage at the time of collection (post-diapause/generation 1, pre-diapause/generation 2, or diapause/generation 2; see Figure 1). The probability of detecting JcDV ("viral prevalence") was assessed using logistic regression, with site and collection stage as predictors and presence/absence of JcDV as the binomial response. Viral loads (log-transformed, relative to an internal control gene) were compared across the same predictors using multiple regression. The intercepts of both models represent individuals collected from the site "MA1" during post-diapause. Thus, odds ratios less than one represent lower odds of viral detection, and estimates less than zero represent lower viral loads, compared to this reference group.

	Viral prevalence			**Viral load**		
Predictors	*Odds Ratio* [*95% CI*]	*z*	*p*	*Estimate* ± *SE*	*t*	*p*
Site (*MA2*)	1.21 [0.57-2.52]	0.51	0.612	1.17 ± 0.50	2.32	**0.023**
Site (*VT1*)	1.28 [0.67-2.48]	0.73	0.463	4.04 ± 0.58	6.92	**<0.001**
Site (*VT2*)	1.54 [0.77-3.08]	1.23	0.220	3.82 ± 0.74	5.17	**<0.001**
Stage (*pre-diapause*)	0.28 [0.15-0.50]	-4.21	**<0.001**	-1.72 ± 1.05	-1.64	0.105
Stage (*diapause*)	0.44 [0.23- 0.80]	-2.62	**0.009**	-0.32 ± 1.00	-0.32	0.752
n	450			85		
R^2	0.05			0.55		

Figure S1 Absolute quantity of Junonia coenia densovirus in infected individuals across the *Euphydryas phaeton* life cycle based upon host plant use (A) and sampling site (B). Points represent mean whole-body viral quantity [log-transformed viral genomes (vg)] ± SE at each stage (post-diapause/generation 1, pre-diapause/generation 2, or diapause/generation 2). At each site, *E. phaeton* used either the native plant, *Chelone glabra* (VT1-2), or the exotic plant, *Plantago lanceolata* (MA1-2). Mean viral quantity was similar across all stages; however, post-diapause insects using *Plantago* harbored more JcDV than those using *Chelone* (β = -0.41 ± 0.17, t = -2.4, p = 0.02), while pre-diapause insects harbored slightly more virus on *Chelone* (β = 0.45 ± 0.31, t = 1.4, p = 0.2).

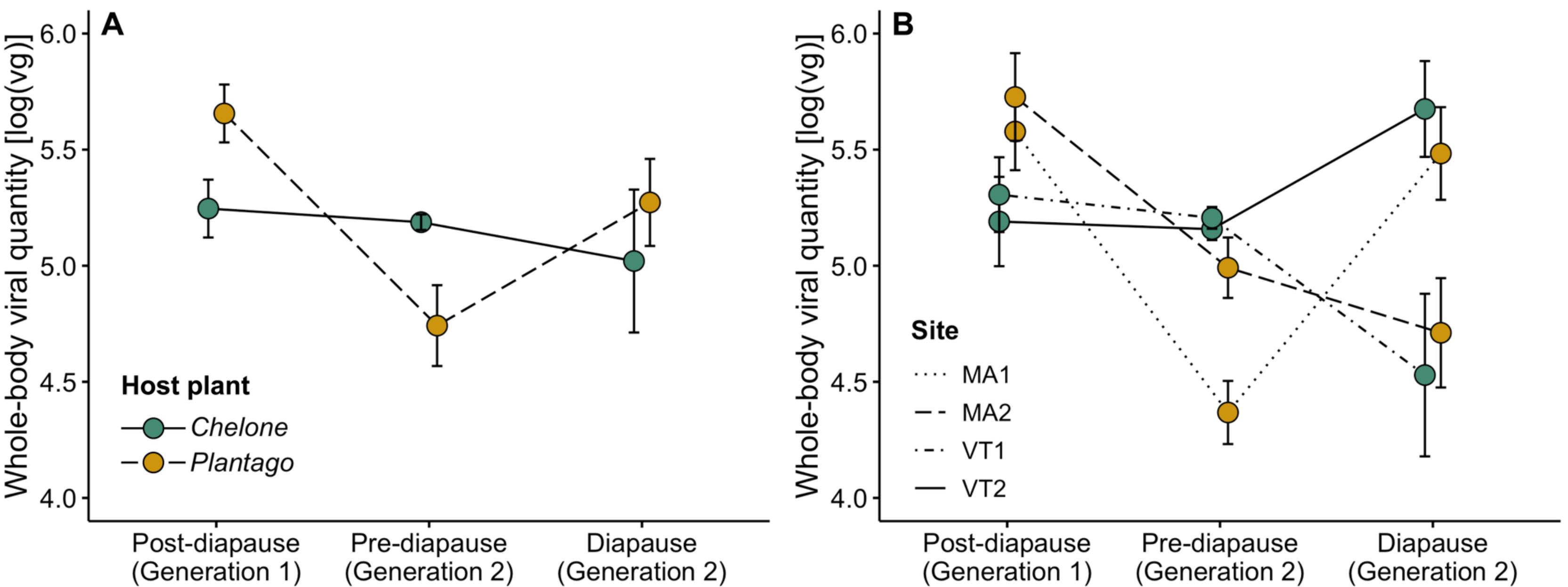
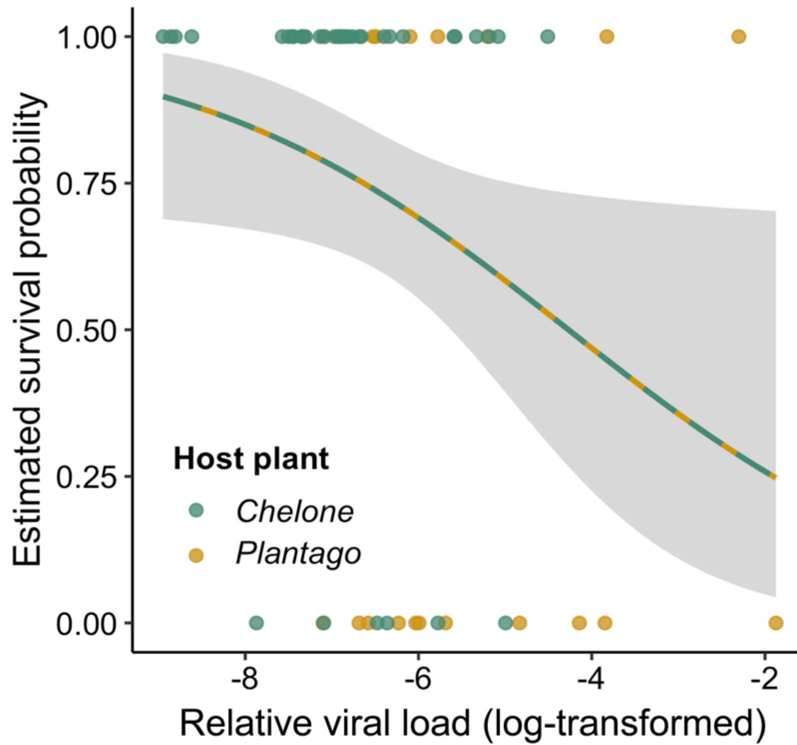


Figure S2 Estimated probabilities of survival to the adult stage in *Euphydryas phaeton* infected with Junonia coenia densovirus, based on relative viral load. Higher JcDV loads corresponded to a lower probability of surviving to the adult stage on both the native host plant, *Chelone glabra,* and the exotic *Plantago lanceolata* (pseudo-R^2 = 0.07, n = 59).



Chapter Three

Use of an exotic host plant reduces viral burden in a native insect herbivore

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ABSTRACT

Incorporation of exotic plant species into the diets of native herbivores is a common phenomenon, influencing interactions with natural enemies and providing insight into the tritrophic costs and benefits of dietary expansion. We evaluated how use of an exotic plant, *Plantago lanceolata*, impacted immune performance, development, and susceptibility to pathogen infection in the neotropical herbivore *Anartia jatrophae* (Lepidoptera: Nymphalidae). Caterpillars were reared on either *P. lanceolata* or a native plant, *Bacopa monnieri*, and experimentally infected with a pathogenic virus, Junonia coenia densovirus. We found that virus-challenged herbivores exhibited higher survival rates and lower viral burdens when reared on *P. lanceolata* compared to *B. monnieri*, though immune performance and development time were largely similar on the two plants. These findings reveal that utilizing an exotic plant can dramatically reduce the vulnerability of a native herbivore to pathogen infection, suggesting diet-mediated protection against disease as a new mechanism driving host range expansion.

1 | INTRODUCTION

Species introductions are a pervasive phenomenon in the modern world, driving substantial ecological and evolutionary change for native species (Mack et al., 2000; Strauss et al., 2006). Exotic plants, in particular, can alter the structure of existing communities and give rise to novel trophic interactions when adopted into the diets of native herbivores (Bezemer et al., 2014; Sunny et al., 2015). For many insect herbivores, exotic plants represent toxic or inferior resources compared to native plants (Yoon and Read, 2016) and may act as "evolutionary traps" for those that colonize them (Keeler and Chew, 2008; Schlaepfer et al., 2005). Alternatively, exotic plants may provide suitable niches for native herbivores and can thereby facilitate population growth or geographic range expansion (Brown et al., 2017; Graves and Shapiro, 2003; Shapiro, 2002). As the incorporation of exotic plants into native diets increases, characterizing the consequences of such dietary expansion for herbivore ecology and evolution represents an important aspect of understanding the ongoing impacts of introduced species on native ecosystems.

Most research on the consequences of exotic host plant use has focused on documenting differences in oviposition preference and larval performance on native and exotic plant species (e.g., Bowers et al., 1992; Forister et al., 2009; Fortuna et al., 2013; Keeler and Chew, 2008). A meta-analysis by Yoon and Read (2016) revealed striking reductions in body mass and survival in Lepidoptera using exotic plants, indicating that dietary expansion may frequently entail costs for herbivore performance. However, it is widely recognized that herbivore fitness on exotic plants is context-dependent and influenced by a broad range of factors beyond suitability for development, including multitrophic interactions (Forister et al., 2020; Price et al., 1980). Consideration of

interactions with natural enemies, alongside development and reproduction, may provide more comprehensive insight into the fitness outcomes of colonizing exotic plants.

Natural enemies are significant agents of mortality for insect herbivores (Hawkins et al., 1997) and can exert differential pressure on populations utilizing native and exotic plants (Feder, 1995; Grosman et al., 2017; Mulatu et al., 2004). Importantly, even if an exotic plant supports relatively poor herbivore development, its use may be advantageous if it provides herbivores with enemy-free space (Bernays and Graham, 1988; Jeffries and Lawton, 1984). For example, exotic plants may be associated with reduced frequency of enemy attack (Fortuna et al., 2013) or may enhance the degree to which herbivores are able to defend themselves. While the importance of incorporating tritrophic interactions into the study of herbivore diet breadth has been acknowledged (Harvey et al., 2010; Lill et al., 2002; Singer and Stireman, 2005), relatively few studies have investigated the role of natural enemies in shaping herbivore fitness on exotic plants, in particular (Fortuna et al., 2013). Fewer still have focused on interactions with pathogens, as opposed to predators or parasitoids (Muchoney et al., 2022) representing a critical knowledge gap.

Substantial research has shown that interactions between insect herbivores and their pathogens can vary dramatically based on host plant use (Cory and Hoover, 2006). Herbivore mortality can vary up to 100-fold when pathogens are ingested on different plant species (Ali et al., 1998; Kouassi et al., 2001), and metrics including speed-of-kill and pathogen yield may also differ (Raymond et al., 2002). As many viral and bacterial entomopathogens must be ingested to establish infection, host plants are often closely involved in the infection process, providing opportunities for direct interactions between plants and pathogens (e.g., on the phylloplane or in the midgut; Felton and Duffey, 1990)

along with indirect interactions mediated by herbivore physiology (Shikano et al., 2010; Yoon et al., 2019). Use of exotic plants, which may differ from native host plants in macronutritional composition, secondary chemistry, and a variety of other traits, may therefore be expected to impact herbivore vulnerability to pathogens in many cases.

One physiological route through which host plant use can impact herbivore susceptibility to pathogens is through dietary effects on the immune response (Smilanich and Muchoney, 2022). Use of different host plants can give rise to significant variation in herbivore immune function (Carper et al., 2019; Diamond and Kingsolver, 2011; Shikano et al., 2010), and as the immune response provides defense against microbial and viral pathogens (Beckage, 2008; Strand, 2008), this variation may contribute to the ability, or inability, of certain plants to provide enemy-free space (Muller et al*.,* 2015). Although considerable progress has been made in documenting the effects of host plant use on entomopathogen infection (see above), relatively few studies have investigated the role of the immune response in mediating these interactions, or the outcomes of host plant-mediated variation in infection and immunity for reproduction (i.e., sublethal effects; Smilanich and Muchoney, 2022). Thus, characterizing the effects of exotic host plants on herbivore resistance to pathogens, immune performance, and reproduction offers potential for insight into the multifaceted costs and benefits of dietary expansion.

In this study, we investigated the tritrophic consequences of exotic host plant use for a native herbivore, *Anartia jatrophae* L. (Lepidoptera: Nymphalidae). This species appears to be in the early stages of incorporating an exotic plant, *Plantago lanceolata* L. (Plantaginaceae), into its host range, and exhibits differential growth and performance when reared on this exotic plant compared to a native host, *Bacopa monnieri* L. Pennell

(Plantaginaceae; Knerl and Bowers, 2013). This species can also be infected by a naturally-occurring pathogen, Junonia coenia densovirus (*Parvoviridae*): a lepidopteran virus capable of infecting several species (Muchoney et al., 2022; Rivers and Longworth, 1968; Smilanich et al., 2018). We surveyed wild populations of *A. jatrophae* to determine viral occurrence and then performed a factorial experiment to evaluate how use of *P. lanceolata* mediates *A. jatrophae* performance through changes in response to viral infection. We addressed two questions: (1) Does exotic plant use impact herbivore immune function and/or vulnerability to viral infection? and (2) Does host plant use mediate the impacts of viral infection on herbivore development, survival, oviposition preference, and fecundity? By simultaneously evaluating susceptibility to a natural pathogen, immune function, and traditional metrics of performance, we provide insight into the tritrophic outcomes of host range expansion for a native insect herbivore.

2 | METHODS

2.1 Herbivore, host plants, and virus

Anartia jatrophae is a neotropical butterfly distributed throughout the southern United States, West Indies, Central America, and much of South America (Silberglied et al., 1979). Caterpillars of this species are oligophagous and have been recorded using host plants from five families (Knerl and Bowers, 2013). In Florida (U.S.), their primary host plant is *B. monnieri* (hereafter, *Bacopa*), a succulent, perennial herb commonly found in wetlands (Rawson 1976). Recently, however, *A. jatrophae* caterpillars in Florida were observed consuming an exotic plant, *P. lanceolata* (hereafter, *Plantago*; Knerl and Bowers, 2013), a perennial herb introduced to North America from Europe during the

19th century (Cavers et al., 1980). This plant has since become naturalized and widespread (USDA, 2021) and has subsequently been incorporated into the diets of several North American lepidopterans (Bowers et al., 1992; Thomas et al., 1987).

Previous research showed that use of *Plantago* entails both positive and negative effects on *A. jatrophae*: larvae took longer to develop on *Plantago* versus *Bacopa,* but exhibited higher pupal weights (Knerl and Bowers, 2013). Importantly, these plants differ considerably in their secondary chemistry and may therefore exert differing effects on *A. jatrophae*'s interactions with natural enemies. *Plantago* leaves contain iridoid glycosides (hereafter, IGs), monoterpenoid secondary metabolites that can be toxic and/or deterrent to non-adapted herbivores (Bowers and Puttick, 1988). *Anartia jatrophae* caterpillars are capable of sequestering and retaining small amounts of IGs into the pupal and adult stages (Knerl and Bowers, 2013), which functions as a form of chemical defense in other sequestering species (Bowers, 1993). However, sequestration of high concentrations of IGs has also been associated with suppression of immune responses in other species of Lepidoptera (Lampert and Bowers, 2015; Laurentz et al., 2012; Smilanich et al., 2009a). In contrast, *Bacopa* does not contain IGs, though it does contain a variety of other secondary compounds (Basak et al., 2016), none of which are known to be sequestered.

Junonia coenia densovirus (hereafter, JcDV), is a nonenveloped, single-stranded DNA virus in the family *Parvoviridae* (*Densovirinae: Lepidopteran protoambidensovirus 1*). JcDV was first identified in *Junonia coenia* (Nymphalidae) but is capable of infecting lepidopterans from multiple families (Mutuel et al., 2010; Rivers and Longworth, 1968). Caterpillars are infected by JcDV via an oral route: viral particles are ingested on host plants and subsequently cross the midgut to replicate in tracheae, hemocytes, visceral

muscles, and epidermis (Mutuel et al., 2010; Wang et al., 2013). JcDV infection may result in hypoxia, disruptions to molting and metamorphosis, and mortality (Mutuel et al., 2010; Smilanich et al., 2018). This study represents the first record of JcDV infection and its effects on survival in *A. jatrophae,* along with the first investigation of the genetic similarity between JcDV found in modern, wild butterflies and the laboratory-propagated isolate that has been employed in many experimental studies of this pathogen (Mutuel et al., 2010; Resnik and Smilanich, 2020; Smilanich et al., 2018; Wang et al., 2013).

2.2 Field survey

To determine whether *A. jatrophae* encounters the focal pathogen (JcDV) in the wild, we collected butterflies (n = 95) from seven locations in southern Florida in April 2017 (see Appendix: Table S1). Though host plant use during the larval stage was not observed, the native *Bacopa* was found to be present at all sampling locations, while the exotic *Plantago* was not observed and has not been recorded in the counties from which butterflies were collected (USDA, 2021). Butterflies were sent live to the University of Colorado, Boulder, where females oviposited on *Bacopa* grown in a greenhouse to establish a colony. Following death, butterflies were frozen and screened for JcDV.

2.2.1 Viral screening

To detect JcDV in wild-collected butterflies, wings were removed and total DNA was extracted from remaining tissues (mass: $\bar{x}$ =18.60 ± 0.46 mg) using Qiagen DNeasy 96 Blood and Tissue Kits (Qiagen, Hilden, North Rhine-Westphalia, Germany) following the Protocol for Purification of Total DNA from Animal Tissues. Extracted DNA was screened for JcDV using quantitative PCR, with primers specific to the VP4 capsid protein gene of JcDV (Wang et al., 2013) and arthropod 28S rDNA primers (Nice et al.,

2009) as an internal control. Samples were screened in duplicate for VP4 and singly for 28S using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) at a reaction volume of 10 µl. Reactions were run on a Bio-Rad CFX96 Optics Module with C1000 Thermal Cycler following the protocols of Muchoney et al. (2022).

