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Efecto del polen nativo chileno y sus compuestos en abejas infectadas con la variante DWV-A del virus de las alas deformadas

Effect of native Chilean pollen and its compounds on bees infected with the DWV-A variant of the Deformed Wing Virus

Tesis para optar al grado de Doctor en Ciencias de la Agronomía

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RESUMEN

La salud de las abejas melíferas (*Apis mellifera*) se ve afectada por la interacción de múltiples estresores, entre los cuales las infecciones virales cumplen un rol central. El virus de las alas deformadas (DWV) es uno de los patógenos más prevalentes, especialmente por su asociación con *Varroa destructor* y su capacidad de persistir como infección subclínica. Su patogenicidad no depende únicamente de la exposición viral, sino que está fuertemente modulada por el estado nutricional y fisiológico del hospedero. En este contexto, el polen emerge como un factor clave de resiliencia, capaz de influir en la carga viral, la sobrevivencia y la respuesta inmune frente a la infección. En este estudio se evaluó el efecto de distintas dietas polínicas y de compuestos bioactivos asociados al polen sobre la infección por DWV-A en abejas melíferas. En una primera etapa, se caracterizaron pólenes monoflorales y poliflorales provenientes de plantas nativas y exóticas de Chile, con énfasis en su composición fitoquímica. Posteriormente, se evaluó el efecto de estas dietas sobre parámetros de infección viral, sobrevivencia y respuesta inmune en abejas inoculadas experimentalmente con DWV-A. Finalmente, se analizó el efecto de un compuesto fenólico seleccionado sobre el desempeño cognitivo de las abejas infectadas. Los pólenes utilizados fueron colectados durante la temporada apícola 2022 en apiarios experimentales ubicados en Chillán y Coihueco (Región de Ñuble) y en Río Bueno (Región de Los Ríos), mientras que los ensayos biológicos se realizaron con abejas provenientes del apiario experimental de la Estación de Investigación “El Nogal” de la Universidad de Concepción, en Chillán, Chile. En términos de caracterización química, el polen monofloral de *Eucryphia cordifolia* presentó las mayores concentraciones de compuestos fenólicos y flavonoides, incluyendo ácido gálico, ácido vainílico, ácido clorogénico, ácido cafeico, quercetina y kaempferol, en comparación con *Rubus ulmifolius* y el polifloral. En los ensayos biológicos, las dietas basadas en polen redujeron significativamente la carga viral en abejas inoculadas con DWV-A, con efectos dependientes del tipo de polen. El polen monofloral de *E. cordifolia* produjo la mayor reducción de carga viral (de $1{,}0 \times 10^{13}$ a $1{,}0 \times 10^{5}$ copias por abeja) y la mayor tasa de sobrevivencia (91 %), mientras que los pólenes de *R. ulmifolius* y polifloral también disminuyeron la carga viral, aunque en menor

magnitud. A nivel inmunológico, las dietas polínicas modularon la expresión de genes asociados a las vías Toll, Imd y RNAi, con una disminución de la expresión de *Cactus* y un aumento de *Dorsal*, *Relish* y *Dicer-like*, respectivamente. Siguiendo esta línea, se evaluó el efecto del ácido clorogénico como compuesto bioactivo representativo, mediante la técnica de Respuesta de Extensión de la Probóscide (PER). La suplementación con ácido clorogénico atenuó los efectos negativos asociados a la infección por DWV-A de manera dependiente de la concentración, mejorando la sobrevivencia y el aprendizaje asociativo. En particular, la sobrevivencia de las abejas infectadas aumentó significativamente, alcanzando valores comparables al control no inoculado en la dosis más alta evaluada. En contraste, las mejoras en aprendizaje y memoria fueron más evidentes a concentraciones intermedias, sugiriendo una respuesta cognitiva no lineal. Estos resultados evidencian que la calidad fitoquímica del polen y sus compuestos bioactivos pueden modular significativamente la dinámica de infección por DWV-A, la respuesta inmune y el desempeño cognitivo en abejas melíferas. Estos hallazgos abren la posibilidad de desarrollar estrategias nutricionales basadas en recursos florales nativos para fortalecer la resiliencia de colonias frente a patógenos virales, con potencial aplicación en manejo apícola sostenible.

ABSTRACT

The health of honey bees (*Apis mellifera*) is affected by the interaction of multiple stressors, among which viral infections play a central role. Deformed wing virus (DWV) is one of the most prevalent pathogens, particularly due to its association with *Varroa destructor* and its ability to persist as a subclinical infection. Its pathogenicity does not depend solely on viral exposure but is strongly modulated by the host's nutritional and physiological status. In this context, pollen emerges as a key resilience factor capable of influencing viral load, survival, and immune responses to infection. In this study, the effect of different pollen-based diets and pollen-associated bioactive compounds on DWV-A infection in honey bees was evaluated. In the first stage, monofloral and polyfloral pollens from native and exotic plant species in Chile were characterized, with emphasis on their phytochemical composition. Subsequently, the effects of these diets on viral infection parameters, survival, and immune response were assessed in bees experimentally inoculated with DWV-A. Finally, the effect of a selected phenolic compound on the cognitive performance of infected bees was analyzed. The pollen samples were collected during the 2022 beekeeping season from experimental apiaries located in Chillán and Coihueco (Ñuble Region) and Río Bueno (Los Ríos Region), while biological assays were conducted using bees from the experimental apiary of the "El Nogal" Research Station of the University of Concepción in Chillán, Chile. Regarding chemical characterization, monofloral pollen from *Eucryphia cordifolia* exhibited the highest concentrations of phenolic compounds and flavonoids, including gallic acid, vanillic acid, chlorogenic acid, caffeic acid, quercetin, and kaempferol, compared with *Rubus ulmifolius* and polyfloral pollen. In biological assays, pollen-based diets significantly reduced viral load in DWV-A-inoculated bees, with effects depending on pollen type. *E. cordifolia* pollen showed the greatest reduction in viral load (from 1.0×10^{13} to 1.0×10^{5} copies per bee) and the highest survival rate (91%), while *R. ulmifolius* and polyfloral diets also reduced viral load, albeit to a lesser extent. At the immune level, pollen diets modulated the expression of genes associated with the Toll, Imd, and RNAi pathways, downregulating *Cactus* and upregulating *Dorsal*, *Relish*, and *Dicer-like*, respectively. Following this line of evidence, chlorogenic acid was evaluated as a representative bioactive compound using the Proboscis

Extension Response (PER) assay. Chlorogenic acid supplementation attenuated the negative effects associated with DWV-A infection in a dose-dependent manner, improving both survival and associative learning. Survival of infected bees increased significantly, reaching levels comparable to the non-inoculated control at the highest dose tested. In contrast, improvements in learning and memory were more pronounced at intermediate concentrations, suggesting a non-linear cognitive response. These results demonstrate that the phytochemical quality of pollen and its bioactive compounds can significantly modulate DWV-A infection dynamics, immune responses, and cognitive performance in honey bees. These findings open the possibility of developing nutrition-based strategies using native floral resources to enhance colony resilience against viral pathogens, with potential applications in sustainable beekeeping management.

INTRODUCCIÓN GENERAL

La abeja melífera (*A. mellifera*) desempeña un rol fundamental en los ecosistemas agrícolas y naturales debido a su contribución a través de la polinización (Reverté et al., 2023). Se estima que aproximadamente el 75% de los cultivos alimentarios presentan algún grado de dependencia de los servicios de polinización, los cuales contribuyen significativamente a la estabilidad y mantenimiento de la biodiversidad vegetal (Klein et al., 2007; Feuerbacher et al., 2025). En términos económicos, el valor global de los servicios de polinización animal ha sido estimado en más de 800 mil millones de dólares anuales, reflejando su impacto directo sobre la seguridad alimentaria y el desarrollo económico mundial (Gebremedhn et al., 2025). De manera más específica, en los Estados Unidos, el valor de los servicios de polinización proporcionados por abejas melíferas se ha estimado en aproximadamente 15 mil millones de dólares anuales para la producción agrícola (USDA, 2024). Asimismo, datos recientes indican que durante el año 2024 los productores agrícolas destinaron más de 400 millones de dólares exclusivamente al arriendo de colmenas para servicios de polinización, actividad que desde 2022 ha superado económicamente a la producción de miel como principal fuente de ingresos para los apicultores (USDA-NASS, 2025).

Sin embargo, a pesar de su importancia ecológica y económica, durante las últimas décadas se ha documentado un incremento en las tasas de mortalidad y debilitamiento de las colonias (Bruckner et al., 2023). Reportes recientes provenientes del U.S. Beekeeping Survey estiman que entre abril de 2024 y abril de 2025 se perdió aproximadamente el 55.6% de las colonias manejadas en Estados Unidos, representando una de las tasas más altas registradas en la última década (Apiary Inspectors of America, 2025). De forma consistente, otros estudios han reportado pérdidas promedio anuales superiores al 60% de las colonias en sistemas comerciales de producción, con impactos directos sobre la disponibilidad de colmenas destinadas a la polinización de cultivos dependientes (Nearman et al., 2025). A nivel regional, estudios recientes en América Latina han evidenciado que las pérdidas invernales de colonias alcanzan en promedio un 20.6%, posicionando a la región en un nivel intermedio entre Europa (12.5%)

y Estados Unidos (40.4%), lo que refleja la magnitud del deterioro sanitario de las colmenas en distintos contextos productivos (Requier et al., 2024).

Este fenómeno ha sido atribuido a la acción combinada de múltiples factores de estrés, que pueden comprometer la inmunocompetencia de las abejas y aumentar la susceptibilidad a enfermedades (Genersch et al., 2010; Chauzat et al., 2016; Gray et al., 2019). Entre estos factores se incluyen la presencia de los patógenos, las deficiencias nutricionales, la pérdida de hábitat, el uso de agroquímicos y la disminución de la diversidad floral disponible (Potts et al., 2010; Goulson et al., 2015; Vanbergen et al., 2018). En este escenario multifactorial, las infecciones virales se han consolidado como uno de los principales determinantes del deterioro de la salud individual y colectiva de las abejas (Claing et al., 2024).

En particular, el virus de las alas deformadas (DWV) destaca como uno de los patógenos más prevalentes y estudiados en *Apis mellifera* a nivel global (Wilfert et al., 2016; Schauer et al., 2023). Su importancia radica no solo en su amplia distribución, sino también en su estrecha asociación con el ácaro *Varroa destructor*, que actúa como vector biológico y contribuye al aumento de las cargas virales en las colonias infestadas (Martin et al., 2012; Wilfert et al., 2016). DWV se presenta como un complejo de variantes genéticas (DWV-A, DWV-B, DWV-C) siendo DWV-A una de las más comunes y persistentes en Chile (Riveros et al., 2020). Este virus es capaz de establecer infecciones subclínicas que afectan funciones críticas sin manifestar necesariamente síntomas visibles como deformaciones de las alas (McMahon et al., 2016; Mordecai et al., 2016).

La patogenicidad del DWV no depende exclusivamente de la exposición viral, sino que está fuertemente modulada por el estado fisiológico y nutricional del hospedero (Alaux et al., 2011). Evidencia creciente indica que abejas con una nutrición deficiente presentan mayores cargas virales, menor sobrevivencia y una respuesta inmune deficiente frente a las infecciones virales. Esto sugiere que la dieta actúa como un modulador clave de la dinámica hospedero–patógeno (DeGrandi-Hoffman et al., 2010; Di Pasquale et al., 2013). En este escenario, la nutrición constituye un componente funcional que influye directamente en la competencia inmunológica de las abejas.

El polen es un recurso floral que las abejas obtienen a partir de las estructuras reproductivas de las plantas (Brodschneider & Crailsheim, 2010). Para estos

insectos constituye una fuente indispensable de proteínas, aminoácidos, lípidos, compuestos bioactivos, vitaminas y micronutrientes, siendo un recurso altamente variable en cuanto a su composición química. Diversos estudios han demostrado que dietas basadas en polen, en comparación con sustitutos artificiales, mejoran la longevidad, el desarrollo glandular y la expresión de genes asociados a la respuesta inmune en abejas adultas (Alaux et al., 2010; Di Pasquale et al., 2013). Esta variabilidad nutricional no solo afecta parámetros fisiológicos generales, sino que también influye en la capacidad de las abejas para tolerar y controlar infecciones virales (Dolezal et al., 2019).

Más allá de su valor nutricional básico, el polen es una fuente compleja de compuestos bioactivos, incluyendo polifenoles, flavonoides, ácidos fenólicos y otros metabolitos secundarios de origen vegetal (Palmer-Young et al., 2019). Estos compuestos han sido ampliamente estudiados en sistemas no asociados a abejas por su actividad antioxidante, inmunomoduladora y antiviral, actuando mediante la regulación del estado redox celular, la interferencia con procesos de replicación viral y la modulación de vías de señalización del hospedero (Zakaryan et al., 2017; Musarra-Pizzo et al., 2021). En abejas melíferas, si bien la evidencia directa aún es limitada, se ha demostrado que ciertos fitoquímicos presentes en el polen pueden inducir la expresión de genes relacionados con detoxificación, inmunidad innata y tolerancia al estrés, lo que sugiere un rol funcional más allá del aporte nutricional (Mao et al., 2013).

En este marco, la calidad química del polen, definida no solo por su contenido proteico sino también por su perfil de compuestos bioactivos, adquiere especial relevancia para la salud de las abejas frente a infecciones virales como el DWV (Palmer-Young et al., 2017). Asimismo, la mayoría de los estudios disponibles se ha centrado en pólenes monoflorales, mezclas controladas o suplementos comerciales, lo que limita la representación de la complejidad nutricional presente en condiciones naturales (Ares et al., 2018). En este contexto, la caracterización del potencial funcional de pólenes nativos sigue siendo incipiente, particularmente en regiones de alta diversidad florística como Chile, donde la flora melífera es altamente heterogénea y aun escasamente estudiada. En particular, la flora nativa chilena constituye una fuente poco explorada de recursos polínicos con perfiles químicos distintivos, cuyo impacto sobre la salud

de las abejas y su capacidad de respuesta frente a infecciones virales aún no ha sido dilucidado (Grimau et al., 2014).

En respuesta a estas brechas, la presente tesis doctoral tiene como objetivo evaluar el efecto del polen nativo chileno y de sus compuestos bioactivos sobre la infección causada por la variante DWV-A en abejas melíferas. A través de un enfoque integrado, esta tesis combina un estudio experimental que analiza el efecto de diferentes dietas polínicas sobre la carga viral, la sobrevivencia y la expresión de genes inmunes, junto con una revisión crítica de la literatura que sintetiza la evidencia disponible sobre el rol de la calidad química del polen en la modulación de infecciones por DWV. Los resultados de esta investigación buscan contribuir a una mejor comprensión de la interacción entre la nutrición, los compuestos vegetales y la salud de *A. mellifera*, aportando bases científicas para el desarrollo de estrategias nutricionales orientadas al fortalecimiento de la resiliencia de las colonias frente a patógenos emergentes y persistentes.

Hipótesis

Los pólenes de plantas nativas chilenas y sus compuestos bioactivos mejoran la salud, sobrevivencia e inmunidad de las abejas infectadas con la variante DWV-A del virus de las alas deformadas.

Objetivo General

Evaluar el efecto de pólenes de plantas nativas chilenas sobre el comportamiento, la sobrevivencia y la respuesta inmune de abejas melíferas (*A. mellifera*) infectadas con DWV-A, mediante bioensayos bajo condiciones experimentales controladas.

Objetivos específicos

1. Analizar la literatura disponible sobre la relación entre la calidad química del polen, la carga viral de DWV y la sobrevivencia de *A. mellifera*.
2. Evaluar el efecto de la ingesta de pólenes nativos en la sobrevivencia, respuesta inmune y disminución de la carga viral de abejas infectadas con la variante DWV-A.

3. Evaluar el efecto de la suplementación de ácido clorogénico sobre la sobrevivencia y el desempeño en tareas de aprendizaje y memoria en abejas (*A. mellifera*) infectadas con la variante DWV-A.

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Capítulo I: From pollen to pathogen defense: how pollen chemical quality impacts Deformed Wing Virus infection and survival in honey bees

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Abstract

Pollen constitutes the primary source of proteins, amino acids, lipids, sterols, vitamins, and minerals for honey bees. However, not all pollen types provide the same resources or have the same biological value. Its chemical composition changes according to botanical origin, geographic location, and environmental conditions. This variability can influence metabolism, the immune system, oxidative balance, and the ability to resist or tolerate infections. This article examines the available evidence on the relationship between pollen chemical quality and the dynamics of Deformed Wing Virus (DWV) infection in *Apis mellifera*. The analysis is approached from molecular, physiological, ecological, and seasonal perspectives. Current findings suggest that more diverse and higher-quality pollen diets are generally associated with greater colony survival and improved health status, although their effects on viral load are more heterogeneous and context-dependent. In some studies, pollen intake is linked to a reduction in DWV, whereas in others viral loads remain stable or even increase despite improvements in survival, physiological condition, or colony performance. These differences suggest that pollen may act not only by enhancing resistance to the virus but also by increasing tolerance to infection-associated damage. The potential role of pollen bioactive compounds, particularly flavonoids and phenolic acids, is also discussed. Nevertheless, evidence of direct antiviral action of these compounds in bees remains limited, as many proposed mechanisms derive from other organisms. This synthesis

provides an integrative perspective on pollen nutrition and its relevance for colony resilience against viral infections..

Keywords: nutrition; *Apis mellifera*; DWV, nutrition; phytochemicals; innate immunity; gut microbiota

1. Introduction

The health of honey bees (Apis mellifera L.) is essential for the functioning of terrestrial ecosystems and global agricultural production because of their central role in the pollination of both crops and wild plants. Pollination by animals underpins the reproduction of nearly 90% of wild flowering plants, contributes to more than 75% of global food crop types, and supports an estimated US$235–577 billion in annual crop production worldwide (Priyadarshana et al., 2024). However, over the past decades, a sustained decline in colony health has been documented across different regions of the world, largely driven by the interaction of multiple stressors, including pathogens, agricultural intensification, pesticide exposure, and reduced floral diversity (Goulson et al., 2015).

Among the biological factors compromising colony stability, viral infections are particularly important because of their high prevalence and cumulative sublethal effects. Deformed wing virus (DWV) has emerged as one of the most important viral pathogens of A. mellifera due to its widespread distribution, persistence, and close association with the mite Varroa destructor, which promotes its transmission and amplification within colonies (Grozinger & Flenniken, 2019). DWV infection has been associated with alterations in development, physiology, behavior, and lifespan, thereby contributing to the progressive weakening of colonies (Penn et al., 2022).

In honey bees, defense against pathogens relies primarily on innate immune mechanisms. At the colony level, these defenses are complemented by forms of social immunity, such as grooming behavior and the removal of compromised individuals, which help limit pathogen transmission (Evans et al., 2006; Simone-Finstrom, 2017). Nevertheless, the effectiveness of these defenses depends strongly on the physiological condition of bees and on the nutritional context in which infection occurs (Ricigliano et al. 2022). Within this framework, nutrition has been proposed to influence not only the activation of immune responses but

also the host capacity to tolerate damage associated with persistent viral infections (Dosch et al., 2021).

