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Nutrition, pesticide exposure, and virus infection interact to produce context-dependent effects in honey bees (*Apis mellifera*)

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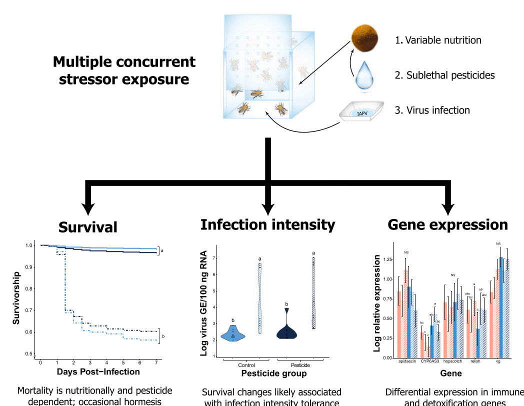
HIGHLIGHTS

- Bees were exposed to concurrent nutritional, pesticidal, and pathogenic stress.
- Bee response to pesticide exposure is frequently dependent on nutritional conditions.
- Pesticide exposure can exacerbate pathogenic infection or induce hormesis.
- All combinations had mixed effects on mortality and gene expression.
- Multi-stressor interactions are complex and can greatly affect bee health.

GRAPHICAL ABSTRACT



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ABSTRACT

Declines in pollinator health are frequently hypothesized to be the combined result of multiple interacting biotic and abiotic stressors; namely, nutritional limitations, pesticide exposure, and infection with pathogens and parasites. Despite this hypothesis, most studies examining stressor interactions have been constrained to two concurrent factors, limiting our understanding of multi-stressor dynamics. Using honey bees as a model, we addressed this gap by studying how variable diet, field-realistic levels of multiple pesticides, and virus infection interact to affect survival, infection intensity, and immune and detoxification gene expression. Although we found evidence that agrochemical exposure (a field-derived mixture of chlorpyrifos and two fungicides) can exacerbate infection and increase virus-induced mortality, this result was nutritionally-dependent, only occurring when bees were provided artificial pollen. Provisioning with naturally-collected polyfloral pollen inverted the effect, reducing virus-induced mortality and suggesting a hormetic response. To test if the response was pesticide specific, we repeated our experiment with a pyrethroid (lambda-cyhalothrin) and a neonicotinoid (thiamethoxam), finding variable results. Finally, to understand the underpinnings of these effects, we measured viral load and expression of important immune and detoxification genes. Together, our results show that multi-stressor interactions are complex and highly context-dependent, but have great potential to affect bee health and physiology.

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1. Introduction

Pollination services, particularly those provided by native and managed bees, are not only critical components of terrestrial landscape ecology (Hung et al., 2018), but also tightly enmeshed within our modern agricultural system (Gallai et al., 2009). As such, sustaining healthy bee populations is of vital importance towards the maintenance of key ecosystem services and agricultural practices, goals which have been made difficult by human-induced environmental stresses (Goulson et al., 2015). Native bee populations continue to decline (Prendergast et al., 2022) and annual rates of managed honey bee colony loss frequently reach 50 % (Aurell et al., 2024), threatening the overall sustainability of beekeeping operations and agricultural practices dependent on their pollination services. Although honey bees differ significantly from native bees in several ecological and physiological respects, they still serve as useful models for studying the effects of pollinator stress response, given their highly tractable nature and the inherent complexity of simulating multi-component environments under controlled settings.

Among the many biotic and abiotic factors aggravated by human-induced environmental change that bees encounter, nutritional stress (Alaux et al., 2010; Brodschneider and Crailsheim, 2010), pesticides (Brandt et al., 2016; Johnson et al., 2010), and pathogens (Grozinger and Flenniken, 2019) have all been identified as causal agents contributing to bee health decline. While each major stressor has been studied individually, the dynamic environment and large foraging range of honey bees mean that effects caused by any singular factor are often highly context-dependent and subject to variation due to interactions with the other factors (Doublet et al., 2015; Harwood and Dolezal, 2020; Stevenson et al., 2017). In fact, for over a decade, many researchers have argued that declines in overall pollinator health, not just honey bees, are due to interactions among all three main stressors (Goulson et al., 2015; Straub et al., 2022). In spite of this, no studies have examined bee responses to concurrent stress from poor nutrition, pesticide exposure, and pathogen infection, instead focusing on at most two paired stressors.

Independently, each of these stressors can result in negative consequences for individual honey bees or their colonies. Honey bee nutrition is complex (Wright et al., 2018); while specialized on pollen and nectar, honey bees are generalists that forage on many species of plants and perform best when able to access a diversity of floral resources (Di Pasquale et al., 2013; Schmidt et al., 1987). However, as landscapes become increasingly urbanized or simplified for agriculture, managed honey bee colonies are increasingly less able to access adequate floral resources, which reduces colony growth and productivity (Alaux et al., 2017; Otto et al., 2017; Zhang et al., 2023). Moreover, lower dietary pollen diversity is associated with reduced immunocompetence (Alaux et al., 2010; Dolezal et al., 2019a), lowered tolerance of pesticides (Barascou et al., 2021), and generally reduced physiological performance (Di Pasquale et al., 2013, 2016). To address declines in food availability for bees and other pollinators, several programs designed to diversify landscapes have incorporated "pollinator habitat" practices. For example, in the United States, the U.S. Conservation Reserve Program (CRP) supports two practices particularly beneficial to pollinators: Pollinator Habitat (CP42) and Prairie Strips (CP43). Prairie strip implementation involves planting buffer strips seeded with native flowering species along the contours of an agricultural field (Wratten et al., 2012). This practice has clear benefits to pollinators, including honey bees (Zhang et al., 2023), by greatly increasing floral diversity while also providing a wide range of other agricultural and environmental co-benefits (Kordbacheh et al., 2020; Schulte et al., 2017). However, one concern is that habitat installations adjacent to conventional agriculture could result in elevated pesticide exposure, thereby creating an ecological trap for bees and other beneficial insects (Hladik et al., 2017; Lee et al., 2001; Mogren and Lundgren, 2016).

This concern is well-founded, as several studies have shown that pesticide exposure, even at sublethal levels, negatively affects the health of many beneficial insects, including honey bees (Chmiel et al., 2020; Doublet et al., 2015; Olaya-Arenas et al., 2020). While there is evidence of interactions between pesticide exposure and nutrition (Barascou et al., 2021; Crone and Grozinger, 2021), even studies on agrochemicals alone pose challenges. In many agricultural areas, multiple active ingredients are used across time and space, including insecticides, herbicides, and fungicides, along with the ostensibly inert adjuvants already mixed into formulated products (Fine et al., 2017; Traynor et al., 2021). Even after selecting a pesticide type for testing, determining an appropriate concentration can be difficult. Field-realistic levels can vary enormously, depending on the application process (Dively and Kamel, 2012; Stoner and Eitzer, 2012) and the substrates from which residues are measured (Krupke et al., 2012; Tong et al., 2018). Thus, even real-world exposure data can be complicated and difficult to interpret. For example, measurements in the CP43 Prairie Strips system revealed ubiquitous, though very low, levels of certain agrochemicals (Hall et al., 2022), but it is not clear if these levels of contamination affect pollinators under different circumstances. In short, it is unclear if the benefits of higher quality nutritional resources outweigh any harm due to low levels of agrochemical residues.