2.2.2 Viral sequencing

To examine the similarity between JcDV detected in *A. jatrophae* and the laboratory-propagated isolate used in the following experiment, we sequenced the primary capsid gene of JcDV (VP4) from wild-collected butterflies. Briefly, DNA from all butterflies that were found to contain JcDV via quantitative PCR underwent nested PCR using external and internal primers for the VP4 gene, which were designed for this study based on the published genome for JcDV (GenBank accession number: KC883978; Pham et al*.,* 2013). PCR products were resolved using agarose gel electrophoresis, and all samples that showed clear bands were purified using QIAquick PCR Purification Kits (Qiagen) and submitted to the Nevada Genomics Center in Reno, NV for Sanger sequencing. Resulting sequences (n = 4) were trimmed and aligned using Unipro UGENE (Okonechnikov et al., 2012) and sequence identity (%) with the JcDV "Oxford" isolate (Pham et al., 2013) was evaluated. See Appendix for details on viral sequencing.

2.3 Laboratory experiment

2.3.1 Experiment overview

To investigate how host plant use impacts *A. jatrophae'*s vulnerability to JcDV infection, we conducted a factorial experiment in which caterpillars were reared in the laboratory on either the native plant, *Bacopa,* or the exotic plant, *Plantago*. Within each group, a subset of individuals was orally inoculated with JcDV at the beginning of the

final larval instar, and a subset of each of the four resulting groups (*Bacopa*/control, *Bacopa*/virus, *Plantago*/control, *Plantago*/virus) underwent immune assays. Caterpillars were then reared out to mortality or adulthood to evaluate the effects of host plant use and viral challenge on development and survival, and those that reached the adult stage underwent mating and oviposition trials to assess fecundity and oviposition preference.

2.3.2 Rearing and dietary treatments

Descendants of *A. jatrophae* collected from Florida in April 2017 (see above) were used to establish a colony at the University of Nevada, Reno in September 2017. Butterflies (n = 75) mated in the laboratory and females oviposited on *Bacopa* grown in a hydroponics system. After hatching, first instar caterpillars were transferred to 1 oz plastic cups and assigned to feed on either *Bacopa* (n = 120) or *Plantago* (n = 138) throughout larval development. Caterpillars were reared in incubators using a 16-hour photoperiod (day temperature: 25°C, night temperature: 20°C) and fed ad libitum with foliage grown in the hydroponics system. Upon molting to the third instar, caterpillars were transferred to individual 2 oz plastic cups and development was monitored from this point onward, with dates of molting, pupation, and eclosion recorded.

2.3.3 Viral challenge

On the day following molting to the sixth instar, approximately half of each host plant group (*Bacopa*: n = 56; *Plantago*: n = 64) was orally inoculated with JcDV. Caterpillars were presented with a 10 mm leaf disc (*Bacopa* or *Plantago,* according to group) with 1.0 x 10^7 viral particles suspended in 1 µl of DI water pipetted onto the surface and allowed to dry. Caterpillars were given 24 hours to consume the leaf disc, and those that did not were excluded from the experiment. Following inoculation, virus-

challenged caterpillars were maintained in an incubator at a separate location from unchallenged controls to avoid cross-contamination between groups.

2.3.4 Immune assays

To evaluate the effects of host plant and viral challenge on herbivore immunity, subsets of all groups underwent immune assessment on the fifth day of the sixth instar (four days following inoculation, for virus-challenged caterpillars). We measured two immune parameters: hemocyte concentrations in the hemolymph and the strength of the melanization response against an abiotic implant. Hemocytes are important mediators of phagocytosis, encapsulation, and melanization of pathogens and parasites (Lavine and Strand, 2002); melanization involves deposition of melanin on the surface of such invaders and production of cytotoxic compounds that contribute to their suppression (Nappi and Christensen, 2005).

To estimate hemocyte concentrations, hemolymph was extracted from caterpillars (n = 78) by piercing the cuticle of the A6 segment using a needle sterilized with 70% ethanol. Hemolymph (4-10 µl) was collected using a pipette and mixed with twice the volume of cold anticoagulant, which was prepared by mixing 0.684 g of EDTA and 0.346 g of citric acid with 180 ml of PBS (Sigma-Aldrich, St. Louis, MO, USA) and adjusting the pH to 7.4 before each use (Triggs and Knell, 2012). Mixtures were refrigerated and analyzed within 8 h of extraction. A 10 µl aliquot of each hemolymph-anticoagulant mixture was pipetted into a Neubauer Bright-Line hemocytometer (Sigma-Aldrich), and hemocytes falling within the central grid were counted using a compound microscope at 400x magnification. The total concentration of hemocytes in the hemolymph (cells/ml)

was extrapolated by multiplying each cell count (cells/100 nl) by a factor of 30,000 to account for sample dilution (2:1) and conversion of units.

To assess the strength of the melanization response, 2 mm nylon monofilaments were sterilized with 70% ethanol and inserted into the abdominal wound immediately following hemolymph extraction (n = 103). Implants were made from abraded nylon fishing line (0.2 mm diameter) knotted at one end to facilitate removal (Rantala and Roff, 2007). Implants were left in the hemocoel for 24 h and then removed, dried, and stored in a freezer. Each implant was photographed at two different angles under 3.2x magnification using a dissecting microscope mounted with a digital camera (Carl Ziess Discovery V.8, AxioCam Software, Oberkochen, Baden-Wurttenburg, Germany). Using the "quick selection" tool in Adobe Photoshop CC 2018 (Adobe Systems Inc., San Jose, California, USA), a mean greyness value (MGV) was generated for each implant photograph. MGV is a measure of greyness on a scale ranging from 0 to 255, where 0 = pure grey and 255 = pure white. Mean MGVs for each implant were transformed into a percentage of melanization [1 – (MGV/maximum MGV)] for ease of interpretation.

2.3.5 Pupation and oviposition trials

Following immune assessment, caterpillars were returned to incubators to complete development. Pupae were weighed and transferred to 32 oz plastic containers for eclosion, and those that survived to adulthood were assigned to mating groups (one female with two males) within their treatment groups. These were housed together in 5L plastic containers and maintained on 10% honey water for three days to allow for mating. Females were then transferred to individual 5L containers, and oviposition preference was assessed by providing a choice of two plants (*Bacopa* or *Plantago*) housed in 2 oz

plastic cups. After 3-4 days, eggs laid on each plant were counted. Oviposition preference index (OPI) was calulcated as the proportion of eggs laid on *Bacopa* [(*Bacopa* eggs - *Plantago* eggs)/total eggs], with OPI = 1 representing total preference for *Bacopa,* OPI = -1 representing total preference for *Plantago* (Keeler and Chew, 2008). Notably, there were no successful oviposition trials involving virus-inoculated females reared on *Bacopa,* likely due to the low number of surviving adults in this group (see Results).

2.3.6 Viral screening and quantification

To quantify postmortem JcDV loads of virus-challenged individuals and verify the absence of JcDV in unchallenged individuals, DNA was extracted from a tissue sample from each insect (*n* = 256) using the protocol above. Whole butterflies (with wings removed) and pupae were used (mass: $\overline{x}$ = 28.7 ± 0.9 mg), whereas an aliquot of homogenized tissue ($\overline{x}$ = 20.0 ± 0.6 mg) was used for each larva. Extracted DNA samples were then screened for JcDV using the quantitative PCR protocol provided above. Viral loads were calculated as $2^{-\Delta Ct}$ (Schmittgen and Livak, 2008), representing the abundance of the JcDV VP4 gene relative to the abundance of the internal control gene [ΔC_t = mean C_t (threshold cycle) for VP4 – mean C_t for 28S] and log-transformed.

2.4 Statistical analyses

All statistical analyses were performed in R version 4.0.4 (R Core Team, 2021), and statistical significance was assessed using an alpha level of 0.05. The effects of host plant species (*Bacopa* or *Plantago*), viral treatment (virus-challenged or control), and their interaction on *A. jatrophae* survival to the adult stage (Y/N) were evaluated using logistic regression. As undergoing immune assessment reduced survival across all groups, assay status (assayed/not assayed) was also included as a predictor. Within the

subset of individuals that were inoculated with JcDV, the probability of postmortem viral detection (Y/N) was compared between host plants using Pearson's chi-squared test, and the relationship between viral detection and survival was examined using logistic regression with host plant, viral detection, and assay status as predictors and survival as the response. Postmortem JcDV loads were then compared using multiple regression with host plant, survival, and assay status as predictors, and the relationship between viral burden and survival was further probed using logistic regression with viral load, host plant, their interaction, and assay status as predictors and survival as the response.

Pre-inoculation development time, defined as the number of days between molting to the third larval instar and the first day of the sixth instar (when inoculation occurred), was compared based on host plant and treatment group assignment (to confirm the absence of bias) using two-way ANOVA. Effects of host plant and viral treatment on post-inoculation development time (days between inoculation and eclosion), pupal time (days in pupal stage), and pupal weight were assessed using separate multiple regression models that included the covariate of assay status. The pupal weight model additionally included sex (M/F) as a predictor to account for observed dimorphism in body size.

Immune responses (hemocyte concentration and melanization) were compared across host plants and viral treatments using separate two-way ANOVAs. The outcomes of immune variation for survival of virus-inoculated individuals were assessed using logistic regression models that included host plant and either hemocyte concentration or melanization as predictors, while relationships between immunity and postmortem viral loads were assessed using multiple regression models with the same predictors. Female fecundity (number of eggs laid over 3-4 days) and oviposition preference (OPI) were

examined using separate multiple regression models, which included host plant and viral treatment as predictors, as well as the number of oviposition days (3-4) as a covariate.

3 | RESULTS

3.1 Field survey

Junonia coenia densovirus was detected in wild *A. jatrophae* butterflies originating from six out of seven sampling locations at an overall frequency of 12% (n = 11 out of 95). Sequences for the primary structural protein gene of JcDV (VP4) amplified from wild-collected *A. jatrophae* (n = 4) exhibited 99-100% identity with the published genome for this pathogen (Figure S1) (Pham et al., 2013), indicating high similarity between the wild isolates of JcDV occurring in *A. jatrophae* populations and the laboratory-propagated isolate utilized for experimental inoculations in the present study. See Appendix for additional details on viral prevalence and sequence diversity.

3.2 Laboratory experiment

3.2.1 Survival

Host plant species did not significantly impact probability of survival to the adult stage in control individuals that were not inoculated with the virus (Figure 1) [odds ratio (OR) = 1.95, 95% confidence interval (CI) = (0.66-6.10), z = 1.2, p = 0.2]. However, there was a significant interaction between host plant and viral treatment on survival, revealing that survival of JcDV challenge was dependent upon larval host plant (Figure 1) [host plant x treatment interaction: OR = 5.73, 95% CI = (1.21-29.01), z = 2.2, p = 0.03]. Specifically, inoculated caterpillars reared on *Bacopa* had 90% lower odds of surviving compared to inoculated caterpillars reared on *Plantago* (Figure 1; Table S2)

3.2.2 Viral detection and loads

Of the individuals that were inoculated with JcDV, 57% harbored a detectable infection at their time of death, indicating that the remaining 43% were able to either avoid, clear, or suppress viral infection below detectable levels. The likelihood of JcDV detection did not differ based on host plant (Figure 2a) ($X^2 = 0.13$, $df = 1$, $p = 0.7$). The absence of a detectable infection was associated with high frequencies of survival on both host plants (Figure 2b), while detection of JcDV was associated with increased mortality in the larval or pupal stages [OR = 0.08, 95% CI = (0.02–0.31), $z = -3.4$, $p = 0.001$].

Within individuals that maintained a detectable infection at their time of death, viral loads were over 200-fold higher in those reared on *Bacopa,* compared to *Plantago* (Figure 2c). This pattern was primarily mediated by the higher frequency of survival on *Plantago* (Figure 2b), as viral loads were substantially lower in individuals that survived to reach the adult stage, compared to those that died as larvae or pupae ($\beta = -3.82 \pm 0.66$, $t = -5.8$, $df = 58$, $p < 0.0001$). In addition, there was a significant negative relationship between viral load and probability of survival to adulthood within individuals reared on *Bacopa* (Figure 2d) [OR = 0.09, 95% CI = (0.00–0.41), $z = -2.0$, $p = 0.04$]. In contrast, 100% of individuals that were reared on *Plantago* and did not undergo immune assays survived inoculation with JcDV. Thus, when accounting for the effect of immune assessment, we documented 0% mortality on *Plantago* regardless of viral burden.

3.2.3 Development and pupal weight

Pre-inoculation development time did not differ between caterpillars consuming *Bacopa* and *Plantago* ($F_{1,250} = 0.58$, $p = 0.4$). Following viral inoculation, the time required to reach the adult stage (final instar through eclosion) again did not differ based

on host plant species (β = 0.14 ± 0.18, t = 0.8, df = 176, p = 0.4), but was significantly accelerated in individuals challenged with JcDV compared to controls (β = -1.30 ± 0.18, t = -7.4, df = 176, p < 0.0001). Individuals that were inoculated with the virus required an average of 1.3 fewer days to reach the adult stage than controls. When examining the duration of the pupal stage in particular, an interaction between host plant use and viral treatment emerged (Figure 3a) (β = -0.70 ± 0.24, t = -3.0, df = 175, p = 0.003). Control individuals reared on *Plantago* spent significantly more time as pupae than those reared on *Bacopa*, but also exhibited a decrease in pupal development time when inoculated with JcDV, whereas individuals reared on *Bacopa* did not. This pattern indicates that host plant effects on the speed of pupal development varied depending on viral exposure.

Pupal weights were significantly higher in individuals reared on *Plantago,* compared to those reared on *Bacopa* (Figure 3b) (β = 18.9 ± 6.3, t = 3.0, df = 180, p = 0.003). In addition, there was a negative effect of viral inoculation on pupal weight (β = -28.9 ± 6.3, t = -4.6, df = 180, p < 0.0001). When accounting for the effects of immune assessment and sexual dimorphism in adult body size, pupal weights were on average 7% greater in individuals reared on *Plantago* than *Bacopa* and 11% greater in controls than individuals that were inoculated with JcDV. In addition, pupal weights were positively correlated with both pre-inoculation development time (Pearson's R_{187} = 0.16, p = 0.02) and post-inoculation development time (Pearson's R_{165} = 0.40, p < 0.0001).

3.2.4 Immune responses

Total hemocyte concentrations in the hemolymph did not differ significantly between caterpillars using *Bacopa* and *Plantago* (Figure 4a) ($F_{1,94}$ = 0.39, p = 0.5). However, larvae inoculated with JcDV exhibited reduced hemocyte concentrations

relative to controls on both plants ($F_{1,94}$ = 5.2, p = 0.02). A similar pattern was found for melanization: inoculation with JcDV reduced the strength of the melanization response (Figure 4b) ($F_{1,100}$ = 6.8, p = 0.01), but there was no significant effect of host plant on this parameter ($F_{1,100}$ = 0.09, p = 0.8). Neither hemocyte concentrations nor melanization score were significantly associated with survival in individuals inoculated with JcDV (Table S3); however, viral loads were greater in individuals with higher melanization scores on *Bacopa* (β = 0.003 ± 0.001, t = 2.1, df = 27, p = 0.04), which may be indicative of increased activation of the melanization response in reaction to higher viral burdens.

3.2.5 Oviposition and fecundity

Female *A. jatrophae* exhibited a strong preference for oviposition on the native plant, *Bacopa,* compared to *Plantago* (mean OPI = 0.996 ± 0.004, n = 32)*,* regardless of the host plant upon which they were reared (β = -0.011 ± 0.009, t = -1.2, df = 30, p = 0.2). There were no successful oviposition trials involving virus-inoculated individuals reared on *Bacopa* due to the low number of surviving adults in this group (see Methods); however, viral treatment had a no effect on OPI in individuals reared on *Plantago* (β = 0.009 ± 0.009, t = 1.0, df = 30, p = 0.3). Notably, only two out of 32 females laid any eggs on *Plantago.* Both of these females had been reared on *Plantago*, and in both cases, the eggs laid on *Bacopa* (n = 91, 49) vastly outnumbered the eggs laid on *Plantago* (n = 1, 3; individual OPIs = 0.989, 0.942). Fecundity, measured as the total number of eggs laid, also did not differ based on larval host plant (Figure 5) (β = 0.51 ± 12.05, t = 0.04, df = 28, p = 0.97) or viral treatment (β = 0.87 ± 11.49, t = 0.08, df =28, p = 0.9).

4 | DISCUSSION

This study demonstrates that use of an exotic host plant can dramatically reduce the vulnerability of a native herbivore to pathogen infection. When reared on *Plantago*, individuals inoculated with JcDV had high survival (100% of individuals that did not undergo immune assays), but the odds of surviving decreased by 90% when reared on *Bacopa* (Figure 1). This lack of susceptibility to JcDV infection in individuals reared on *Plantago* was unexpected, and it may be predicted to promote the incorporation of *Plantago* into the dietary range of *A. jatrophae* in the wild, particularly in populations where exposure to JcDV is high. Importantly, our preliminary field survey confirmed that JcDV is present throughout wild *A. jatrophae* populations, and that the "wild" virus encountered by these herbivores is genetically similar to the virus used in our experiment (Figure S1). Moving forward, gaining a deeper understanding of the prevalence of this pathogen throughout *A. jatrophae*'s range, particularly in regions where *Plantago* is present, represents an important step toward understanding how this tritrophic benefit of exotic host plant use may shape the ecology and evolution of this native herbivore.

The stark difference in survivorship between individuals reared on the native and exotic plants raises the question of whether individuals consuming *Plantago* were able to avoid the establishment of infection, which is referred to as "qualitative resistance" or "anti-infection resistance" (De Roode et al., 2011). However, since a similar proportion of virus-inoculated individuals showed no detectable infection when reared on each host plant (ca. 43%; Figure 2a), it is unlikely that the ability to avoid infection was host plant dependent. Whether due to avoidance or clearance, these individuals experienced high survival to adulthood, while the majority of mortality was observed in virus-challenged individuals that maintained detectable infections (Figure 2b). Within this cohort, JcDV

loads were lower in individuals reared on *Plantago* (the majority of which survived to adulthood) compared to *Bacopa,* where loads were higher and primarily detected in larvae and pupae (Figure 2c). Thus, herbivores using the exotic plant harbored reduced viral burdens by the time that they died. We also documented a negative relationship between viral load and survival in individuals reared on *Bacopa* (Figure 2d)*,* indicating that the higher viral burdens experienced by herbivores using this plant were a primary causal agent in their mortality. Given these patterns, it is likely that using *Plantago* provides *A. jatrophae* with protection against JcDV via "quantitative resistance," or the ability to reduce pathogen burden upon infection (De Roode and Lefèvre, 2012).

Use of the exotic plant could reduce viral burdens in *A. jatrophae* through a number of different routes. First, ingesting JcDV on *Plantago* foliage may decrease the effective dose of viral particles that initially establish infection via the midgut, which may be considered an "imperfect form" of anti-infection resistance (De Roode *et al.* 2011). Certain phytochemicals have been found to directly interfere with pathogen infectivity in other lepidopteran systems (Felton and Duffey, 1990; Keating et al., 1990); however, if this were the case with *Plantago*, we would have expected to observe greater differences in infection probability between the two host plants (Figure 2a). The second, and more likely scenario, is that consuming *Plantago* suppresses JcDV replication once infection has been established, as postmortem loads were 200-fold lower on this plant (Figure 2c). As lower viral loads on *Plantago* were primarily mediated by higher survival to the adult stage (Figure 2b), and JcDV is known to impair pupation (Mutuel et al., 2010), we can hypothesize that individuals using this plant were better able to suppress JcDV during the larval stage, thereby increasing their chance of reaching and surviving metamorphosis.