In this context, the nutrition of honey bees encompasses the acquisition and utilization of essential macro- and micronutrients that sustain individual growth, development, and vital functions. Carbohydrates, obtained from nectar, represent the primary energy source and contribute to oxidative balance and antimicrobial defense (Fernandes et al., 2022). Pollen, on the other hand, provides proteins, amino acids, lipids, sterols, vitamins, minerals, and bioactive compounds that support tissue development and immune competence, although its nutritional quality varies according to botanical origin and environmental conditions (Kolayli et al., 2024; Ansaloni et al., 2025).

Despite growing interest in the links between nutrition and honey bee health, the specific role of pollen chemical quality in shaping DWV infection outcomes remains insufficiently integrated. Available evidence is dispersed across studies focused on nutrition, immune function, oxidative stress, gut microbiota, and seasonal ecology, and is often difficult to compare because of differences in experimental systems, life stages, and response variables. In this systematic review, the available evidence linking pollen chemical quality to DWV infection dynamics and survival in *A. mellifera* is examined across molecular, physiological, ecological, and seasonal scales. Attention is given to the role of bioactive pollen compounds, especially flavonoids and phenolic acids, while explicitly distinguishing evidence derived from honey bee studies from mechanistic hypotheses proposed in other viral systems. This review aims to integrate these fragmented lines of evidence, identify the main knowledge gaps, and provide a critical framework for understanding how pollen chemical quality may shape DWV outcomes and honey bee survival under different biological and ecological contexts.

2.Methodology

This review was conducted as a structured narrative synthesis given the substantial heterogeneity in viral inoculation methods, host age, diet composition, and outcome metrics in the literature. Comprehensive research was performed in Web of Science, Scopus, PubMed, Google Scholar, and journal databases using combinations of terms related to bee nutrition, DWV infection, immune gene

expression, pollen chemistry, phytochemicals, and microbiota. The primary search window covered studies published between 2000 and 2026, with older references retained only when they provided foundational information on honey bee immunity or viral biology. Priority was given to peer-reviewed experimental studies evaluating pollen, pollen substitutes, bee bread, floral phytochemicals, or defined dietary compounds in relation to DWV or other honey bee viruses. Studies were grouped by experimental scale, dietary treatment, pathogen context, and response variables, allowing mechanistic and ecological evidence to be integrated qualitatively and comparatively.

3.Deformed Wing Virus (DWV) in *Apis mellifera*: biology, transmission, and pathological effects

Deformed wing virus (DWV) is one of the most widely distributed viral pathogens in *Apis mellifera* and is currently recognized as one of the central components within the set of factors underlying colony weakening and collapse at a global scale (Martin & Brettell, 2019). Its high prevalence, even in colonies that do not show clinical signs, reflects its remarkable ability to establish persistent and subclinical infections, which can be maintained for prolonged periods within bee populations (Gisder & Genersch, 2021). This characteristic contributes to the persistence of DWV within honey bee populations and helps explain why its impact is often expressed progressively and cumulatively, rather than as isolated events of massive mortality (Lamas et al. 2026).

DWV belongs to the family *Iflaviridae* and corresponds to a non-enveloped, positive-sense single-stranded RNA virus, whose genomic organization follows the characteristic pattern of picorna-like viruses (de Miranda & Genersch, 2010). Its genome is translated as a single polyprotein that is subsequently processed to give rise to structural and non-structural proteins, including an RNA-dependent RNA polymerase, a helicase, and a 3C-like protease, all of which are indispensable for viral replication and virion assembly (Reuscher et al. 2023). In recent years, advances based on reverse genetics have made it possible to deepen the understanding of the replicative biology of DWV; however, relevant gaps remain regarding its cellular tropism and the host factors that modulate the efficiency of viral replication (Woodford & Evans, 2021). At the population level,

DWV should not be understood as a homogeneous entity, but rather as a complex of closely related genetic variants. To date, at least four main variants have been described, DWV-A, DWV-B, DWV-C and DWV-D, in addition to multiple recombinant forms whose relative frequencies vary as a function of space and time (Mordecai et al., 2016; de Miranda et al., 2022).

DWV transmission can occur through multiple routes, including vertical transmission from the queen to the offspring, through drone semen, horizontal transmission between individuals by trophallaxis, and through contact with infected pupae (Amiri et al., 2016; Posada-Florez et al. 2021). Nevertheless, the most consequential change in the epidemiology of this virus has been its association with vector-mediated transmission by *Varroa destructor*, a process that significantly increases transmission efficiency and promotes abrupt increases in viral load. In addition, this route is associated with a greater probability of clinical disease manifestation (Martin & Brettell, 2019; Piou et al., 2022). The association between DWV and *Varroa* has therefore redefined not only the intensity of infection, but also its pathological and epidemiological consequences within colonies (Doublet et al. 2024). In this sense, it has been described that DWV replication can occur in diverse bee tissues, and that tissue tropism constitutes a key component for understanding the breadth of the physiological effects associated with infection (Gusachenko et al., 2021). When high viral loads are reached during pupal development, particularly in the presence of *Varroa* parasitism, evident clinical manifestations may appear, including the characteristic deformed wing phenotype (Wu et al., 2021). However, in most adult bees, DWV persists as a covert infection, with sublethal consequences that include reduced longevity, lower survival, and impaired foraging performance (Benaets et al., 2017). Therefore, the biological importance of DWV lies not only in its capacity to generate visible clinical signs, but also in its ability to silently and persistently compromise colony physiology and functioning.

4.Immune system of honey bees and its modulation by diet quality

Since the pathological manifestation of DWV depends not only on viral load but also on the host's ability to resist and tolerate infection, it is essential to consider the organization of the immune system in *A. mellifera* and the nutritional factors that modulate its function. Honey bees rely exclusively on an innate immune system to combat pathogens, as they lack antibody-based adaptive immunity. This system is composed of humoral and cellular mechanisms highly conserved in insects, allowing bees to recognize, limit, and tolerate infections caused by bacteria, fungi, and viruses (Evans et al., 2006). The effectiveness of these responses is not constant but is closely linked to the physiological state of the individual, which depends largely on the availability and quality of nutritional resources (Alaux et al., 2010; Di Pasquale et al., 2013). However, some studies have shown that adequate pollen nutrition does not necessarily reduce DWV virus titers and that, under certain conditions, viral loads may remain unchanged or even increase (Alaux et al., 2011; Corona et al., 2023). From the perspective of host defense theory, these contrasting outcomes can be interpreted within the resistance–tolerance framework (Medzhitov et al., 2012). Resistance mechanisms reduce pathogen burden, whereas tolerance mechanisms reduce the physiological consequences of infection without necessarily decreasing viral load. This distinction is particularly relevant for DWV because improved nutrition may enhance bee survival and colony performance even when viral titers remain unchanged (Branchiccela et al, 2019).

Pathogen recognition in *A. mellifera* occurs through pattern recognition receptors (PRRs), which detect conserved molecular structures of microorganisms and activate intracellular signaling cascades (Larsen et al., 2019). Among the main immune pathways described in bees are Toll, Imd, JAK/STAT, and JNK, which regulate the expression of immune genes and the production of antimicrobial peptides (AMPs) such as defensin, abaecin, apidaecin, and hymenoptaecin (Evans et al., 2006; Evans & Spivak, 2010).

Against RNA viruses such as DWV, the primary antiviral defense in bees is RNA interference (RNAi), a mechanism highly conserved in insects (Swevers et al., 2018). During viral replication, double-stranded RNA (dsRNA) intermediates are generated, recognized by the enzyme Dicer-2 and processed into small interfering RNAs (siRNAs) (Sabin et al., 2013). These siRNAs are incorporated

into the RISC complex, where Argonaute-2 directs the specific degradation of viral RNA, thereby reducing viral replication (Brutscher et al., 2015). The activity of the RNAi pathway is energetically demanding, and its efficiency is thus directly sensitive to nutritional status (Roger et al., 2017). Together with the production of antimicrobial peptides and the activation of immune signaling pathways, RNAi is primarily associated with resistance because it contributes directly to limiting viral replication. A non-specific antiviral response induced by dsRNA has also been described in *A. mellifera*, capable of activating immune genes without exact sequence matching (Niu et al., 2014).

Along with humoral responses, bees possess cellular immunity mechanisms mediated by hemocytes, which participate in phagocytosis, encapsulation, and melanization (Strand, 2008). Melanization, regulated by the enzyme phenoloxidase, generates reactive oxygen species as by-products, implying a risk of collateral tissue damage if not properly controlled (González-Santoyo & Córdoba-Aguilar, 2012). This balance between immune activation and damage control is particularly relevant during persistent infections such as DWV and represents an interface where pollen-derived antioxidants may play a meaningful physiological role. Such processes are more closely aligned with tolerance mechanisms, as they may reduce infection-associated damage and improve host survival even when viral loads remain unchanged (Dosch et al., 2021).

The activation and maintenance of immune responses represent a significant energetic cost (Roger et al., 2017). In this context, poor or low-diversity pollen is associated with reduced immune capacity and increased susceptibility to viral infections, whereas access to high-quality pollen improves general physiological condition and tolerance to pathogenic stress (Belsky & Joshi, 2019). The effectiveness of these immune pathways, both humoral and cellular, depends not only on their genetic integrity, but on the availability of the energetic and molecular substrates that pollen provides. Dietary quality thus emerges as a transversal determinant of immune function in A. mellifera, ultimately shaping the margin of resistance and tolerance available against DWV infection.

5.Pollen chemical quality as a determinant of defense against deformed wing virus (DWV) in *Apis mellifera*

In *A. mellifera*, pollen constitutes the primary dietary source of proteins, lipids, sterols, vitamins, minerals, and a wide range of bioactive compounds (Brodschneider & Crailsheim, 2010). These elements provide the chemical substrates necessary to sustain the vital processes related to the health and defense of the host organism (Walton & Dolezal, 2021). Unlike nectar, whose role is primarily energetic, pollen supplies the essential compounds that become especially critical under pathogen pressure (Vaudo et al., 2016). Beyond the concentration of nutrients, pollen quality is also influenced by the balance among macronutrients, particularly protein-to-lipid (P) ratios. Honey bees can selectively forage on pollen resources that better match their nutritional requirements, and access to diverse floral communities may facilitate nutritional balancing through complementary pollen sources (Vaudo et al., 2020). The magnitude of this nutritional dependence becomes evident when considering the scale at which colonies consume pollen. A colony can consume between 15 and 55 kg annually, with individual adult bee daily consumption ranging from 3.4 to 5.4 mg depending on age (Ansaloni et al., 2025). The period of greatest pollen demand corresponds to the phases of active brood rearing and population growth. To rear a single worker larva, between 124 and 187.5 mg of pollen are required, providing between 25 and 37.5 mg of protein depending on the available protein content (Hrassnigg & Crailsheim, 2005). This demand falls primarily on nurse bees, which during their first 10 days of age consume large quantities of pollen to drive the development and secretory activity of their hypopharyngeal glands (Lazarov et al., 2025). These glands produce the main protein fraction of royal jelly, distributed via trophallaxis to larvae and the queen. Therefore, the nutritional status of nurse bees is not merely an individual matter, but a determining factor of population dynamics.

These figures reflect not only the energetic investment that pollen represents for the colony, but also its indispensable role in sustaining all major physiological functions (Qiao et al., 2024). However, pollen composition is far from uniform. Protein levels ranging from 2.5% to 61% have been reported in pollen collected by honey bees (Roulston et al., 2000). This variability in quantity and quality can represent a challenge for the nutritional homeostasis of the colony (Rodríguez-

Pólit et al., 2023). Nutritional deficits are directly reflected in lifespan, disease susceptibility, and body weight. In this regard, Di Pasquale et al. (2013) found that bees fed higher-quality protein pollen exhibited more developed hypopharyngeal glands, higher vitelogenin levels, and greater pathogen tolerance, suggesting that the nutritional quality of pollen directly modulates both the physiology and defensive capacity of nurse bees. Similarly, Branchiccela et al. (2019) found that nutritional stress associated with a diet based primarily on Eucalyptus grandis pollen compromised colony strength, observing a reduction in brood and adult bee populations. While supplemented colonies showed higher levels of RNA virus infection, these were generally low and without a clear negative impact. The authors suggest that improved nutrition may favor a more active physiological machinery that promotes viral replication, while also enhancing the colonies' ability to cope with infection. Furthermore, lower *Nosema* infection in supplemented colonies may have reduced competition for nutritional resources, potentially contributing to greater DWV replication. In the long term, nutritional stress hindered population recovery in spring, while supplemented colonies maintained better growth dynamics and seasonal resilience. This suggests that pollen quality not only determines the nutritional status of the colony but may also condition both resistance and tolerance responses to viral pathogens.

In this context, a previous study from our research group (García Domínguez et al., 2025) evaluated the effect of diets based on pollen of different botanical compositions on DWV-A viral load, survival, and immune gene expression in experimentally inoculated bees. The results showed that native *Eucryphia cordifolia* pollen drastically reduced viral load from 1.0×10^{13} to 1.0×10^{5} copies per bee, along with a survival rate of 91%. Furthermore, pollen diets modulated the expression of immune-related genes, reducing *Cactus* expression while upregulating *Dorsal*, *Relish*, and *Dicer-like*.

At the colony level, DeGrandi-Hoffman et al. (2020) demonstrated that colonies supplemented with pollen developed larger populations and survived longer than unsupplemented ones, although *V. destructor* and DWV levels increased similarly in both groups throughout the season. This suggests that the primary benefit of pollen does not lie in directly reducing pathogen load, but in strengthening colony resilience against infection. As shown in Table 1, these

findings indicate that pollen quality influences susceptibility to DWV across multiple levels of biological organization, from nurse bee physiology and gland development to immune gene expression, brood quality, population dynamics, and seasonal colony resilience. Consequently, pollen should be understood not only as a nutritional resource but also as a key modulator of host–pathogen interactions in *A. mellifera*, with its quality and diversity shaping the colony's physiological capacity to tolerate viral infections such as DWV. Nevertheless, stored pollen (bee bread) may also contribute to viral transmission within the colony. Bee bread can harbor infective DWV particles and may facilitate viral persistence, particularly in colonies with high DWV prevalence, a condition frequently associated with *V. destructor* infestation (Saleh et al., 2024).

Table 1. Effects of pollen quality and pollen-based diets on DWV infection outcomes and related health traits in honey bees

Study focus	Diet or pollen treatment	Experimental system	Response variables	Pathogen(s) evaluated	Main findings	Reference
Diet type and DWV load	Natural pollen, protein supplements, or sucrose syrup	Individual bees (laboratory)	Protein levels, hypopharyngeal gland acini size, viral titer	DWV	DWV titers were lowest in pollen-fed bees and highest in sucrose-fed bees; viral load declined over time in bees fed pollen.	DeGrandi-Hoffman et al., 2010
Pollen supplementation and colony-level viral load	Colonies in an *Eucalyptus grandis* plantation with or without polyfloral pollen patty supplementation	Colonies (field)	Colony strength, *Nosema* infection level, brood, RNA virus load	RNA viruses, *Nosema* spp.	Supplemented colonies showed higher DWV and ABPV titers but were stronger; viral titers remained below threshold for colony decline	Branchiccela et al., 2019
Effect of pollen nutrition on gene expression in healthy and Varroa-parasitized honey bees (nutrigenomics)	Pollen + sugar syrup vs. sugar syrup only	Laboratory conditions	Transcriptome (digital gene expression / DGE-tag profiling); nutrient-sensing pathways, metabolic genes, longevity genes, antimicrobial peptide genes	Varroa destructor associated viral populations	Pollen activated nutrient-sensing, metabolic and immune pathways; however, the virus-promoting effects associated with Varroa parasitism persisted despite pollen consumption..	Alaux et al., 2011
Contrasting pollen-based diets and viral load	Pollen-based diets from different floral sources	Individual bees (laboratory)	Immune gene expression, survival, viral load	DWV-A	Pollen diets reduced the viral load of DWV-A from 10^{13} to 10^{5}–10^{6} copies/bee and	García Domínguez et al., 2025

					increased survival rates to 91%. In addition, they modulated the expression of immune genes.	
Pollen, Varroa, and DWV via behavioral maturation genes	Pollen-supplemented vs. pollen-free diet in *Varroa*-infested bees	Individual bees (laboratory)	Lifespan, Vitellogenin, Juvenile Hormone Esterase, immune-related gene expression, DWV load	DWV (*Varroa*-associated)	Pollen increased the lifespan of mite-infested bees by reversing premature behavioral maturation induced by *Varroa* and was associated with higher AMP expressions and lower DWV load.	Frizzera et al., 2022
Field pollen supplementation and colony demography	Supplemental pollen feeding	Colonies (field)	Colony population, brood, survival; DWV and *Varroa* levels	Varroa *destructor* and DWV	Pollen-fed colonies were larger, had more brood, and survived longer, whereas DWV and *Varroa* levels were similar between fed and unfed colonies. The survival benefit was therefore associated with improved colony growth rather than with a consistent reduction in pathogen or mite levels.	DeGrandi-Hoffman et al., 2020
Diet protein composition and viral outcomes	Artificial diets: free amino acids vs. intact proteins	Caged bees (laboratory)	Vitellogenin, MRJP1, survival	DWV	Improved nutritional biomarkers (vg and mrjp1 expression), with contrasting effects on	Tapia-Rivera et al., 2025

					DWV dynamics depending on whether nutrients were supplied as intact proteins or free amino acids.	
Artificial diets and DWV expression	Artificial diets varying in protein composition and digestibility	Individual bees (laboratory)	Protein metabolism, molecular health markers, DWV relative expression	DWV	Diet composition was associated with differences in protein digestibility, host molecular markers, and DWV relative expression	Frunze et al., 2024
Diet type and DWV load	Natural pollen, protein supplements, or sucrose syrup	Individual bees (laboratory)	Protein levels, hypopharyngeal gland acini size, viral titer	DWV	DWV titers were lowest in pollen-fed bees and highest in sucrose-fed bees; viral load declined over time in bees fed pollen.	DeGrandi-Hoffman et al., 2010

Note: Comparisons across studies should be interpreted with caution because of differences in experimental systems, viral strains, life stages, and response variables evaluated

6. Pollen phytochemicals as context-dependent modulators of DWV outcomes in *Apis mellifera*

Pollen is not only a source of essential nutrients for *Apis mellifera*, but also a chemically complex matrix of secondary metabolites with potentially important effects on host physiology and pathogen responses. Among the most relevant compounds are polyphenols, terpenoids, phytosterols, alkaloids, and other aromatic metabolites, whose abundance and composition vary markedly with botanical origin, floral diversity, and environmental conditions (Gámbaro et al., 2025; Tlak Gajger & Cvetkovikj, 2025). Phenolic compounds are particularly prominent in this context because they are frequently detected in pollen and have been associated with antioxidant activity and other biological properties that may influence bee health (Rodríguez-Pólit et al., 2023). In honey bees, the relevance of pollen-derived phytochemicals extends beyond their simple presence in floral resources. Current evidence suggests that these compounds may modulate immune function, oxidative balance, and physiological resilience, thereby affecting the host context in which viral infection develops. At the molecular level, phytochemicals derived from nectar and pollen have been associated with changes in immune-related gene expression, including the regulation of antiviral effectors and signaling pathways linked to Toll- and Imd-related responses (Barroso-Arévalo et al., 2019; Parekh et al., 2021). These observations support the idea that floral secondary metabolites may influence antiviral defense in bees, although the magnitude and biological significance of these effects likely depend on the compound involved and the physiological condition of the host.