While honey bee nutrition and pesticide exposure can be highly variable depending on the landscape and agricultural system, high pathogen pressure has become effectively ubiquitous in many parts of the world. Although honey bees encounter a variety of diseases caused by bacteria, fungi, or microsporidians, viruses vectored by the ectoparasitic mite *Varroa destructor* result in the most damage (Traynor et al., 2020). *V. destructor* can transmit many different viruses, each of which results in different symptoms in infected bees. Virus levels fluctuate dramatically across the year, but most colonies exhibit some level of virus infection much of the time (Crone and Grozinger, 2021; Faurot-Daniels et al., 2020) and these infections can cause a host of problems for bee health and survival (Brutscher et al., 2015; Chen and Siede, 2007). Additionally, infection status can interact with pesticide exposure (Harwood and Dolezal, 2020; Morfin et al., 2020) or nutritional stress (Crone and Grozinger, 2021; Dolezal et al., 2019a) to further influence survival outcomes. Thus, while nutritional stress, pesticide exposure, and pathogen infection all play a role in bee health and have been shown to interact in studies of paired stressors (Al Naggar and Paxton, 2021; Dolezal et al., 2019a; Tesovnik et al., 2020), how bees respond when presented with representatives of all three stresses at once remains to be determined.

To address this major gap, we subjected adult honey bee workers to multiple combinations of varying diet types, pesticide exposure, and viral infection to simulate the tripartite interactions that could plausibly occur in a field-realistic environment. Using a series of cage bioassays, we provided bees with variable nutrition (naturally-collected pollen vs. artificial pollen supplement), exposed them to insecticides and fungicides at a range of concentrations sourced directly from prairie strips exposure data (Hall et al., 2022), and infected them with Israeli acute paralysis virus (IAPV), a common dicistrovirus found in honey bees (Chen et al., 2014) that serves as a useful model for pathogen stress due to the distinct, well-characterized locomotory and dose-dependent responses it induces (Carrillo-Tripp et al., 2016; Dolezal et al., 2019a; Hsieh et al., 2020a). Through these experiments, we assessed the interactive effects of these stressors on honey bee survival and also began to determine which molecular pathways may be responsible by measuring immune and detoxification gene expression. We hypothesized that naturally-collected pollen would improve the survival of bees exposed to pesticides or pathogens and that the combination of nutritional stress and pesticide exposure would result in additive or synergistic negative effects. While our data are consistent with this hypothesis, we also observed hormetic responses to some pesticide treatments, i.e., improvement of bee survival, that reveal the intricacies of these interactions and underline the challenges present in understanding how bees respond to complex stressors in their environment.

2. Methods

2.1. Honey bee source and cage assay procedure

All bees used in experiments were sourced from colonies managed at the University of Illinois Urbana Champaign apiaries (GPS coordinates: 40.072927, −88.219327) according to standard management protocols. All source colonies were derived from queens marketed as Italian (*Apis mellifera ligustica)* and were regularly monitored and treated for varroa mites to reduce the risk of excessive background virus infections. Cage bioassays were performed using a modified protocol adapted from Hsieh et al. (2020a). For any given experimental assay preparation, a minimum of five frames of worker honey bee pupae were collected from at least three different colonies and placed into eclosion boxes stored in a 34 °C incubator set to 50 % relative humidity. After 24 h, the frames were removed from the eclosion boxes and newly emerged bees were brushed into a single collection container. Aged-matched bees from all source colonies were gently hand-mixed in the container to minimize age and colony-source effects and were then haphazardly distributed into acrylic cages with 35 bees per cage and eventually separated into 20–25 cages per experimental treatment.

2.2. Oral virus and sucrose inoculation

Following distribution of bees, all cages were returned to incubator conditions and immediately supplied with a small weigh boat containing either 600 μl of 30 % sucrose solution mixed with an IAPV inoculum or an equivalent quantity of unadulterated sucrose solution. The IAPV stock solution used to generate the diluted assay inocula was produced in honey bee pupae using the protocol described in Hsieh et al. (2020b) and contained an estimated 1.23×10^7 IAPV genome equivalents per 100 ng RNA at over 99 % purity containing only trace amounts of deformed wing virus, sacbrood virus, and black queen cell virus. IAPV inocula concentrations were selected based on preliminary assays performed prior to each set of cage experiments to determine a dose that would produce a 40–60 % mortality rate, resulting in inocula concentrations ranging between 0.1 and 1.5 %. After all inocula had been consumed (approximately 12–14 h), a 30 % sucrose feeder tube was added, allowing bees to consume sugar water ad libitum for the duration of the experiment.

2.3. Diet type and oral pesticide exposure treatments

At the same time the 30 % sucrose feeder was added, each cage received one of two different 200 mg balls of dietary protein (Azzouz-Olden et al., 2018) mixed with 67 % sugar syrup at a 65:35 ratio. We selected two protein sources to serve as alternative types of nutrition: MegaBee® (MegaBee), an artificial protein supplement commonly used by beekeepers, and honey bee-collected polyfloral pollen (CC Pollen High Desert Bee Pollen Granules, C. C. Pollen Company, Phoenix, AZ). Protein balls were removed and replaced every 24 h to prevent desiccation. Both diet types are frequently used for honey bee bioassays (Azzouz-Olden et al., 2018; Crone and Grozinger, 2021; DeGrandi-Hoffman et al., 2016) and the commercially available polyfloral pollen has been previously independently verified to be pesticide-free (Iverson et al., 2019). Although MegaBee is consumed by bees at rates comparable to that of pollen patties (DeGrandi-Hoffman et al., 2010), the complex micronutrient composition of wild-caught pollen means that it is difficult to replicate in a nutritional supplement. For clarity, MegaBee and the polyfloral pollen are hereby referred to as “protein supplement” and “pollen”, respectively.

To simulate oral pesticide exposure through consumption of contaminated pollen resources, caged bees were fed one of three different pesticides or pesticide mixes by combining the protein sources with the target pesticide or an equivalent volume of acetone to serve as a control. We chose to test the effects of lambda-cyhalothrin, thiamethoxam, and a mixture containing chlorpyrifos, azoxystrobin, and pyraclostrobin (hereby shortened to “chlorpyrifos+F”), as each of these pesticides represent a major class of synthetic xenobiotics that are frequently used in large volumes in agricultural settings (pyrethroids, neonicotinoids, and organophosphates mixed with fungicides, respectively). Each of these pesticide classes are neurotoxins with similar modes of action that seek to overstimulate invertebrate nervous systems by acting upon different molecular components: pyrethroids target voltage-dependent sodium channels (Vijverberg and Vanden Bercken, 1990), neonicotinoids bind to nicotinic acetylcholine receptors (Taillebois and Thany, 2022), and organophosphates prevent the natural degradation activities of acetylcholinesterase (O'Brien, 1963). Concentrations for all pesticides (expressed as μg active ingredient per kg diet) were selected to simulate field-realistic levels that honey bees can feasibly encounter and were based on either plant tissue residues collected from established prairie strips (chlorpyrifos 24 μg/kg; azoxystrobin 130 μg/kg; pyraclostrobin 20 μg/kg) (Hall et al., 2022) or other agricultural residues (lambda-cyhalothrin 280 μg/kg (Dolezal et al., 2016); thiamethoxam 70 μg/kg (Dively and Kamel, 2012; Henry et al., 2012)). Analytical grade versions of all pesticides were purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in appropriate quantities of acetone to create experimental dose solutions.

