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RESEARCH ARTICLE

Pollen protein and lipid content influence resilience to insecticides in honey bees (*Apis mellifera*)

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ABSTRACT

In honey bees (*Apis mellifera*), there is growing evidence that the impacts of multiple stressors can be mitigated by quality nutrition. Pollen, which is the primary source of protein and lipids in bee diets, is particularly critical for generating more resilient phenotypes. Here, we evaluated the relationship between pollen protein to lipid (P:L) ratio and honey bee insecticide resilience. We hypothesized that pollen diets richer in lipids would lead to increased survival in bees exposed to insecticides, as pollen-derived lipids have previously been shown to improve bee resilience to pathogens and parasites. Furthermore, lipid metabolic processes are altered in bees exposed to insecticides. We fed age-matched bees pollen diets of different P:L ratios by altering a base pollen by either adding protein (casein powder) or lipid (canola oil) and simulating chronic insecticide exposure by feeding bees an organophosphate (chlorpyrifos). We also tested pollen diets of naturally different P:L ratios to determine whether the results were consistent. Linear regression analysis revealed that mean survival time for bees fed altered diets was best explained by protein concentration (P=0.04, adjusted R^2=0.92), and that mean survival time for bees fed natural diets was best explained by the P:L ratio (P=0.008, adjusted R^2=0.93). Our results indicate that higher dietary P:L ratios have a negative effect on bee physiology when combined with insecticide exposure, while lower P:L ratios have a positive effect. These results suggest that protein and lipid intake differentially influence insecticide response in bees, laying the groundwork for future studies of metabolic processes and development of improved diets.

KEY WORDS: Macronutrients, Nutrition, Pesticide, Stress response, Survival, Gene expression, Regression

INTRODUCTION

Animals, including insects, balance their intake of macronutrients to optimize their fitness (Raubenheimer and Simpson, 1997). Different ratios of macronutrients are needed to optimize development, growth, reproduction or longevity, and thus an insect's nutritional requirements may shift over their lifetime (Lee et al., 2008; Raubenheimer et al., 2014; Simpson et al., 1995). Similarly, if insects are infected or stressed, their nutritional requirements change to address these challenges (Boggs, 2009; Cotter et al., 2011). While these shifts in nutritional requirements have been demonstrated in several species, the underlying mechanisms by which different macronutrients support different physiological or organismal outcomes are often not well understood.

Honey bee diets and nutritional requirements appear to be quite dynamic. Honey bees obtain their nutrients from pollen (protein, lipids and micronutrients) and nectar (carbohydrates) (Wright et al., 2018). Pollen protein to lipid (P:L) ratios can vary widely across plant species, and honey bees collect pollen with a broad range of P:L ratios (Vaudo et al., 2018; DeGrandi-Hoffman et al., 2018; Jones, 2020). Previous studies have demonstrated that pollen-based diets improve survival in honey bees exposed to parasites, pathogens and pesticides, while poor nutrition synergizes with pathogen or insecticide stress to exacerbate negative health outcomes (Annoscia et al., 2017; Schmehl et al., 2014; Barraud et al., 2020; Dolezal and Toth, 2018; Tosi et al., 2017). Furthermore, secondary plant compounds found in honey, which are likely derived from pollen, upregulate honey bee detoxification genes and increase metabolism of certain insecticides (Mao et al., 2013; Liao et al., 2017). Though pollen diets can contribute to resilient phenotypes, there can be considerable variation in outcomes of honey bees fed pollen from different plant species, as well as diverse pollen diets compared with single species (DiPasquale et al., 2013; Dolezal et al., 2019). The factors that contribute to variation in outcomes in bees fed pollen from different plant species have not been determined, but it is likely that protein and lipid content play an important role.

Pollen is broadly composed of protein (2–60%), lipids (1–20%) and secondary metabolites (Roulston and Cane, 2000; Roulston et al., 2000; Stevenson, 2020). The lipid detection method most commonly used, the sulfo-phospho-vanillin (SPV) assay, can only determine the concentration of total lipids and does not delineate between different categories, each of which is uniquely important for honey bee physiology (Cheng et al., 2011). Pollen-derived lipids include sterols, polyunsaturated fatty acids (PUFAs), free fatty acids and hydrocarbons (Van Handel, 1985), all of which play different roles in insect physiology and health. For example, sterols are key components of cell membranes and hormone precursors (Jing and Behmer, 2020). The major PUFAs, omega-3 and omega-6 fatty acids, are important for insect neurological health, and are also key components of cell membranes (Arien et al., 2015). Free fatty acids are stored as triglycerides by insects in the fat bodies, where they can be used to meet energy or stress demands, synthesize phospholipids and waxes, or serve as a precursor to pheromones (Arrese and Soulages, 2010). Finally, hydrocarbons make up the insect cuticle, and function as pheromone components in nestmate recognition (Dani et al., 2005). Again, the SPV assay can only determine the concentration of total lipids and does not delineate between different lipid types (Cheng et al., 2011). Thus, though different types of lipids serve different functions in insects, we use the term 'lipids' to refer to total pollen lipids that encompass all of these categories unless otherwise specified.