Some of the strongest evidence in bees comes from studies showing that dietary phytochemicals can alter responses associated with DWV infection. Palmer-Young et al. (2017) reported that phytochemicals naturally present in nectar and pollen stimulated antimicrobial peptide expression in adult bees and reduced DWV levels in young bees under experimental conditions. Likewise, Lu et al. (2020) showed that caffeine increased the expression of immune-related genes and reduced DWV copy number in *A. mellifera*. More broadly, dietary phytochemicals have also been linked to increased longevity and improved tolerance to pathogens, reinforcing the view that floral chemistry may shape infection outcomes not only through effects on pathogen load, but also through enhanced host resilience (Bernklau et al., 2019).

Beyond immune modulation, pollen-derived phytochemicals may influence host–pathogen interactions through several non-exclusive mechanisms. They may contribute to antioxidant protection, help maintain redox homeostasis during infection, and support physiological functions that are otherwise compromised under viral challenge (Liao et al., 2021). They may also interfere more directly with viral processes, although evidence for this possibility remains much stronger in non-bee systems than in honey bees themselves (Brutscher et al., 2015; Hsieh et al., 2020). Because direct mechanistic evidence in the honey bee–DWV system is still limited, much of the current functional interpretation relies on studies from other RNA virus models, where polyphenols have been shown to act at different stages of the viral cycle. For example, polyphenol-rich extracts can reduce viral infectivity at the pre-entry stage by altering capsid integrity or viral adsorption (Magnavacca et al., 2022), whereas compounds such as quercetin and luteolin have been reported to inhibit RNA-dependent RNA polymerase activity in positive-sense RNA viruses, including SARS-CoV-2 (Munafò et al., 2022; Agrawal et al., 2020). In the context of DWV, however, these mechanisms should be regarded as plausible hypotheses rather than demonstrated effects.

Evidence generated directly in bees nevertheless suggests that plant-derived compounds can meaningfully alter DWV-related outcomes. Boncristiani et al. (2021) highlighted the capacity of natural products, including flavonoids, to modulate viral infections in pollinators through both immunomodulatory and putative antiviral effects. Pascual et al. (2022) further showed that supplementation with grape pomace rich in polyphenols was associated with reduced DWV load, improved survival, and activation of immune-related genes in adult honey bees. These findings are especially relevant because they indicate that pollen-associated phytochemicals may affect DWV not only indirectly through general improvements in host condition, but also through more specific changes in infection-associated physiology.

At the same time, the biological relevance of pollen phytochemicals cannot be inferred solely from their occurrence in pollen. Their effects are shaped by compound identity, dose, post-ingestion transformation, and the broader biological context in which they are consumed. Structurally related compounds may differ substantially in their antipathogenic activity, and floral products can affect bee pathogens through both direct and host-mediated pathways (Fitch et al., 2022). Host-plant chemistry may also alter pathogen susceptibility through interactions with immune regulation and

microbiota composition (Sanaei & de Roode, 2025). This complexity is reinforced by evidence showing that the phytochemical profiles of larvae and adult bees differ from those of their pollen diet, suggesting that metabolism, selective transfer, and biotransformation strongly influence actual host exposure (Vidkjær et al., 2024).

Thus, the role of pollen-derived phytochemicals should not be viewed as a uniform antiviral effect, but rather as a context-dependent modulation of infection outcomes emerging from the interaction among floral chemistry, host physiology, oxidative balance, microbiota, and environmental stress. From this perspective, the novelty of current research lies not simply in identifying phytochemicals in pollen, but in understanding how these compounds shape the physiological environment in which DWV persists, replicates, and causes damage. Pollen phytochemicals therefore emerge as candidate modulators of infection outcome rather than universally acting antiviral agents (Figure 1). This interpretation provides a more integrative framework for explaining why chemically distinct diets may lead to different patterns of viral load, survival, and tolerance in *A. mellifera* and highlights the need for bee-specific mechanistic studies that move beyond extrapolation from non-apicultural viral systems

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appear to depend on both the specific compound involved and the physiological status of the bees.

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Nevertheless, structural studies of DWV have provided insights into potential viral targets that could be susceptible to chemical interference. The high-resolution structure of the DWV virion revealed a surface-exposed P domain containing conserved putative catalytic residues that may participate in receptor binding and viral entry, as well as conserved RNA-binding sites involved in virion stability and assembly (Škubník et al., 2017). Although direct evidence of phytochemical interactions with these targets is currently lacking, these structural insights provide a useful mechanistic framework for future studies investigating how pollen-derived compounds may affect DWV infectivity. Studies in other RNA virus models have shown that polyphenol-rich

extracts can reduce viral infectivity at the pre-entry stage by altering capsid integrity or viral adsorption (Magnavacca et al., 2022), whereas compounds such as quercetin and luteolin have been reported to inhibit RNA-dependent RNA polymerase activity in positive-sense RNA viruses, including SARS-CoV-2 (Munafò et al., 2022; Agrawal et al., 2020). In the context of DWV, however, these mechanisms should be regarded as plausible hypotheses rather than demonstrated effects.

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At the same time, the biological relevance of pollen phytochemicals cannot be inferred solely from their occurrence in pollen. Their effects are shaped by compound identity, dose, post-ingestion transformation, and host physiological condition, infection status, and gut microbiota composition (Santhiravel et al., 2022; Kwon et al., 2023). In particular, the biological activity of flavonoids and other polyphenols is strongly influenced by their molecular structure. Variations in hydroxylation patterns, glycosylation, methylation, and overall molecular conformation can affect bioavailability, antioxidant capacity, immunomodulatory properties, and potential antiviral activity (Kumar & Pandey, 2013; Panche et al., 2016). Consequently, individual flavonoids and polyphenols may differ substantially in their biological activity. Therefore, antiviral or immunomodulatory effects observed for specific compounds cannot be assumed for all members of the same chemical class. Floral products can affect bee pathogens through both direct and host-mediated pathways, including effects on immune function and gut microbiota composition (Fitch et al., 2022; Sanaei & de Roode, 2025). This complexity is reinforced by evidence showing that the phytochemical profiles of larvae and adult bees differ from those of their pollen diet, suggesting that metabolism, selective transfer, and biotransformation strongly influence actual host exposure (Vidkjær et al., 2024). Viewed within the resistance–

tolerance framework, pollen-derived phytochemicals may influence DWV outcomes through both mechanisms. Some compounds may contribute to resistance by limiting viral replication or enhancing antiviral immune responses, whereas others may promote tolerance by reducing oxidative damage, supporting physiological homeostasis, and improving survival despite persistent infection.

Thus, the role of pollen-derived phytochemicals should not be viewed as a uniform antiviral effect, but rather as a context-dependent modulation of infection outcomes emerging from the interaction among floral chemistry, host physiology, oxidative balance, microbiota, and environmental stress. From this perspective, the novelty of current research lies not simply in identifying phytochemicals in pollen, but in understanding how these compounds shape the physiological environment in which DWV persists, replicates, and causes damage. Pollen phytochemicals therefore emerge as candidate modulators of infection outcome rather than universally acting antiviral agents (Figure 1). This interpretation provides a more integrative framework for explaining why chemically distinct diets may lead to different patterns of viral load, survival, and tolerance in *A. mellifera* and highlights the need for bee-specific mechanistic studies that move beyond extrapolation from non-apicultural viral systems.

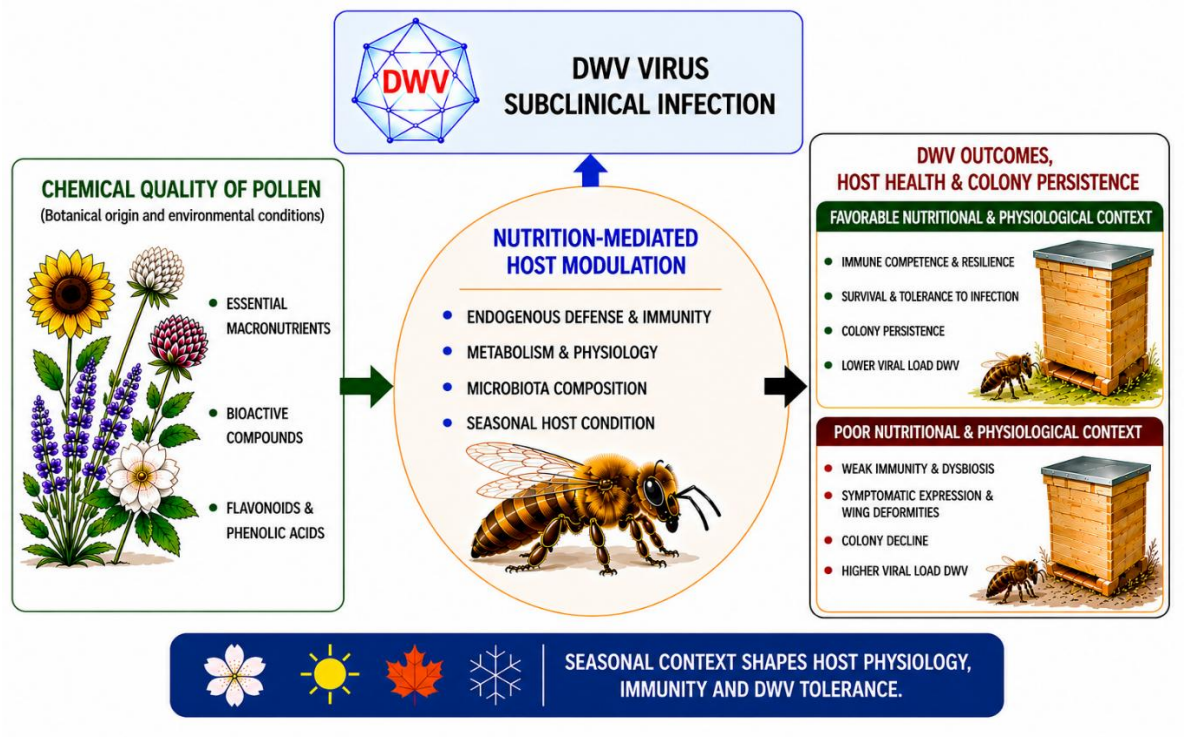

Figure 1. Conceptual model showing how pollen chemical quality influences honey bee immunity, DWV viral load, and colony health.

Direct mechanistic evidence for the effects of pollen-derived phytochemicals in the *A. mellifera*–DWV system remains limited. Therefore, much of the current functional interpretation is informed by studies conducted in other RNA virus models. Table 2 summarizes selected pollen-derived phytochemicals with reported antiviral activity in non-bee systems, highlighting their proposed mechanisms of action and their plausible relevance to DWV infection in honey bees. In this context, these mechanisms should be interpreted as working hypotheses rather than as demonstrated antiviral effects in bees.

Table 2. Pollen-derived phytochemicals with antiviral activity reported in non-bee systems: potential mechanistic hypotheses for future studies of DWV in honey bees.

Compound	Chemical classification	Viral models studied	Reported antiviral mechanism	Affected stage of viral cycle	Relevance to DWV in honey bees	References
Quercetin	Flavonol	SARS-CoV-2, rhinovirus, influenza, and other RNA viruses	RdRp inhibition; interference with endocytosis pathways; redox and immune modulation.	Viral entry, genome replication, translation	Plausible candidate for modulation of viral replication and host oxidative balance in DWV-infected bees	Agrawal et al. (2020)
Chlorogenic acid	Phenolic acid	Influenza A (H1N1/H3N2) and other viruses	Neuraminidase inhibition; reduced viral replication; antioxidant and anti-inflammatory activity.	Viral release/spread , replication	Potential modulator of oxidative stress and infection-associated physiological damage rather than a demonstrated direct antiviral in bees	Ding et al. (2017)
Luteolin	Flavone	Coronavirus, influenza, enterovirus, RSV; SARS-CoV-2	Inhibition of viral entry and replication; modulation of the host response.	Viral entry, genome replication	Plausible candidate for interference with viral replication and for immunomodulatory effects	Lu et al. (2023); Munafò et al. (2022)

					in the honey bee–DWV system	
Kaempferol	Flavonol	Influenza, coronavirus, hepatitis B, enterovirus	Inhibition of viral entry or fusion and replication; modulation of host pathways.	viral entry, replication	Potential contributor to host resilience and antiviral defense, although evidence in bees remains indirect	Periferakis et al. (2023)
Apigenin	Flavone	Enterovirus 71	Inhibition of viral IRES activity; modulation of the JNK pathway; suppression of replication.	Genome replication, viral translation	Plausible mechanistic candidate for modulation of viral translation-related processes, pending bee-specific evidence	Lv et al. (2014)
Myricetin	Flavonol	SARS-CoV	Inhibition of the viral helicase nsP13 and other viral enzymatic activities	Genome replication, viral protein processing	Hypothetically relevant to DWV replication through interference with viral enzymatic functions, but not demonstrated in bees	Yu et al. (2012)
Pinocembrin	Flavanone	Zika virus and other RNA viruses	Inhibition of post-entry processes; reduction of viral RNA and viral protein synthesis	Post-entry replication	Plausible candidate for limiting intracellular viral replication and associated cellular stress in bees	Lee et al. (2019)

Caffeic acid	Hydroxycinnamic acid	HCV, HBV, HSV-1	Antioxidant activity and inhibitory effects on viral replication	Viral replication, host antioxidant response	More likely relevant as a modulator of oxidative balance and host condition during DWV infection than as a confirmed direct antiviral	Pavlíková et al. (2022)
Ferulic acid	Hydroxycinnamic acid	Influenza, VSR	Antioxidant activity and modulation of NF-κB/host inflammatory response	The host's response to oxidative stress and inflammation	Plausibly relevant to tolerance mechanisms by limiting infection-associated oxidative and inflammatory damage	Antonopoulou et al. (2022)
Rutin	Flavonol	EqHV-8, SARS-CoV-2	Reduction of viral infection and oxidative stress; activation of the Nrf2/HO-1 pathway	Viral replication, antioxidant response	Potential candidate for improving tolerance to DWV through antioxidant and cytoprotective effects	Chen et al. (2024)
Naringenin	Flavanone	SARS-CoV-2, Zika, dengue and other RNA viruses	Putative inhibition of 3CLpro; reduced viral internalization/entry; decreased viral titter in flaviviruses; anti-	Viral entry, post-entry replication	Plausible multifunctional candidate linking antiviral and host-protective effects, although evidence for DWV remains indirect	Tutunchi et al. (2020)

			inflammatory modulation			

***Note*:** Mechanisms described for mammalian RNA viruses are presented as plausible working hypotheses, not as demonstrated effects in the *Apis mellifera*–DWV system

7. Seasonal and ecological influences on diet quality, DWV load, and bee survival

Honey bees exhibit seasonal variation in their tolerance to viral infections. The biological impact of DWV depends not only on the presence of the virus or on viral load, but also on the health status of the host and the environmental context in which the infection occurs (Dowell, 2001). This perspective is consistent with general frameworks in disease ecology, according to which seasonality simultaneously modulates pathogen dynamics, host susceptibility, and the environmental conditions that mediate their interaction (Altizer et al., 2006). In this sense, seasonality implies changes in the body condition of individuals and in colony organization. Differences between summer and winter bees include variations in fat body reserves, metabolism, microbiota, transcriptomic profiles, and immunocompetence (Dostálková et al., 2021). In this context, tolerance to DWV should be understood as a dynamic trait, conditioned by the moment of the annual cycle in which infection occurs (Bresnahan et al., 2022). This framework is especially relevant for DWV, whose epidemiology is closely linked to *V. destructor*, but is not explained exclusively by the presence of the mite. The interaction between *Varroa* and DWV has driven the amplification, spread, and changes in virulence of this pathogen system on a global scale (de Miranda & Genersch, 2010; Wilfert et al., 2016; Traynor et al., 2020). Longitudinal studies have shown that viral abundance, pathogen co-occurrence, and colony vulnerability change seasonally, with overwintering being a particularly critical period (Faurot-Daniels et al., 2020). Seasonality can also influence the population structure of DWV itself. Temporal changes have been documented in the relative prevalence of viral variants and in their association with colony losses, especially in relation to the dynamics between DWV-A and DWV-B (Grindrod et al., 2021; Paxton et al., 2022). Evidence from colony-level metagenomic analyses further indicates that DWV populations can undergo seasonal genetic shifts within individual colonies, highlighting the dynamic nature of this virus beyond broader population-level patterns (Thaduri et al., 2021). Given that some genotypes differ in virulence, transmission, and persistence, these transitions can modify not only the epidemiology of the virus, but also the biological consequences of infection at the colony level (Norton et al., 2021). Within this scenario, the floral landscape

and the seasonal availability of pollen form part of the ecological context that modulates the host-pathogen relationship. The composition and diversity of collected pollen change throughout the year and according to land use, affecting the nutritional basis that sustains both individual bee and the colony (Lau et al., 2019). In intensive agricultural landscapes, colonies often face an alternation between floral abundance and scarcity, with effects on their health and their ability to cope with stressors (Zhang et al., 2020). Thus, seasonal tolerance to DWV should not be interpreted only as an intrinsic property of the host, but rather as the result of the interaction among the time of year, the context of infection, and the quality of the nutritional environment.

This way of understanding tolerance is aligned with the distinction between resistance and tolerance as alternative defense strategies against disease (Medzhitov et al., 2012). Applied to *A. mellifera*, this suggests that colonies may pass through periods of the year in which they do not necessarily control infection more effectively but do differ in their ability to maintain vital functions and survive in the face of viral infection. In this sense, seasonality emerges as an ecological organizer of DWV pathogenicity and raises relevant applied implications, since if tolerance to the virus changes throughout the year and depends on the ecological context of the host, then floral landscape management, resource availability during critical periods, and timely *Varroa* control could contribute not only to limiting viral transmission, but also to reducing the probability that infection results in severe damage or colony loss (Gregorc & Sampson, 2019).