2.4. Survivorship monitoring and sampling

Mortality within each cage was recorded at 12-h intervals for the first 72 h of each experiment, after which the frequency of recording was reduced to every 24 h for the remainder of the seven-day assay. Dead bees were removed following each recording to prevent repeated count errors. Three live bees were collected from each cage between 24 and 36 h-post-inoculation (hpi) and immediately frozen on dry ice for eventual viral titer and gene expression quantification. See Fig. 1 for a graphical depiction of the survivorship assay timeline.

In total, seven sets of cage assay experiments were performed split across the three pesticide treatment groups using a full factorial design by crossing each protein source with each pesticide treatment (chlorpyrifos+F-supplement, chlorpyrifos+F-pollen; lambda-supplement, lambda-pollen; etc.). After observing significant survival differences in the two preceding cage experiments, a third chlorpyrifos+F experiment was run using both protein sources (chlorpyrifos+F-supplement/pollen) to evaluate potential interactive effects of nutrition. Complete details for each experiment are provided in Table 1 and will hereafter be abbreviated to their respective experiment numbers (e.g., Experiment 1 = chlorpyrifos+F-supplement, Experiment 2 = chlorpyrifos+F-pollen, etc.).

2.5. RNA extraction and RT-qPCR viral titer quantification

RNA extraction and subsequent viral titer quantification protocols are identical to those described in Hsieh et al. (2020a). Briefly, whole-body RNA was extracted from bees representing ten cages per treatment per cage experiment by pooling two of three bees sampled from each cage using TRIzol (Life Technologies, Carlsbad, CA, USA) extraction and amplified with a Power SYBR® Green RNA-CT™ 1-Step Kit (Applied Biosystems, Foster City, CA, USA) for absolute quantification within a 384-well Quantstudio 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each RT-qPCR reaction consisted of 5 μl of 2× SYBR Green RT-PCR mix, 0.08 μl of RT enzyme mix, 0.5 μl of the forward and reverse primers, 2.42 μl of nuclease-free water, and 2 μl of the extracted RNA template for a total volume of 10 μl.

2.6. Gene selection and expression quantification

Following a preliminary screening of eleven potential target genes belonging to honey bee immune, detoxification, or nutritional pathways, five genes with expression levels that varied between initial

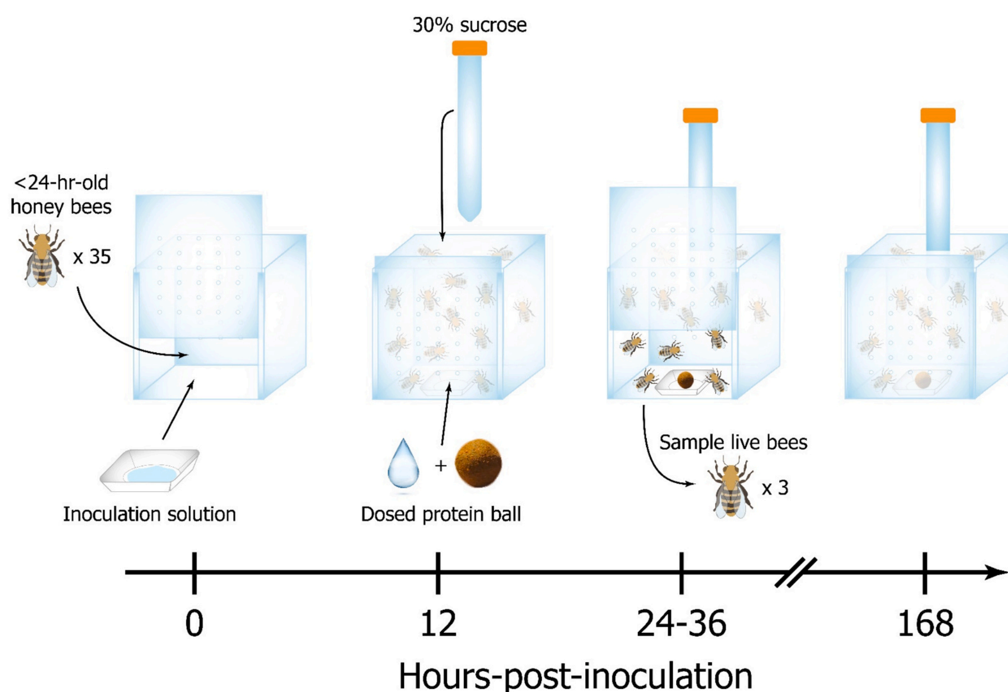

Fig. 1. Diagrammatic representation of the cage bioassay survivorship timeline. 35 bees aged under 24-h were inserted into acrylic cube cages and provided with 600 μl of an inoculation solution containing either IAPV or unadulterated sucrose. 12 h after insertion, a protein ball dosed with either acetone or pesticide is inserted into the cage and subsequently refreshed every 24 h. 30 % sucrose solution is provided ad lib via a gravity feeder. 3 live bees per cage are sampled between 24 and 36-h-post-incoulation. Mortality is recorded every 12 h for the first 3 days and then subsequently every 24 h until the termination of each experiment at 7 days.

Table 1
Details for all cage bioassay experiments performed using a full factorial design across variable nutrition, pesticide, and virus stressors.

Experiment #	Diet types	Pesticide	Virus inoculation	Cages / treatment	Bees / cage	Associated figures
1	Artificial supplement	Chlorpyrifos+F	IAPV	25	35	Figs. 2A, 3A, 5A
2	Natural pollen	Chlorpyrifos+F	IAPV	25	35	Figs. 2B, 3B, 5B
3	Artificial supplement, Natural pollen	Chlorpyrifos+F	IAPV	20	35	Figs. 2C, 3C, 5C
4	Artificial supplement	Lambda-cyhalothrin	IAPV	20	35	Figs. 2D, 3D
5	Natural pollen	Lambda-cyhalothrin	IAPV	20	35	Figs. 2E, 3E
6	Artificial supplement	Thiamethoxam	IAPV	20	35	Figs. 2F, 3F
7	Natural pollen	Thiamethoxam	IAPV	20	35	Figs. 2G, 3G

treatments were selected as targets for further expression quantification: *apidaecin, CYP6AS3, hopscotch, relish,* and *vg*. The relative expression of all target genes was measured using identical kits, instruments, thermocycler conditions, and extracted bee RNA as those used for viral titer quantifications. Expression levels of target genes were normalized to the geometric mean of four reference genes (*ache2, gapdh, rps18, tif*) selected for their stability under IAPV infection (Deng et al., 2020) and relative expression calculations were performed utilizing the $2^{-\Delta\Delta Ct}$ (Livak) method with the control treatments (no pesticide and no virus) designated as the calibrator samples. Primer sequences for all target and reference genes are displayed in Table S1.