Host plant use may indirectly influence entomopathogen burdens through its impacts on various aspects of herbivore physiology, including growth and development. In contrast to previous research with *A. jatrophae* (Knerl and Bowers, 2013), we found only partial evidence of slower development on *Plantago,* relative to *Bacopa*: larval development time was similar on the two plants, while pupal development was slower on *Plantago* (Figure 3a) (see also Lampert et al., 2014). Our results were consistent with Knerl and Bowers' finding of higher pupal weights on *Plantago* (Figure 3b). In addition, inoculation with the virus accelerated development and resulted in smaller pupae (Figure 3b). Accelerated development in response to JcDV infection has been documented in another nymphalid butterfly (Smilanich et al., 2018). Whether faster development is an advantageous strategy on the part of the herbivore (Agnew et al., 2000), or a form of host manipulation that benefits the virus, remains to be determined, and the two possibilities are not mutually exclusive. If herbivores are able to suppress or clear JcDV through metamorphosis, as proposed by Mutuel et al. (2010), then reaching pupation or eclosion as quickly as possible may maximize an infected herbivore's chance of reproducing. Though individuals reared on *Plantago* were able to accelerate pupal development in response to viral challenge (Figure 3a), they ultimately reached eclosion within a similar time frame to those reared on *Bacopa,* suggesting that developmental differences are unlikely to explain the pattern of reduced viral loads documented on the exotic plant.

Another avenue through which host plant use may influence herbivore resistance to entomopathogens is through enhancement or suppression of immune responses. In this study, however, variation in immunocompetence did not appear to mediate differences in vulnerability to JcDV on the two plant species. Both hemocyte concentrations (Figure 4a)

and the strength of the melanization response (Figure 4b) were similar in caterpillars using *Bacopa* and *Plantago,* and neither parameter influenced the likelihood of survival in virus-inoculated individuals (Table S3). Thus, while eco-immunological research has provided many examples of variation in immune performance across Lepidoptera utilizing different host plants (e.g., Decker et al., 2020; Diamond and Kingsolver, 2011; Ojala et al., 2005; Smilanich et al., 2009), this system provides a contrasting example. Similarity in immune performance on the two plants is surprising, given the marked differences in secondary chemistry between these two species: *Plantago* leaves contain iridoid glycosides, which have been linked to suppression of immune responses in three other butterfly species (Lampert and Bowers, 2015; Muchoney et al., 2022; Richards et al., 2012; Smilanich et al., 2009a). However, these species are all specialists that sequester IGs in high concentrations, whereas *A. jatrophae* sequesters lower levels of IGs (Knerl and Bowers, 2013) that may be insufficient to elicit an immunosuppressive effect.

As diet-mediated variation in development rate and immunity provided limited explanatory power into patterns of quantitative resistance to JcDV in *A. jatrophae*, additional research will be necessary to elucidate how individuals using *Plantago* are able to suppress viral replication and increase their likelihood of surviving infection. As the specific routes through which the immune response contributes to defense against JcDV are unclear (Muchoney et al., 2022; Resnik and Smilanich, 2020; Smilanich et al., 2018), it is possible that immune parameters that were not measured in this study contribute to defense against JcDV and are enhanced in larvae consuming *Plantago.* In addition, the role of plant chemistry in mediating herbivore-virus interactions warrants consideration (Cory and Hoover, 2006). As previously noted, *A. jatrophae* is capable of sequestering

IGs when feeding on *Plantago* but not *Bacopa,* representing a potentially consequential physiological difference within the context of tritrophic interactions (Knerl and Bowers, 2013; Lampert et al., 2014). Previous research with *Euphydryas phaeton,* a nymphalid that exhibits a high degree of IG sequestration, revealed a negative relationship between sequestration and JcDV loads (Muchoney *et al.* 2022), suggesting that IG sequestration may provide an additional, non-immunological form of defense against this virus.

In summary, this study provides evidence of a clear tritrophic fitness benefit that can arise through the adoption of an exotic plant into the diet of a native insect herbivore. We discovered that consuming *Plantago* provides *A. jatrophae* with a major survival advantage when faced with JcDV infection (Figure 1), though the mechanism underlying this pattern remains unclear. We found little evidence to indicate that this benefit was accompanied by fitness-related costs: development time (Figure 3a), immunocompetence (Figure 4), and fecundity (Figure 5) were largely similar on the two plant species, while pupal weights were higher on the exotic plant (Figure 3b). We conclude that *Plantago* represents: (1) a suitable resource for supporting *A. jatrophae* development in the absence of JcDV infection, and (2) a superior resource for supporting *A. jatrophae* development when JcDV is present, both of which could facilitate population growth or geographic expansion in this species (Shapiro, 2002). Despite the apparent suitability of *Plantago*, it is important to note that females exhibited an unwavering preference for oviposition on the native *Bacopa* across treatments. However, this may be explained by the novelty of this plant to the lineage of herbivores used for this experiment, which were collected from populations where *Plantago* did not appear to be present. The acceptability of *Plantago* to ovipositing females should be further resolved using no-choice assays, and

through the study of populations where both host plants are present. To conclude, this study illustrates that host plant identity can dramatically impact herbivore interactions with their natural enemies (Cory and Hoover, 2006; Kaplan et al., 2016; Ode, 2006). Examination of interactions with higher trophic levels, including predators, parasites, and pathogens, may therefore offer valuable opportunities for insight into both the evolution of herbivore host range and the ecological impacts of exotic plants on native ecosystems.

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AUTHOR CONTRIBUTIONS

The study was conceived by AMS and MDB, and all authors contributed to experimental design. ALC and NDM conducted the field survey and maintained insect

colonies, and viral sequencing was performed by NDM under the guidance of MBT and MO. NDM performed the laboratory experiment, analyzed data, and wrote the manuscript, and all authors contributed substantially to revisions.

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FIGURE LEGENDS

Figure 1 Effects of larval host plant species and viral treatment on survival of *Anartia jatrophae* to the adult stage. Individuals reared on the native plant, *Bacopa monnieri,* exhibited a reduced frequency of survival to adulthood when inoculated with Junonia coenia densovirus during the final larval instar (n = 56), compared to controls (n = 57). Individuals reared on the exotic plant, *Plantago lanceolata,* exhibited similarly high survival rates across treatment groups (virus-inoculated: n = 64; controls: n = 67).

Figure 2 Effects of larval host plant species on infection and resistance parameters in *Anartia jatrophae* inoculated with Junonia coenia densovirus. (A) Postmortem detection frequencies of JcDV in individuals reared on either the native plant, *Bacopa monnieri,* or the exotic plant, *Plantago lanceolata.* (B) Frequencies of survival to the adult stage based on postmortem detection of JcDV and larval host plant species. (C) Postmortem viral loads of individuals reared on *Bacopa* or *Plantago*. Points represent mean ± SE and are provided for individuals that died prior to reaching the adult stage (larva/pupa), those that survived to reach the adult stage (adult), and the overall means for each host plant group. (D) Relationship between postmortem viral load and estimated survival probability in individuals reared on *Bacopa* or *Plantago*. Points represent individual observations.

Figure 3 Effects of larval host plant and inoculation with Junonia coenia densovirus on pupal development time and pupal weights in *Anartia jatrophae*. Points represent mean ± SE. (A) Controls reared on the native plant, *Bacopa monnieri,* exhibited faster pupal

development than those reared on the exotic plant, *Plantago lanceolata*. However, pupal development time was similar between the two plants in virus-inoculated individuals. (B) Pupal weight was greater in individuals reared on *Plantago* than those reared on *Bacopa* and was additionally reduced in virus-inoculated individuals, relative to controls.

Figure 4 Immune responses of *Anartia jatrophae* caterpillars based on host plant and inoculation with Junonia coenia densovirus. Points represent mean ± SE. (A) Hemocyte concentrations in the hemolymph did not differ between caterpillars using *Bacopa monnieri* and *Plantago lanceolata.* However, caterpillars that were inoculated with JcDV exhibited reduced hemocyte densities, relative to controls. (B) Melanization of an abiotic implant (nylon monofilament) did not differ based upon host plant use, while inoculation with JcDV had a negative impact on melanization across both host plant groups.

Figure 5 Effects of larval host plant species and inoculation with Junonia coenia densovirus on fecundity of *Anartia jatrophae* females. Points represent mean ± SE. The total number of eggs laid over 3-4 days did not differ significantly between control females that were reared on the native host plant, *Bacopa monnieri,* or the exotic host plant, *Plantago.* In addition, the number of eggs laid by females reared on *Plantago* were similar across virus-inoculated individuals and controls. There were no successful oviposition trials involving virus-inoculated individuals that were reared on *Bacopa.*