8.Gut microbiota as an intermediary between pollen chemical quality and DWV outcomes in *Apis mellifera*

In *A. mellifera*, the relationship between pollen chemical quality and deformed wing virus (DWV) infection should be interpreted considering the gut microbiota, which may act as an important intermediary between diet and host response (Motta & Moran, 2024). The honey bee gut microbiome is relatively simple and is concentrated mainly in the hindgut, where a core set of bacterial taxa predominates, including *Lactobacillus, Bifidobacterium*, *Bombilactobacillus*, *Gilliamella*, and Snodgrassella, whereas other taxa such as *Bartonella*, *Commensalibacter,* and *Frischella* are often considered more variable or

transient members (Bonilla-Rosso & Engel, 2018). This microbial community contributes to digestion, metabolism, and immune regulation, and its composition is shaped by diet, season, and environmental conditions (Meehan & O'Toole, 2025). In general, diets derived from diverse floral sources tend to support more stable and functionally diverse gut communities, whereas monofloral or artificial diets are more often associated with simplified microbiota (Powell et al., 2023). Within this framework, pollen-derived bioactive compounds may influence host–pathogen interactions not only at the systemic level but also through changes in the intestinal environment. Experimental evidence shows that dietary phytochemicals such as caffeine, gallic acid, p-coumaric acid, and kaempferol can increase gut microbial diversity and alter the abundance of taxa such as *Snodgrassella* and *Lactobacillus*, although these effects appear to be compound-specific (Geldert et al., 2021). Similarly, Braglia et al. (2025) showed that pollen from different botanical origins significantly modified the composition of the core gut microbiota and was associated with changes in immune-related hemolymph markers, highlighting the importance of pollen quality as a determinant of host physiological status. Other studies have also linked dominant symbionts, particularly *Gilliamella apicola*, to improved digestive efficiency, intestinal barrier function, and overall physiological condition (Kwong & Moran, 2016). Although these studies do not directly assess DWV infection, they provide a mechanistic basis for considering microbiota as a mediator of diet-dependent variation in infection outcome (Kwong et al., 2017). A complementary perspective was provided by Svobodová et al. (2023), who compared Varroa-surviving and Varroa-susceptible honey bee populations using microbial association networks. Their results showed marked differences in microbiota assembly between the two groups and suggested that microbiome structure may contribute to resilience against viral infections, although the study was based on associative rather than mechanistic evidence. This interpretation is especially relevant because the gut microbiota appears to contribute more to infection tolerance than to direct viral elimination. Microbiota stability and functionality are strongly influenced by diet, and pollen phenolics may reach the gut in partially transformed forms that selectively affect microbial composition and metabolism (Ozdal et al., 2016). Similar interactions have been described in other organisms, although their specific outcome depends on compound identity, concentration, and the pre-

existing microbial community (Bešlo et al., 2023). In honey bees, microbial biotransformation of dietary compounds has been documented mainly in relation to nutrient metabolism and host physiology, rather than as a direct antiviral mechanism (Khumalo et al., 2025). From the perspective of DWV infection, microbiota therefore seems more likely to support infection tolerance than to directly suppress viral replication (Kwong et al., 2017). This framework may help explain why bees fed chemically richer diets often show lower viral loads and improved survival. At least part of this pattern could reflect a better basal physiological state mediated by microbiota, rather than a direct antiviral effect exerted by gut bacteria themselve. This interpretation is consistent with studies showing that disruption or absence of the gut microbiota is associated with broad changes in host gene expression affecting immunity, metabolism, development, and behavior (Raymann & Moran, 2018). Nevertheless, in the honey bee–DWV system, current evidence does not support a direct causal role of the microbiota in controlling viral replication. Rather, the gut microbiome should be viewed as part of a broader diet-dependent physiological network through which pollen chemical quality may shape tolerance to infection, survival, and colony health in honey bees.

9.Limitations and gaps in knowledge

Despite increasing evidence that pollen chemical quality can influence DWV infection outcomes in honey bees, important methodological and conceptual limitations still prevent the development of robust and predictive models. One of the main challenges is the high phytochemical variability of pollen, which arises from taxonomic, environmental, and seasonal differences (Bryś et al., 2021). This variability complicates comparisons among studies and limits reproducibility. The concentration of flavonoids and other bioactive metabolites can vary considerably according to the botanical and geographical origin of bee pollen, making it challenging to directly compare biological effects among studies (Gercek et al., 2021; El Ghouizi et al., 2023).

A second major limitation lies in the heterogeneity of experimental designs used to evaluate the effects of pollen and bioactive compounds on DWV. Studies differ in viral inoculation procedures, developmental stages examined, laboratory

versus field settings, control diets, and concentrations of phenolic compounds, often preventing direct comparison of results (Dubois et al., 2020; Pascual et al., 2022). In addition, many experiments rely on complex plant extracts or pollen mixtures whose chemical composition is only partially characterized, which limits the identification of the metabolites responsible for the observed responses (Qiao et al., 2024).

Knowledge of the bioavailability and metabolism of flavonoids and phenolic acids in bees also remains surprisingly limited. It is still largely unknown how these compounds are absorbed, biotransformed, distributed, or stored, and which concentrations they reach in physiologically relevant tissues such as the hypopharyngeal glands, brain, or gut (Vidkjær et al., 2024). This uncertainty makes it difficult to connect dietary composition with actual host exposure and biological effect. The interaction between phenolic compounds and the gut microbiota adds further complexity, since microbial biotransformation may generate secondary metabolites capable of influencing host physiology and immunity, yet the underlying mechanisms remain poorly resolved (Motta & Moran, 2024). The lack of integrated metabolomic approaches therefore remains a key obstacle to establishing causal links among pollen chemistry, microbial activity, and DWV modulation.

Another unresolved issue concerns the dose-dependent effects of polyphenols. Although these compounds are often discussed in relation to antioxidant and protective functions, some studies indicate that high concentrations may act as pro-oxidants and induce oxidative or cytotoxic effects (Andrés et al., 2023). This pattern is consistent with hormetic responses, in which the effects of bioactive compounds vary according to dose and biological context (Calabrese, 2008). This suggests that the biological activity of pollen phytochemicals cannot be interpreted independently of dose, diet matrix, or physiological context. In parallel, intercolony variability in responses to supplemental diets indicates that genetic, and possibly epigenetic, factors may also influence antiviral responses, although these sources of variation are rarely incorporated into the same experimental framework (Penn et al., 2022).

A further gap concerns the translation of laboratory findings to field conditions. Many studies report beneficial or antiviral-associated effects of supplemental diets under controlled conditions, but their expression in real colonies is shaped

by seasonality, floral variability, varroa mite level, pathogen co-occurrence, and environmental stress (Noordyke et al., 2021). As a result, effects detected in caged-bee assays cannot be directly extrapolated to colony-level resilience in complex landscapes.

Overall, these limitations indicate that the relationship between pollen chemical quality and DWV cannot yet be understood through single-factor explanations. Progress in this field will require more standardized experimental designs, chemically well-characterized diets, integrated metabolomic and microbiome analyses, and field-scale studies capable of capturing seasonal and ecological variation. Addressing these gaps will be essential not only to clarify how pollen chemistry shapes DWV outcomes, but also to support the development of evidence-based nutritional strategies to improve colony resilience under increasingly challenging environmental conditions.

10.Conclusions

The available evidence indicates that the outcome of deformed wing virus (DWV) infection in *A. mellifera* is shaped not only by viral pressure, but also by the nutritional and physiological context in which infection occurs. Within this framework, pollen chemical quality emerges as an important determinant of host condition, influencing immune function, oxidative balance, and the capacity of bees to tolerate infection. Chemically rich and diverse pollen diets are more consistently associated with improved survival and physiological performance than with uniform reductions in viral load, suggesting that their main contribution may lie in enhancing host resilience rather than in acting as direct antiviral factors.

Together, these findings support the view that pollen quality influences DWV outcomes through a combination of resistance- and tolerance-related mechanisms, with the latter appearing more consistently associated with improved survival and colony performance. This interpretation also highlights the potential role of the gut microbiota as an intermediary through which pollen-derived compounds may influence host physiology and infection outcomes, although the specific mechanisms involved remain insufficiently resolved.

At the same time, current knowledge is constrained by the high phytochemical variability among pollen sources, the heterogeneity of experimental designs, the

limited understanding of compound bioavailability and metabolism in bees, and the difficulty of extrapolating laboratory findings to field conditions. Future research should therefore combine chemically characterized diets, bee-specific mechanistic studies, microbiota and metabolomic approaches, and field-scale experiments that incorporate seasonal and ecological variation. Such advances will be essential to clarify how pollen chemical quality shapes DWV outcomes and to support the development of evidence-based nutritional strategies aimed at strengthening colony resilience under increasingly challenging environmental conditions.

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Capítulo II: Comparative study of pollen-based diets on viral load, survival, and expression of immune genes in honey bees infected with the DWV-A virus

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Abstract

Honeybee populations have experienced alarming declines, often associated with the presence of pathogens such as deformed wing virus (DWV). In this context, pollen is essential for the health and survival of bees. The biodiversity of Chilean native plants offers a unique opportunity to find natural resources with antiviral properties. In this sense, the effect of diets based on pollen of different botanical compositions on the reduction of viral load, the increase in survival and immune response of bees inoculated with the DWV-A was evaluated. Phenolic profiles and antioxidant properties were also studied. The results indicated that in honeybees treated with native Eucryphia cordifolia pollen significantly reduced the DWV-A load from 1.0x10 ^{13} to 1.0x10 ^{5} copy number per bee, meanwhile, that polyfloral and monofloral *Rubus ulmifolius* pollen reduced DVW-A load from 1.0x10 ^{13} to 1.0x10^{6} copy number per bee. The highest survival rate (91%) corresponded to bees that consumed E. cordifolia pollen; however, there were no significant differences detected with other pollen sources. Pollen diets modulated the expression of four immune-related genes, reducing Cactus expression while up-regulating Dorsal, Relish, and Dicer-like. Native *E. cordifolia* pollen showed high concentrations of gallic acid, vanillic acid, 3,4-dimethoxybenzyl, vanillin, chloro genic acid, caffeic acid, quercetin, and kaempferol. Principal component analysis (PCA) revealed a separation between pollens according to their botanical composition, with greater distinction between

non-native polyfloral and monofloral pollen of *R. ulmifolius* compared to native monofloral pollen of *E. cordifolia*. These findings highlight the antiviral value of pollens.

Keywords: Nutrition; pollen; antioxidants; immunostimu lants; antiviral; DWV

1. Introduction

Concern about declining Apis mellifera L. populations continues to be a topic of relevance in the scientific and beekeeping community (Powney et al., 2019). The possible causes of these declines are multiple and complex, which include factors such as climate change, pesticide use, habitat losses, declining floral diversity, parasites, and pathogens (Goulson et al., 2015; Siviter et al., 2021). The combination of these factors has created an environment that poses major challenges to bee health and survival (Budge et al., 2015). Global colony losses of A. mellifera reported in the last decade in North America, Europe, Asia (Japan), Middle East and more recently in Latin America (Requier et al., 2024). Winter is considered the season wherein the honey bees are more sus ceptible to a range of parasites and pathogens (Barroso-Arevalo et al., 2019; Kulhanek et al., 2017). In this sense, studies have described deformed wing virus (DWV) and its vector Varroa destructor as responsible for winter mortality (Frizzera et al., 2023; McMahon et al., 2016). DWV has vertical transmission from the queen to the eggs, horizontal transmission through trophallaxis, direct contact with infected pupae, or feeding by V. destructor, which is the pri mary vector (Amiri et al., 2018; Posada-Florez et al., 2021; Zhao et al., 2019). Three variants with major epidemiological relevance have been identified: DWV-A, DWV-B, and DWV-C, which have been detected in different countries with varying prevalen ces (Brasesco et al., 2021; de Souza et al., 2019; Kevill et al., 2019). Wherein the DWV-A is the most preva lent variant (71%) in Chilean apiaries (Riveros et al., 2020).

As a line of defense against pathogens, nutrition emerges as a key factor in improving the resilience and health of honey bees (Jovanovic et al., 2023). Adequate and diverse nutrition strengthens their immune system, improves longevity, and increases resistance to stressors. In this sense, honey bees depend on plants for nectar and pollen, essential elements in their diet (Di

Pasquale et al., 2013; Ricigliano et al., 2022). Pollen-provided colonies have been found to have lower pathogen infections and a higher overwintering survival rate (DeGrandi- Hoffman et al., 2010, 2016). In addition, the role of pollen in the activation and regulation of certain pathways of the innate immune system of these insects has been demonstrated (Frizzera et al., 2022). Among the pollen constituents, the profile of phen olic compounds has been studied (Bridi et al., 2022). This analysis has been important to understanding their bioactive, biological, and pharmacological prop erties (Albishi et al., 2019). Several natural compounds, including flavonoids, phenolic acids, and their derivatives, have been identified (Puerto et al., 2015). Pollen from native plants may be an abundant source of these bioactive compounds (Viteri et al., 2022). In addition, native plants, being an integral part of their ecosystem, interact with local fauna, including bees. In this context, Chile is recognized for its endemism and biodiversity of native plants (Bridi et al., 2019). This is a favorable scenario for identifying food resources and phytochemical treatments capable of mitigating DWV infection. In this study, the effects of pollen diets with different botanical compositions (polyfloral, non-native mono floral, and native monofloral) on viral load, survival, and immune response of bees inoculated with the DWV-A variant were evaluated. Additionally, the phenolic profiles and bioactive properties of the pollens were analyzed to identify their relevant compounds.

2. Materials and methods

2.1 Collection and botanical analysis of pollen samples

The pollen used in the study were collected during the 2022 beekeeping season in experimental apiaries located in Chillan (36°35'58"S 72°04'51"W; sample BP1), and Coihueco (36°37'00"S-71°50'00"W) sample BP2), Ñuble Region, and Río Bueno (40°26'18"S 72°30'43"W; sample BP3), Los Ríos Region, south-central Chile. The corbicular pollen was collected using pollen traps placed at the entrance of the honey bees hive for 20 days. A subsample (10 g) of the collected material was used for botanical identification and classification geographic origin according to Chilean Standard Normative NCh3255-2011; service made by the Palynology Section of the Laboratory of Entomology, Universidad Austral de Chile. Thus, sample BP1 was classified as polyfloral pollen composed mainly of

four botanical non-native species such as Brassica campestris (32.77%), Trifolium pratense (30.40%), Rubus ulmifolius (14.18%) and Lotus uliginosus (10.13%) (Table S1, supplementary material). The sample BP2 was classified as non-native monofloral pollen dominated by Rubus ulmifolius (70.20%). On the other hand, sample BP3 was classified as monofloral native pollen dominated by Eucryphia cordifolia (79.60%) (Table S1, supplementary material). Another subsample of each pollen (10 g) was used for proximate chemical analysis at the Animal Nutrition Laboratory of the Agronomy Faculty of the Universidad de Concepción (Table S2, supplementary material). The rest of pollen samples were stored at -20^{o}C until used in the bioassays.

2.2 Preparation of DWV-A Inoculum

The DWV-A was isolated from symptomatic honey bees from colonies infected with DWV, following the methodology described by Gusachenko et al. (2020). In brief, a group of 20 honey bees was homogenized in a Stomacher 80 Lab Blender (Seward, London, UK) with phosphate-buffered saline solution (1X PBS) for 90 s at high speed in a Stomacher 80 laboratory homogenizer (Seward, London, United Kingdom). Then, the samples were centrifuged twice at 1500 × g for 10 min at 4°C, followed by 10000 × g for 10 min at the same temperature. Afterward, the supernatant was filtered and purified with a 0.22 µm filter (PES, Merck Millipore, Darmstadt, Germany). Finally, for RNA extraction, cDNA synthesis and subsequent quantification of the viral load of the inoculum, 200 µL of the supernatant were used. The rest of the inoculum was stored at -80°C.

2.3 Bioassays

2.3.1 Effect of pollen type on the decrease of viral load, expression of immune system genes and survival of DWV-A infected bees

The honey bees used in this study were collected from hives of the experimental apiary at the 'El Nogal' research station (36°35′58″ S, 72°04′51″ W) of the University of Concepción, Chillán, Chile. Brood combs with capped worker cells were moved to the Apicultural Pathology Laboratory and maintained under controlled conditions (30°C; 50-60% RH). Prior to experimentation, colony health status was assessed using molecular techniques following the Vargas et al. (2017) protocol. We performed qPCR screening for honey bee pathogens

including *Nosema ceranae*, *Lotmaria passim*, Chronic Bee Paralysis Virus (CBPV), Acute Bee Paralysis Virus (ABPV), and Deformed Wing Virus (DWV). After the qPCR screening, we selected for lab experiments only three colonies that were pathogen-free or with low load pathogen level (Table S3, Supplementary material).

To determine the effect of pollen diet on the viral load and expression of genes associated with the immune system of honey bees, newly emerged bees (1-2 days old) were carefully collected from brood combs and divided into five treatments using a randomized design. The treatments include bees inoculated with DWV-A, and treated with six grams of (1) Polyfloral pollen (Pol-DWV-A), (2) *R. ulmifolius* pollen (RU-DWV-A), and (3) *E. cordifolia* pollen (EC-DWV-A). Two controls group treated with pollen substitute (Arismendi et al., 2018) were included: (4) inoculated bees (I-DWV-A, positive control), and (5) non-inoculated bees (N-DWV-A, negative control). All treatments were provided with 60% sucrose solution *ad libitum*. Each treatment consisted of 70 bees per plastic cage (base = 8 cm diameter, mouth = 10 cm diameter, and height = 15 cm) and four cages per treatment. In DWV-A treatments, each honey bee was orally inoculated (Porrini et al., 2013) with 5 µL of a viral suspension (1×10^9 viral copy number per bee) mixed with 50% sucrose syrup (Silva et al., 2021). Bees that did not consume the inoculum were discarded from the trial. After that, the DWV-A inoculated and non-inoculated honey bees were maintained under controlled conditions in a growth chamber (30°C; 50-60% RH) (Diego et al., 2024).

To determine the viral load and the expression of genes associated with the immune system, five bees were collected at 0, 5-, 10-, 15- and 20-days post-inoculation (Silva et al., 2021). The day 0 sample was used to evaluate the initial inoculation load, as well as the basal virus load in non-inoculated bees.

Survival of honey bees exposed to different pollen diets and inoculated and non-inoculated with DWV-A was determined following the above methodology with some modifications. In brief, each treatment consisted of 100 bees per cage and four cages per treatment. Daily counts were made for 20 days to record the number of dead honey bees, and the dead individuals were removed from each cage.

2.4 RNA extraction and cDNA synthesis

For RNA extraction and cDNA synthesis, the methodology described by Riveros et al. (2020) was used. The five bees collected in each sampling times (5, 10, 15 and 20-days post-inoculation) were triturated in PBS-buffered saline (5 mL) and then, 200 µL of the macerate was collected. Subsequently, 500 µL of TrizolTM (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was added and RNA extraction was performed using an E.Z.N.A. ®Total RNA Kit I (Omega BioTek, Norcross, GA, USA). In addition, RNA quality and yield were determined using a spectrometer (Infinite 200 PRO NanoQuant, Tecan Group, Männedorf, Switzerland). For the reverse transcription reaction, 1 µg of DNAase-treated total RNA was used to synthesize first-strand cDNA with the M-MLV reverse transcriptase enzyme (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA samples were then stored at −20 °C until further analysis.

2.5 Quantification by real-time PCR (qPCR)

For quantification of DWV-A viral load and expression of immune-related genes (Cactus, Dorsal, Relish, Dicer-like) of A. mellifera, specific primers were used (Table S4, supplementary material) (Evans, 2006). The PCR reaction was carried out using 1× KAPA SYBR FAST Universal 2× qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA), according to the supplier's instructions. Each sample was brought to a final volume of 15 µL, which included 20 ng cDNA, 530 nM of each primer and sterile filtered molecular grade water. The reaction thermal conditions were 95 °C for 3 min, followed by 40 cycles at 95 °C for 5 s, 60 °C for 15 s and 72 °C for 15 s (Diego et al., 2024). Real-time PCR assays were performed on an Agilent AriaMx Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA), and data were analyzed using Agilent AriaMx 1.5 software (Stratagene, Agilent Technologies, Santa Clara, CA, USA).