2.7. Statistical analyses

All statistical analyses were conducted in the R statistical environment, version 4.3.2 (R Core Team, 2023). Survival analyses were performed using Cox proportional-hazards modeling via the survival (version 3.5–7 (Therneau, 2023)) package and visualized using the survminer (version 0.4.9 (Kassambara et al., 2021)) package. Pairwise comparisons within all Cox models were corrected with Bejamini-Hochberg corrections to reduce Type I errors. Viral titers between treatments in each set of cage experiments obtained via RT-qPCR were compared with Kruskal-Wallis rank sum tests after failing to meet normality and homogeneity-of-variance assumptions (dplyr package, version 1.1.4 (Wickham et al., 2023)). Subsequent pairwise differences were determined using Wilcoxon rank sum tests and multiple comparisons were corrected with Benjamini-Hochberg corrections. Relative expression of the five target immune/detoxification genes within each set of cage experiments were log-transformed to allow for easier comparison before subjecting them to *t*-tests or linear mixed effect models using the lme4 package (version 1.1–35.1 (Bates et al., 2015)), depending on the number of treatment groups being compared. Interplate variation within genes was accounted for by designating it as a random factor within the mixed effect models. Significant effects of specific treatments and stressors were determined with post-hoc comparisons and Tukey adjustments via the emmeans package (version 1.10.0 (Lenth, 2024)). All survivorship curves, violin plots, and bar chart graphics were generated in R via the survminer or ggplot2 (version 3.4.4 (Wickham, 2016)) packages.

3. Results

3.1. Chlorpyrifos+F exposure

Two general patterns emerged from the survivorship assay data that remained consistent across all tested pesticides: (1) in the absence of additional stressors (i.e., virus inoculation), consumption of field-relevant pesticide doses did not result in significant differences in

survival compared to acetone vehicle controls, and (2) virus inoculation always significantly reduced survival compared to sucrose-inoculated treatments (Fig. 2A-G).

3.1.1. Chlorpyrifos+F – cage assay survivorship (Experiments 1–3)

Bees fed artificial protein supplement and exposed to chlorpyrifos+F and virus had significantly reduced survival compared to bees exposed to virus alone (Experiment 1) (Cox proportional-hazards; HR = 1.198, $p < 0.0001$) (Fig. 2A). However, when bees were instead fed naturally-collected polyfloral pollen, the same exposure scenario resulted in a trend in the opposite direction. Bees that consumed both chlorpyrifos+F and virus experienced apparently 4 % greater survival than those that consumed virus alone (Experiment 2) (Cox proportional-hazards; HR = 0.95, $p = 0.182$) (Fig. 2B). A subsequent, independent chlorpyrifos+F experiment in which bees were fed either diet type reinforced the trends observed in both previous independent diet trials (Experiment 3). Among the virus-inoculated bees, those fed pollen and chlorpyrifos+F survived at rates significantly higher than did those fed only pollen (Cox proportional-hazards; HR = 0.894, $p = 0.04$) and bees fed protein supplement and chlorpyrifos+F again experienced significantly lower survival than those fed the supplement only (Cox proportional-hazards; HR = 1.11, p = 0.04) (Fig. 2C).

The effect of diet was therefore measurable only when bees were exposed to *both* chlorpyrifos+F and virus. Without both stressors, neither diet type significantly altered survival (Cox proportional-hazards; HR = 0.991, $p = 0.787$), but, when both virus and pesticides were present, pollen consumption greatly improved survival compared to that of the protein supplement (Cox proportional-hazards; HR = 1.145, $p = 0.0001$) (Fig. 2C).

3.1.2. Chlorpyrifos+F – virus titers

RT-qPCR confirmed the successful viral inoculation of virus-treated bees in Experiments 1–3 but revealed no significant effect of diet or pesticide on viral titers. On average, bees that consumed the virus inoculum contained viral loads multiple orders of magnitude greater than those that consumed the unadulterated dose (i.e., those with only pre-existing background virus levels) (Fig. 3A-C), though this pattern was not consistent among all experiments. The titers of virus-inoculated control bees in Experiment 1 were not significantly different from titers in either sucrose-inoculated group (control $p = 0.336$; pesticide $p =$

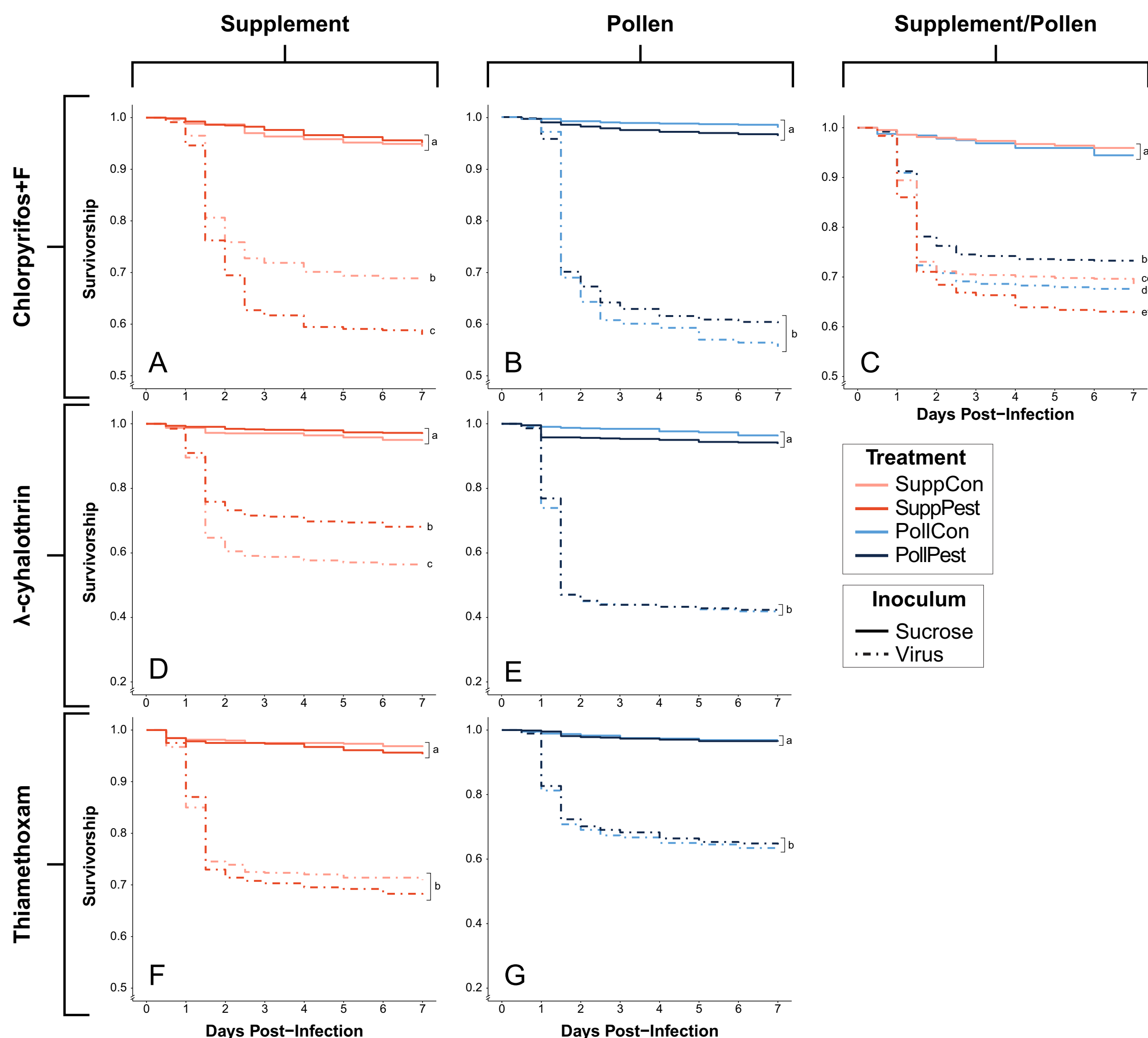

Fig. 2. (A)-(G) Survival of caged honey bees subjected to varying diet-pesticide-virus combinations (Experiments 1–7, respectively). Diet-pesticide treatment combinations are represented by color (“Supp” = artificial protein supplement, “Poll” = pollen, “Con” = acetone controls, “Pest” = pesticide) and inoculation status by line type (solid = sucrose, dash = virus). Pesticide type is labeled to the left of each bracket. Letters denote significant differences ($p < 0.05$) between treatments within each panel (pairwise Cox proportional-hazards models, Benjamini-Hochberg correction), (A)-(B) n = 25 cages per treatment, (C)-(G) n = 20 cages per treatment.