FIGURES

Figure 1

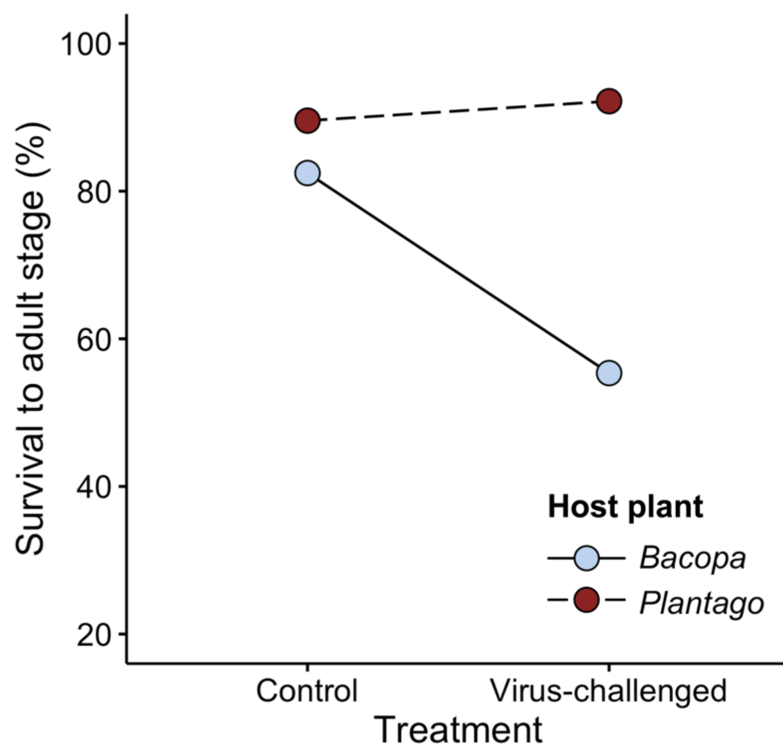


Figure 2

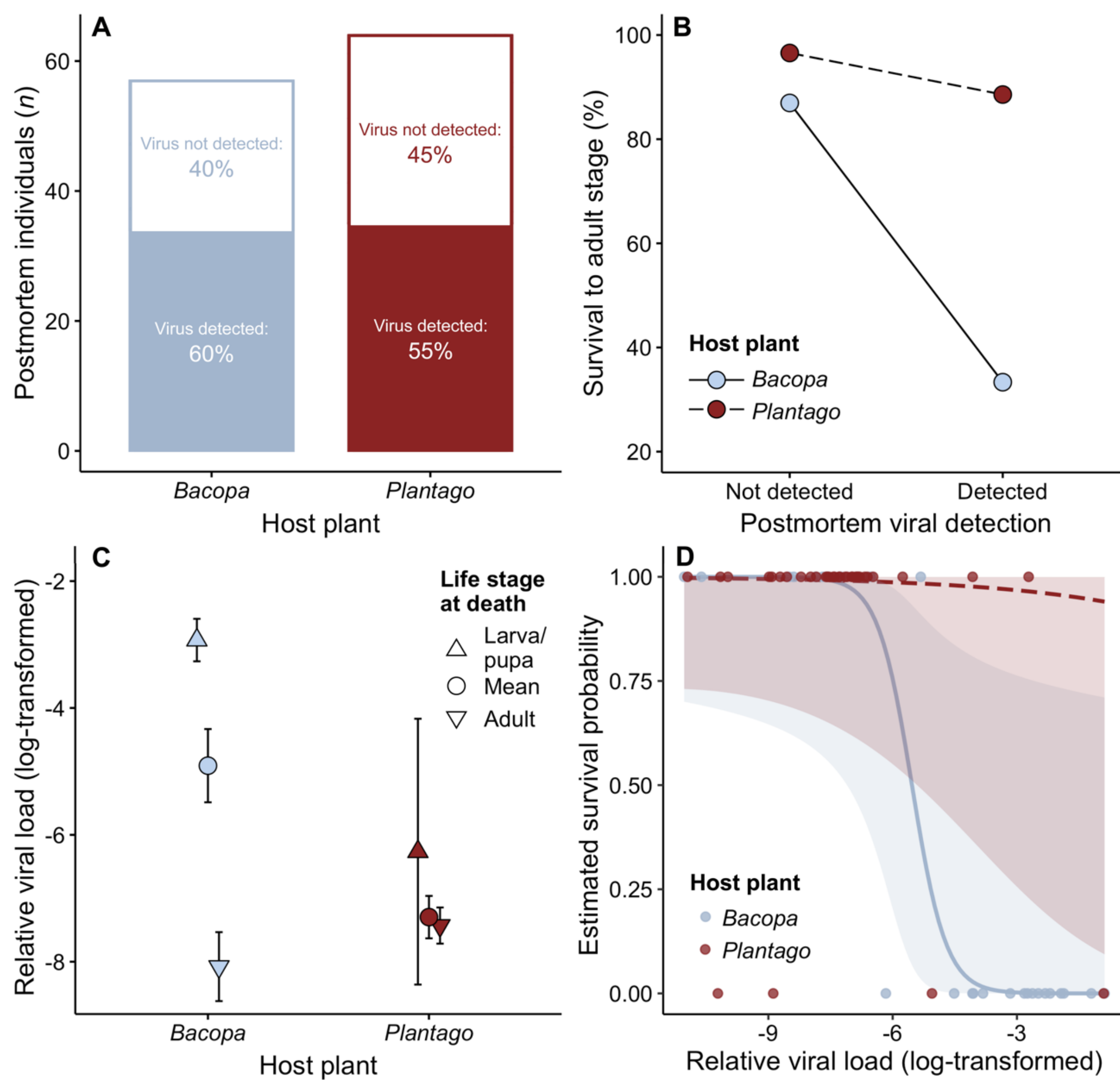


Figure 3

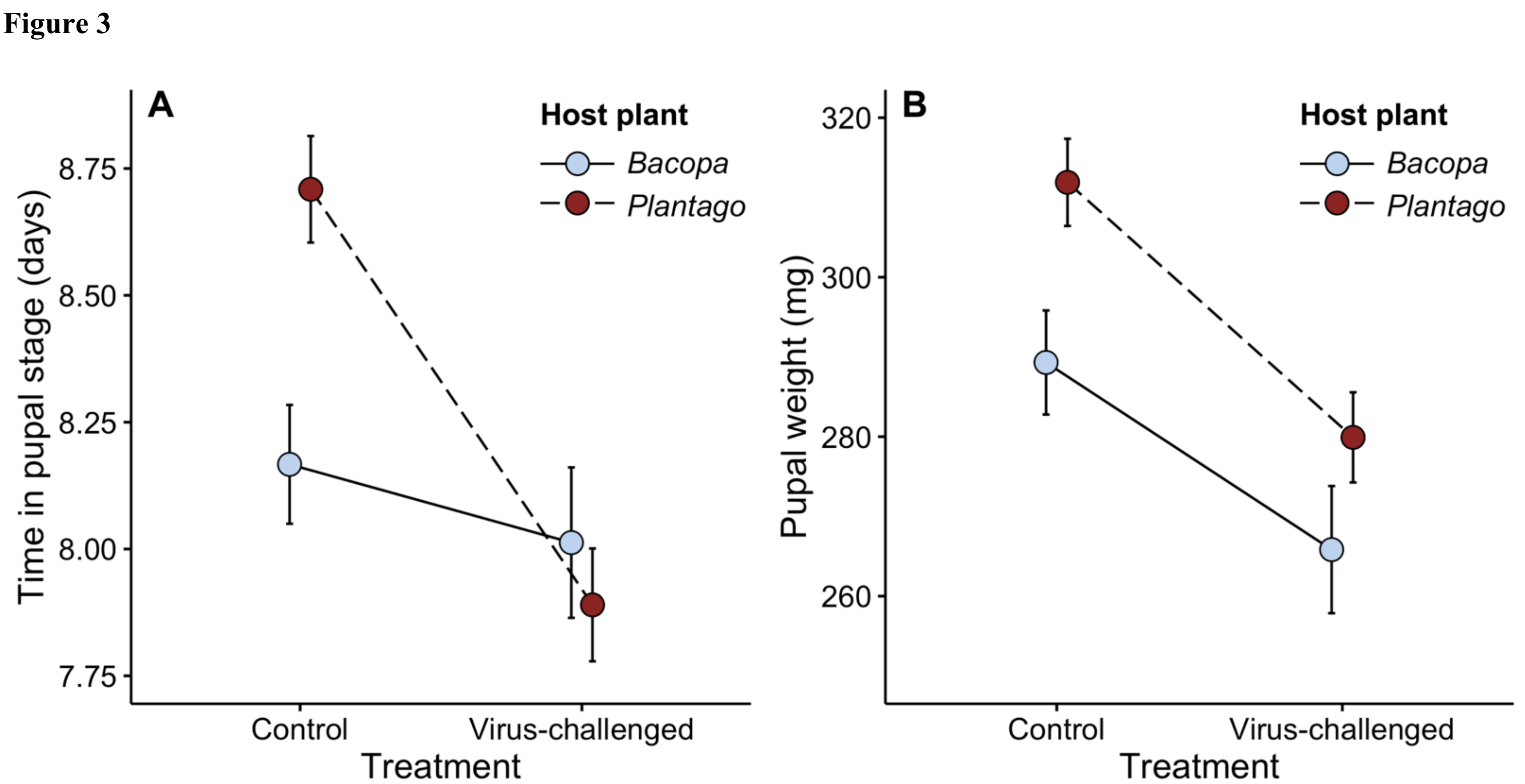


Figure 4

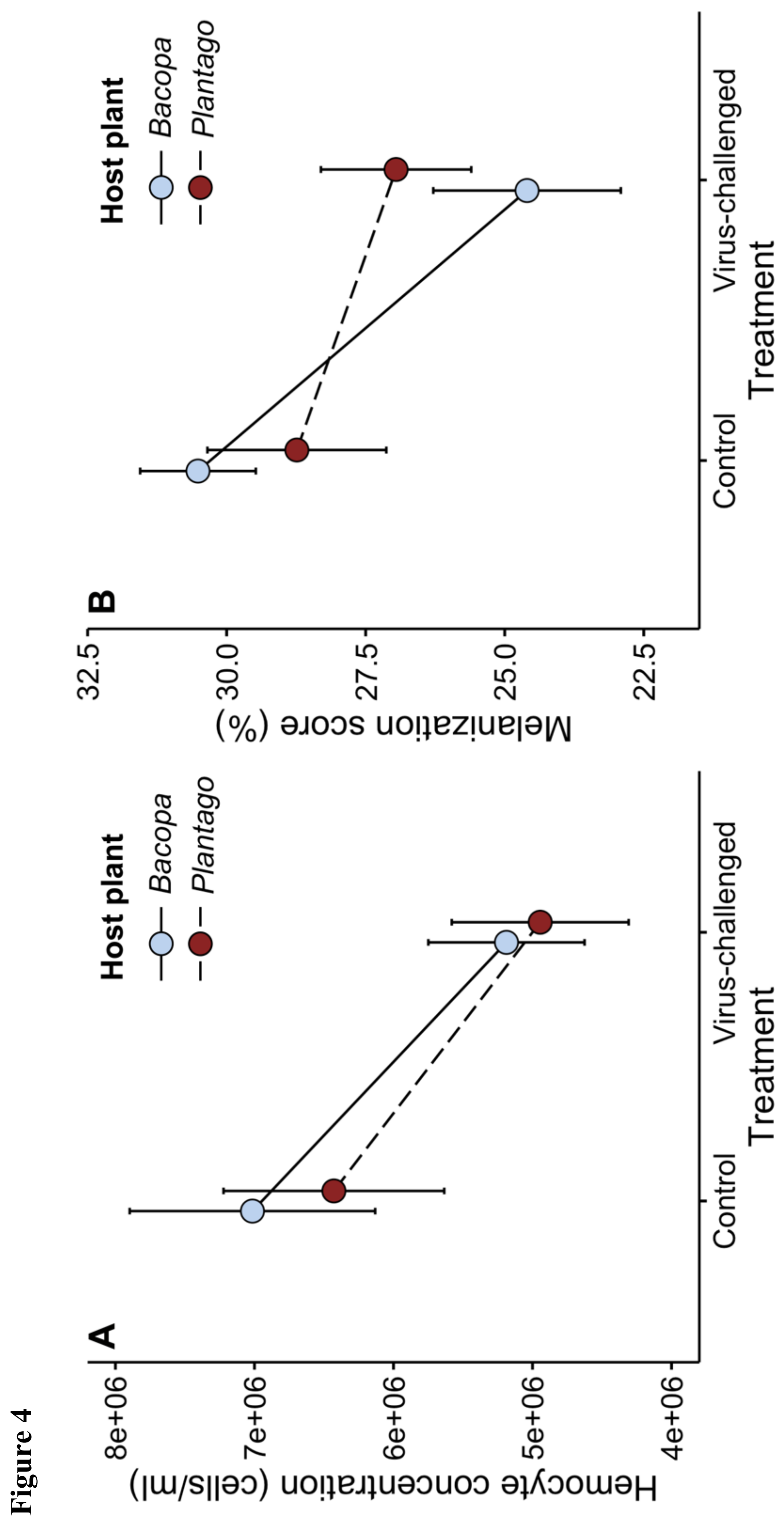
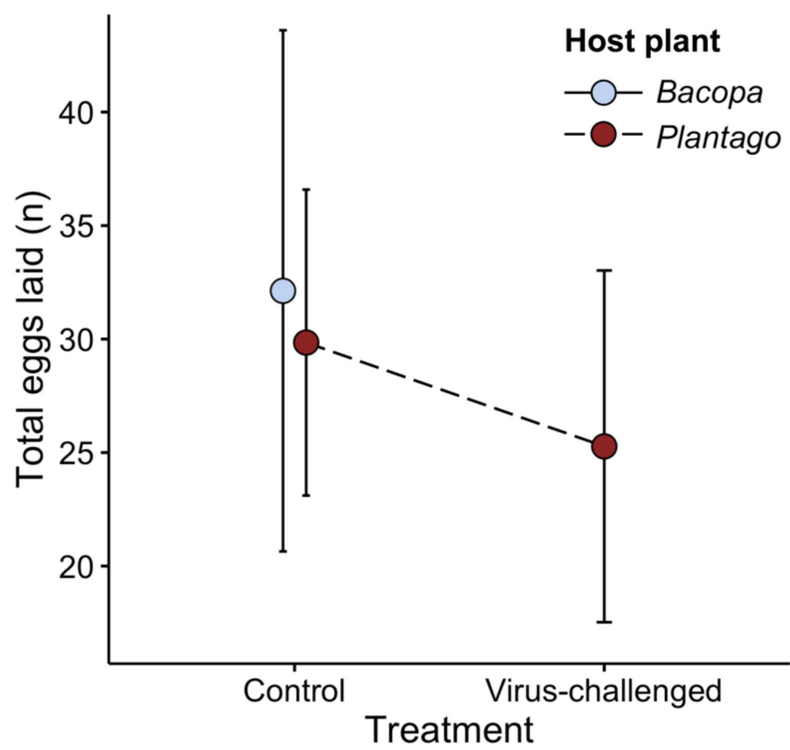


Figure 5



APPENDIX

Supplemental Methods: Viral amplification and sequencing

To examine the similarity between JcDV detected in *Anartia jatrophae* and the laboratory-propagated isolate used in this study, we sequenced the primary capsid gene of JcDV (VP4) from wild-collected butterflies. Extracted DNA from all butterflies found to contain JcDV through quantitative PCR (n = 12; see protocol in Section 2.2.2) underwent nested PCR using external and internal primers for the VP4 gene designed based on the published genome for JcDV (GenBank accession number: KC883978; Pham et al., 2013). This nested approach was utilized in order to maximize amplification of the VP4 gene, as the overall concentration of viral DNA was low within each sample of butterfly DNA.

The first round of PCR was performed using external primers (forward: 5'-ACGCTCCACATAACTCGCAA-3'; reverse: 5'-GGTGCACTAGTAGCAGTGGG-3') with GoTaq® Flexi DNA Polymerase, Green GoTaq® Flexi Buffer, DNA Nucleotide Mix (10 mM), and magnesium chloride (all from Promega, Madison, WI, USA) at a total reaction volume of 25 µl. Reactions were run on a Bio-Rad T100 Thermal Cycler using the following protocol: initial denaturation at 95°C for 2 minutes; followed by 45 cycles of 95°C for 30 seconds (denaturation), 57°C for 30 seconds (annealing), and 72°C for 50 seconds (extension); followed by a final extension step at 72°C for 5 minutes.

Each PCR product from the first round then underwent a second round of nested PCR using internal primers (forward: 5'-TCCTAGTTCTTCCGGAGCAA-3'; reverse 5'-TGATCTATCAATACCCCATCCAAGT-3') using the same reagents and PCR protocol as above, with the exception of a modified annealing temperature of 60°C. PCR products from the second round were then visualized using gel electrophoresis on 1% agarose gels

stained with ethidium bromide. All samples that showed clear bands (*n* =4) were purified using QIAquick PCR Purification Kits (Qiagen) and submitted to the Nevada Genomics Center in Reno, NV for Sanger sequencing. Resulting sequences were trimmed and aligned using Unipro UGENE (Okonechnikov et al., 2012) (Figure S1), and sequence identity (%) with the JcDV "Oxford" isolate (Pham et al., 2013) was evaluated.

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Table S1 Sampling details for *Anartia jatrophae* butterflies collected from the wild in Florida, USA in April 2017. Butterflies from each site were screened for the presence of Junonia coenia densovirus. Sample sizes for butterflies screened (n = 95 total) and butterflies in which the virus was detected (n = 11 total) are provided for each site.

Site ID	**Butterflies screened (*n*)**	**Virus present (*n*)**	**Latitude**	**Longitude**
AB	2	1	27.20791	-81.34508
CP	3	0	26.86702	-80.62888
LG	3	1	27.61612	-80.38095
MF	9	2	26.98291	-82.27898
OS	26	4	26.62451	-81.36967
PR	27	2	26.56415	-81.32753
SI	25	1	26.57371	-81.37381

Table S2 Effects of host plant species and viral inoculation on survival of *Anartia jatrophae* individuals. Logistic regression was used to evaluate the effects of host plant species (*Bacopa monnieri* or *Plantago lanceolata*), treatment (inoculated with Junonia coenia densovirus or control), immune assessment (Y/N), and the interaction between host plant species and treatment on probability of survival to the adult stage (Y/N). Larval host plant species did not significantly impact survival in control individuals; however, the interaction between host plant and treatment indicates that inoculation with JcDV had a negative effect on survival in individuals using the native *Bacopa,* but no effect on survival in individuals using the exotic *Plantago*. Additionally, undergoing immune assessment during the larval stage reduced survival across all groups.

Predictor	*Odds Ratio*	*95% CI*	*z*	*p*
Host plant (*Plantago*)	1.95	0.66 – 6.10	1.19	0.234
Treatment *(virus*)	0.26	0.10 – 0.67	-2.73	**0.006**
Immune assayed (*yes*)	0.11	0.04 – 0.25	-4.99	**<0.001**
Host plant (*Plantago)* x treatment (*virus*)	5.73	1.21 – 29.01	2.17	**0.030**
n	244			
Tjur's R^2	0.27			

Table S3 Effects of host plant species and immune responses on survival and viral burdens of *Anartia jatrophae* following inoculation with Junonia coenia densovirus. Logistic regression was used to evaluate the effects of host plant (*Bacopa monnieri* or *Plantago lanceolata*), each larval immune parameter (hemocyte concentration in the hemolymph or melanization of an abiotic implant), and their two-way interactions on probability of survival to the adult stage ("Survival). Postmortem viral burdens were compared across the same predictors using multiple regression ("Viral load"). Neither hemocyte concentrations nor melanization score were significantly associated with survival. Viral load was positively associated with melanization score in individuals reared on *Bacopa,* while hemocytes were not associated with viral load on either plant.

	Survival			**Viral load**		
Predictors	*Odds Ratio [95% CI]*	*z*	*p*	*Estimate ± SE*	*t*	*p*
Host plant (*Plantago)*	0.92 [0.01 – 179.57]	-0.03	0.974	-1.22 ± 4.02	-0.30	0.764
Hemocyte concentration	0.999 [0.997–1.000]	-1.71	0.088	0.0011 ± 0.0015	0.73	0.475
Host plant x hemocytes	1.001 [0.999 – 1.003]	0.89	0.375	-0.0009 ± 0.0017	-0.52	0.605
n	51			27		
R^2	0.27			0.29		
	Survival			**Viral load**		
Predictors	*Odds Ratio [95% CI]*	*z*	*p*	*Estimate ± SE*	*t*	*p*
Host plant (*Plantago)*	4.11 [0.36 – 119.53]	0.95	0.342	-2.84 ± 2.15	-0.30	0.197
Melanization score	0.999 [0.998–1.001]	-0.49	0.627	0.0032 ± 0.0015	0.73	**0.042**
Host plant x melanization	1.001 [0.997 – 1.005]	0.60	0.551	-0.0014 ± 0.0026	-0.52	0.599
n	58			31		
R^2	0.25			0.44		

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Figure S1 Multiple alignment comparing sequences of the Junonia coenia densovirus VP4 gene isolated from wild *Anartia jatrophae* butterflies (OS08, OS24, AB01, OS25) to the published genome for this pathogen (KC883978 REF; Pham et al., 2013). Polymorphism was evident at only one site (208) out of the 448 nucleotide-long amplicon, indicating 99-100% sequence identity across all isolates. The "M" present in the KC883978 sequence (location 9) refers to the occurrence of adenine (A) or cytosine (C).

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AJ VP4 OS08    CTACTTCTATTGACGTTGAAATGGCTATGTCATTACCTGGAACTGGTTCTGGAACATCATCTGGAGGAGGCAACACTTCAGGTCAAGAGGTTTATGTAAT   100
AJ VP4 OS24    CTACTTCTATTGACGTTGAAATGGCTATGTCATTACCTGGAACTGGTTCTGGAACATCATCTGGAGGAGGCAACACTTCAGGTCAAGAGGTTTATGTAAT   100
AJ VP4 AB01    CTACTTCTATTGACGTTGAAATGGCTATGTCATTACCTGGAACTGGTTCTGGAACATCATCTGGAGGAGGCAACACTTCAGGTCAAGAGGTTTATGTAAT   100
AJ VP4 OS25    CTACTTCTATTGACGTTGAAATGGCTATGTCATTACCTGGAACTGGTTCTGGAACATCATCTGGAGGAGGCAACACTTCAGGTCAAGAGGTTTATGTAAT   100
KC883978 REF   CTACTTCTMTTGACGTTGAAATGGCTATGTCATTACCTGGAACTGGTTCTGGAACATCATCTGGAGGAGGCAACACTTCAGGTCAAGAGGTTTATGTAAT   100

AJ VP4 OS08    TCCTCGTCCATTTTCGAACTTTGGTAAAAAATTAAGTACTTATACAAAGTCTCATAAATTTATGATATTTGGTCTTGCCAATAATGTTATTGGACCTACA   200
AJ VP4 OS24    TCCTCGTCCATTTTCGAACTTTGGTAAAAAATTAAGTACTTATACAAAGTCTCATAAATTTATGATATTTGGTCTTGCCAATAATGTTATTGGACCTACA   200
AJ VP4 AB01    TCCTCGTCCATTTTCGAACTTTGGTAAAAAATTAAGTACTTATACAAAGTCTCATAAATTTATGATATTTGGTCTTGCCAATAATGTTATTGGACCTACA   200
AJ VP4 OS25    TCCTCGTCCATTTTCGAACTTTGGTAAAAAATTAAGTACTTATACAAAGTCTCATAAATTTATGATATTTGGTCTTGCCAATAATGTTATTGGACCTACA   200
KC883978 REF   TCCTCGTCCATTTTCGAACTTTGGTAAAAAATTAAGTACTTATACAAAGTCTCATAAATTTATGATATTTGGTCTTGCCAATAATGTTATTGGACCTACA   200

AJ VP4 OS08    GGTACTGGTACAACAGCTGTAAATCGTTTAATTACAACTTGTTTGGCTGAAATTCCATGGCAGAAATTGCCTTTGTATATGAACCAATCTGAATTTGATT   300
AJ VP4 OS24    GGTACTGGTACAACAGCTGTAAATCGTTTAATTACAACTTGTTTGGCTGAAATTCCATGGCAGAAATTGCCTTTGTATATGAACCAATCTGAATTTGATT   300
AJ VP4 AB01    GGTACTGATACAACAGCTGTAAATCGTTTAATTACAACTTGTTTGGCTGAAATTCCATGGCAGAAATTGCCTTTGTATATGAACCAATCTGAATTTGATT   300
AJ VP4 OS25    GGTACTGATACAACAGCTGTAAATCGTTTAATTACAACTTGTTTGGCTGAAATTCCATGGCAGAAATTGCCTTTGTATATGAACCAATCTGAATTTGATT   300
KC883978 REF   GGTACTGGTACAACAGCTGTAAATCGTTTAATTACAACTTGTTTGGCTGAAATTCCATGGCAGAAATTGCCTTTGTATATGAACCAATCTGAATTTGATT   300

AJ VP4 OS08    TATTACCTCCTGGTAGTAGAGTAGTTGAATGTAATGTTAAAGTAATATTCAGAACTAATCGTATTGCATTTGAGACTAGTTCAACTGCTACTAAACAAGC   400
AJ VP4 OS24    TATTACCTCCTGGTAGTAGAGTAGTTGAATGTAATGTTAAAGTAATATTCAGAACTAATCGTATTGCATTTGAGACTAGTTCAACTGCTACTAAACAAGC   400
AJ VP4 AB01    TATTACCTCCTGGTAGTAGAGTAGTTGAATGTAATGTTAAAGTAATATTCAGAACTAATCGTATTGCATTTGAGACTAGTTCAACTGCTACTAAACAAGC   400
AJ VP4 OS25    TATTACCTCCTGGTAGTAGAGTAGTTGAATGTAATGTTAAAGTAATATTCAGAACTAATCGTATTGCATTTGAGACTAGTTCAACTGCTACTAAACAAGC   400
KC883978 REF   TATTACCTCCTGGTAGTAGAGTAGTTGAATGTAATGTTAAAGTAATATTCAGAACTAATCGTATTGCATTTGAGACTAGTTCAACTGCTACTAAACAAGC   400

AJ VP4 OS08    TACATTGAATCAAATATCTAATTTACAAACTGCTGTTGGATTAAATAA   448
AJ VP4 OS24    TACATTGAATCAAATATCTAATTTACAAACTGCTGTTGGATTAAATAA   448     X  non-conserved
AJ VP4 AB01    TACATTGAATCAAATATCTAATTTACAAACTGCTGTTGGATTAAATAA   448     X  ≥50% conserved
AJ VP4 OS25    TACATTGAATCAAATATCTAATTTACAAACTGCTGTTGGATTAAATAA   448
KC883978 REF   TACATTGAATCAAATATCTAATTTACAAACTGCTGTTGGATTAAATAA   448
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Chapter Four

Dose-dependent dynamics of densovirus infection in two nymphalid butterfly species utilizing native or exotic host plants

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ABSTRACT

Insects are attacked by a diverse range of pathogens in the wild. In herbivorous species, larval host plants can play a critical role in mediating susceptibility to infection. Characterizing plant-mediated effects on herbivore-pathogen interactions may provide insight into patterns of infection across wild populations. In this study, we investigated the effects of host plant use on entomopathogen infection across a range of doses in two North American butterflies: *Euphydryas phaeton* (Nymphalidae) and *Anartia jatrophae* (Nymphalidae). Both of these herbivores recently incorporated the same exotic plant, *Plantago lanceolata* (Plantaginaceae)*,* into their host range and are naturally infected by the same pathogen, Junonia coenia densovirus (*Parvoviridae*) in wild populations. We performed two factorial experiments in which *E. phaeton* and *A. jatrophae* were reared on either *P. lanceolata* or a native host plant and inoculated with a low, medium, or high dose of the virus. Overall, we found that the outcomes of infection were highly dose-dependent, with higher viral doses resulting in faster time-to-death, greater mortality, and higher viral burdens. In *E. phaeton*, however, neither survival nor viral burdens varied depending upon the host plant that was consumed. In contrast, host plant use had a strong effect on viral load in *A. jatrophae,* with the exotic plant appearing to enhance resistance to infection. Together, these results illustrate that host plant use may exert a stronger influence on resistance to infection in certain systems, relative to others, highlighting the importance of investigating plant-herbivore relationships within a tritrophic framework.

1 | INTRODUCTION

Pathogens are a ubiquitous and integral component of ecosystems worldwide, exerting powerful influences on the ecology and evolution of the organisms that they infect (Gulland, 1995) and the broader communities that they inhabit (Dobson and Hudson, 1986; French and Holmes, 2020; Paseka et al., 2020). In insects, pathogens can play a major role in regulating population cycles (Dwyer et al., 2004; Myers and Cory, 2013) and have been linked to declines and/or management concerns in economically significant species (Cameron et al., 2011; Cox-Foster et al., 2007; Eilenberg and Jensen, 2018). Though insects are attacked by a diverse range of pathogens, including bacteria, fungi, viruses, and protozoa (Anderson and May, 1981; Briggs et al., 1995), research on entomopathogens has historically been concentrated on their potential use as biological control agents (Lacey et al., 2015; Moscardi, 1999), with less attention given to their influence in "natural" or unmanaged systems (but see Alger et al., 2018; Altizer et al., 2000; Cory and Myers, 2009 for examples). Such systems offer unique opportunities for insight into the role of ecological factors in shaping insect-pathogen interactions (Cory, 2010; De Roode et al., 2008), with applications to both basic and applied insect research.