Dietary lipids and lipid metabolic processes may play a general role in stress responses in honey bees. Honey bees under different types of stress mobilize lipids, specifically triglycerides, from fat

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bodies to meet energetic demands, particularly when the insect stress hormone octopamine is circulated to coordinate the stress response (Even et al., 2012). Nutrient-stressed honey bees have also been found to have smaller fat bodies but, to our knowledge, there has not yet been a study quantifying fat bodies in honey bees fed an excess of lipids (Toth et al., 2005). Annoscia et al. (2017) demonstrated that pollen-based lipids were important for the survival of honey bees parasitized by *Varroa* mites and reduced viral titers in these bees. The authors proposed that fatty acids from pollen are needed to fuel the energetic and immune pathways responding to stress from parasitization and infection. Bees deprived of dietary lipids or that have reduced lipid metabolism exhibit more rapid maturation from nursing to foraging (Toth et al., 2005). This accelerated maturation has been associated with a general stress response that can lead to unbalanced colony demographic structures, and ultimately colony collapse (Barron, 2015). However, others have proposed that lipids can be harmful to bees in higher quantities: in bumble bees, a higher dietary intake of lipids is associated with increased mortality and reduced feeding (Ruedenauer et al., 2020). Overall, lipids seem to be an important macronutrient in bee diets, but the requirements for lipids, or the type of lipid, likely vary with context or bee species.

The role of dietary lipids and lipid metabolic processes in responding to and potentially increasing resilience to insecticide stress has not been well studied. Lipid metabolic processes seem to be generally regulated by insecticide exposure in insects, particularly in the case of insecticides that target acetylcholine signaling pathways, such as neonicotinoids and organophosphates (https://irac-online.org/modes-of-action/, accessed 2020). Altered lipid metabolism in bees exposed to neonicotinoid insecticides may be due to an increased energetic cost of detoxification (Derecka et al., 2013). Additionally, bees exposed to nicotine, which also targets acetylcholine signaling pathways, show an increase in lipid metabolism and an abundance of lipid metabolites (Rand et al., 2015). Lipid storage is also influenced by the presence of excess reactive oxygen species (ROS), which are often generated by insecticide stress in insects (Lukaszewicz-Hussain, 2010). Martelli et al. (2020) demonstrated that low doses of a neonicotinoid insecticide in *Drosophila melanogaster* induced ROS, triggering an accumulation of lipids in droplet form in the fat bodies (an internal reaction to prevent excess peroxidation), and altered metabolic processes, which the authors propose to be a consistent response across many insect species. However, it remains to be determined whether these changes in lipid metabolic processes increase resilience to insecticides or are a downstream negative effect of insecticide exposure. There is some evidence that providing bees with dietary lipids may improve their resilience to insecticides. Honey bees fed pollen and sucrose had higher survival when exposed to an organophosphate than bees that were fed sucrose alone (Schmehl et al., 2014). However, when honey bees were provided with a non-pollen source of protein, survival was not improved, suggesting that other elements in pollen, such as lipids, are important for organophosphate tolerance (Schmehl et al., 2014).

In addition to lipids, insects also balance protein intake for optimal development and reproduction, with diets high in protein leading to shorter lifespans (Lee et al., 2008; Le Couteur et al., 2016). Too much protein, or the wrong source of protein, has also been found to be harmful to honey bees (Pirk et al., 2010). To maintain optimal functioning, essential amino acids (EAAs) are balanced with carbohydrates depending on age, with workers gradually needing a lower amount of EAAs as they transition from nurses to foragers (Paoli et al., 2014). Total protein level and EAA composition have also been found to be important for immune response, brood production and overwintering survival in honey bees (Tritschler et al., 2017; Schmickl and Crailsheim, 2001; DeGrandi-Hoffman et al., 2016). Interestingly, protein supplements for honey bees currently on the market have not been found to increase brood production, increase the number of adult bees or decrease *Nosema* infection (Mortensen et al., 2019). Therefore, while it is clear that dietary protein is important for honey bees, it is likely that bees have context-specific needs for specific EAAs, dietary protein concentration and P:L ratios.