0.058) (Kruskal-Wallis, $\chi^2 = 12.795$, df = 3, $p = 0.005$; Wilcoxon rank-sum) (Fig. 3A), though this lack of differences was not reflected in Experiment 2, where both virus-inoculated groups produced significantly greater viral titers than their sucrose-inoculated counterparts (control $p = 0.0008$; pesticide p = 0.0008) (Kruskal-Wallis, $\chi^2 = 22.168$, df = 3, $p < 0.0001$; Wilcoxon rank-sum) (Fig. 3B).

The virus titer patterns in Experiments 1–2 were for the most part similar to those in Experiment 3, though the magnitude of titer differences between virus- and sucrose-inoculated groups was overall reduced. Sucrose-inoculated control bees of both diet types contained significantly fewer viral copies than any of the virus-inoculated bees, with lower titers in supplement-fed bees than in those fed pollen ($p = 0.008$) (Kruskal-Wallis, $\chi^2 = 37.342$, df = 5, p < 0.0001; Wilcoxon rank-sum) (Fig. 3C). Viral titers between all virus-inoculated treatments were statistically comparable, with the exception of the supplement-fed pesticide bees, which had significantly greater viral loads than the pollen-fed control bees ($p = 0.031$) (Wilcoxon rank-sum) (Fig. 3C).

3.1.3. *Chlorpyrifos+F – gene expression*

Gene expression measurements were performed on bees collected from Experiments 1–3 based on the elevated level of interactions observed between diet, virus, and pesticide compared to those in Experiments 4–7. We first examined the gene expression of sucrose control bees exposed to chlorpyrifos+F to isolate any potential influences of diet. Significant differences were detected in the expression of two of five targeted genes, with pollen-fed bees displaying higher relative expression of *CYP6AS3* and *hopscotch* than supplement-fed bees (1.88-fold and 1.32-fold increases, respectively) (*CYP6AS3*: *t*-test, t-stat = 3.001, df = 18, p = 0.008; *hopscotch*: t-test, t-stat = 3.545, df = 18, $p = 0.002$) (Fig. 4). Subsequent analyses among all remaining collected bees also showed significant differences in the relative expression of *CYP6AS3* and *hopscotch*, as well as *relish*, with no significant variation detected in *apidaecin* nor *vg* across any treatments. Overall, *CYP6AS3* displayed the greatest level of expression variation between treatments and among experiments. Although no differences were detected between treatments in Experiment 2 (Fig. 5B), sucrose-inoculated pesticide bees in Experiment 1 had the lowest levels of *CYP6AS3* expression among all treatments, while sucrose-inoculated control bees had the highest (linear mixed model, F-stat = 11.024, df = 3, $p < 0.0001$) (Fig. 5A). This expression pattern is somewhat mirrored in Experiment 3, in which the absence of pesticide increases *CYP6AS3* expression regardless of diet type, though this effect does still interact with virus presence (linear mixed model, F-stat = 10.301, df = 5, p < 0.0001) (Fig. 5C). Linear mixed models revealed that in general, pesticide and diet both significantly affect *CYP6AS3* expression, with pesticide exposure decreasing expression and pollen consumption increasing expression relative to the protein supplement (Tables S2, S4).

Relish expression was less consistent across experiments compared to *CYP6AS3*. In Experiments 1–2, sucrose-inoculated pesticide bees displayed lower *relish* expression than all other treatments, though this

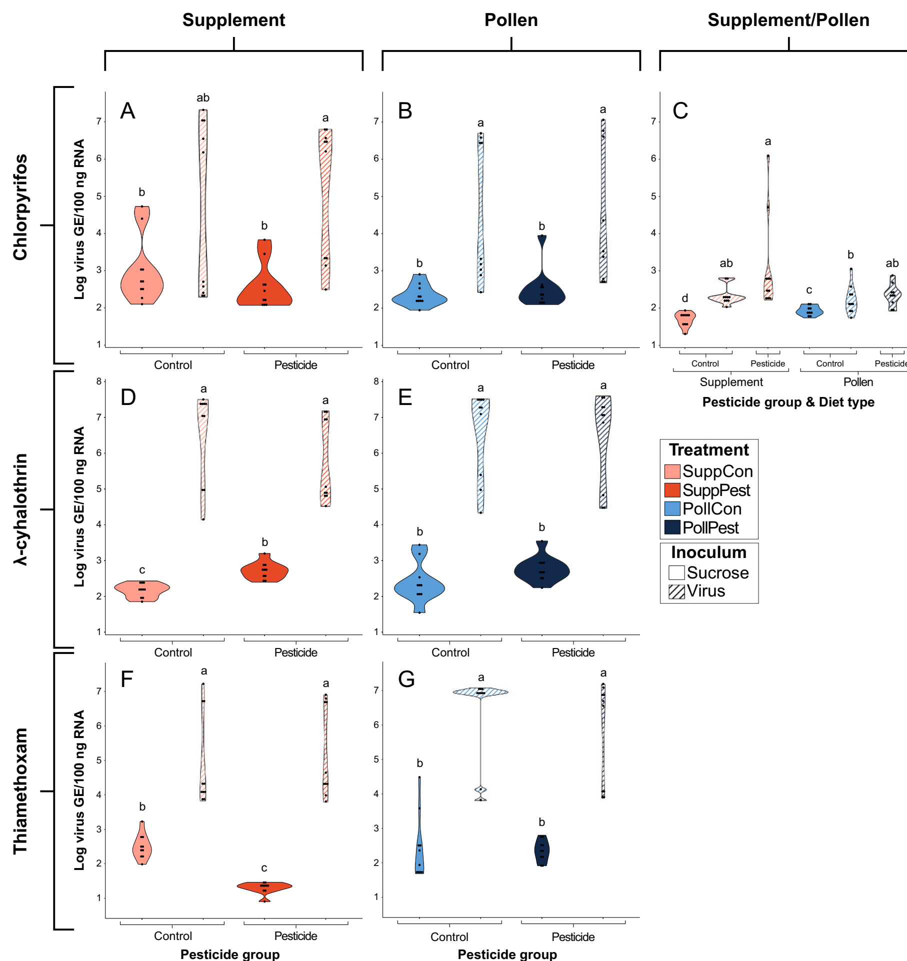

Fig. 3. (A)-(G) IAPV viral loads of bees sampled from cage survivorship assays (Experiments 1–7, respectively), expressed as mean ± SE log viral genome equivalents per 100 ng RNA. Diet-pesticide treatment combinations are represented by color ("Supp" = supplement, "Poll" = pollen, "Con" = acetone controls, "Pest" = pesticide) and inoculation status by pattern (solid = sucrose, hatch = virus). Pesticide type is labeled to the left of each bracket. $n = 10$ pools of 2 bees each from different cages per treatment. Letters denote significant differences ($p < 0.05$) between treatments within each panel (Kruskal-Wallis, Wilcoxon rank-sum, Benjamini-Hochberg correction).