Much research on ecological sources of variation in insect-pathogen relationships has highlighted the importance of larval host plants in mediating infection in herbivorous species (reviewed in Cory and Hoover, 2006). Both inter- and intraspecific variation in host plant quality, which may include aspects of nutritional and/or secondary chemistry, can impact susceptibility to infection in the herbivores that consume them (Duffey et al., 1995; Shikano et al., 2010). In well-studied entomopathogens, including baculoviruses and the bacterium *Bacillus thuringiensis,* it has been repeatedly demonstrated that host

mortality, pathogen replication, and speed-of-kill can vary markedly depending on the chemistry and/or identity of the host plant consumed by the herbivore (Ali et al., 1998; Duffey et al., 1995; Kouassi et al., 2001; Raymond et al., 2002). In baculovirus systems, much attention has been given to the potential for direct inhibition of pathogens by host plant constituents within the midgut (e.g., Felton and Duffey, 1990; Hoover et al., 1998). However, host plants may also mediate herbivore-pathogen interactions through indirect effects, including impacts of macronutrients and phytochemicals on herbivore immune responses (Smilanich and Muchoney, 2022) or overall body condition (Shikano et al., 2010) both prior to and following infection. Though it is clear that host plant use can impact susceptibility to infection in many cases, the majority of research on this subject has focused on a small suite of pathogens and host species (Cory and Hoover, 2006). Non-model systems offer exciting opportunities to investigate the generalizability of these patterns across diverse pathogen taxa and gain insight into the broader role of bottom-up effects in regulating insect-pathogen interactions in wild populations.

In this study, we examined the role of host plant use in mediating interactions between insect herbivores and a pathogenic virus, Junonia coenia densovirus (hereafter, JcDV). JcDV is a nonenveloped, single-stranded DNA virus in the family *Parvoviridae* (*Densovirinae: Lepidopteran protoambidensovirus 1*) that infects hosts within the order Lepidoptera. Insects become infected by JcDV during the larval stage through ingestion of viral particles on host plants, which enter the hemocoel via the midgut and replicate in tracheae, hemocytes, visceral muscles, and epidermis (Mutuel et al., 2010; Wang et al., 2013). Infection results in hypoxia and disruptions to molting and metamorphosis, with mortality depending upon the dose that is ingested (Mutuel et al., 2010; Smilanich et al.,

2018). Though capable of infecting Lepidoptera in multiple families in a laboratory setting (Mutuel et al., 2010; Resnik and Smilanich, 2020; Rivers and Longworth, 1968), the prevalence, host range, and ecological impact of this pathogen in wild herbivore populations is only recently beginning to be characterized (Muchoney et al., 2022).

Our research has documented the occurrence of JcDV across wild populations of two North American butterfly species, *Euphydryas phaeton* Drury (Nymphalidae), the Baltimore checkerspot (Muchoney et al., 2022), and *Anartia jatrophae* L. (Nymphalidae), the white peacock (Muchoney et al., unpublished data). Both of these herbivores recently incorporated the same exotic plant, *Plantago lanceolata* L. (Plantaginaceae) (hereafter, *Plantago*), into their dietary ranges, and exhibit differences in growth and performance when utilizing this exotic species, compared to native host plant species (Bowers et al., 1992; Brown et al., 2017; Knerl and Bowers, 2013; Lampert et al., 2014; Stamp, 1979). Notably, *Plantago* contains iridoid glycosides (IGs), a class of terpene-derived secondary metabolites that are toxic and/or deterrent to many herbivores (Bowers and Puttick, 1988) and play an important role in mediating tritrophic interactions (Bowers, 1991; Dyer and Bowers, 1996; Smilanich et al., 2009a; Theodoratus and Bowers, 1999). Both *E. phaeton* and *A. jatrophae* are able to consume and sequester these phytochemicals; however, *E. phaeton* is a specialist on plants containing IGs and sequesters these compounds in high concentrations (Bowers, 1980), whereas *A. jatrophae* consumes plants with or without IGs, and sequesters lower amounts (Knerl and Bowers, 2013; Lampert et al., 2014).

The recent colonization of *Plantago* by both *E. phaeton* and *A. jatrophae* has provided the opportunity to investigate how interactions with the focal viral pathogen, JcDV, vary depending on host plant use. In a field survey of *E. phaeton* caterpillars, we

found that JcDV loads of naturally infected individuals were higher in populations using *Plantago,* compared to the primary native host plant for this species, *Chelone glabra* L. (hereafter, *Chelone*; Plantaginaceae) (Muchoney et al., 2022), presenting the question of whether use of the exotic host plant increases susceptibility to this pathogen. In contrast, JcDV-infected *A. jatrophae* exhibited reduced viral loads and higher survival when using *Plantago*, compared to the native host plant *Bacopa monnieri* L. Pennell (hereafter, *Bacopa*; Plantaginaceae), in a laboratory setting (Muchoney et al., unpublished data), suggesting that using the exotic plant may improve resistance to infection in this species.

In this study, we investigated the effects of host plant use on herbivore-virus interactions across a range of viral doses, aiming to understand whether consuming an exotic host plant, *Plantago,* enhances or reduces resistance to viral infection in the focal herbivore species. We performed two laboratory experiments in which herbivores were reared on either *Plantago* or a native host plant (*Chelone* for *E. phaeton; Bacopa* for *A. jatrophae*) and orally challenged with one of three doses of JcDV. Our specific goals differed slightly between the two herbivores. In *E. phaeton,* we aimed to determine the effects of host plant use on survival and postmortem viral burdens following different doses. As the influence of host plant use on survival and postmortem viral burdens was previously characterized in *A. jatrophae* and found to be substantial (Muchoney et al., unpublished data), we aimed to determine how pathogen replication varies over time on the two plant species. By evaluating variation in the dynamics and outcomes of infection in two herbivores using either native or exotic host plants, we provide insight into the role of diet in mediating the impacts of a naturally occurring pathogen on its insect hosts.

2 | METHODS

2.1 Overview of experiments

To examine the effects of host plant use on herbivore-virus interactions across a range of doses, two factorial laboratory experiments were performed, the first focusing on *E. phaeton* and the second focusing on *A. jatrophae.* In both experiments, herbivores were reared on either the exotic host plant, *Plantago,* or a native host plant (*Chelone* for *E. phaeton; Bacopa* for *A. jatrophae*) and orally inoculated with one of three doses of purified JcDV [low: 1.0×10^7 viral genomes (vg), medium: 1.0×10^9 vg, or high: 5.1×10^{10} vg]. The lowest viral dose employed in these experiments was found to result in 20% mortality of *A. jatrophae* in a previous study (Muchoney et al., unpublished data); thus, sequentially higher doses were selected for the "medium" and "high" treatments in order to examine the outcomes of more severe JcDV infections. Notably, the highest dose used in these experiments (5.1×10^{10} vg) was similar to a dose that resulted in 80% pre-adult mortality of final-instar larvae in another lepidopteran species, *Spodoptera frugiperda* (5.0×10^{10} vg; Mutuel et al., 2010) and was therefore expected to result in high mortality.

In both experiments, caterpillars were orally inoculated with their assigned viral dose on the first day following molting to the final (sixth) larval instar. In the *E. phaeton* experiment (Experiment 1), JcDV-challenged caterpillars were subsequently reared out until death in the larval, pupal, or adult stage to assess the effects of host plant species on survival, development, and postmortem viral loads (i.e., viral yield) at different doses. In contrast, JcDV-challenged individuals in the *A. jatrophae* experiment (Experiment 2) were sacrificed at varying time points following inoculation in order to gain insight into the time course of viral replication in herbivores reared on different host plant species.

2.2 Experiment 1: *Euphydryas phaeton*

Euphydryas phaeton caterpillars used in this experiment were the offspring of individuals collected from wild populations in Barnstable, MA and Montpelier, VT in May 2017. Caterpillars were reared at the University of Colorado, Boulder on either the native host plant, *Chelone,* or the exotic host plant, *Plantago,* throughout pre-diapause development (larval instars 1-3) and underwent obligate overwintering diapause at this laboratory during the fourth instar. In late May 2018, caterpillars were transferred to the University of Nevada, Reno, where they emerged from diapause and resumed feeding on their respective host plant species. From this point onward, caterpillars were reared in incubators in individual 2 oz plastic cups using a 16-hour photoperiod (day temperature: 25°C, night temperature: 20°C) and fed ad libitum with *Chelone* or *Plantago* foliage. *Plantago* leaves were collected from the wild in Reno, NV, while *Chelone* leaves were collected from Montpelier, VT and stored in a refrigerator. Leaf surfaces were sterilized prior to feeding by soaking in 5% bleach solution for 10 min and rinsing thoroughly.

Caterpillars reared on each host plant species were randomly assigned to one of four treatment groups: the low, medium, or high viral dose (n = 9-11 per host plant for each dose) or a control group (no virus; n = 11 per host plant). On the first day following molting to the sixth instar, caterpillars in the low-, medium-, and high-dose groups were orally inoculated with their assigned dose of JcDV. Each caterpillar was presented with a 10 mm leaf disc (*Chelone* or *Plantago,* according to host plant group) with 1.0×10^7, 1.0×10^9, or 5.1×10^9 viral genomes suspended in 1 µl of DI water pipetted onto the surface and allowed to dry. Caterpillars were given 24 h to consume the leaf disc, and those that did not were either re-inoculated the following day or excluded from the experiment.

Following inoculation, caterpillars continued to be fed according to their host plant group and were checked daily to monitor development and survival time. Control insects were maintained in an incubator at a separate location from JcDV-challenged insects to avoid cross-contamination between groups. In addition, frass was collected from each JcDV-challenged caterpillar on days 1-5 following inoculation in order to investigate the amount of viral DNA that was excreted following each dose. Sterile technique was used between handling of each insect and frass sample, which entailed soaking instruments in DNA-Erase (MP Biomedicals, Santa Ana, CA, USA), a 30% solution of bleach, and a 70% solution of ethanol. Individuals that reached the pupal stage were weighed and transferred to 32 oz plastic containers with mesh lids for eclosion, and butterflies were maintained on a diet of 10% honey water until death. Following death in the larval, pupal, or adult stage, all individuals were promptly frozen for viral screening.

2.3 Experiment 2: *Anartia jatrophae*

Anartia jatrophae caterpillars used in this experiment were obtained from a colony maintained at the University of Colorado, Boulder, which originated from wild butterflies collected from Florida in April 2017. Insects from this colony were shipped to the University of Nevada, Reno (UNR), and a mixture of first- and third-generation UNR offspring were used for the experiment. Approximately half of experimental insects were reared on the native host plant, *Bacopa* ($n = 185$), while the other half were reared on the exotic host plant, *Plantago* ($n = 225$), throughout larval development. Though *Bacopa*- and *Plantago*-fed individuals were present in both generation sets, the first generation primarily consisted of *Plantago*-fed larvae, while the third generation primarily consisted of *Bacopa*-fed larvae, due to variation in host plant availability. Caterpillars were reared

in incubators under the same conditions described for Experiment 1 (above) and fed ad libitum with sterilized *Bacopa* or *Plantago* foliage. *Bacopa* was grown in a greenhouse at the University of Colorado, Boulder and stored in a refrigerator, whereas *Plantago* was either collected from the wild in Reno, NV or grown in a hydroponics system at UNR.

Caterpillars reared on each host plant species were randomly assigned to receive either the low, medium, or high dose of JcDV (n = 61-87 per host plant for each dose) on the first day after molting to the sixth instar. Oral inoculations were performed following the protocol described for Experiment 1 (above), after which larvae continued to be fed according to their host plant group. Beginning on day two following inoculation, subsets of caterpillars from each dose and host plant group were freeze-killed each day until day six post-inoculation (n = 8-14 per day). An additional subset of each group was sacrificed on the first day following pupation (n = 4-9) to examine viral burdens in pupae.

2.4 Viral screening and quantification

To detect and quantify JcDV infection in *E. phaeton* and *A. jatrophae*, total DNA was extracted from a tissue sample from each insect using Qiagen DNeasy 96 Blood and Tissue Kits (Qiagen, Hilden, North Rhine-Westphalia, Germany) following the Protocol for Purification of Total DNA from Animal Tissues. Whole caterpillars and pupae were homogenized using a Qiagen TissueLyser II and DNA was extracted from a 20 mg aliquot of tissue, whereas whole bodies (with wings removed) were used for butterflies.

Extracted DNA was screened for JcDV using quantitative PCR, with primers specific to the VP4 capsid protein gene of JcDV (Wang et al., 2013) and arthropod 28S rDNA primers (Nice et al., 2009) as an internal control. DNA samples were screened in duplicate for both VP4 and 28S using iTaq Universal SYBR Green Supermix (Bio-Rad,

Hercules, CA, USA) at a volume of 10 µl. Reactions were run on a Bio-Rad CFX96 Optics Module with C1000 Thermal Cycler following the protocols of Muchoney *et al.* (2022). Viral loads were calculated as $2^{-\Delta Ct}$ (Schmittgen and Livak, 2008), representing the abundance of the JcDV VP4 gene relative to the abundance of the internal control gene [ΔC_t = mean C_t (threshold cycle) for VP4 – mean C_t for 28S], and log-transformed.

To detect and quantify JcDV in *E. phaeton* frass, total DNA was extracted from each sample following the protocol described above. For frass samples weighing less than 20 mg, whole samples were used, while a 20 mg aliquot of homogenized frass was used for larger samples. Extracted DNA was screened in duplicate for the JcDV VP4 gene using the qPCR protocol above, and viral quantity was estimated using a standard curve. Briefly, a stock solution of JcDV was serially diluted and used to generate a standard curve over seven orders of magnitude (1.0×10^3 to 1.0×10^9 viral genomes), which was used to calculate absolute quantity of JcDV in each sample of extracted DNA based on C_t values for the VP4 gene [expressed as log-transformed viral genomes (vg)].

2.5 Statistical analyses

2.5.1. Experiment 1: Euphydryas phaeton

All statistical analyses were performed in R version 4.1.0 (R Core Team, 2021). The dose-dependent effect of JcDV on *E. phaeton* survival was evaluated using logistic regression, with viral dose (log-transformed viral genomes), host plant species (*Chelone* or *Plantago*), and their interaction as predictors and survival to the adult stage (Y/N) as the response. Based on this model, the dose required to kill 50% of insects prior to the adult stage (LD_{50}) was calculated. Using the 'survival' package (Therneau et al., 2022), a Cox proportional hazard model was fitted to assess the effects of viral treatment (low,

medium, or high dose) and host plant on survival following inoculation, and Kaplan-Meier survival analysis was used to illustrate daily survivorship across viral doses.

The probability of detecting JcDV (Y/N) in virus-challenged insects following death in the larval, pupal, or adult stage was compared across viral doses using logistic regression, and the relationship between viral detection and survival was examined using Pearson's chi-squared test. Pupal mass and adult longevity (n days between eclosion and death) were compared between butterflies that maintained a detectable infection and those that did not using two linear regression models, which included sex as a predictor. Within the subset of insects that harbored a detectable infection, multiple regression was used to evaluate the effects of viral dose, host plant species, the interaction between dose and host plant, and life stage at death (larva, pupa, or adult) on postmortem viral load.

The probability of detecting JcDV (Y/N) in the frass of virus-challenged larvae on days 1-5 following inoculation was evaluated using mixed-effects logistic regression, with viral dose and day as fixed effects and random intercepts for each individual larva. Viral quantities in frass were compared across host plant species and doses using a linear mixed-effects model, which included dose, host plant, day, and the interaction between dose and host plant as fixed effects, with random intercepts for individuals. Both mixed-effects models were fitted using the 'lme4' package (Bates et al., 2015), with p-values for the linear mixed-effects model generated using 'lmerTest' (Kuznetsova et al., 2017).

Finally, the relationship between initial viral dose, postmortem viral load, and the quantity of virus excreted in the frass was investigated using a multiple regression model, which included viral dose, mean viral quantity in frass, and their two-way interaction as predictors, with postmortem load in each insect as the response. To probe this interaction,

a Johnson–Neyman interval for significance of the conditional effect of JcDV quantity in frass on viral load in insects was calculated with the 'interactions' package (Long, 2019).

2.5.2 Experiment 2: Anartia jatrophae

The probability of detecting JcDV (Y/N) in *A. jatrophae* caterpillars following inoculation was compared across viral doses, host plant species (*Bacopa* or *Plantago*) and day sacrificed (day 2-6 post-inoculation) using logistic regression. For the subset of caterpillars that harbored a detectable infection, multiple regression was used to evaluate the effects of viral dose, host plant species, and the interaction between dose and host plant species on viral load. Viral loads were also compared between individuals that were sacrificed during the larval stage and those that were sacrificed as pupae using a multiple regression model, which evaluated the effects of viral dose, host plant species, life stage (larva or pupa), and the interaction between host plant species and life stage. The effects of host plant species and viral dose on the body mass of caterpillars and pupae at their time of sacrifice were investigated using separate multiple regression models, which included viral dose, host plant, and day post-inoculation (for larvae only) as predictors.

3 | RESULTS

3.1 Experiment 1: *Euphydryas phaeton*

3.1.1 Survival and longevity

In *E. phaeton* challenged with JcDV, survival to the adult stage decreased with viral dose [odds ratio (OR) = 0.11, 95% confidence interval (CI) = (0.03-0.26), $z = -4.3$, $p < 0.0001$], with 100% mortality observed in individuals that received the highest dose (Figure 1). In contrast, 100% of uninfected controls successfully reached the adult stage

(Appendix: Figure S1). This negative relationship between viral dose and survival was highly consistent across individuals reared on the two host plants (Figure S1) [odds ratio on *Chelone*: 0.10 (0.02-0.32); odds ratio on *Plantago*: 0.12 (0.02-0.37)], and the LD_{50} for sixth instar *E. phaeton* on both plants was estimated to be 1.0 x 10^8 viral genomes (vg).

Survival time following inoculation was also reduced at higher viral doses (Figure 1a) [medium dose: Cox proportional hazard ratio (HR) = 27.4, 95% CI = (6.2, 121.4), z = 4.4, $p < 0.0001$; high dose: HR = 28.4, 95% CI = (6.2, 129.6), $z = 4.3$, $p < 0.0001$] but did not differ based upon host plant species [HR = 1.4, 95% CI = (0.7-2.7), $z = 1.0$, p = 0.3]. Individuals that received the lowest dose of JcDV survived for an average of 38 days, successfully reaching the adult stage in 90% of cases (Figure 1b), compared to 15 and 13 days in the medium- and high-dose groups, respectively. Though survival time was similar following medium and high viral doses, individuals that received the highest dose were more likely to die during the larval stage, while a greater proportion of those that received the medium dose died as pupae (Figure 1b) ($X^2 = 9.2$, $df = 1$, $p = 0.002$).