The relative expression of each gene was calculated using the Pfaffl (2006) method (equation N°5) after normalization with the endogenous gene β-actin (Yang and Cox-Foster, 2005). The group of bees non-inoculated with DWV-A and treated with pollen substitute (N-DWV-A) was used as the control sample for calculating relative expression levels of all experimental groups.

For absolute quantification of DWV-A, a standard curve was used from the purified PCR product (Wizard®VR SV gel and PCR clean up system, Promega, Madison, WI, USA), belonging to the viral target sequence (Riveros et al., 2020). The purified amplicon was then quantified by spectrophotometry (EpochTM Microplate Spectrophotometer, BioTek, Winooski, VT, USA) to calculate the copy number, according to Wu et al. (2017). In brief, linear standard curves (95-100% efficiency) were generated using a serial dilution (1.0 × 101 to 1.0 × 109) of the viral copy number of the purified cDNA. Ct values were then plotted against copy number values (log10) (Silva et al., 2021). Thus, the sample copy number was estimated using the Ct values and compared with the linear equation of the standard curve and the normalization values of the β-actin gene (Yang and Cox-Foster, 2005). To normalize viral load, β-actin Ct values were also converted to absolute quantities by generating a standard curve using purified PCR products of the β-actin sequence. The purified amplicon was quantified to calculate the number of β-actin copies per µL of cDNA. A linear standard curve was then constructed from serial dilutions of the purified amplicon (cDNA), followed by plotting the Ct values of the samples against the $\log_{10}$-transformed copy number values of β-actin. Afterwards, data were expressed as the number of copies of DWV-A per bee (log10-transformed) by considering the dilutions that were performed in the cDNA synthesis and qPCR reaction.

2.6 Characterization, quantification of phenolic compounds and antioxidant properties of pollen

2.6.1 Preparation of extracts

To carry out the extraction of phenolic compounds, 0.5 g of each freeze-dried pollen was treated with 5 mL of methanol/water/formic acid (50:49:1, v/v/v). The acidified hydromethanolic extracts were then placed in ultrasound for 60 min and left to stand for 24 h at 4oC. The next day, ultrasound was used again for 60 min and then centrifuged at 3500 rpm for 5 min. Finally, the supernatant was collected and filtered (0.22 µm) and then stored at -20oC (Ferreres et al., 2009).

2.7 Total polyphenol content (TPC) and antioxidant activity of pollen extracts

Total polyphenol content (TPC) was determined by the Folin-Ciocalteu method adapted to microscale (Singleton and Rossi, 1965). Briefly, 25 µL of the pollen extracts, 25 µL of 0.5N Folin-Ciocalteu reagent and 200 µL of H2O type 1 were added and incubated for 5 min at 25oC in the dark. Then 25 µL of 10% sodium carbonate (Na2CO3) was added and incubated for 60 min at 25oC. The absorbance was measured at 765 nm through a Synergy H1 hybrid multimode 96-well microplate reader (Biotek, Winooski, VT, USA). Four replicates were performed, and the results were expressed in mg of gallic acid equivalents per 100 g (mg GAE 100 g-1).

The antioxidant activity of the pollen extracts was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and oxygen radical absorbance capacity (ORAC) assays following the methodology described by Gu et al. (2019). The antioxidant activity by DPPH assay was evaluated by measuring the variation in absorbance at 517 nm after 30 min of reaction with the radical at a temperature of 25oC, while the ORAC antioxidant activity was estimated by measuring the changes in fluorescence during 90 min of reaction with the radical at excitation and emission wavelength of 485 and 520 nm at 37oC. Both assays were performed with a Synergy H1 hybrid multimode 96-well microplate reader (Biotek, Winooski, VT, USA). Results were expressed as µmol Trolox g-1. Four replicates were performed for each analysis.

2.8 Identification and quantification of phenolic compounds in acidified hydroalcoholic extracts of pollen by HPLC-DAD.

Identification and quantification of phenolic compounds was carried out using a Hitachi HPLC-DAD system (Hitachi technologies, MERCK, Darmstadt, Germany). The separation was carried out on a Kromasil C18 column (250 mm x 4.6 mm, particle size 5 mm; Phenomenex, Macclesfield, UK). The mobile phase consisted of two solvents: (A) water/formic acid (99:1, v/v) and (B) acetonitrile, with a flow rate of 1 mL min-1. The gradient varied with 6 % solvent B, reaching 25 % at 46 min, 50 % at 59 min, maintained up to 60 min and returned to initial conditions for the next 5 min. Compounds were identified and quantified by comparison with standards on a diode array detector (DAD) (MERCK, Darmstadt,

Germany). Chromatograms were recorded at 280 and 320 nm. Results were expressed as mg 100 g-1 DW.

2.9 Data analysis

Honey bee survival curves were plotted using a Kaplan–Meier estimator, considering live bees observed at the end of the experiment as censored data. Differences between survival curves were estimated by log-rank tests and p values were corrected with the Holm–Bonferroni method (Holm, 1979) for pairwise multiple comparisons.

Since the viral load and relative expression gene data were not normally distributed, they were transformed to log 10 to meet the assumptions of para metric tests. To evaluate the differences in viral load over time (0, 5, 10, 15, and 20-d post-inoculation) and between treatments (bees inoculated with DWV- A and treated with different pollen types), a nested analysis of variance (Nested ANOVA) was performed. Then, the Bonferroni test ($p < 0.05$) was run to separate the means between treatments.

Considering that the relative gene expressions of Cactus, Dorsal, Relish, and Dicer-like were correlated in bees inoculated and non-inoculated with DWV-A and treated pollens, a nested multivariate analysis of variance (MANOVA) was performed. Thus, the rela tive gene expression was considered the response variable. Sampling time post-inoculation (5, 10, 15, and 20 d) and DWV-A inoculated bees exposed to pollen types ([pol-DWV-A, RU-DWV-A, EC-DWV-A, I- DWV-A (positive control) and N-DWV-A [negative control]) were considered independent variables, with the pollen type treatments nested in the sam pling time post-inoculation. The Bonferroni test ($p < 0.05$) was then run to separate the means between treatments.

TPC, antioxidant activity and phenolic compound concentration were analyzed using a one-way ANOVA and then, Tukey's test ($p < 0.05$) for multiple comparisons. Also, a principal component analysis (PCA) was performed to compare the phenolic profiles of pollens in relation to survival and viral load reduction in bees. All analyses were performed in STATISTICA® version 7 statistical package for Windows®.

3. Results

3.1 Effect of pollen type on viral load, survival and expression of immune system genes in bees

Viral load varied significantly (nested ANOVA F = 51.90, df = 20, 75; p < 0.001) between experimental groups (Figure 1). These differences were observed during all post-inoculation intervals. Regardless of the type of pollen consumed by the worker honey bees (native and non-native), a significant decrease in viral load was observed compared to the inoculated bees with DWV-A, and treated with pollen substitute, at 20 d post-inoculation. Thus, worker honey bees treated with polyfloral and monofloral *R. ulmifolius* pollen reduce significantly DWV-A load from 1.0 × 10^{13} to 1.0 × 10^{6} copy number per bee, meanwhile monofloral *E. cordifolia* pollen reduce the DWV-A load to 1.0 × 10^{5} copy number per bee at the end of the experiment (Figure 1).

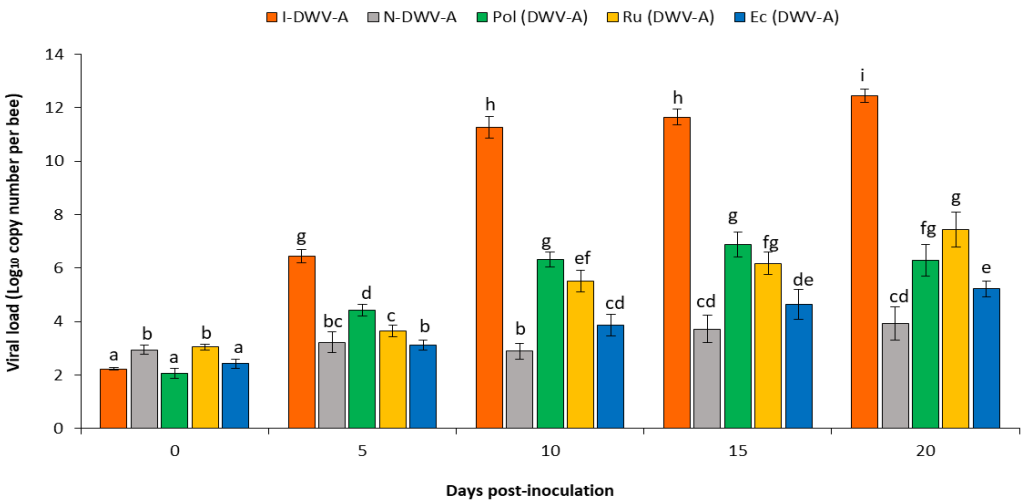

Figure 1. Viral load of honey bees inoculated and non-inoculated with DWV-A and exposed to native and non-native pollen, measured at 0, 5, 10, 15, and 20d post-inoculation. I-DWV-A, N-DWV-A, M, Ru, and Ec represent inoculated control, non-inoculated control, polyfloral, non-native monofloral *R. ulmifolius*, and native monofloral *E. cordifolia* pollen, respectively. Different letters indicate significant differences according to the Bonferroni test (p < 0.05).

Bees inoculated with the DWV-A and exposed to different pollen diets showed a significantly higher survival rate compared to the inoculated control (I-DWV-A vs. N-DWV-A, Log-rank test value = 4.238, p = 0.001; I-DWV vs. Pol-DWV-A, Log-

rank test value = 4.972, p= 0.001; I-DWV vs. Ru-DWV-A, Log-rank test value = 4.213, p= 0.001, and I-DWV vs Eu-DWV-A, Log-rank test value = 5.768, p= 0.001). The results indicated a survival rate of 85% and 89% for bees inoculated with DWV-A, and exposed to monofloral *R. ulmifolius* and polyfloral pollen, respectively (Figure 2). Similarly, the group of bees inoculated and fed with native monofloral pollen of *E. cordifolia* showed the highest survival rate at 91%, while bees inoculated with DWV-A and treated with pollen substitute, almost half died (46%) at the end of the experiment.

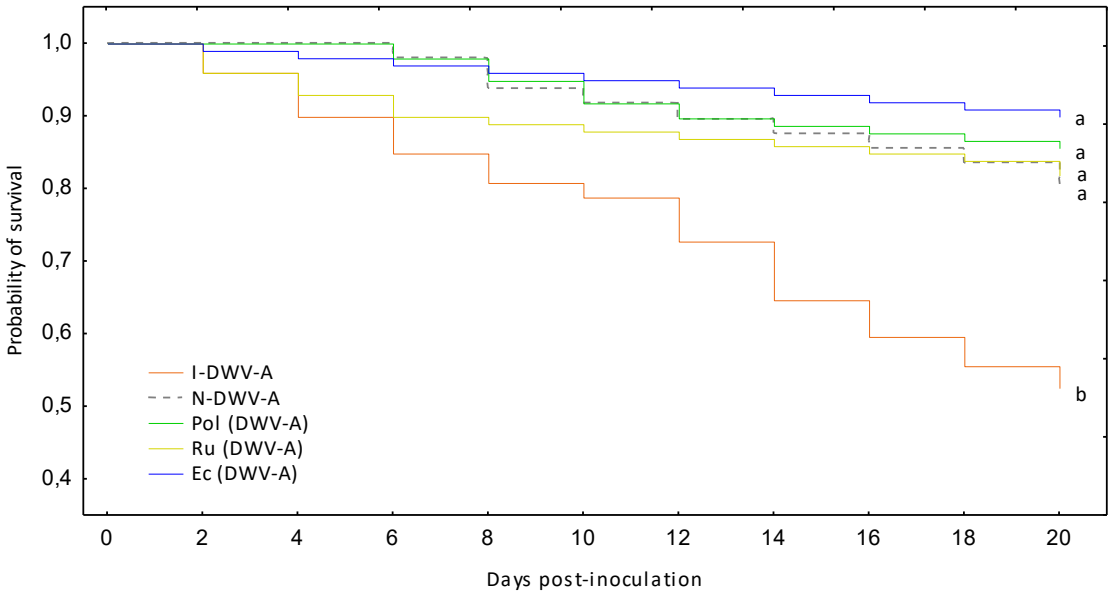

Figure 2. Kaplan–Meier survival curves distribution of worker honey bees inoculated (I-DWV-A) and non-inoculated (N-DWV-A) with DWV-A and exposed to polyfloral pollen (Pol), non-native pollen from R. ulmifolius (Ru) and native monofloral pollen from *E. cordifolia* (Ec). Different letters indicate significant differences between treatment groups according to the Log-rank test with Holm-Bonferroni correction.

Regarding gene expressions of Cactus, Dorsal, Relish and Dicer-like, there were detected significant differences in all genes analyzed. Nevertheless, DWV-A inoculated bees exposed to pollen types were influenced significantly by sampling time post-inoculation, and therefore, in the expression of these genes related to immune system (MANOVA Wilks = 0.005; F = 10.39; df = 64, 225; p < 0.001).

The relative gene expression by Cactus was significantly lower in the pollen-consuming bee groups compared to the inoculated control (Figure 3a). However, bees inoculated with DWV-A and fed with monofloral native *E. cordifolia* pollen, maintained significantly lower Cactus gene expression compared with other

pollen types at almost end of experiment (Figure 3a). On day 20 post-inoculation, bees treated with polyfloral pollen showed a reduction in Cactus gene expression to 7-fold relative to the I-DWV-A control group. Similarly, bees fed with *R. ulmifolius* and *E. cordifolia* pollen exhibited reduced expression levels of 10-fold and 24-fold, respectively.

Worker bees inoculated with DWV-A (I-DWV-A) and treated with pollen substitute showed down-regulation of *Dorsal* gene expression compared to the non-inoculated control group (N-DWV-A) (Figure 3b). In contrast, bees fed with different pollen types exhibited *Dorsal* expression levels like or higher than those observed in the N-DWV-A group throughout the experiment. Notably, bees fed with native monofloral *E. cordifolia* pollen showed increased *Dorsal* gene expression, reaching a 4-fold increase on day 5 and a 7-fold increase on day 10 relative to the I-DWV-A group (Figure 3b). In comparison to bees fed with polyfloral and non-native *R. ulmifolius* pollen, *E. cordifolia* treatment led to a 1.83-fold and 1.74-fold higher *Dorsal* expression on days 5 and 10, respectively (Figure 3b).

The results also revealed significant differences in the relative expression of the Relish gene among the different treatments (Figure 3c). In the group of bees inoculated with DWV-A and not treated with pollen, the Relish gene expression was down-regulated compared to the bees that were non-inoculated with DWV-A (negative control group) throughout the duration of the experiment (Figure 3c). On the contrary, bees inoculated with DWV-A and treated with pollen, increased significantly Relish gene expression and some case, more than non-inoculated bees with DWV-A. Bees exposed to monofloral pollen from native *E. cordifolia* showed greatest increases in the Relish gene expression, being significantly higher at some sampling times (e.g. 15 days post-inoculation) than the other pollens in the study (Figure 3c). Honey bees inoculated with DWV-A increased *Relish* gene expression over time when treated with pollen, reaching average fold changes of 4.5 and 3.75 with *E. cordifolia*, polyfloral and *R. ulmifolius* pollen, respectively, compared to bees that were inoculated with DWV-A treated with pollen substitute (I-DWV-A) (Figure 3c).

Dicer-like gene expression levels showed significant differences between experimental groups (Figure 3d). DWV-A inoculated bees and treated with pollen substitute (I-DWV-A), showed a significant increase of Dicer-like gene expression

and compared with control group (N-DWV-A) at 5- and 10-days post-inoculation. Interestingly, at the same time, bees inoculated with DWV-A and treated with the different types of plant pollen showed a significant up-regulation of Dicer-like expression when compared to DWV-A inoculated and treated with pollen substitute (Figure 3d). Ever more, worker honey bees inoculated with DWV-A and treated with the native *E. cordifolia* pollen, showed a substantially higher increase in the gene expression than the other two types of pollen used in the experiment, especially at 5, 10 and 15 post-inoculation (Figure 3d). Thus, during the first 15 days post-inoculation, DWV-A infected bees fed with *E. cordifolia* pollen exhibited the highest expression levels of the Dicer-like gene, showing a 1.43-fold increase compared to inoculated bees that did not receive pollen (I-DWV). Moreover, this expression was 1.20-fold higher than the average expression observed in bees fed with polyfloral and *R. ulmifolius* pollen at the first 15 days DWV-A post-inoculation. These differences were not as marked at 20 days post-inoculation; however, DWV-A inoculated bees and treated with polyfloral and *E. cordifolia* pollen continued to show the highest levels of expression of the Dicer-like gene (Figure 3d).

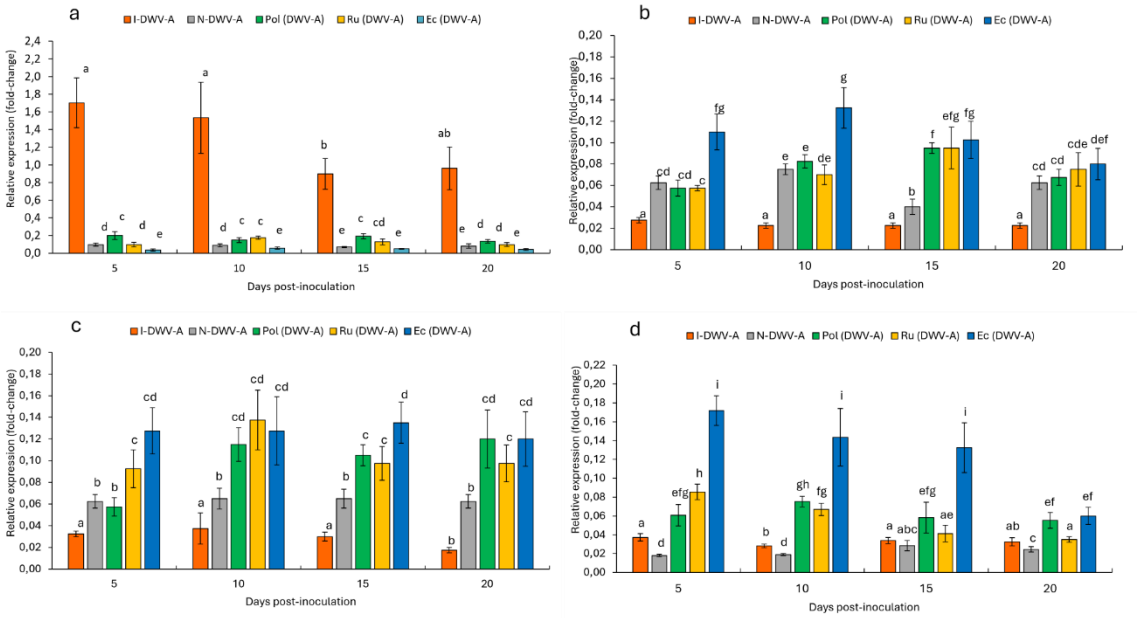

Figure 3. Relatives' expressions levels of (a) Cactus; (b) Dorsal; (c) Relish and (d) Dicer-like gene in honey bees inoculated and non-inoculated with DWV-A and exposed to native and non-native pollen, measured at intervals of 5, 10, 15, and 20 days post-inoculation. I-DWV-A, N-DWV-A, Pol, Ru, and Ec represent inoculated control, non-inoculated control, polyfloral pollen, no native monofloral from *R. ulmifolius*, and native monofloral from *E. cordifolia*, respectively. Different letters indicate significant differences according to the Bonferroni test ($p < 0.05$).