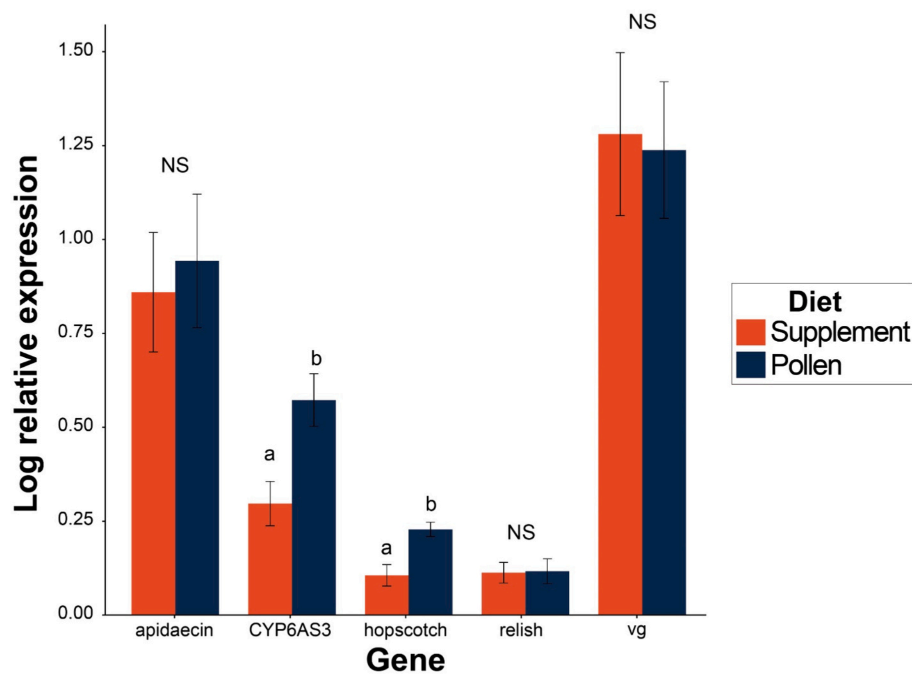

Fig. 4. The effect of diet type on log relative expression of immune and detoxification genes in honey bees exposed to a mixture of chlorpyrifos, azoxystrobin, and pyraclostrobin. Relative expression measured using RT-qPCR and normalized against the geometric mean of reference genes *ache2, gapdh,* and *tif*. Letters denote significant differences ($p < 0.05$) between diet types within each gene (*t*-test), $n = 10$ pools of 2 bees each per diet treatment, error bars represent ± SE.

trend was only significant in Experiment 1 (linear mixed model, F-stat = 5.55, df = 3, $p = 0.003$) (Fig. 5A, B). Correspondingly, linear mixed models reveal that both pesticide and virus presence significantly affect expression levels, though again, only in Experiment 1 (Table S2). However, these patterns are not repeated in Experiment 3; instead, only diet is shown to significantly affect expression, with supplement consumption elevating *relish* expression compared to pollen consumption (linear mixed model, F-stat = 10.772, df = 1, p = 0.002). Additionally, diet was found to significantly interact with both pesticide and virus (Table S4), resulting in large levels of overlap in expression between most treatments (Fig. 5C).

Like expression of *CYP6AS3* and *relish*, *hopscotch* expression in sucrose-fed pesticide bees was significantly lower than in all other treatments in Experiment 1 (linear mixed model, F-stat = 7.936, df = 3, $p = 0.0003$) (Fig. 5A), but neither this pattern nor any other difference in *hopscotch* expression was detected between treatments in any of the other chlorpyrifos+F experiments (Fig. 5B, C).

3.2. Lambda-cyhalothrin exposure

3.2.1. Lambda-cyhalothrin – cage assay survivorship (experiments 4–5)

Bees exposed to lambda-cyhalothrin produced survivorship assay results unlike those of bees exposed to chlorpyrifos+F. When fed protein supplement, bees exposed to lambda-cyhalothrin and virus survived at significantly higher rates than those exposed to virus alone (Experiment 4) (Cox proportional-hazards; HR = 0.821, $p < 0.0001$) (Fig. 2D). When bees were instead fed pollen, lambda-cyhalothrin consumption had no effect on the survival of virally inoculated bees (Experiment 5) (Cox proportional-hazards; HR = 0.989, $p = 0.78$) (Fig. 2E). Due to the comparatively lower level of interaction between diet and pesticide, additional testing of concurrent diet effects like that of the chlorpyrifos+F trials was not performed.

3.2.2. Lambda-cyhalothrin – virus titers

Bees collected from Experiments 4–5 produced similar viral titer patterns to those from Experiments 1–3, albeit with less variance within treatment groups. No differences were detected within inoculation treatments across both experiments, suggesting a general lack of effect of lambda-cyhalothrin on virus titers, with the exception of Experiment 4: sucrose-inoculated pesticide bees produced significantly higher virus titers than control bees (sucrose p < 0.0001) (Kruskal-Wallis, $\chi^2 = 33.771$, df = 3, p < 0.0001; Wilcoxon rank-sum) (Fig. 3D).

3.3. Thiamethoxam exposure

3.3.1. Thiamethoxam – cage assay survivorship (Experiments 6–7)

Both diet type and pesticide exposure had negligible effects on survival in Experiments 6–7. Virus-inoculated bees exposed to thiamethoxam did not have significantly different survival compared to their sucrose-inoculated counterparts when fed protein supplement (Experiment 6) (Cox proportional-hazards; HR = 1.046, $p = 0.376$) (Fig. 2F) or pollen (Experiment 7) (Cox proportional-hazards; HR = 0.975, $p = 0.645$) (Fig. 2G).

3.3.2. Thiamethoxam – virus titers

Bees exposed to thiamethoxam in Experiments 6–7 produced nearly identical virus titer patterns to those collected from Experiments 4–5, with the exception of Experiment 6: sucrose-inoculated pesticide bees contained significantly lower virus titers than control bees (sucrose p < 0.0001; Kruskal-Wallis, $\chi^2 = 33.045$, df = 3, p < 0.0001; Wilcoxon rank-sum) (Fig. 3F), rather than greater.

4. Discussion

Honey bee colonies regularly experience combined effects of poor nutrition, pesticide exposure, and virus infection in field environments, but the high level of interaction among such factors makes predicting specific outcomes challenging. Our findings provide further evidence of this challenge while also highlighting the importance of context by demonstrating the range of responses that can occur in survival of honey bees when they are subjected to varying combinations of the three major stressors.