3.1.2 Viral detection and loads in insects

The likelihood of detecting JcDV in virus-challenged individuals following death in the larval, pupal, or adult stage increased with dose [OR = 3.8, 95% CI = (1.7, 16.3), z = 2.6, $p = 0.009$]. All individuals that received the highest dose harbored a detectable infection at their time of death, compared to 95% of the medium-dose group and 60% of the low-dose group (Figure 2a). This pattern was mediated by the higher frequency of survival in these groups (see Figure 1), as JcDV was detected in 100% of individuals that died during the larval or pupal stages, but only 55% of individuals that reached the adult stage ($X^2 = 17.0$, $df = 1$, $p < 0.0001$). Though patterns of viral detection were similar on

the two host plant species (Figure 2a), a greater proportion of butterflies that had been reared on *Plantago* maintained a detectable infection after receiving the lowest dose, compared to *Chelone* [OR = 4.50, 95% CI = (0.65-43.41), $z = 1.5$, $p = 0.1$]. Virus-challenged butterflies that maintained a detectable infection exhibited lower pupal masses than those that did not ($\beta = -41.7 \pm 16.6$, $t = -2.5$, $df = 15$, $p = 0.02$), though adult longevity was similar between the two groups ($\beta = 0.95 \pm 2.97$, $t = 0.3$, $df = 15$, $p = 0.8$).

Within the subset of insects that harbored detectable infections, viral loads also varied according to dose (Figure 2b). There was a positive relationship between initial dose and postmortem load ($\beta = 0.9 \pm 0.3$, $t = 2.6$, $df = 43$, $p = 0.02$), which did not differ based on host plant species (host plant x dose interaction: $\beta = -0.2 \pm 0.4$, $t = -0.7$, $df = 43$, $p = 0.5$). In addition, individuals that died as larvae and pupae harbored substantially higher viral burdens than those that reached the adult stage (Figure S2) (larvae: $\beta = 6.3 \pm 1.0$, $t = 6.2$, $df = 43$, $p < 0.0001$; pupae: $\beta = 8.0 \pm 0.9$, $t = 8.6$, $df = 43$, $p < 0.0001$).

3.1.3 Viral detection and loads in frass

JcDV was detected in frass samples from 100% of virus-challenged caterpillars and detected on all five sampling days in 76% of individuals. Overall, the likelihood of detecting JcDV in frass increased with viral dose [OR = 2.15, 95% CI = (1.19-3.88), $z = 2.5$, $p = 0.01$] and decreased slightly across days following inoculation [OR = 0.68, 95% CI = (0.46-1.00), $z = -2.0$, $p = 0.05$]. The amount of JcDV in each frass sample mirrored this pattern: viral quantity was strongly influenced by the dose that was ingested (Figure 3a) ($\beta = 0.68 \pm 0.11$, $t = 6.4$, $df = 50$, $p < 0.0001$) and decreased over time following inoculation ($\beta = -0.65 \pm 0.04$, $t = -15.7$, $df = 192$, $p < 0.0001$). The relationship between

viral dose and the amount of virus excreted in the frass did not differ based on host plant use (Figure S3) (dose x host plant interaction: $\beta = 0.08 \pm 0.15$, $t = 0.6$, $df = 50$, $p = 0.6$).

The postmortem viral load of each insect was positively associated with both the initial dose that was ingested ($\beta = 11.5 \pm 1.7$, $t = 6.9$, $df = 41$, $p < 0.0001$) and the average amount of JcDV that was excreted in frass ($\beta = 15.9 \pm 2.7$, $t = 6.0$, $df = 41$, $p < 0.0001$). Notably, there was also a negative interaction between viral dose and viral content in frass ($\beta = -1.50 \pm 0.26$, $t = -5.9$, $df = 41$, $p < 0.0001$). This interaction indicates that when larvae consumed lower doses of JcDV, viral quantity in frass was a strong predictor of the postmortem viral burden of the insect; however, this relationship attenuated at higher doses (Figure 3b). The threshold dose at which frass was no longer positively associated with viral load was calculated as 1.1×10^{10} viral genomes (Johnson-Neyman interval); above this value, viral quantity in frass was not a reliable indicator of infection burden.

3.2 Experiment 2: *Anartia jatrophae*

3.2.1 Viral detection and loads in insects

In virus-challenged *A. jatrophae*, JcDV was detected in 95% of individuals that were sacrificed as larvae (2-6 days post-inoculation) and 100% of individuals that were sacrificed following pupation. The likelihood of viral detection did not differ based on viral dose [OR = 1.13, 95% CI = (0.81-1.60), $z = 0.7$, $p = 0.5$] or across days following inoculation [OR = 0.81, 95% CI = (0.59-1.10), $z = -1.3$, $p = 0.2$], but was greater in caterpillars that were reared on the exotic host plant, *Plantago*, compared to the native host plant, *Bacopa* (Figure 4a) [OR = 8.6, 95% CI = (2.3-55.7), $z = 2.8$, $p = 0.005$].

In caterpillars that harbored detectable infections, viral loads increased according to dose (Figure 4b) ($\beta = 0.58 \pm 0.06$, $t = 9.6$, $df = 307$, $p < 0.0001$) and were substantially

higher in larvae reared on *Bacopa* than those reared on *Plantago* (β = -1.0 ± 0.19, t = -5.6, df = 307, p < 0.0001) across all doses and sampling days (Figure S4). Altogether, larvae that consumed the native *Bacopa* exhibited 12-fold higher loads than those that consumed the exotic *Plantago.* In addition, infection loads decreased only slightly over time following inoculation (Figure 5a) (β = -0.11 ± 0.07 t = -1.7, df = 307, p = 0.08). However, individuals that were sacrificed as pupae harbored lower viral burdens than those that were sacrificed as larvae (Figure 5b). This decrease in load following pupation was more pronounced in insects reared on *Bacopa* (β = -2.82 ± 0.43 t = -6.6, df = 346, p < 0.0001) compared to *Plantago* (β = -0.75 ± 0.37, t = -2.0, df = 346, p = 0.04) (Figure S4).

3.2.2 Caterpillar growth and pupal mass

Caterpillar body mass was higher in individuals that developed for a longer period of time before sacrifice (β = 28.0 ± 4.0, t = 7.0, df = 304, p < 0.0001), increasing by 39% between day 2 and day 6 post-inoculation, but did not vary depending on viral dose (β = -3.6 ± 3.7, t = -1.0, df = 304, p = 0.3). However, larvae reared on *Plantago* were larger across all sampling days (β = 38.7 ± 11.4, t = 3.4, df = 304, p = 0.0007), with 16% higher overall mass. A similar pattern was evident in individuals that were sacrificed as pupae: pupal mass was 65% higher on *Plantago* than *Bacopa* (β = 145.0 ± 33.8, t = 4.3, df = 34, p = 0.0001) but was not impacted by viral dose (β = -3.1 ± 10.4 t = -0.3, df = 34, p = 0.8).

4 | DISCUSSION

4.1 Experiment 1: *Euphydryas phaeton*

In *E. phaeton,* the outcomes of JcDV infection were highly dose-dependent, with higher viral doses resulting in faster time-to-death (Figure 1a), greater mortality during

the larval and pupal stages (Figure 1b), and higher postmortem viral burdens (Figure 2b). However, patterns of survival (Figure S1) and viral loads (Figure 2b) were largely similar in herbivores reared on the two host plant species, suggesting that host plant use does not strongly mediate resistance to infection in this species within a controlled environment. Our previous research documented higher JcDV burdens on the exotic plant, *Plantago,* compared to the native *Chelone,* in field-collected *E. phaeton* during the post-diapause stage (Muchoney et al., 2022; Muchoney et al., unpublished data), raising the question of whether using *Plantago* increases *E. phaeton'*s vulnerability to this pathogen. However, when naturally infected individuals were reared out to assess survivorship, we did not discover differences in the ability to survive infection on the two plants. The findings of this study are consistent with this result, indicating that both the severity of infection (Figure 2b) and its outcomes for survival and development (Figure 1a-b) are primarily determined by the dose that is ingested, rather than the host plant that is consumed.

Notably, the primary difference observed between the two plants in *E. phaeton* involved the frequency with which JcDV was detected in inoculated insects following their death, which was higher in *Plantago*-fed individuals that survived to the adult stage after receiving the lowest viral dose (Figure 2a). Assuming viral infection was initially established after inoculation, this pattern may indicate a reduced capacity to suppress or clear infection before reaching the adult stage. Alternatively, it is possible that a greater proportion of *Plantago*-fed individuals initially contracted infection, while *Chelone*-fed individuals were better able to avoid or limit its establishment (i.e., qualitative resistance; De Roode et al., 2011). Though this pattern was only evident at the lowest viral dose, we found that post-mortem loads of individuals that received this dose [median (IQR): -6.7 (-

7.5, -5.1)] were highly similar to those of JcDV-infected individuals collected from the wild in two different studies [-6.5 (-7.2, -5.7); Muchoney et al., 2022] [-6.7 (-7.1, -5.7); Muchoney et al., unpublished data]. Thus, the "low" dose employed in this study (1.0 x 10^7 vg) likely represents a field-relevant dose for *E. phaeton.* As maintaining a detectable infection into the adult stage entailed a cost for performance (lower pupal weight), we may expect this sublethal effect to be more prevalent in populations using *Plantago,* compared to *Chelone*. In addition, higher incidence of infection in adults may provide increased opportunity for vertical transmission of the virus on *Plantago*, though the efficiency with which JcDV is transmitted to offspring is currently unknown.

Together, these results suggest that higher viral burdens observed in the wild on *Plantago* may be the product of additional abiotic or biotic factors that differ between populations utilizing the two plants, rather than intrinsic differences in the herbivore's ability to resist infection. One possibility is that JcDV occurrence in the environment (i.e., on host plant surfaces) is higher on *Plantago,* thereby influencing the amount of virus that is ingested throughout development. As host plant surface chemistry (Young et al., 1977), architecture (Duffey et al., 1995), and habitat (Raymond et al., 2005) have all been shown to influence entomopathogen persistence on the phylloplane, this possibility warrants investigation. However, we discovered that herbivores challenged with a field-relevant dose of JcDV were more likely to harbor an infection following metamorphosis on *Plantago* (Figure 2a), which could give rise to sublethal effects and contribute to high viral burdens in populations using *Plantago* if this pathogen is transmitted to offspring.

4.2 Experiment 2: *Anartia jatrophae*

In contrast to the patterns documented in *E. phaeton*, JcDV infection appears to be strongly modulated by host plant use in *A. jatrophae.* While viral burdens varied as expected according to dose that was ingested (Figure 5a), they were also consistently higher in caterpillars reared on *Bacopa,* compared to the exotic plant, *Plantago,* across a range of infectious doses (Figure 4b) and across time following inoculation (Figure S4), indicating that viral replication was suppressed in larvae consuming this exotic plant. These findings corroborate the results of our previous study within this system, which found that postmortem loads were reduced, and survival was dramatically enhanced, in individuals using *Plantago* following inoculation with the lowest dose of JcDV used in the present study (Muchoney et al., unpublished data). Together, these results support the hypothesis that survival of viral infection is improved when *A. jatrophae* is reared on *Plantago,* relative to *Bacopa,* due to an enhanced ability to suppress pathogen burden or proliferation following infection (i.e., quantitative resistance; De Roode & Lefèvre 2012).

The mechanisms underlying this improved resistance remain unknown. Our previous research documented similar patterns of immunocompetence in *A. jatrophae* reared on *Bacopa* and *Plantago* (see also Lampert et al., 2014)*,* though the contributions of specific immune parameters to defense against JcDV appear to be complex and require further study (Muchoney et al., 2022; Resnik and Smilanich, 2020; Smilanich et al., 2018). It is therefore possible that immune responses that have not yet been quantified in *A. jatrophae* contribute to defense against JcDV and are enhanced in larvae consuming *Plantago.* In addition, differences in the chemistry of the two host plants may play a role in mediating resistance (Cory and Hoover, 2006). As previously noted, *Plantago* contains iridoid glycosides, a class of secondary metabolites that are highly consequential for

multitrophic interactions (Bowers, 1991; Dyer and Bowers, 1996; Smilanich et al., 2009a), whereas the native plant *Bacopa* does not contain these compounds. Previous research indicates that sequestration of IGs may contribute to defense against JcDV: in wild-collected *E. phaeton,* there was a negative relationship between the amount of IGs sequestered from host plants and JcDV loads (Muchoney *et al.* 2022), and survival of JcDV infection in another nymphalid butterfly, *Junonia coenia*, was higher on *Plantago lanceolata,* compared to a congeneric species containing lower concentrations of IGs, *P. major* (Smilanich et al., 2018). As *A. jatrophae* is capable of sequestering IGs when consuming *Plantago* (Knerl and Bowers, 2013)*,* the possibility that IG sequestration is directly or indirectly linked to suppression of JcDV replication is a compelling one.

Surprisingly, the frequency with which JcDV was detected in *A. jatrophae* on days 2-6 following inoculation was higher in caterpillars reared on *Plantago* (98%) than *Bacopa* (91%) (Figure 4a)*,* while no difference in postmortem detection was found in our previous study (Muchoney et al., unpublished data). Overall, the virus was detected in a higher proportion of caterpillars in the days following inoculation (95% in the present study) than following death (57%; Muchoney, et al., unpublished data), indicating that JcDV is highly infective in this species at the experimental doses, but that some insects are able to effectively clear infection by the time they die. In addition, JcDV was detected in 100% of individuals sacrificed during the pupal stage on both plants. Thus, although initial establishment of infection was higher on *Plantago,* these larvae maintained lower viral burdens than those using *Bacopa* (Figure 4b)*,* which is highly consequential for survival outcomes in this species (Muchoney, et al., unpublished data; see also Figure 1).

4.3 Viral infection dynamics in wild hosts

Beyond providing insight into the role of host plants in mediating viral infection, these experiments afforded opportunities to compare the dynamics of JcDV infection in two wild hosts of this pathogen (*E. phaeton* and *A. jatrophae*) to its effects in the model host in which it has primarily been studied, *Spodoptera frugiperda* (Mutuel et al., 2010; Wang et al., 2013). Overall susceptibility to infection appears to be higher in final-instar *E. phaeton,* compared to *S. frugiperda* (Figure 1a) (e.g., 100% mortality at the high dose, compared to 80% in *S. frugiperda*; Mutuel et al., 2010), and slightly lower in *E. phaeton* than *A. jatrophae* (10% mortality at the lowest dose, compared to 20% in *A. jatrophae*; Muchoney et al., unpublished data). Our results are consistent with previous findings that mortality primarily occurs before or during pupation at high doses (Figure 1b) (Mutuel et al., 2010); however, JcDV infection did not appear to persist into the adult stage in *S. frugiperda*, whereas it was detected in 55% of *E. phaeton* butterflies (Figure 2a).

Viral quantities excreted in *E. phaeton* frass closely mirrored the doses that were ingested by their hosts, particularly on the first day following inoculation (Figure 3a). This result is consistent with the research of Mutuel et al. (2010), which demonstrated that a low proportion of JcDV (0.1% of viral particles) crosses the midgut epithelium to establish in host tissues, and that there is an accumulation of virus in the midgut that is eliminated within 24 hours of inoculation. In addition, postmortem viral loads of insects were positively associated with the amount of JcDV that was excreted in the frass at low to medium viral doses (Figure 3b), suggesting that viral content in frass may be a reliable indicator of the severity of infection that is established in the insect host. At the highest dose, however, herbivores experienced uniformly high postmortem burdens that were not correlated with frass content, which may be the product of the relatively low proportion

of particles required to establish infection at this over-dose. Altogether, characterizing the dynamics of viral excretion in herbivore frass represents an important component of understanding horizontal transmission via the fecal-to-oral route in these systems.

4.4 Conclusions

These results provide insight into the relative roles of host plant use and pathogen dose in mediating the outcomes of infection in herbivorous insects. Together, the patterns documented in *E. phaeton* and *A. jatrophae* provide an interesting contrast, illustrating that host plant identity may exert a stronger influence on resistance to infection in certain systems, relative to others. Use of the exotic host plant, *Plantago,* did not impact JcDV burdens or mortality in *E. phaeton,* though the potential for sublethal effects in infected adults may differ between the two host plants. In contrast, host plant use had a substantial effect on viral burdens in *A. jatrophae,* with *Plantago* appearing to enhance resistance to infection. These experiments contribute examples from a relatively understudied group of pathogens, the densoviruses (François et al., 2016), to a rich literature demonstrating that host plant use can, but does not always, influence interactions between herbivores and their natural enemies (Cory and Hoover, 2006; Kaplan et al., 2016; Ode, 2006). These experiments, conducted within controlled settings at field-relevant doses, provide critical context for field-based studies documenting patterns of infection across wild populations. In addition, characterizing the role of host plants in mediating vulnerability to infection provides insight in the tritrophic costs and benefits of utilizing different plant species, with implications for understanding the influence of pathogens on herbivore host range.

AUTHOR CONTRIBUTIONS

The study was conceived by AMS, NDM, and AW, and all authors contributed to experimental design. NDM conducted Experiment 1 (*E. phaeton*), AW conducted Experiment 2 (*A. jatrophae*), and NDM and AW performed viral screening. NDM analyzed data and wrote the manuscript, and all authors contributed to revisions.

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FIGURE LEGENDS

Figure 1 Effects of Junonia coenia densovirus dose on survival time and development in *Euphydryas phaeton* (Experiment 1). Results include individuals reared on both the native host plant, *Chelone glabra,* and the exotic host plant, *Plantago lanceolata.* (A) Kaplan–Meier survival plot of individuals inoculated with either a low (n = 18), medium (n = 19), or high (n = 19) dose of JcDV. Time-to-death was faster in insects inoculated with the medium and high viral doses, compared to the lowest viral dose. (B) Life stage at death following inoculation with a low, medium, or high dose of JcDV. A relatively high proportion of individuals that received the lowest dose survived to reach the adult stage, while mortality during the larval and pupal stages increased at higher viral doses.

Figure 2 Effects of host plant species on postmortem detection frequencies and viral burdens of *Euphydryas phaeton* inoculated with a low, medium, or high dose of Junonia coenia densovirus (Experiment 1). (A) Frequency of viral detection in JcDV-challenged insects following death in the larval, pupal, or adult stage. Points represent % infected individuals reared on the native host plant, *Chelone glabra,* or the exotic plant, *Plantago lanceolata*. (B) Postmortem viral loads of JcDV-challenged insects following death in the larval, pupal, or adult stage. Points represent mean load (log-transformed, relative to an internal control gene) ± SE on the native or exotic host plant. Patterns of viral detection and loads were similar on the two plant species, though infection was detected in a higher proportion of individuals reared on *Plantago* following inoculation with the lowest dose.

Figure 3 Detection of Junonia coenia densovirus in *Euphydryas phaeton* frass following inoculation with a low, medium, or high dose of JcDV (Experiment 1). Results include individuals reared on the native host plant, *Chelone glabra,* and the exotic host plant, *Plantago lanceolata.* (A) Daily amount of JcDV excreted in frass. Points represent mean quantity of JcDV (log-transformed number of viral genomes) ± SE on days 1-5 following inoculation. Viral content of frass increased with viral dose and decreased slightly across days following inoculation. (B) Relationship between the quantity of JcDV excreted in frass (averaged across days 1-5 post-inoculation) and the postmortem viral load of each insect. There was a positive association between these two parameters in individuals inoculated with low and medium doses, but not in those inoculated with the highest dose.