3.2 Chemical characterization of pollens

The total polyphenols content and antioxidant activity of the pollen extracts varied significantly among the pollen samples (One-way ANOVA F = 149.06; df = 2, 9; p <0.001) (Table 1). The non-native monofloral pollen extract of R. ulmifolius showed the higher TPC (603.91 mg GAE 100 g-1) values than other pollen extracts (Table S4). Similarly, the DPPH value also showed significant difference between pollen samples (One-way ANOVA F = 6.69; df = 2, 9; p < 0.02); DPPH values ranged from 677.17 µmol Trolox g-1 (E. cordifolia) to 799.98 µmol Trolox g-1 (Polyfloral) (Table 1). On the other hand, the ORAC test showed no significant differences (One-way ANOVA F = 2, 70; df = 2, 9; p < 0.12) between pollen extracts (Table 1).

Table 1. Total polyphenols content (TPC) and antioxidant activity of extracts of pollens. Different letters in the same column indicate significant differences, according to Tukey's test (p ≤ 0.05).

Pollen	TPC mg GAE 100 g-1	DPPH µmol Trolox g-1	ORAC µmol Trolox g-1
Polyfloral	482.86b	799.98a	294.19a
R. ulmifolius	603.91a	731.09ab	296.54a
E. cordifolia	419.35c	677.17b	248.88a

The concentrations of phenolic compounds varied significantly between pollen samples (Table 2). The native monofloral pollen of E. cordifolia showed eight compounds in higher concentrations, these include gallic acid, vanillic acid, 3,4 dimethoxybenzyl, vanillin, chlorogenic acid, caffeic acid, quercetin and kaempferol. It was found that E. cordifolia showed approximately 10 times more chlorogenic acid and 5 times more kaempferol and 3,4 dimethoxybenzyl than all other pollen (Table 2). In the case of polyfloral pollen, the highest concentrations of 3,4-DHPglycol, 3-hydroxytyrosol, 2,6-dimethoxyphenol and Q 3-rutinoside were found. On the contrary, only two compounds showed a higher concentration in the non-native monofloral pollen of R. ulmifolius: 4-methylcatechol and ferulic acid (Table 2).

Table 2. Quantification (mg/100g-1DW) of phenolic compounds in acidified hydromethanolic extracts of pollens by HPLC-DAD. Different letters in the same row indicate significant differences, according to Tukey's test ($p \leq 0.05$).

Classification	Compound	Pol	Ru	Ec	p-values
Phenolic acid derivates					
	gallic acid	9.32 b	5.53 c	12.47 a	0.001
	p-tyrosol	26.77 a	21.64 b	13.62 c	0.001
	vanillic acid	4.83 a	3.70 a	4.90 a	0,18
	3,4 dimethoxybenzyl	25.12 b	10.13 c	62.40 a	0.001
	vanillin	6.53 c	4.65 b	11.16 a	0,001
Catechols					
	3,4-DHPglycol	125.42 a	118,86 b	71.87 c	0.001
	3-hydroxytyrosol	17.85 a	11.83 b	2.73 c	0.001
	4-methylcatechol	13.77 c	157.92 a	47.59 b	0.001
	2,6-dimethoxyphenol	98.26 a	21.47 b	6.41 c	0.001
Hydroxycinnamic acids					
	chlorogenic acid	4.20 c	9.30 b	58.45 a	0.001
	caffeic acid	4.46 ab	3.41 b	5.90 a	0.002
	p-coumaric acid	14.22 a	11.05 b	7.65 c	0,001
	ferulic acid	63.63 b	87.43 a	17.19 c	0.001
Flavonols					
	Q 3-rutinoside	32.46 a	30.73 a	14.24 b	0.001
	myricetin	34.63 b	118.61 a	72.29 c	0.001
	Quercetin	28.73 b	42.62 a	44.52 a	0.006
	Kaempferol	2.74 b	3.33 b	20.41 a	0.001
Others					
	5-HMF	4.33 a	4.92 a	-	0.095
Total identified		517.28 b	667.14 a	473.82 c	

The results revealed clear groupings for the different pollen types, indicating that the specific chemical composition of each pollen has a significant impact on honey bee survival and viral load in infected bees (Figure 4). Monofloral pollen of native E. cordifolia showed an association with phenolic compounds such as chlorogenic acid, 3,4-dimethoxybenzyl, gallic acid, caffeic acid, vanillic acid, vainillin and kaempferol suggesting that these compounds could contribute to improved bee survival and decreased DWV-A load on them. On the other hand,

polyfloral pollen was associated with compounds such as Q3-ruthinoside, p-tyrosol, 3,4-DHPglycol, 3-hydroxytyrosol, 2,6-dimethoxyphenol and p-coumaric acid, while the non-native monofloral pollen of R. ulmifolius was associated with ferulic acid, 4-methylcatechol and myricetin suggesting that they have no effect or are not related to the decrease of viral load on inoculated honey bees. In general, a separation between pollens according to their origin and botanical composition was observed, with a greater distinction between the polyfloral and non-native monofloral of R. ulmifolius with respect to the native monofloral pollen from E. cordifolia (Figure 4).

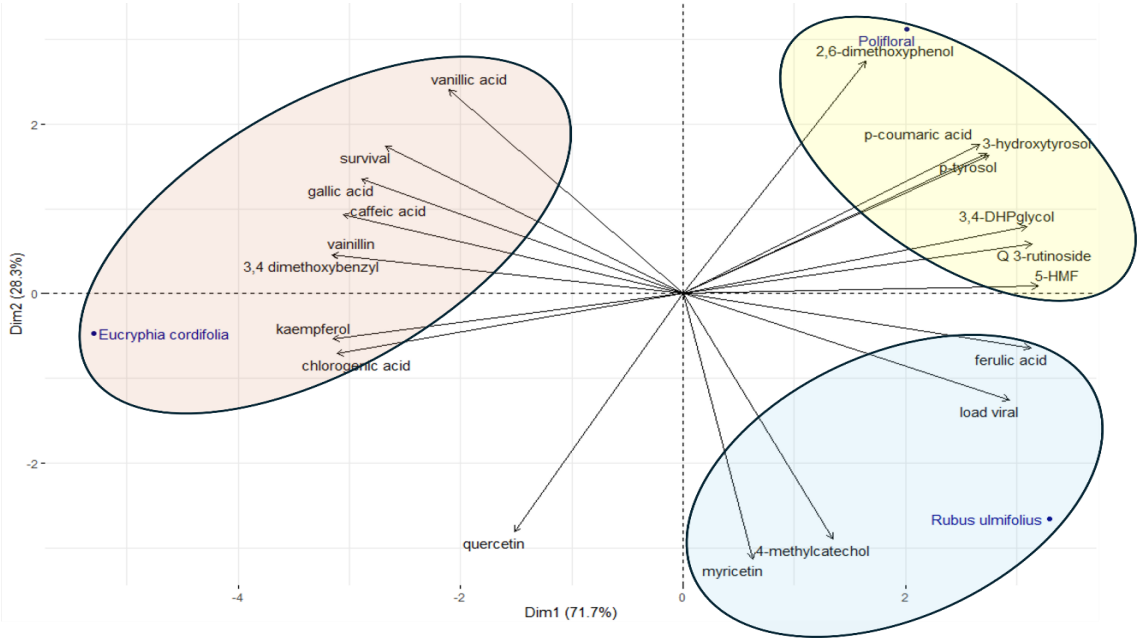

Figure 4. Principal Component Analysis (PCA) biplot of metabolites in different pollens (polyfloral, R. ulmifolius, and E. cordifolia), used for test their effect on survival, immunity and viral load on honey bees infected with DWV-A. The ellipses represent groupings of samples according to their chemical composition. The arrows indicate the contribution of each chemical compound to the variability explained by the principal components, highlighting the differences in chemical profiles among treatments.

4. Discussion

In this study we showed that the viral load of the DWV-A variant decreased in bees fed with pollen while it increased in the inoculated control untreated with plant pollens (Figure 1). This effect was evident in the survival assay, where in the worker bees that consumed plant pollen, showed a better survival rate. This

effect was more notable in bees fed the native pollen diet from E. cordifolia (Figure 2). In fact, no differences were observed with the uninoculated control, supporting the effect of native E. cordifolia pollen in reducing the viral titles of the DWV-A variant.

Protein content has been shown to be an important factor in reducing pathogen load and enhancing immune system response (Castelli et al., 2020). However, the native monofloral pollen from E. cordifolia showed the lowest levels (Table S2). Therefore, the results of viral load and survival could be associated with the presence of phenolic compounds in higher concentrations in this type of pollen (Table 2). Recently, it has been reported that supplementation with grape marc powder, rich in phenolic compounds, modulates the immune response against the DWV-A (Pascual et al., 2022). In this regard, authors describe a decrease in viral replication in bees when incorporating pollen into the diet (Tritschler et al., 2017). Conversely, a positive correlation between DWV titles and the availability of pollen at the colony level has been reported (Corona et al., 2023). On the other hand, the protective effect and potential synergistic effects of phytochemicals against bee parasites have been demonstrated (Palmer-Young et al., 2017).

In this study, significant variation was observed in the expression of the Cactus gene (IkB), related to the inhibition of transcription factors in Toll and Immunodeficiency (Imd) signaling pathways (Figure 3a). Specifically, down-regulation of Cactus in worker bees that consumed pollen, compared to the inoculated control group, suggests degradation of this inhibitor and induction of the expression of Dorsal and Relish (NF-κB) transcription factors (Figures 3b and 3c). In contrast, in infected but not plant pollen-treated bees, the elevated expression of Cactus acts as an inhibitor blocking the activation of the expression of these genes and suppressing the bee's defense response. These findings suggest that pollen consumption activates Cactus-mediated immune pathways, generating a protective effect against DWV-A variant infection. Furthermore, a positive correlation between these transcription factors and the production of antimicrobial peptides (AMPs) has been observed (Barroso-Arévalo et al., 2019). AMPs have been described as key components in the immune response against DWV (Nazzi and Pennacchio, 2018). On the other hand, when we analyzed a gene involved in the RNA interference (RNAi) system, considered one of the most important antiviral gene silencing mechanisms in bees (Brutscher et al., 2017),

there was observed that the expression of the Dicer-like gene was up-regulated in honey bees inoculated with DWV-A and treated with pollens (Figure 3d). This suggests that pollen may also enhance immune defense through this pathway(Niu et al., 2016).

The study of plants as a source of phytochemicals is an area of research in constant growth (Thakur and Nanda, 2020). There are reports on the improvement of survival and reduction of the parasitic load in honey bees (Arismendi et al., 2018; Bernklau et al., 2019). In addition, they can improve memory and learning abilities and even confer tolerance to stressors (Lu et al., 2020). In this regard, by analyzing the content of bioactive compounds in pollen samples, it was observed that the concentration of bioactive compounds varied significantly depending on the type of pollen (Table 1 and 2). The non-native monofloral pollen extract from R. ulmifolius showed the highest TPC (603.91 mg GAE 100g-1), while lower values were observed for the native monofloral pollen of E. cordifolia (419.35 mg GAE 100g-1). The highest DPPH values was observed in the polyfloral pollen extract (799.98 µmol Trolox g-1) and the lowest in the E. cordifolia pollen extract (677.17 µmol Trolox g-1). In this regard, values have been reported in TPC pollen samples between 1600.40 and 4100.17 mg GAE 100g-1, and DPPH values between 810 and 2930 µmol Trolox g^{-1}, higher than those observed in this study (Mărgăoan et al., 2021). On the contrary, the TPC and ORAC values here were similar those reported in pollen samples from the Central Region of Chile (Bridi et al., 2019; Oyarzún et al., 2020).). In this sense, Muñoz et al., (2020), suggest that the bioactivity of pollen is affected by botanical origin, geographic area, and climatic conditions.

Another relevant aspect of the study is the identification of phenolic compounds in higher concentrations in the native pollen of E. cordifolia, including many flavonoids (Table 1). There was detected higher concentrations of chlorogenic acid, kaempferol, quercetin, caffeic acid, p-coumaric acid, and ferulic acid compared to those reported in most of the pollens analyzed in the Southern Region of Chile (Bridi et al., 2022). There is evidence that many flavonoids have the capacity to reduce viral load and inhibit virus replication (Sharifi-Rad et al., 2018; Fahmy et al., 2020). The antiviral activity of flavonoids has been attributed to the ability to inhibit viral proteins, such as proteases and viral replication proteins (Ninfali et al., 2020). Additionally, the structural differences among

flavonoids have been implicated in their antiviral activity, suggesting their potential to modulate the immune response to viral infections. For instance, the antiviral activity of flavonoids has been described as associated with the regulation of enzymes involved in the production of reactive oxygen species (ROS) in cells (Zakaryan et al., 2017; Dias et al., 2021). In this sense, it has been described that viruses need cellular machinery to replicate in the host cell (Grozinger and Flenniken, 2019). Therefore, flavonoids could interfere with the virus ability to utilize cellular resources, reducing the production of new viral particles (Amatore et al., 2015). In this sense, the different responses observed in the expression of immune system genes in inoculated bees could be explained by the chemical composition of the pollen, as evidenced by the principal component analysis (Figure 4). Here, the native pollen of E. cordifolia groups differently from the rest of the plant pollens, suggesting that compounds such as chlorogenic acid, 3,4-dimethoxybenzyl, gallic acid, caffeic acid, vanillic acid, vanillin, and kaempferol might provide more effective antiviral protection against DWV-A. Moreover, it is interesting that one of the main flavonoids reported in this study, quercetin, has been described as an inhibitor of RNA-dependent RNA polymerase (RdRP), successfully suppressing DWV viral replication and infection in an in vitro system (Wu et al., 2021). Another compound present in higher concentration in the native pollen of *E. cordifolia*, kaempferol, has been described as having antiviral activity against rhinoviruses (Kwon et al., 2020). Therefore, certain specific compounds in pollen can be key to improving bees' tolerance to pathogens (Palmer-Young et al., 2022), and it is possible to expect that a high intake of these compounds present in the pollen could help reduce and control viral replication. In addition, previous studies have demonstrated that the quality and diversity of pollen extend bee health while reducing viral infections (Annoscia et al., 2017). This supports the hypothesis that pollen chemistry is important for honeybees' ability to resist different pathogen infections.

When talking about pollen diversity, it is expected that pollen collected and/or stored within the colony comes from different floral sources (Malagnini et al., 2022). Therefore, it was expected that polyfloral pollen would present the best results in survival parameters, immunity and its effect on viral load, but surprisingly, a monofloral pollen, E. cordifolia, presented the best results in these measured parameters. In this regard, it is possible that there are iconic floral

sources, such as E. cordifolia, that meet and provide needs that allow bees to protect themselves against key pathogens such as DWV-A. For this reason, it is key to know the compounds that these pollens have and their possible functions as antimicrobial and immunostimulant elements and therefore, their potential uses in the control of DWV-A and other pathogens of importance in bee health.

5. Conclusions

Pollen-based diets significantly reduced viral load in bees inoculated with the DWV-A variant, with native E. cordifolia pollen showing the highest effectiveness. Additionally, treatment with pollen notably improved the survival and immune response of infected bees, with E. cordifolia yielding the best results in both parameters. E. cordifolia pollen exhibited high concentrations of phenolic compounds with antiviral properties, including gallic acid, vanillic acid, 3,4-dimethoxybenzyl, vanillin, chlorogenic acid, caffeic acid, quercetin, and kaempferol, which may be responsible for the observed effects. Principal component analysis revealed a clear separation between different pollen types based on their botanical composition, highlighting that the biodiversity of native Chilean plants, particularly E. cordifolia, offers promising natural resources to protect bee health against viral infections like DWV-A.

Author contribution

Conceptualization: M.V. Methodology: R.G., D.S., F.N., S.V. Formal analysis: M.V., N.A., MDL-B., M.G. Resources: M.V. Data curation: R.G., D.S, F.N. Writing-original draft: R.G. Writing-review & editing: R.G., D.S., F.N., M.D.L-B., N.A., M.G., S.V., M.V. Project administration: M.V. Funding acquisition: M.V. All co-authors reviewed the final version and approved the manuscript before submission.

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Data availability statement

Raw data of qPCR analysis is available at https://doi.org/10.5281/zenodo.16777252

Supplementary material

Table S1. Palynological analysis of bee pollen samples.

Table S2. Proximate chemical analysis of pollen samples.

Table S3. Ct-value (qPCR) in honey bee colonies screening for bee pathogens.

Table S4. Nucleotide sequence of Apis mellifera defense gene and deformed wing virus (DWV-A) primers used in qRT-PCR.

Conflict of interest

The authors declare that they have no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Table S1. Palynological analysis of bee pollen samples (BP).

Pollen sample	Classification	Dominant species (>45%)	Secondary species (16–45%)	Other species (3-15%)
BP1	No native Polyfloral		*Brassica campestris* 32.77% *Trifolium pratense* 30.40%	*Rubus ulmifolius* 14.18% *Lotus uliginosus* 10.13% Other species 12.52%
BP2	No native monofloral	*Rubus ulmifolius* (Ru) 71%	Other species 29 %	
BP3	Native monofloral	*Eucryphia cordifolia* (Eu) 75%	Other species 25%	

Table S2. Proximate chemical analysis of pollen samples.

Pollen	Protein %	Dry Matter %	Humidity%	Total, Ash %	Etherea l Extract %	Crude Fiber %	Carbohydrate s T. %
Polyfloral	27,50	83,49	16,51	4,28	6,49	0,90	44,32
R. ulmifolius	25,32	82,72	17,28	3,00	7,05	0,62	46,73
E. cordifolia	21,65	83,04	16,96	2,26	4,79	1,73	52,61

Table S3. Ct-value (qPCR) in honey bee colonies screening for bee pathogens.