Among the tested pesticide types, the organophosphate insecticide chlorpyrifos mixed with the fungicides azoxystrobin and pyraclostrobin

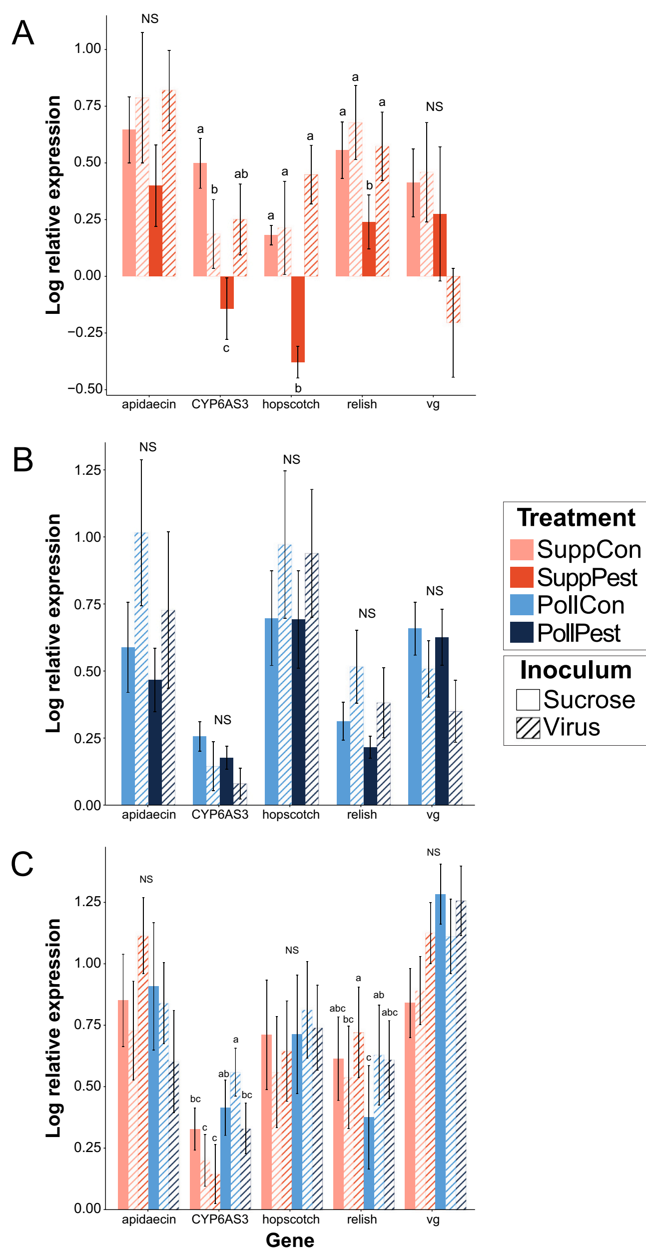

Fig. 5. (A)-(C) The effect of diet type on log relative expression of immune and detoxification genes in honey bees subjected to varying diet-pesticide-virus combinations (Experiments 1–3, respectively). Diet-pesticide treatment combinations are represented by color ("Supp" = supplement, "Poll" = pollen, "Con" = acetone control, "Pest" = chlorpyrifos-azoxystrobin-pyraclostrobin mixture) and inoculation status by pattern (solid = sucrose, hatch = virus). Relative expression measured using RT-qPCR and normalized against the geometric mean of reference genes *ache2, gapdh, rps18*, and *tif*. Letters denote significant differences ($p < 0.05$) between diet types within each gene (linear mixed effects models, Tukey HSD), $n = 10$ pools of 2 bees each per diet treatment, except (C), where $n = 9$ pools of 2 bees each per diet treatment for *relish* and *vg*, error bars represent ± SE.

demonstrated the greatest level of interactivity with variable diet and virus inoculation. Such elevated interactivity could be explained, in part, by the greater number of distinct components in the field-derived cocktail, as well as the potential negative non-target effects fungicides can have on honey bees (Barascou et al., 2021; Degrandi-Hoffman et al., 2015). Notably, though, exposure to neither this agrochemical cocktail nor any of the other pesticides significantly influenced survival without an additional viral stressor also present (Fig. 2A-C). This outcome occurred even though the concentrations for these agrochemicals were intentionally set to match the highest detected levels found in pollen collected by bees located in prairie strips (Hall et al., 2022), thereby representing a possible, yet extreme, exposure scenario for bees in these pollinator habitats. This extreme scenario not only failed to affect survival in the absence of virus inoculation but actually induced hormesis, a phenomenon in which low levels of an otherwise harmful substance produces a beneficial effect in living organisms (Cutler and Rix, 2015), though it is unlikely that such an effect would continue if the pesticide concentrations were increased. Sublethal exposure effects are complicated and, critically, hormesis was dependent on which diet type the bees consumed. When virus-inoculated bees were fed an artificial protein supplement, chlorpyrifos+F exposure worsened the effects of viral infection (Fig. 2A,), but when the bees were instead fed natural pollen, the same level of pesticide exposure actually increased their survival relative to sucrose-inoculated controls (Fig. 2B). These survival patterns provide clear evidence of dietary influence and are further reinforced by Experiment 3, in which, much like the effect of pesticide, diet type does not significantly affect survival unless bees are also exposed to both pesticide and virus (Fig. 2C). In essence, the effects of all three major stressors—variable nutrition, pesticide exposure, and viral infection—can be interdependent.

This conditional impact of diet suggests that there is physiological interplay between diet and chlorpyrifos+F exposure, a hypothesis which is further supported by the gene expression analyses. We observed that pollen consumption is associated with the upregulation of two of the five target genes, *CYP6AS3*, a member of the CYP3 clade of the cytochrome P450 monooxygenase superfamily broadly associated with the detoxification of xenobiotics such as synthetic pesticides or phytochemicals (Li et al., 2007; Mao et al., 2009), and *hopscotch*, a member of the Jak/STAT immune pathway that is conserved across many insect systems (Evans et al., 2006) (Fig. 4). *CYP6AS3* and *hopscotch*, along with *relish*, an immune gene found in the Imd pathway (Evans et al., 2006), are all consistently differentially expressed in other chlorpyrifos+F trials (Fig. 5A, C), although the introduction of multiple pesticide and virus treatments may obscure the singular contributions of individual stressors to overall expression. Mixed effect modeling allowed us to distinguish overall trends within differentially expressed genes and provides insight into which molecular processes these external stressors may stimulate. Consumption of natural pollen boosts the expression of *CY6PAS3* (Table S4), while artificial supplement consumption has a similar effect on *relish* expression (Table S4). Diet type also interacts with virus and/or pesticide (Table S4), further illustrating the context-dependent stressor interactions as demonstrated in the survival assays. This high degree of variability underlines the complexity associated with nutrition in that no single diet type is guaranteed to stimulate immune gene response.

Virus inoculation and chlorpyrifos+F exposure both resulted in differential expression of several target genes, but the direction of influence was not always consistent with our original predictions. Bees inoculated with virus displayed elevated *hopscotch* and *relish* expression (Tables S2, S4), as was expected given their well-established roles in antiviral pathways (Evans et al., 2006; Iverson et al., 2019) and similar activation by other infectious microbes (Tesovnik et al., 2020). However, bees exposed to chlorpyrifos+F exhibited *decreased* expression of the detoxification gene *CYP6AS3* (Fig. 5A, C; Tables S2, S4). Although *CYP6AS3* is upregulated in honey bees exposed to other organophosphates such as coumaphos (Schmehl et al., 2014), both coumaphos (Chaimanee et al., 2016) and chlorpyrifos (Agrebi et al., 2024) have been shown to reduce expression of other closely related CYP genes belonging to the same clade. Still, chlorpyrifos consumption not only failed to upregulate *CYP6AS*, but also resulted in a hormetic effect, increasing the survival of virus-inoculated pesticide bees relative to virus-inoculated controls (Fig. 2B, C). Hormesis, though more thoroughly studied in pest insects (Cutler et al., 2022; Guedes and Cutler, 2014), has often been documented in beneficial species including pollinators or predatory insects

used in biocontrol (Cutler and Rix, 2015). Compounds known to be toxic at high doses such as naturally occurring aflatoxins (Johnson et al., 2012) and the folivore deterrents caffeine and nicotine (Köhler et al., 2012; Wright et al., 2013) all stimulate positive responses in honey bees via increasing adult lifespans or improving memory retention when applied at low concentrations, despite acting upon honey bee physiology through different avenues.