Figure 4 Effects of host plant species on detection frequencies and viral burdens of *Anartia jatrophae* caterpillars inoculated with a low, medium, or high dose of Junonia coenia densovirus (Experiment 2). Results include individuals sacrificed on days 2-6 following inoculation. (A) Frequency of viral detection in JcDV-challenged individuals following sacrifice. Points represent % infected individuals reared on the native host plant, *Bacopa monnieri,* or the exotic plant, *Plantago lanceolata*. (B) Viral loads of JcDV-challenged larvae following sacrifice. Points represent mean JcDV load (log-transformed, relative to an internal control gene) ± SE in individuals reared on the native or exotic host plants. JcDV was detected in a higher frequency of larvae reared on *Plantago,* while viral burdens were substantially higher on *Bacopa* across all doses.

Figure 5 Variation in viral loads across time in *Anartia jatrophae* caterpillars inoculated with a low, medium, or high dose of Junonia coenia densovirus (Experiment 2). Results include individuals reared on the native host plant, *Bacopa monnieri,* and the exotic plant, *Plantago lanceolata.* (A) Viral loads of JcDV-challenged larvae following sacrifice at days 2-6 following inoculation. Points represent mean load (log-transformed, relative to an internal control gene) ± SE. (B) Viral loads of JcDV-challenged individuals following sacrifice in either the larval stage (averaged over days 2-6 following inoculation) or the pupal stage (day 1 following pupation). Points represent mean load (log-transformed, relative to an internal control gene) ± SE. Infection loads decreased slightly over time following inoculation but were lower in pupae, relative to larvae, across all doses.

FIGURES

Figure 1

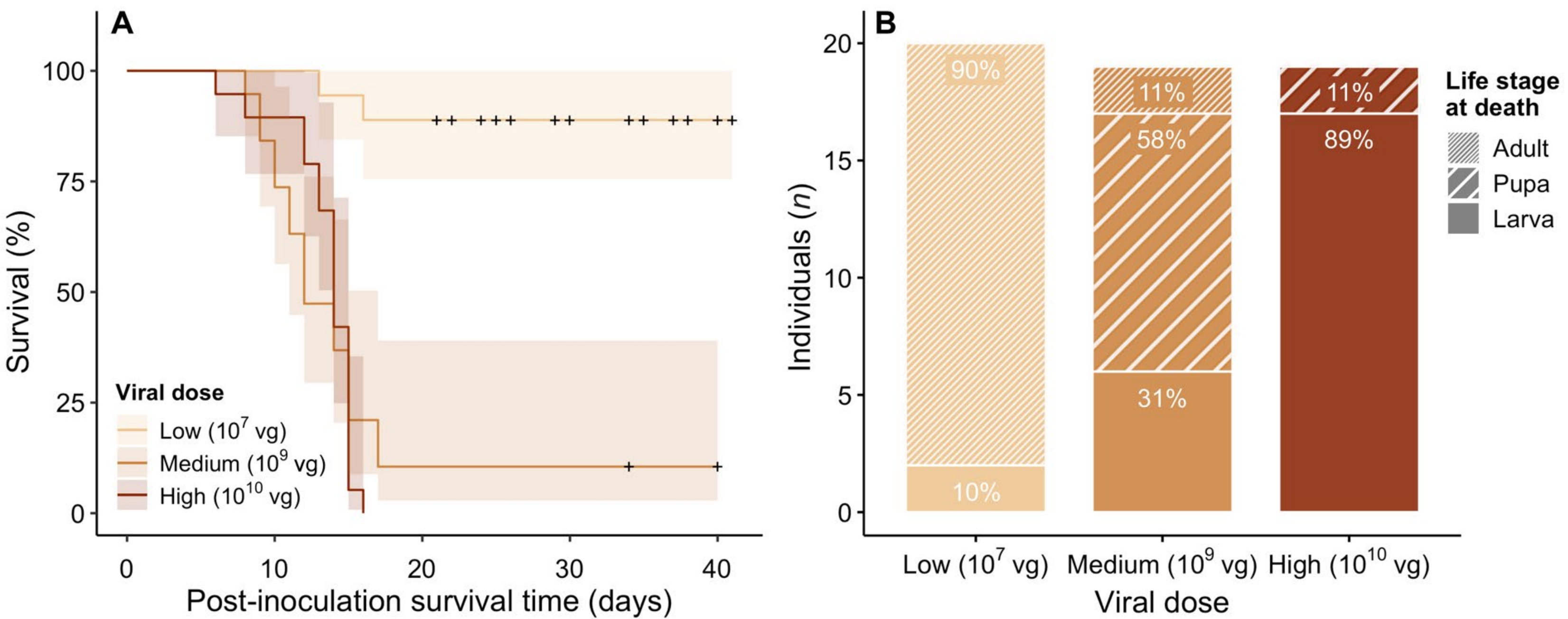


Figure 2

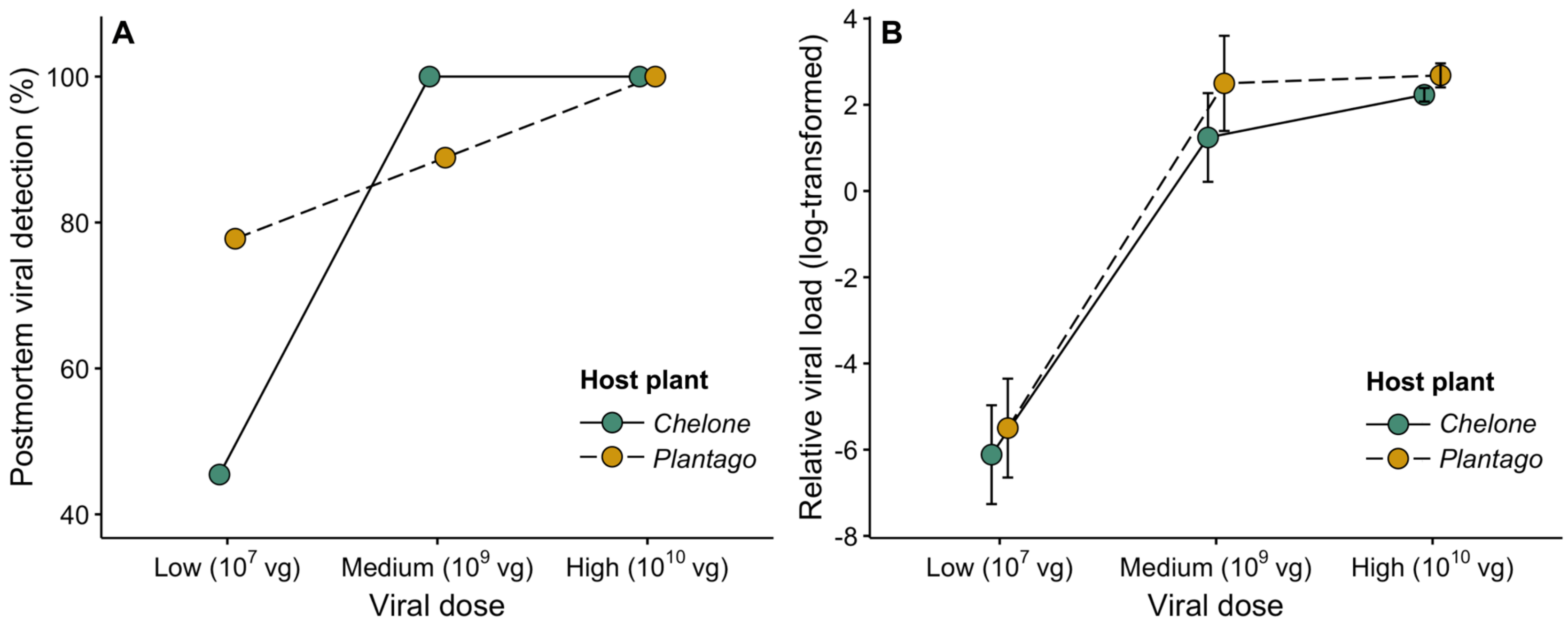


Figure 3

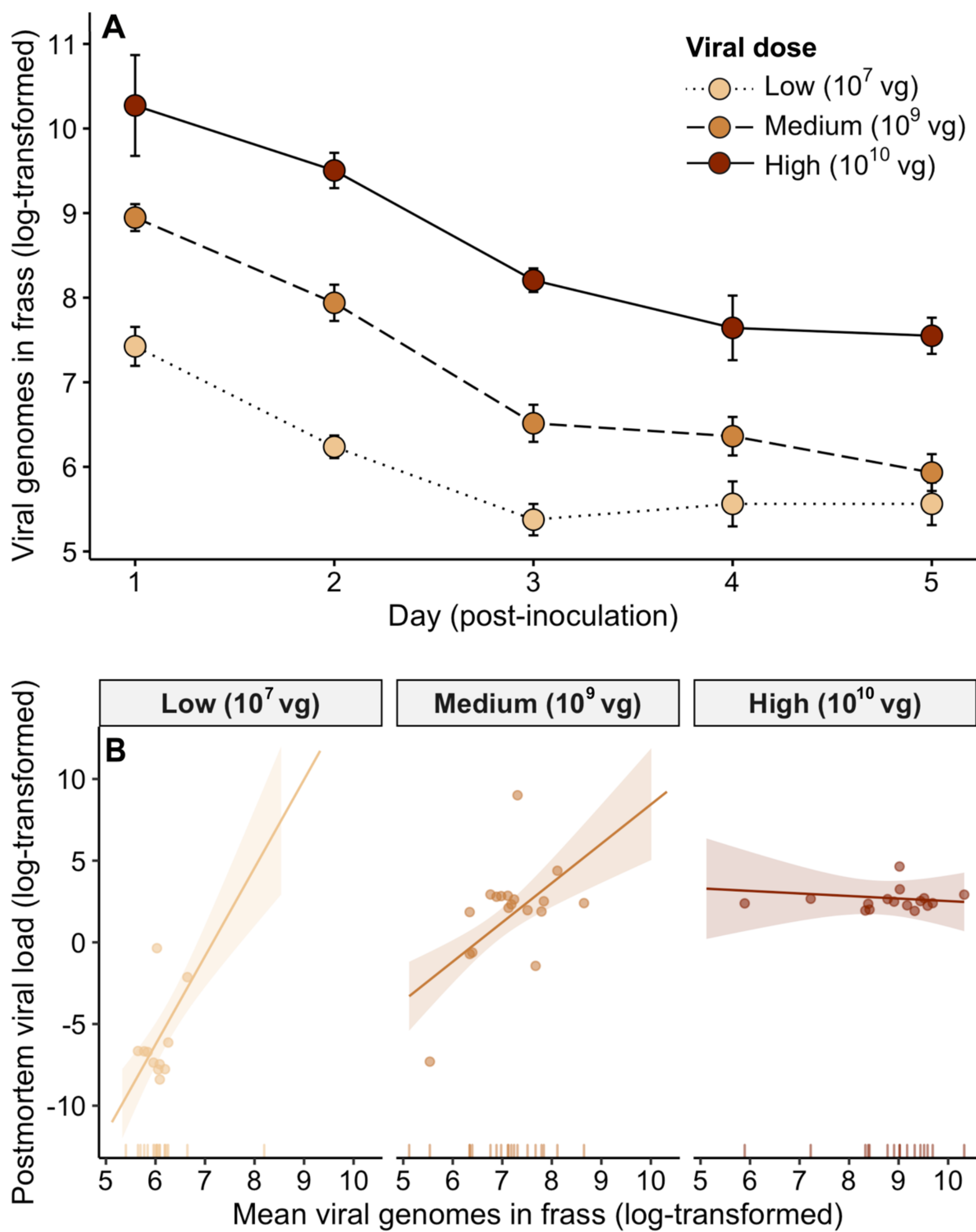


Figure 4

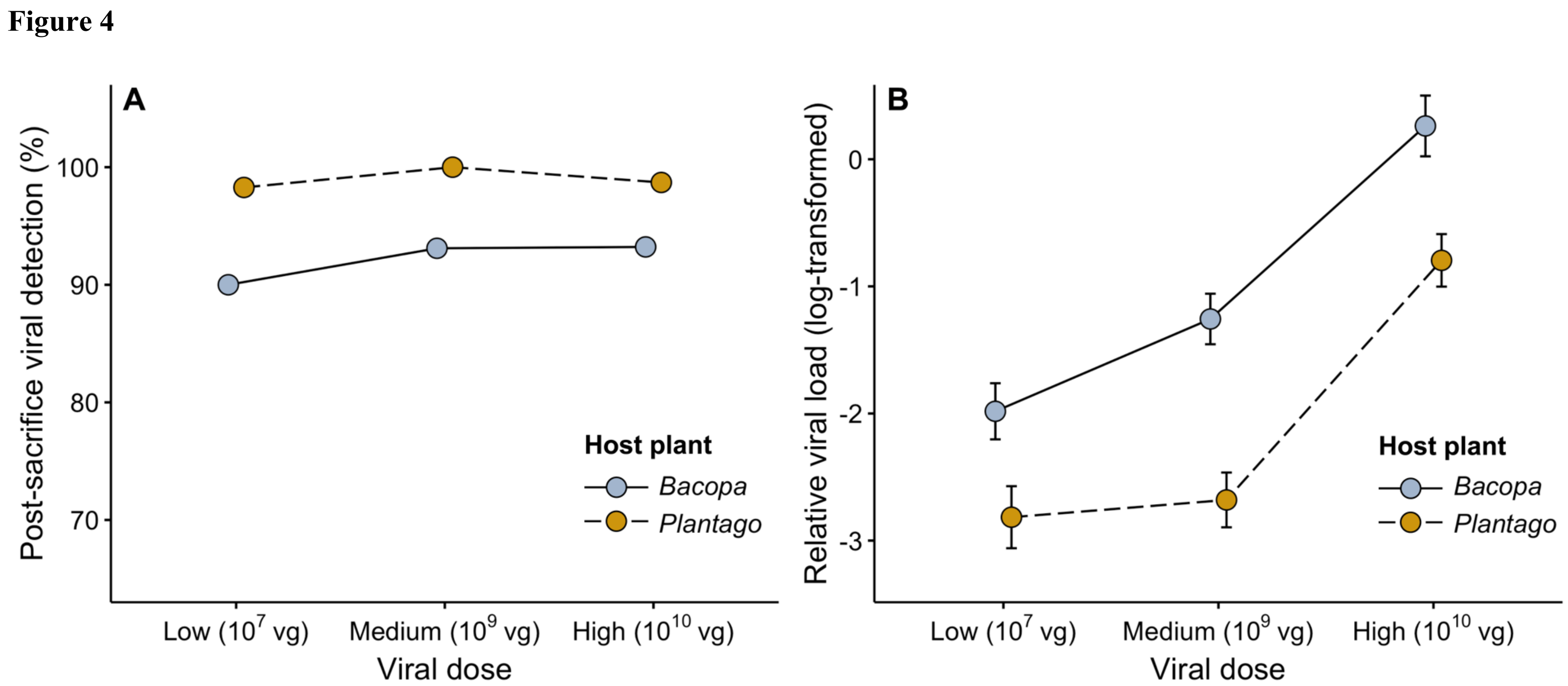
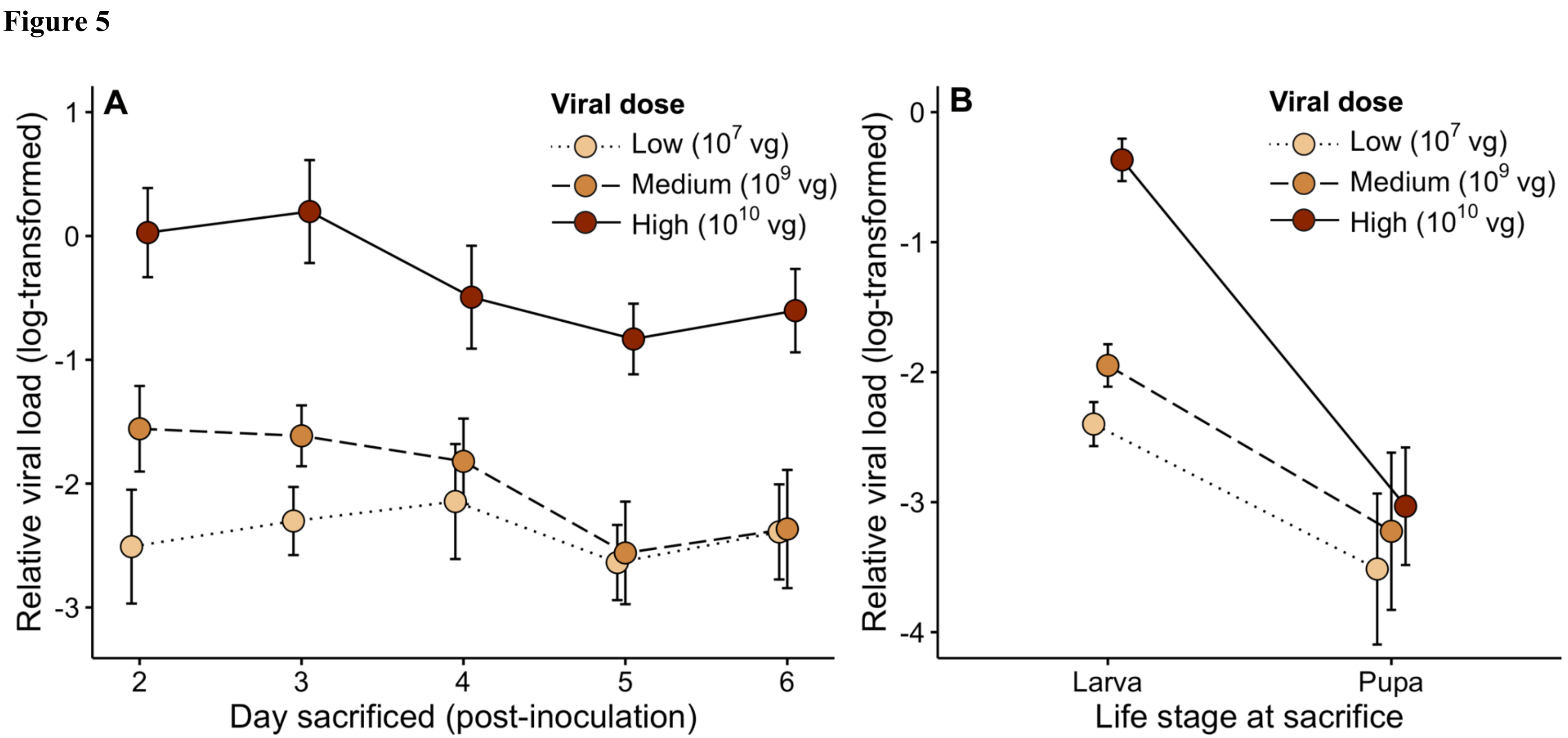


Figure 5



APPENDIX

Figure S1 Effect of host plant species on survival of *Euphydryas phaeton* following inoculation with a low, medium, or high dose of Junonia coenia densovirus, compared to unchallenged controls (Experiment 1). Points represent frequencies of survival to the adult stage in individuals reared on either the native plant, *Chelone glabra* (*n* = 37), or the exotic *Plantago lanceolata* (*n* = 31). Survival was high in controls and decreased at higher doses, but was similar in individuals reared on the two plants across all treatments.