ID Number Beehive	*Nosema cerenae*	*Lotmaria passim*	CBPV	ABPC	DWV	Selection
1	30.86	35.19	20.67	27.60	27.18	No selected
2	32.53	No ct	30.72	27.74	24.33	No selected
3	31.94	No ct	26.74	30.05	21.76	No selected
4	35.95	39.08	29.00	22.91	**7.98**	**Selected for Innoculum preparation**
5	31.15	39.30	30.90	26.23	23.00	No selected
6	32.67	35.62	30.68	25.82	25.26	No selected
7	34.33	No ct	24.91	22.64	25.83	No selected
8	33.09	36.56	24.60	25.34	30.38	No selected
9	30.36	No ct	21.74	23.25	27.26	No selected
10	32.57	35.11	28.37	25.44	28.02	No selected
11	No ct	39.16	28.29	28.98	24.53	No selected
12	32.44	38.03	21.63	30.87	25.00	No selected
13	38.86	No ct	26.31	29.19	**10.84**	**Selected for Innoculum preparation**
14	36.83	37.32	22.48	24.80	21.27	No selected
15	38.13	37.64	28.48	27.92	26.24	No selected
16	**37.98**	No ct	**35.16**	**33.17**	**32.51**	**Selected for experiment**

17	No ct	36.83	30.93	20.38	25.73	No selected
18	38.21	35.21	21.41	28.91	27.87	No selected
19	31.92	38.67	26.86	30.07	28.24	No selected
20	No ct	No ct	25.65	22.45	26.15	No selected
21	31.85	36.16	26.72	24.68	24.28	No selected
22	33.65	35.97	24.69	22.45	25.36	No selected
23	No ct	37.48	22.60	29.54	29.05	No selected
24	33.56	39.73	24.89	30.67	29.27	No selected
25	34.75	38.48	30.12	21.32	26.46	No selected
26	No ct	**38.97**	**35.43**	**33.86**	**36.54**	**Selected for experiment**
27	**37.52**	No ct	**35.71**	**34.46**	**33.96**	**Selected for experiment**
28	26.24	36.04	21.42	20.99	24.29	No selected
29	31.03	37.30	25.27	22.44	20.47	No selected
30	31.94	38.34	25.43	26.28	22.73	No selected
31	33.06	38.77	27.75	24.06	22.10	No selected
32	31.10	No ct	27.01	28.11	20.18	No selected
33	26.94	No ct	25.84	27.54	26.67	No selected
34	37.34	No ct	24.48	28.87	24.09	No selected
35	25.59	No ct	24.51	24.79	23.13	No selected
36	33.44	No ct	22.30	27.95	24.67	No selected
37	31.40	No ct	29.54	24.75	23.03	No selected
38	37.69	37.92	21.37	26.39	29.69	No selected
39	34.36	35.20	21.05	23.02	28.34	No selected
40	No ct	No ct	24.95	23.21	28.80	No selected
41	No ct	37.43	25.20	23.21	20.62	No selected
42	36.59	No ct	22.80	20.66	26.63	No selected
43	31.87	36.54	20.39	29.14	25.71	No selected
44	35.44	35.44	29.56	27.43	21.58	No selected

Ct > 30, Low pathogen level; Ct < 15, high pathogen level

Table S4. Nucleotide sequence of *Apis mellifera* defense gene and deformed wing virus (DWV-A) primers used in qRT-PCR.

Primers	Sequences		References
DWV-A	F	TATCTTCATTAAAGCCACCTGGAA	(Yang and Cox-Foster, 2005)
	R	TTTCCTCATTAACTGTGTCGTTGAT	
β-Actin	F	ATGCCAACACTGTCCTTTCTGG	(Yang and Cox-Foster, 2005)
	R	GACCCACCAATCCATACGGA	
Cactus	F	CACAAGATCTGGAGCAACGA	(Evans, 2006)
	R	GCATTCTTGAAGGAGGAACG	
Relish	F	GCAGTGTTGAAGGAGCTGAA	(Evans, 2006)
	R	CCAATTCTGAAAAGCGTCCA	
Dorsal	F	CTCATCGGAAGACATGACAGTGA	(Zhao et al., 2019)
	R	TGAATTCAAAGCCAGTTCGAAAA	
Dicer-like	F	AGCAGTAGCTGATTGTGTGGA	(Zhao et al., 2019)
	R	TGAAGGATGTGTAAACGCCTGT	

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Capítulo III: El ácido clorogénico mejora la sobrevivencia y el rendimiento cognitivo en abejas melíferas infectadas con el virus de las alas deformadas (DWV-A).

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Estado: No enviada

Resumen

El virus de las alas deformadas es uno de los patógenos más prevalentes en las abejas melíferas y se asocia con efectos subletales que comprometen la cognición y el comportamiento. En este estudio, evaluamos si el ácido clorogénico modula la sobrevivencia, el aprendizaje asociativo y la retención de la memoria en abejas inoculadas experimentalmente con DWV-A. Las abejas recién emergidas fueron inoculadas oralmente con DWV-A y alimentadas durante 10 días con jarabe suplementado con concentraciones crecientes de ácido clorogénico (12,5, 25, 50 y 100 ppm). La sobrevivencia se monitorizó durante 20 días después de la inoculación y el rendimiento cognitivo se evaluó mediante el condicionamiento del reflejo de extensión de la probóscide (PER). La infección por DWV-A redujo la sobrevivencia y afectó el aprendizaje asociativo y la memoria a corto plazo en comparación con el control no inoculado. La suplementación con ácido clorogénico mejoró la sobrevivencia, observándose el efecto más claro a 100 ppm, y alcanzó valores estadísticamente comparables a los del control no inoculado. El rendimiento cognitivo mostró una respuesta no lineal, observándose la mayor recuperación del aprendizaje y la memoria con 25 ppm. Las respuestas al estímulo sin refuerzo se mantuvieron bajas en todos los tratamientos, lo que indica una discriminación olfativa preservada. En conjunto, estos resultados indican que el ácido clorogénico atenuó el deterioro funcional asociado al DWV-A en las condiciones de este estudio de laboratorio y respaldan una evaluación más exhaustiva de los compuestos fenólicos derivados de

plantas como componentes de estrategias nutricionales para mejorar la resistencia de las abejas melíferas al estrés viral.

Palabras claves: DWV; *Apis mellifera*; compuesto bioactivo

Introducción

El virus de las alas deformadas (DWV) es una de las principales amenazas para la salud de las abejas melíferas (*A. mellifera*) debido a su prevalencia mundial, amplia distribución y estrecha asociación con *Varroa destructor* (Paxton, 2022; Lopes et al., 2024). Más allá de sus manifestaciones clínicas evidentes, el DWV también puede producir efectos subletales persistentes que afectan el desempeño individual y alteran procesos esenciales para la función y viabilidad de la colonia (Pizzorno et al., 2021; Ferreira et al., 2025). Cabe destacar que las infecciones subclínicas pueden causar alteraciones funcionales incluso en ausencia de síntomas morfológicos visibles y también pueden modificar las preferencias florales de las abejas (Benaets et al., 2017; Power et al., 2021). El DWV puede afectar la capacidad de respuesta sensorial y rasgos cognitivos como el aprendizaje y la memoria, mientras que estudios transcriptómicos y neurofisiológicos sugieren que estos efectos están asociados con cambios en la expresión génica cerebral y la función neuronal (Doussot et al., 2024; Loughran et al., 2025). Dado que tales alteraciones pueden reducir la orientación y la eficiencia en la búsqueda de alimento, sus consecuencias pueden extenderse más allá del individuo y, en última instancia, comprometer el desempeño de la colonia (Klein et al., 2017; Kevill et al., 2019).

Por lo tanto, rasgos funcionales como la sobrevivencia y el aprendizaje proporcionan un marco integrador para evaluar el impacto biológico de la infección por DWV (Gómez-Moracho et al., 2017). Al mismo tiempo, la nutrición ha surgido como un factor clave que moldea las respuestas de las abejas a múltiples factores de estrés, incluidos los patógenos virales (Alaux et al., 2010; DeGrandi-Hoffman et al., 2016). En este contexto, los compuestos fenólicos derivados de plantas han atraído una atención creciente debido a su abundancia en recursos florales y sus potenciales efectos bioactivos en la fisiología de las abejas (Hýbl et al., 2021; Riddick et al., 2024). Entre ellos, el ácido clorogénico (CGA) es particularmente relevante debido a su amplia presencia en tejidos

vegetales y a sus reconocidas propiedades antioxidantes y bioactivas bajo condiciones de estrés metabólico e infeccioso (Del Rio et al., 2010; Clifford et al., 2020; Naveed et al., 2018). Si bien el CGA ha sido detectado en polen y néctar de diversas especies vegetales, sus efectos funcionales en *A. mellifera*, particularmente bajo infección por DWV, aún no han sido suficientemente caracterizados (García Domínguez et al., 2025). Estas propiedades lo posicionan como un compuesto de interés en el contexto de la interacción entre nutrición y estrés fisiológico en abejas, aunque la evidencia experimental disponible sobre su impacto en la respuesta a infecciones virales sigue siendo limitada. En este contexto, existe una brecha de conocimiento respecto de cómo compuestos fenólicos dietarios como el CGA pueden modular la fisiología del hospedero frente a infecciones virales y sus consecuencias asociadas en el desempeño cognitivo y la sobrevivencia en abejas melíferas.

2. Materiales y métodos

2.1 Evaluación del condicionamiento olfativo mediante la técnica del reflejo de extensión de la probóscide (PER).

Las abejas melíferas utilizadas en este estudio fueron obtenidas del apiario experimental de la estación “El Nogal” (36°35′58″ S, 72°04′51″ W), de la Universidad de Concepción, Chillán, Chile. Los cuadros que contenían cría operculada de obreras fueron transferidos al Laboratorio de Patología Apícola y mantenidos bajo condiciones controladas (30 °C; 50–60% HR) hasta la emergencia. El estado sanitario de las colonias fue previamente evaluado mediante qPCR, siguiendo el protocolo descrito por Vargas et al. (2017), para la detección de *Nosema ceranae*, *Lotmaria passim*, CBPV, ABPV y DWV. Posteriormente, se seleccionaron tres colonias libres o con baja carga de patógenos para ser utilizadas en los bioensayos.

Para evaluar el efecto del ácido clorogénico sobre el aprendizaje asociativo, se recolectaron abejas recién emergidas (1–2 días de edad) a partir de cuadros con cría operculada y se asignaron aleatoriamente a seis tratamientos experimentales: CN (control no infectado), CI (abejas infectadas con DWV-A sin tratamiento) y cuatro grupos de abejas infectadas suplementadas con ácido clorogénico a diferentes concentraciones: T3 (12,5 ppm), T4 (25 ppm), T5 (50 ppm) y T6 (100 ppm). Las abejas se mantuvieron bajo condiciones controladas y fueron alimentadas ad libitum con una solución de sacarosa al 60%

suplementada con las respectivas concentraciones de ácido clorogénico según el tratamiento.

Cada tratamiento estuvo compuesto por 50 abejas por jaula plástica (base = 8 cm de diámetro, parte superior = 10 cm de diámetro, altura = 15 cm), de las cuales se seleccionaron 10 individuos para los ensayos de condicionamiento. En los tratamientos infectados, cada abeja fue inoculada oralmente con 5 µL de una suspensión viral de DWV-A (1 × 10^9 copias virales por abeja) mezclada con jarabe de sacarosa al 50% (Porrini et al., 2013). Las abejas que no consumieron completamente el inóculo fueron excluidas del ensayo.

Después de un período de 10 días post-inoculación, las abejas fueron sometidas al ensayo de condicionamiento olfativo utilizando la técnica del reflejo de extensión de la probóscide (PER) (Smith et al., 2014). Antes del condicionamiento, las abejas fueron inmovilizadas individualmente en soportes y alimentadas hasta la saciedad con solución de sacarosa, permitiendo un período de aclimatación antes del inicio del experimento.

El protocolo de condicionamiento consistió en la presentación alternada de dos estímulos olfativos: un estímulo condicionado reforzado (CS+, olor asociado a una recompensa de sacarosa) y un estímulo no reforzado (CS−, olor sin recompensa). Cada abeja fue sometida a una secuencia de 12 presentaciones en el orden (A-B-B-A-B-A-A-B-A-B-B-A), donde A corresponde al CS+ y B al CS−. Para el análisis del aprendizaje asociativo, solo se consideraron las respuestas PER al CS+, correspondientes a seis ensayos de condicionamiento (C1–C6). La respuesta PER fue registrada como la presencia (1) o ausencia (0) de extensión de la probóscide durante la presentación del estímulo.

2.1.1 Estimulación olfativa

Los estímulos olfativos utilizados para los ensayos de condicionamiento y memoria fueron 1-hexanol (2 M; Sigma-Aldrich, St. Louis, MO, EE. UU.) y 2-octanona (2 M; Fluka, Sigma-Aldrich, St. Louis, MO, EE. UU.). Ambos compuestos han sido ampliamente utilizados en estudios previos sobre aprendizaje olfativo y percepción de olores en abejas melíferas (Thorn and Smith, 1997; Wright et al., 2005; Wright et al., 2009; Smith et al., 2014). Los olores fueron diluidos en aceite mineral pesado (Sigma-Aldrich, St. Louis, MO, EE. UU.).

Los cartuchos de olor consistieron en jeringas de tuberculina de 1 mL (BD Medical, Franklin Lakes, NJ, EE. UU.), equipadas con un tubo corto de silicona (Cole-Parmer, Vernon Hills, IL, EE. UU.) como constricción en el extremo ancho. Diez microlitros de la solución odorante fueron depositados sobre una tira de papel filtro (Whatman 114, Sigma-Aldrich, St. Louis, MO, EE. UU.), la cual fue colocada dentro del cartucho. El cartucho fue conectado al sistema automatizado de entrega de olores mediante un tubo acoplado al extremo angosto y posicionado de manera que el extremo ancho quedara aproximadamente a 2 cm de las antenas de la abeja durante el condicionamiento.

La presentación de los estímulos se realizó mediante un sistema automatizado de entrega de olores controlado por una plataforma basada en Arduino, programada para activar la apertura de una válvula solenoide, redirigiendo un flujo de aire (~400 mL/min) a través del cartucho de olor. Durante la estimulación, el flujo de aire atravesaba el cartucho, transportando el aire cargado con el olor hacia las antenas de la abeja. Un sistema continuo de extracción, ubicado aproximadamente a 5 cm detrás del individuo, eliminaba el olor del área de condicionamiento después de cada ensayo, asegurando una exposición temporalmente discreta a cada estímulo.

2.2 Evaluación de la memoria (pruebas de retención)

Posteriormente al condicionamiento, la retención de memoria fue evaluada a los 10 y 30 min mediante pruebas de evocación no reforzadas. En cada momento, las abejas fueron expuestas tanto al estímulo condicionado previamente reforzado durante el entrenamiento (CS+) como al estímulo no reforzado (CS−), sin entrega de sacarosa. Las respuestas fueron registradas como presencia (1) o ausencia (0) del reflejo de extensión de la probóscide (PER). La discriminación fue interpretada como una mayor probabilidad de respuesta al CS+ en comparación con el CS−.

2.3 Preparación del inoculo de DWV-A

El DWV-A fue aislado a partir de abejas melíferas sintomáticas provenientes de colonias infectadas con DWV, siguiendo la metodología descrita por Gusachenko et al. (2020). En breve, un grupo de 20 abejas fue homogeneizado en un Stomacher 80 Lab Blender (Seward, Londres, Reino Unido) con solución salina tamponada con fosfato (PBS 1X) durante 90 s a alta velocidad en un

homogeneizador de laboratorio Stomacher 80 (Seward, Londres, Reino Unido). Posteriormente, las muestras fueron centrifugadas dos veces a 1500 × g durante 10 min a 4 °C, seguidas de una centrifugación a 10.000 × g durante 10 min a la misma temperatura.

Luego, el sobrenadante fue filtrado y purificado mediante un filtro de 0,22 µm (PES, Merck Millipore, Darmstadt, Alemania). Finalmente, para la extracción de ARN, síntesis de cDNA y posterior cuantificación de la carga viral del inóculo, se utilizaron 200 µL del sobrenadante. El resto del inóculo fue almacenado a −80 °C.

2.4 Sobrevivencia de abejas tratadas y no tratadas con ácido clorogénico

La sobrevivencia de las abejas expuestas a los distintos tratamientos con ácido clorogénico, tanto inoculadas como no inoculadas con DWV-A, se determinó siguiendo la metodología descrita por García-Domínguez et al. (2025). En breve, cada tratamiento consistió en 100 abejas por jaula y cuatro jaulas por tratamiento, para un total de 400 abejas por grupo experimental. Se realizaron recuentos diarios durante 20 días post inoculación para registrar el número de abejas muertas, y los individuos muertos fueron retirados de cada jaula.

2.5 Análisis de datos

El aprendizaje asociativo evaluado mediante el condicionamiento del reflejo de extensión de la probóscide (PER) fue analizado utilizando modelos lineales generalizados mixtos binomiales (GLMM) con enlace logit, considerando la respuesta PER (presencia/ausencia) como variable dependiente. Para el análisis del aprendizaje, se incluyeron como efectos fijos el tratamiento, el número de ensayo de condicionamiento (C1–C6) y su interacción, mientras que la identidad de la abeja se incorporó como efecto aleatorio para considerar las mediciones repetidas. Las respuestas PER en las pruebas de retención de memoria (0/1) se analizaron mediante GLMM binomiales con tratamiento, tiempo (10 vs. 30 min), tipo de estímulo (CS+ vs. CS−) y sus interacciones como efectos fijos, incluyendo nuevamente la identidad de la abeja como efecto aleatorio. La significancia de los efectos fijos en ambos modelos se evaluó mediante pruebas de χ^2 de Wald.

Las curvas de sobrevivencia de las abejas fueron representadas utilizando el estimador de Kaplan–Meier, considerando como datos censurados las abejas vivas al final del experimento. Las diferencias entre las curvas de sobrevivencia

se estimaron mediante pruebas de log-rank y los valores de p fueron corregidos mediante el método de Holm–Bonferroni (Holm, 1979) para comparaciones múltiples por pares.

3. Resultados

3.1 Aprendizaje asociativo (PER)

El aprendizaje asociativo, evaluado mediante el condicionamiento del reflejo de extensión de la probóscide (PER), se vio significativamente afectado por el tratamiento durante la inoculación experimental con DWV-A (Figura 1). Las abejas infectadas y no tratadas (CI) mostraron un bajo porcentaje de respuestas PER a lo largo de los ensayos de condicionamiento en comparación con el control no inoculado (CN), el cual presentó un aumento progresivo de las respuestas condicionadas (Figura 1). El análisis estadístico reveló un efecto significativo del tratamiento sobre la probabilidad de respuesta PER durante el condicionamiento (Wald $\chi^2 = 18.20$, $p = 0.003$), así como un efecto significativo del número de ensayo ($p = 0.003$), lo que indica un aumento general de la probabilidad de respuesta a medida que avanzaban los ensayos. La interacción entre tratamiento y ensayo no fue significativa ($p = 0.831$). Las abejas infectadas suplementadas con ácido clorogénico mostraron una recuperación parcial del rendimiento de aprendizaje (Figura 1). El tratamiento de 25 ppm produjo el efecto más marcado, con una probabilidad de respuesta PER significativamente mayor que la observada en el control infectado (CI) ($p = 0.028$). En este tratamiento, la curva de aprendizaje se aproximó a la del control no inoculado en los ensayos finales. Los tratamientos de 12,5, 50 y 100 ppm mostraron valores intermedios de aprendizaje, superiores a los del control infectado. Sin embargo, estas diferencias no fueron estadísticamente significativas ($p > 0.05$). Estos resultados indican que la suplementación con ácido clorogénico atenuó el deterioro del aprendizaje asociativo observado en abejas inoculadas con DWV-A, con el efecto más claro detectado a 25 ppm.