To assess if the effects we observed were specific to our particular pesticide treatment derived from prairie strip sampling or a more generalized response to most pesticides, we performed similar experiments with lambda-cyhalothrin and thiamethoxam, both of which belong to different pesticide classes. However, the physiological mechanisms behind the previously observed hormesis remain unclear and are not evidently predictable based on diet types or pesticide class, as seen from the results of the survival assays—supplement-fed bees exposed to lambda-cyhalothrin exhibited a similar hormetic response (Fig. 2D) to that of the pollen-fed chlorpyrifos+F bees (Fig. 2B). One potential explanation could be that the low concentrations of pesticides stimulated immune responses in the honey bees that improved overall longevity, whereas the macro and micronutrients present in the consumed protein sources provided a level of nutritional buffering that helped to counteract any otherwise negative effects. Similar interactions between diet and xenobiotic exposure are well-documented in the literature (Liao et al., 2020; Tosi et al., 2017), although the exact macromolecules and ways in which they interact with lambda-cyhalothrin or chlorpyrifos+F remain to be explored. This hypothesis is partially supported by the gene expression analyses, which show that the detoxification and immune genes *CYP6AS3* and *relish* are significantly or marginally affected by pesticide presence, and that in both cases diet type further influences the direction of effect (Table S4). Although chlorpyrifos+F exposure downregulates *CYP6AS3* expression, more "generalist" CYP genes, such as the CYP9Q enzymes (Maiwald et al., 2023; Mao et al., 2011), may be better suited for chlorpyrifos+F metabolism and therefore among the group of genes upregulated by such exposures.

The results of the viral titer quantifications, though consistent with previous studies investigating paired stressor interactions (Dolezal et al., 2019a; Hsieh et al., 2020a), do not provide any further insight into the mechanisms behind virus infection tolerance in honey bees. Exposure to any of the three tested pesticides did not affect viral titers of virus-inoculated bees and, despite the statistical differences between sucrose-inoculated treatment groups in several experiments (Fig. 3C, D, F), it is unlikely that these differences are biologically relevant. In all cases, mean virus titers of sucrose-inoculated bees bordered on the range of the detectable limits of the absolute quantification method employed and were ultimately of negligible physiological consequence (Amiri et al., 2019; Carrillo-Tripp et al., 2016). The bimodal distribution of viral titers measured in virus-inoculated bees as well as the lack of differences between the titers of sucrose- and virus-inoculated bees from the chlorpyrifos+F experiments (Fig. 3A-C) can likely both be attributed to the sampling regime, the natural progression of IAPV replication in honey bees, and survivor bias (Dolezal et al., 2019a; Prayugo, 2024). Bees that survive IAPV inoculation typically contain highly reduced titers by 36-h-post-inoculation (Harwood and Dolezal, 2020; Prayugo, 2024), leading to an increased possibility of selecting bees with low titer counts if sampled near the end of the 24–36-hpi collection window. Consequently, none of the tested pesticides significantly affect virus infection intensity in any direction, a result that both supports (Al Naggar and Paxton, 2021) and contradicts (Di Prisco et al., 2013) previous investigations of the effects of pesticide exposure on virus infection in honey bees. The inconsistency among studies is likely a result of the countless different virus-pesticide combinations, but our findings at least suggest that changes in survival are due to the ability of the bees to tolerate variable virus infection intensities rather than directly reduce viral copy counts, though evidence suggests that honey bees can employ a combination of both virus management strategies (Lu et al., 2023).

The aggregated results of our study on the interactive effects of variable diet, pesticide exposure, and virus infection have broader implications for the management practices of honey bees, particularly for those located in pollinator habitats adjacent to agricultural sites, including prairie strips (CP43). Such habitats could potentially act as ecological traps (Hladik et al., 2017; Lee et al., 2001; Mogren and Lundgren, 2016), attracting honey bees and other pollinators to these hotspots of floral abundance where they will invariably be exposed to sublethal levels of pesticides (Hall et al., 2022), which could further interact with extant viral infections. Our bioassays simulated these environmental stressor interactions in the laboratory and showed that, critically, consumption of prairie-strip-relevant levels of pesticides in the absence of virus inoculation does not significantly reduce honey bee survival. This finding reflects favorably on the use of prairie strips as pollinator habitats, as the multitude of nutritional and ecological benefits gained from prairie strips (Kordbacheh et al., 2020; Schulte et al., 2017) could conceivably outweigh potential negative effects associated with sublethal pesticide exposure. However, while our results are generally indicative of the greater ability of natural pollen to ameliorate the negative influences of pesticides and virus infection than artificial protein supplements, the results of any single pesticide class cannot be broadly generalized to all pesticides. Our observed interactions between pesticides and other factors could be partially attributed to dose-dependent effects and have potential to vary greatly at higher or lower pesticide concentrations. Caution must therefore be exercised against absolute interpretations of interaction studies, especially given the dynamic environments honey bees inhabit, which are often subject to temporal (Dolezal et al., 2019b; Hsieh et al., 2020b) and climatic variation (Rajagopalan et al., 2024; Zapata-Hernández et al., 2024). Lastly, our study has begun to determine the molecular underpinnings of the variable responses of honey bees to interactive stressors. We found no evidence of biologically relevant effects of pesticide exposure on viral infection intensity but have identified several differentially regulated immune and detoxification genes, the pathways for which would be suitable for further investigation. Future directions could include RNA-seq analyses to provide a more complete image of the gene families involved and help elucidate the seemingly counterintuitive results of *CYP6AS3* expression.

5. Conclusions

Our study demonstrates that, even across a comparatively small set of variable stressors, interactions between biotic and abiotic factors within the honey bee system are highly complex. Altering any factor within the diet-pesticide-virus paradigm can lead to unexpected phenotypic responses. Although our findings are specific to the honey bee system, the same forces examined likely produce equally complex and difficult-to-predict interactions in native pollinator species. Honey bees are well-studied model organisms and frequently act as surrogate species in risk assessments (Gradish et al., 2019; Uhl et al., 2019), but, given the comparative dearth of relevant research in native pollinators, the applicability of our findings to these species should not be assumed. As such, gaining an improved understanding of these interactions may be key to predicting best management practices for sustainable pollinator management, especially during periods of increased pesticide and virus exposure risk. Upscaling our bioassays to field colony sizes and testing interactive effects across a greater seasonal range can help achieve this goal by identifying which dietary protein sources maximize nutritional buffering against stressors known to decrease survivorship in a less artificial setting.

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CRediT authorship contribution statement

Edward M. Hsieh: Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Adam G. Dolezal:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Adam G. Dolezal reports financial support was provided by Foundation for the Food and Agriculture Research. Edward M. Hsieh reports financial support was provided by North American Pollinator Protection Campaign. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

I have shared a link to my data/code in a Dryad repository in the 'Attach File' step

Science of the Total Environment - Nutrition, pesticides, and virus (Original data) (Dryad)

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.175125.

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