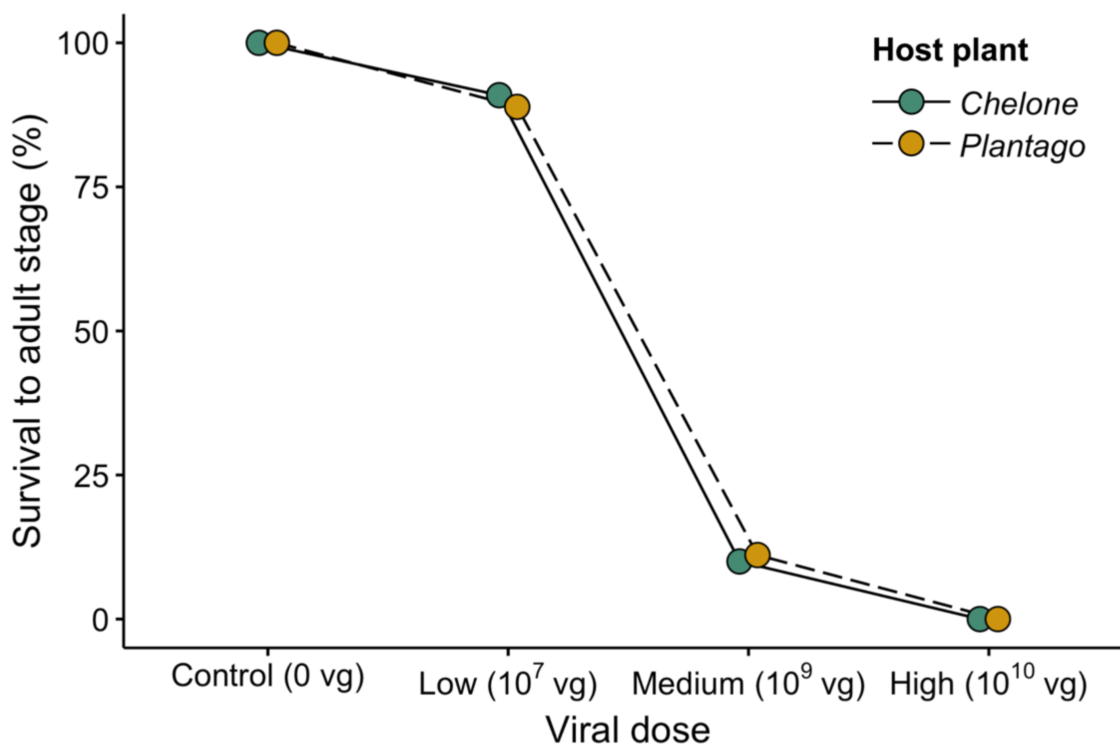


Figure S2 Effect of host plant species on viral loads of *Euphydryas phaeton* following death in the larval, pupal, or adult stage (Experiment 1). Results include individuals inoculated with a low, medium, or high dose of Junonia coenia densovirus. Points represent mean postmortem viral load (log-transformed, relative to an internal control gene) ± SE of individuals reared on the native host plant, *Chelone glabra,* or the exotic plant, *Plantago lanceolata*. JcDV-challenged individuals that died as larvae and pupae harbored higher viral burdens than those that survived to reach the adult stage, and viral loads were similar in individuals reared on the two host plant species across all stages.

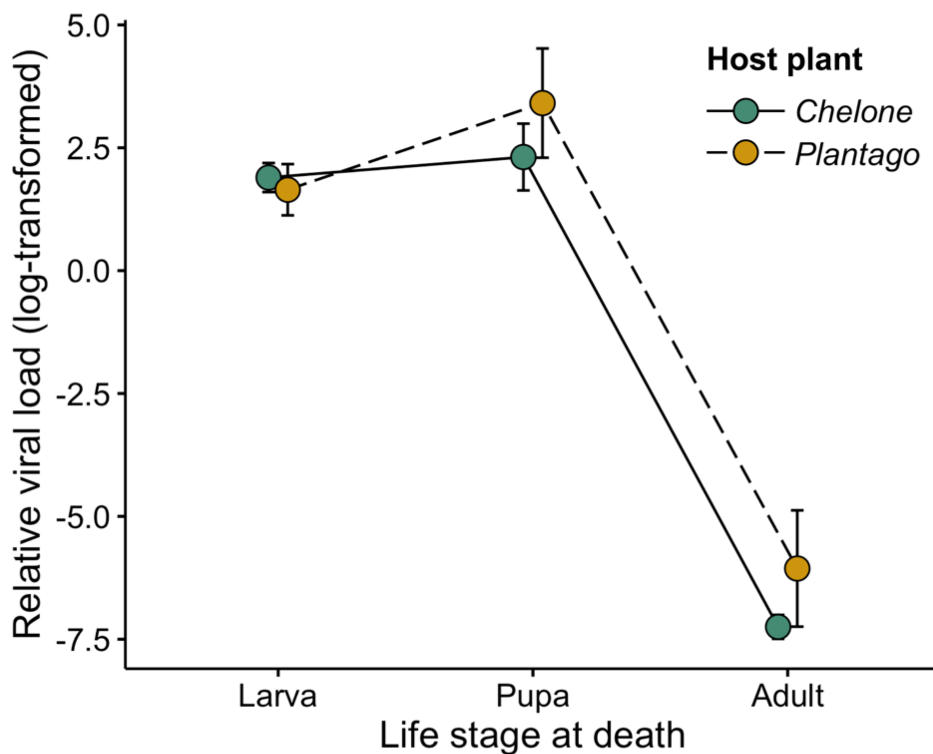


Figure S3 Effects of host plant species on viral quantities excreted in *Euphydryas phaeton* frass following inoculation with Junonia coenia densovirus (Experiment 1). Points represent mean quantity of JcDV (log-transformed number of viral genomes) ± SE in individuals reared on the native host plant, *Chelone glabra,* or the exotic host plant, *Plantago lanceolata.* (A) Amount of JcDV in frass following inoculation with a low, medium, or high dose of JcDV. Results are averaged across days 1-5 following inoculation. (B) Amount of JcDV in frass on days 1-5 following inoculation. Results are averaged across viral doses. Viral content of frass increased with dose and decreased across days following inoculation, but did not differ based upon host plant use.

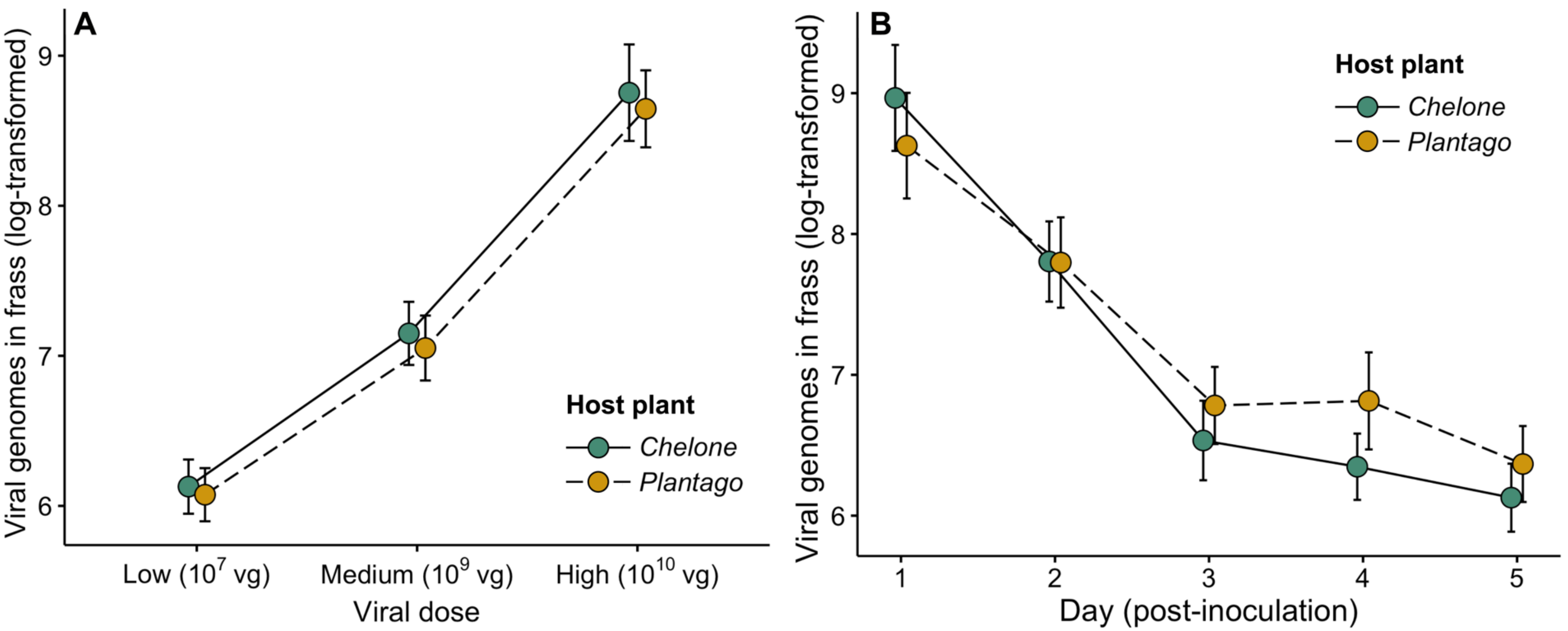
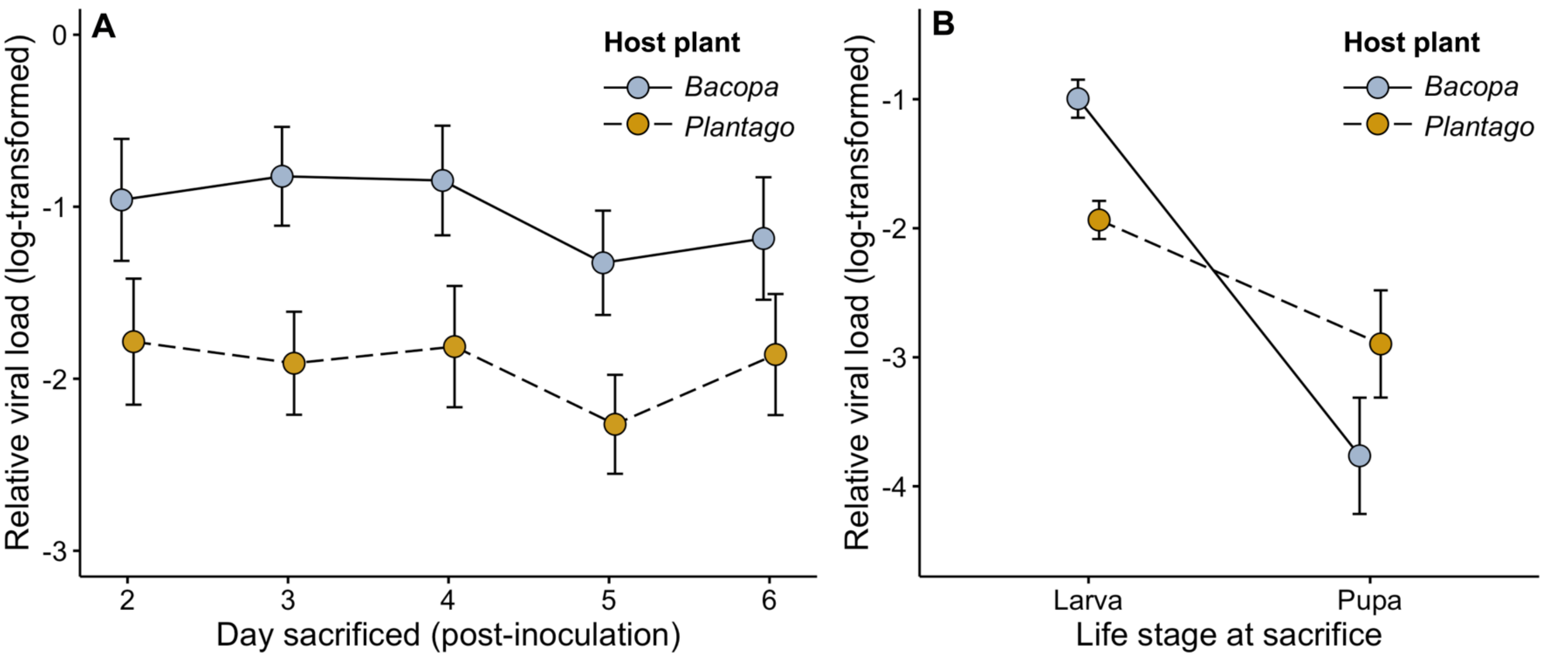


Figure S4 Variation in viral loads across time in *Anartia jatrophae* caterpillars reared on the native host plant, *Bacopa monnieri,* or the exotic plant, *Plantago lanceolata* (Experiment 2). Results include individuals inoculated with a low, medium, or high dose of Junonia coenia densovirus, and points represent mean load (log-transformed, relative to an internal control gene) ± SE. (A) Viral loads of larvae sacrificed at days 2-6 following inoculation. JcDV loads were higher in larvae reared on *Bacopa* across all days. (B) Viral loads of individuals sacrificed in either the larval stage (days 2-6) or the pupal stage (day 1 following pupation). JcDV loads were lower in pupae, relative to larvae, on both host plants, though this decrease was greater in magnitude on *Bacopa*.



Conclusions

As exotic plants continue to persist outside of their native ranges (van Kleunen et al., 2015), their incorporation into the diets of native herbivores will likely continue to occur. Beyond providing insight into the impacts of introduced plant species on native organisms (Tallamy et al., 2021), these contemporary examples of host range evolution provide natural experiments through which to investigate the complex suite of ecological factors shaping herbivore persistence on novel host plants (Forister et al., 2020). The goal of this dissertation was to evaluate the role of host plant use in mediating interactions between insect herbivores and their natural enemies and, by extension, to gain a deeper understanding of the context-dependence of herbivore fitness and persistence on different host plants in the wild (Singer and Stireman, 2005). I employed approaches from the fields of ecological immunology (Schulenburg et al., 2009), chemical ecology (Dyer et al., 2018), and disease ecology (Campos-Herrera and Lacey, 2018) to investigate the tritrophic outcomes of host range expansion for two native herbivores, *Euphydryas phaeton* and *Anartia jatrophae.* I focused particularly on their interactions with a virus, Junonia coenia densovirus (JcDV), as the impacts of naturally occurring pathogens are understudied in many systems (Williams, 2018). This research illustrated that use of an exotic plant (in these cases, *Plantago lanceolata*) can give rise to multifaceted changes in herbivore defenses against, and interactions with, their natural enemies, which may be expected to influence herbivore persistence on novel resources in wild populations.

Through field- and laboratory-based investigations with *E. phaeton* (Chapters 1, 2, and 4), I found that use of the exotic host plant resulted in: (1) suppression of certain

immune parameters, which could influence susceptibility to a range of pathogens and parasites beyond the focal virus (JcDV) and (2) differential composition of sequestered iridoid glycosides, which may reduce the efficacy of chemical defense against predators (Bowers, 1980) and potentially compromise the strength of immune defenses (Chapter 1; see also Richards et al., 2012; Smilanich et al., 2009). An important challenge will be to elucidate the outcomes of these defensive differences for *E. phaeton*'s vulnerability to different types of natural enemies, as relationships between immune assays and disease outcomes are not always straightforward (Adamo, 2004). As an example of this, I found that *E. phaeton*'s ability to survive JcDV infection was similar on the exotic and native host plants, suggesting that additional factors (e.g., phytochemical sequestration) may compensate for immunosuppression on *P. lanceolata* when this virus is encountered.

In addition, JcDV burdens were found to be higher in field-collected *E. phaeton* using the exotic plant, compared to the native plant, during the post-diapause stage in two studies (Chapters 1-2), though resistance to infection was similar on the two host plants within a controlled environment (Chapter 4). These findings highlight the importance of studying herbivore-pathogen interactions in both laboratory and field settings and suggest that using the exotic plant may entail either: (1) greater exposure to JcDV in the wild, or (2) increased susceptibility to JcDV in the wild, which could be mediated by ecological, physiological, or behavioral factors that were not present in the laboratory. Both of these possibilities warrant further study. Altogether, these differences in immunity, chemical defense, and viral infection represent potential tritrophic costs of host range expansion that may be predicted to limit *E. phaeton*'s ability to persist on the exotic plant. However,

the example of JcDV illustrates that *P. lanceolata* may represent a functionally suitable resource for supporting *E. phaeton* development within certain tritrophic contexts.

Contrasting results were documented in *A. jatrophae*, for which the exotic plant conferred a clear fitness advantage within the context of pathogen infection. Herbivores reared on the exotic plant exhibited a substantially higher likelihood of surviving JcDV infection, compared to those reared on the native plant (Chapter 3), which was apparently mediated by suppression of viral replication (Chapter 4). The mechanisms underlying this enhanced resistance have not yet been determined, as measured immune responses were similar on the two host plants. Fruitful avenues for future research include determining the impacts of ingesting and/or sequestering iridoid glycosides on JcDV infection, as well as examining the role that developmental differences (i.e., slower growth and greater body weight on *P. lanceolata*; Knerl and Bowers, 2013) may play in resistance to JcDV.

These findings ultimately indicate that the exotic plant represents a superior resource for supporting *A. jatrophae* development when JcDV is present, which may be predicted to promote persistence on this plant, particularly in areas where exposure to JcDV is high (i.e., enemy-free space; Jeffries and Lawton, 1984). Potential outcomes of such persistence could include an expansion of *A. jatrophae*'s geographic range or local abundance (Graves and Shapiro, 2003), or even a shift in oviposition behavior such that *P. lanceolata* becomes preferred over native host plants in certain populations (Singer et al., 1993). Moving forward, corroborating these results through field-based research, and gaining a deeper understanding of the prevalence and impact of JcDV throughout wild *A. jatrophae* populations, represent important steps toward elucidating how this tritrophic benefit of exotic plant use may impact host range evolution in this native herbivore.

In conclusion, this research highlights the importance of considering the evolution of herbivore host range within a tritrophic framework (Bernays and Graham, 1988; Lill et al., 2002; Singer and Stireman, 2005). Though an herbivore's capacity to recognize and develop on an exotic plant is an undeniably important aspect of colonization, it is clear that persistence on these novel resources can involve complex ecological contingencies (Forister et al., 2020; Fortuna et al., 2013; Yoon et al., 2019). Here, I have highlighted one such contingency that has received little attention within studies of herbivore host range evolution: host plant mediated variation in susceptibility to pathogens. Importantly, the examples provided by *E. phaeton* and *A. jatrophae* contribute to substantial bodies of literature demonstrating that herbivore immunity (Smilanich and Muchoney, 2022) and resistance to pathogens (Cory and Hoover, 2006) can vary dramatically based on host plant use, suggesting that these tritrophic effects may be common. Thus, while the use of exotic plants frequently entails reductions in herbivore growth and survival (Yoon and Read, 2016), I have demonstrated that exotic plants may in some cases represent suitable or even superior resources, compared to native plants, when the impacts of pathogens are considered. While vulnerability to infectious disease represents one of many dimensions of herbivore performance that can differ between native and exotic host plants (Forister and Wilson, 2013), this research provides important insight into the context-dependence of herbivore fitness in wild settings. Given the increasing accessibility of molecular tools for detecting pathogens in insect hosts (Campos-Herrera and Lacey, 2018), evaluation of interactions with pathogens may continue to provide exciting opportunities to investigate the ecological factors facilitating, or constraining, herbivore host range evolution.

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