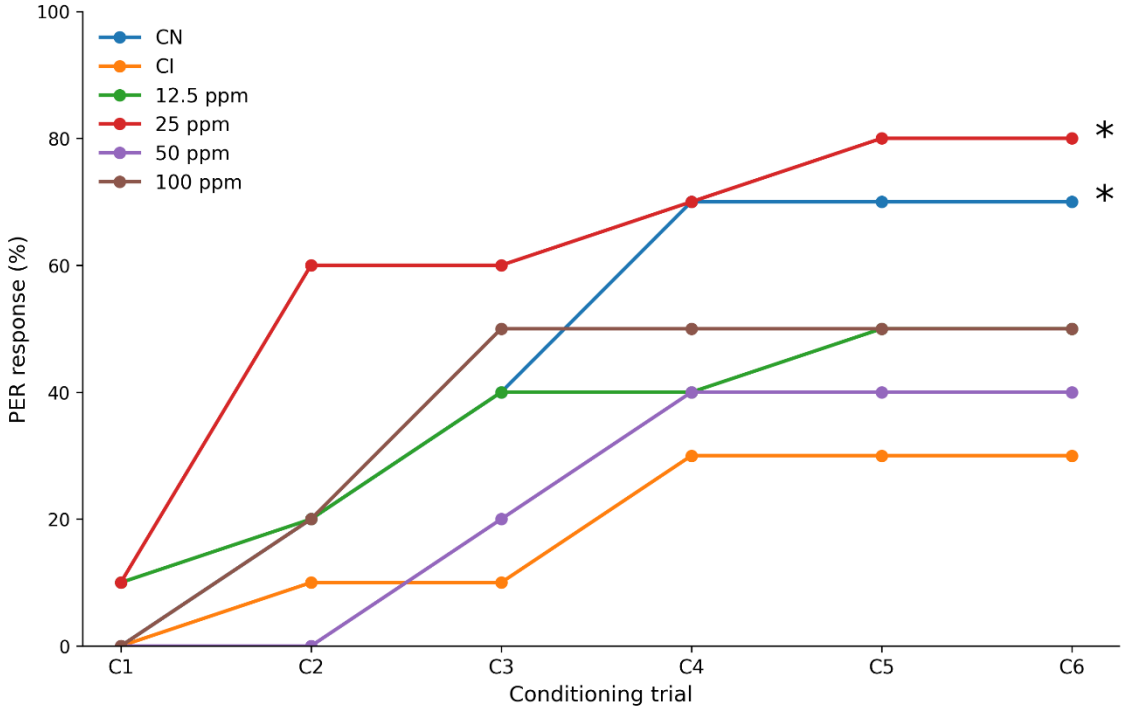

Figura 1. Porcentaje de respuestas del reflejo de extensión de la probóscide (PER) al estímulo condicionado (EC+) en ensayos de condicionamiento sucesivos (C1-C6). CN representa abejas control no inoculadas, CI representa abejas inoculadas con DWV-A sin tratamiento, y 12,5, 25, 50 y 100 ppm representan abejas inoculadas con DWV-A suplementadas con ácido clorogénico. El asterisco (*) en C6 indica una diferencia significativa en comparación con el control infectado (CI) ($p < 0,05$).

3.2 Evaluación de la memoria a corto plazo

La retención de la memoria se evaluó a los 10 y 30 minutos después del condicionamiento olfativo mediante presentaciones sin recompensa del estímulo condicionado reforzado (EC+) y del estímulo no reforzado (EC-). El análisis mediante modelos lineales mixtos generalizados binomiales reveló un efecto significativo del tratamiento (Wald $\chi^2 = 13,62$, gl = 5, $p = 0,018$) y del tipo de estímulo (EC+ frente a EC-; Wald $\chi^2 = 79,44$, gl = 1, $p < 0,001$), así como una interacción significativa entre ambos factores (Wald $\chi^2 = 11,27$, gl = 5, $p = 0,046$), lo que indica que la capacidad de discriminar entre estímulos dependía del tratamiento. En contraste, el tiempo de evaluación (10 vs. 30 min) no tuvo un efecto significativo en la respuesta PER (Wald $\chi^2 = 0,39$, gl = 1, $p = 0,532$) (Figura 2).

Las abejas del grupo control no inoculado (CN) mantuvieron una alta proporción de respuestas al CS+ y una baja proporción de respuestas al CS-, lo que indica una memoria asociativa y una capacidad de discriminación intactas. Por el contrario, las abejas infectadas no tratadas (CI) mostraron una marcada reducción en las respuestas al CS+, consistente con un deterioro de la memoria a corto plazo bajo la inoculación experimental con DWV-A. La suplementación con ácido clorogénico restauró parcialmente el rendimiento de la memoria en las abejas infectadas de manera dosis-dependiente. En particular, el tratamiento de 25 ppm mostró la mayor recuperación en la respuesta al CS+, alcanzando valores comparables a los del grupo control no inoculado y superando claramente a los del grupo control infectado. Los tratamientos de 12,5, 50 y 100 ppm mostraron respuestas intermedias, superiores a las del grupo control infectado pero inferiores a las del tratamiento de 25 ppm. En todos los tratamientos, las respuestas al EC- se mantuvieron bajas, lo que indica que las diferencias observadas estaban asociadas con el rendimiento de la memoria asociativa en lugar de con respuestas no específicas.

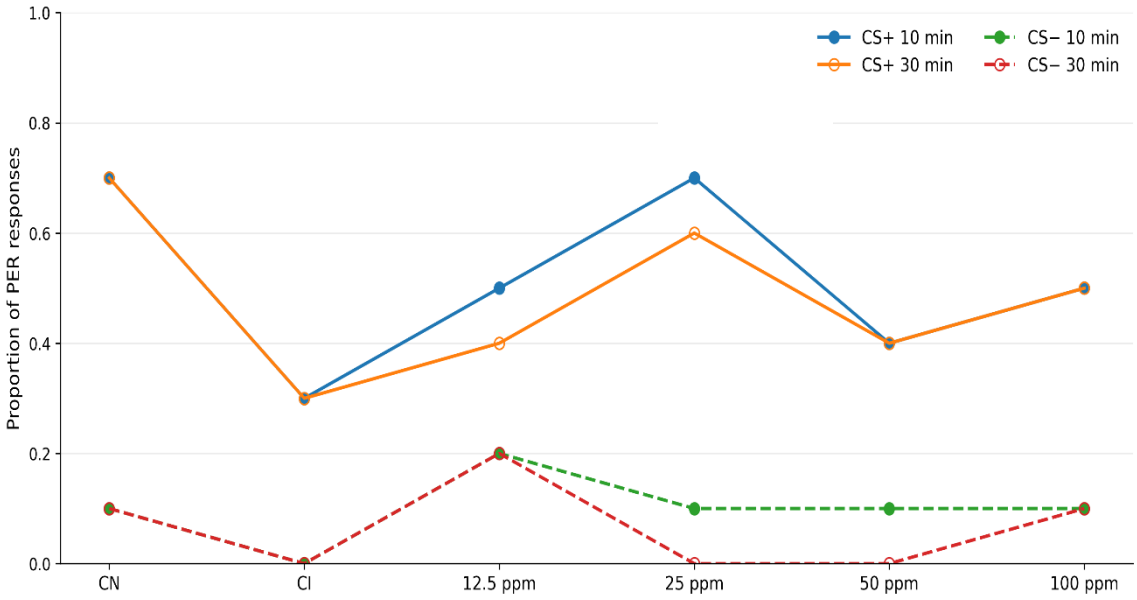

Figura 2. Proporción de respuestas PER a CS+ y CS- evaluadas 10 y 30 min después del condicionamiento olfativo. CN representa abejas control no inoculadas, CI representa abejas infectadas con DWV no tratadas, y 12,5, 25, 50 y 100 ppm representan abejas infectadas con DWV suplementadas con ácido clorogénico.

3.3 Sobrevivencia de las abejas

La infección por DWV-A redujo la sobrevivencia en comparación con el grupo control no infectado (CN) (CN vs. CI: estadístico de prueba = -5,89, p < 0,001). Entre las abejas infectadas, la suplementación con ácido clorogénico se asoció con una mayor sobrevivencia; sin embargo, solo el tratamiento de 100 ppm difirió significativamente del grupo infectado no tratado (CI) (CI vs. 12,5 ppm: estadístico de prueba = 1,27, p = 0,821; CI vs. 25 ppm: estadístico de prueba = 2,36, p = 0,079; CI vs. 50 ppm: estadístico de prueba = 2,00, p = 0,182; CI vs. 100 ppm: estadístico de prueba = 4,40, p < 0,001). Entre los tratamientos con ácido clorogénico, la dosis de 100 ppm mostró la mayor sobrevivencia y alcanzó valores estadísticamente comparables a los del CN (CN vs. 100 ppm: estadístico de prueba = -1,70, p = 0,447), mientras que los grupos de 12,5, 25 y 50 ppm se mantuvieron significativamente por debajo del control no infectado (CN vs. 12,5 ppm: estadístico de prueba = -4,66, p < 0,001; CN vs. 25 ppm: estadístico de prueba = -3,86, p < 0,001; CN vs. 50 ppm: estadístico de prueba = -4,285, p < 0,001). En general, estos resultados indican que el ácido clorogénico mejoró la sobrevivencia en abejas infectadas con DWV-A, observándose el efecto protector más claro a 100 ppm (Figura 3).

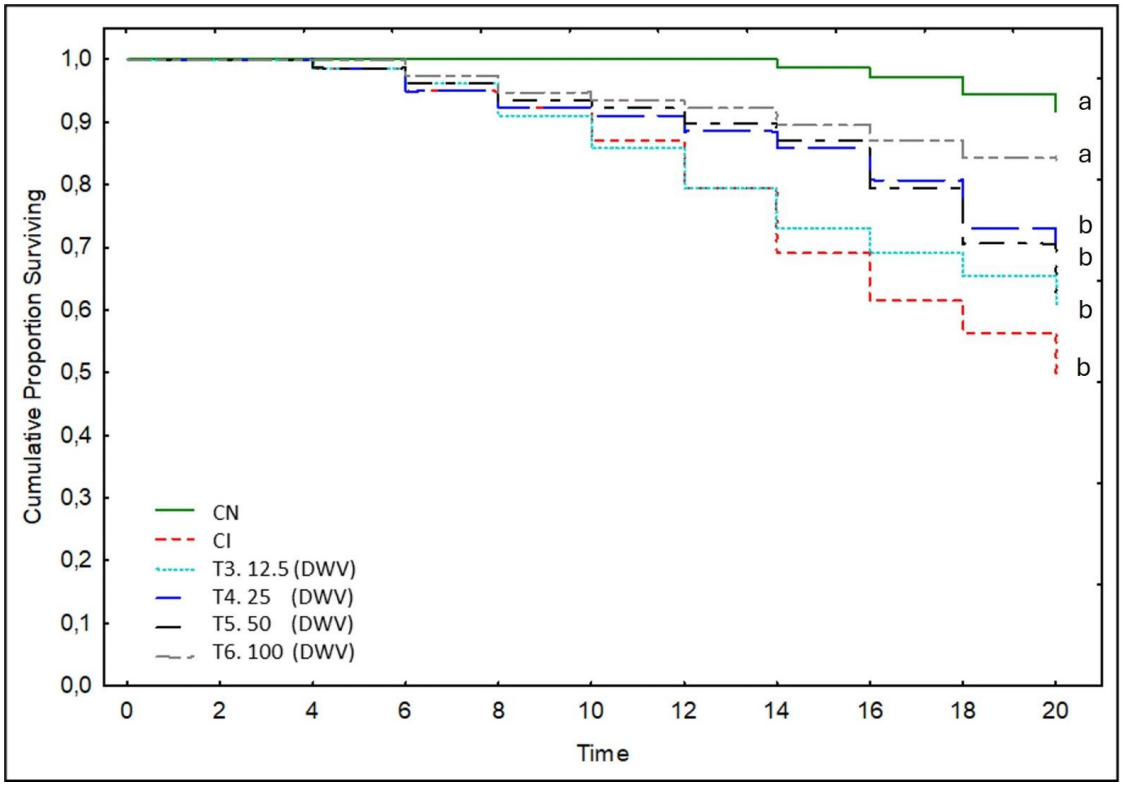

Figura 3. Curvas de sobrevivencia acumulada de abejas infectadas con DWV-A bajo diferentes tratamientos con ácido clorogénico. CN: control no infectado; CI: infectado sin tratamiento; T3: 12,5 ppm; T4: 25 ppm; T5: 50 ppm; T6: 100 ppm.

4. Discusión

En este estudio, evaluamos el efecto del ácido clorogénico sobre la sobrevivencia, el aprendizaje asociativo y la retención de memoria en abejas inoculadas experimentalmente con el Virus de las Alas Deformadas (DWV-A). Los resultados muestran que la inoculación con DWV-A comprometió tanto la sobrevivencia como el rendimiento cognitivo, y que la suplementación con ácido clorogénico atenuó estos efectos, aunque la respuesta difirió entre los distintos endpoints biológicos.

La reducción de la sobrevivencia observada en las abejas infectadas no tratadas es consistente con estudios previos que reportan el impacto del DWV tanto a nivel individual como de colonia (Deboutte et al., 2024). En este contexto, la suplementación con ácido clorogénico se asoció con una mayor sobrevivencia bajo condiciones de infección experimental, aunque solo se observó significancia estadística a 100 ppm en comparación con el control infectado. Asimismo, esta dosis alcanzó valores de sobrevivencia comparables al control no inoculado, sugiriendo una marcada reducción del daño funcional asociado a DWV-A. En contraste, los tratamientos de 12,5, 25 y 50 ppm no mostraron diferencias significativas respecto al grupo infectado no tratado (Figura 3). Estos hallazgos sugieren que el beneficio más claro en sobrevivencia se evidencio a la concentración más alta evaluada.

En relación con el aprendizaje asociativo y la memoria, nuestros resultados confirman que la inoculación con DWV-A afectó negativamente tanto la adquisición del aprendizaje asociativo como la retención de la información vinculada al estímulo reforzado (Figuras 1 y 2), en concordancia con estudios previos que han asociado la infección por DWV con déficits en tareas de aprendizaje y memoria (Tang et al., 2021; Ferreira et al., 2025). La suplementación con ácido clorogénico permitió una recuperación parcial de estas funciones, observándose el efecto más consistente de mejora cognitiva con la dosis de 25 ppm. Las respuestas al estímulo no reforzado (CS−) se mantuvieron bajas en todos los tratamientos, lo que indica una discriminación

olfativa conservada y respalda la interpretación de que los efectos observados se relacionan con el aprendizaje y la memoria, más que con un aumento generalizado de la excitabilidad o la respuesta conductual (Riveros et al., 2022; Szymański et al., 2024).

Los efectos beneficiosos del ácido clorogénico sobre la sobrevivencia y la cognición podrían estar relacionados con sus propiedades antioxidantes y bioactivas, descritas en otros sistemas biológicos bajo condiciones de estrés metabólico e infeccioso (Liang & Kitts, 2015; Bao et al., 2018; Zheng et al., 2022). En abejas, los compuestos fenólicos han sido propuestos como componentes de la nutrición funcional que contribuyen a la resiliencia frente a estresores ambientales y biológicos, incluyendo patógenos (Tlak Gajger et al., 2025). Aunque en este estudio no se evaluaron directamente marcadores de estrés oxidativo, carga viral ni vías neuronales o inmunes, la evidencia previa sobre el ácido clorogénico y polifenoles relacionados proporciona plausibilidad biológica para los efectos funcionales observados (Kwon et al., 2010; Oboh et al., 2013; Burkard et al., 2025).

La divergencia entre la dosis asociada al mayor efecto en sobrevivencia (100 ppm) y la vinculada a la mayor respuesta cognitiva (25 ppm) sugiere una respuesta no lineal dependiendo de la variable biológica considerada. Este patrón es compatible con respuestas tipo hormesis descritas para compuestos fenólicos, en las que dosis intermedias pueden maximizar ciertos beneficios funcionales, mientras que dosis más altas son requeridas para otros (Eseberri et al., 2022; Calabrese et al., 2024).

Estos resultados indican que la suplementación con ácido clorogénico se asoció con una reducción del deterioro funcional relacionado con DWV-A en abejas bajo las condiciones de laboratorio evaluadas. El objetivo de este estudio fue evaluar la sobrevivencia y el rendimiento cognitivo como endpoints funcionales, con el fin de proporcionar una caracterización funcional inicial. En concordancia con ello, la carga viral final, los marcadores de estrés oxidativo y la expresión génica asociada a respuestas inmunes o neuronales no fueron incluidos en el presente análisis. Estudios futuros que incorporen estas variables serán necesarios para determinar si los efectos observados se asocian con actividad antiviral, regulación redox, neuroprotección o una combinación de estos procesos, y para

definir mejor la contribución de los fenoles derivados del polen a la resiliencia frente a patógenos en abejas melíferas.

5.Conclusiones

La suplementación con ácido clorogénico atenuó los efectos negativos asociados con la inoculación experimental con DWV-A de manera dependiente de la concentración, mejorando tanto la sobrevivencia como el desempeño relacionado con el aprendizaje asociativo. La sobrevivencia de las abejas infectadas aumentó significativamente con la suplementación con ácido clorogénico, alcanzando valores comparables al control no inoculado en la dosis más alta probada. Por el contrario, las mejoras en el aprendizaje y la memoria fueron más evidentes en una concentración intermedia, lo que sugiere una respuesta cognitiva no lineal al tratamiento. Por tanto, estos resultados sugieren que el ácido clorogénico puede ser un candidato prometedor para futuras investigaciones dirigidas a fortalecer la resiliencia de las abejas melíferas frente a la infección viral, aunque los mecanismos involucrados aún deben dilucidarse.

Contribuciones de los autores: Conceptualización, M.V.; metodología, M.V., R.G.D.; análisis formal, M.V., N.A., R.G.D.; investigación, M.V., R.G.D.; recursos, M.V.; redacción del borrador original, R.G.D.; revisión y edición, M.V., R.G.D., M.D.L., N.A.; supervisión, M.V.; obtención de financiación, M.V. Todos los autores han leído y aprobado la versión publicada del manuscrito.

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Conclusiones generales

Los resultados de esta tesis demuestran que la patogenicidad del DWV-A en *A. mellifera* está fuertemente condicionada por el contexto nutricional, y particularmente por la calidad química del polen consumido. Las dietas basadas en polen no solo mejoran la sobrevivencia y la respuesta fisiológica de las abejas infectadas, sino que, dependiendo de su composición fitoquímica, también pueden contribuir a disminuir la carga viral.

Dentro de los recursos evaluados, el polen nativo de *E. cordifolia* mostró el mayor potencial protector frente al DWV-A, asociado a una composición rica en compuestos fenólicos de posible acción antiviral e inmunomoduladora. A su vez, se sugiere que metabolitos específicos como el ácido clorogénico pueden atenuar los efectos subletales de la infección causada por el virus DWV, mejorando tanto la sobrevivencia como las funciones cognitivas afectadas por el virus.

Esta tesis establece que la nutrición no debe considerarse un factor complementario, sino un componente central en la dinámica de la infección por DWV-A. Además, estos resultados abren nuevas perspectivas tanto para la comprensión mecanística de la interacción abeja–virus–dieta como para el diseño de estrategias nutricionales basadas en recursos florales nativos que contribuyan al fortalecimiento de la resiliencia de las colonias.