Here, we tested the effect of dietary P:L ratio on honey bee resilience to an organophosphate insecticide. Throughout this paper, nutritional P:L ratios (1:1, 5:1, etc.) are used to refer to diets with different parts of protein per part of lipid. P:L ratios are not dependent on the concentration of protein or lipid in a diet, but represent the relationship between amounts of macronutrients. This macronutrient ratio concept is used in nutritional research to understand a species' broad nutritional requirements, which is needed before narrowing down more specific micronutrient needs (Raubenheimer et al., 2009). We refrained from testing tolerance or resistance directly because of the difficulty of supporting such a hypothesis at this stage of our understanding of the interactions between nutrition and insecticide stress. We instead chose to test survival rate, which we are equating with the term 'resilience' in this context. We focused on the organophosphate chlorpyrifos, because it was found consistently and at high levels in honey bee workers, wax and pollen in a previous national survey, and has been used previously in dietary stress studies (Mullin et al., 2010; Schmehl et al., 2014). Chlorpyrifos inhibits acetylcholinesterase and causes an accumulation of acetylcholine in neuronal synapses, leading to paralysis and death (Fukuto, 1990). The increased lipid metabolism found when bees were exposed to other acetylcholinesterase inhibitors may also occur under chlorpyrifos exposure, and, if so, increased dietary lipids may lead to increased survival of exposed bees. Therefore, we hypothesized that pollen diets with low P:L ratios would lead to increased survival in honey bees exposed to organophosphates. We also hypothesized that the ideal range of P:L ratio could be reached by adding protein (casein powder) or lipid (canola oil) to pollen.

To test our hypotheses, we fed age-matched honey bees pollen diets of different P:L ratios by altering a base pollen with a ratio of ∼5.5:1 and simulated chronic insecticide exposure by feeding bees chlorpyrifos dissolved in sucrose. Survival was recorded over a 12 day period to determine the best diet for honey bees exposed to chlorpyrifos. These trials will hereafter be referred to as 'altered diet trials'. We also conducted the same survival trials with multifloral pollen diets of naturally different P:L ratios to determine whether results would be consistent in a more realistic scenario, hereafter referred to as 'natural diet trials'. Data from these bioassays was used to fit regression models to predict the best diet for improving survival of honey bees exposed to organophosphates. In addition, candidate genes were evaluated for differential expression between groups of honey bees fed altered diets to understand the underlying effects of each treatment group.

MATERIALS AND METHODS

Honey bees

Honey bees, *Apis mellifera* Linnaeus 1758, were sourced from 10 different colonies from Pennsylvania State University-managed apiaries in State College, PA, USA, and surrounding areas. Colonies were either from Italian or Carniolan lineages. Queens inseminated by a single drone were sourced from Honey Bee Insemination Services (Coupeville, WA, USA) or were instrumentally

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inseminated by Grozinger lab personnel (K. Anton). Naturally mated queens were reared from local stock. Colonies were inspected each week to prevent queen supersedure. Colonies were treated for mites with formic acid when thresholds were above 3 individuals per ethanol shake.

Pollen diets

For the altered diet trials, a base honey bee-collected pollen from Arizona deserts (CC Pollen Co., Phoenix, AZ, USA) was altered to have different ratios by either adding protein (casein powder, Sigma Aldrich) or lipid (canola oil) as in Vaudo et al. (2016). See Table S1 for all diet recipes. Canola oil was used to increase lipid content because it has a relatively low omega-6 to omega-3 ratio, which has been shown to be optimal for honey bee health (Arien et al., 2015). Prior to mixing in additives, pollen was irradiated to inactivate any viruses present, and though we did not test pollen for pesticides, this brand has been used previously as a no-pesticide control diet (McArt et al., 2017). We then ground pollen in a standard coffee grinder and mixed in casein powder or canola oil before adding sucrose to make a pollen paste.

For the natural diet trials, pollen was collected from Pennsylvania State University-managed apiaries by using pollen traps at the entrances of honey bee colonies. This pollen represented a blend of spring and summer flowering plant species from the region. Pollen was collected from March to August of 2020 and screened for P:L ratio by using a modified Bradford and SPV assay as in Vaudo et al. (2016). Values ranged from 1.3:1 to 13.3:1, and diets used (Table 1) were chosen to give the largest spread between ratios.

Altered and natural diet insecticide bioassays

For both the altered and natural diet insecticide bioassays, 1 day old honey bees were sorted into cages created from plastic cups and Petri dishes. Feeders created from 1.5 ml centrifuge tubes were used to provide 50% sucrose solution to bees throughout the experiment. On days 1–4, all bees were fed unadulterated sucrose, and on days 5–12 all treatments except the control were fed chlorpyrifos sucrose solution. The chlorpyrifos (Sigma Aldrich PESTANAL® analytical standard, St Louis, MO, USA) dose was chosen based on preliminary testing to determine the approximate median lethal dose (LD_{50}) when honey bees were fed sucrose only (data not shown). In 2019, the dose used for the altered diet experiments was 5.8 ppm, and in 2020 the dose used for the natural diet experiments was 11.6 ppm. Differences in concentration by year were due to different LD_{50} values for each set of colonies. Although using different doses could impact the interpretation of results, we chose to use the equivalent LD_{50} with each set of colonies to maintain the same level of stress in each set of experiments. Honey bees also received pollen diets mixed with sucrose for the 12 day mortality monitoring period.

Table 1. List of diet protein and lipid concentrations for each set of experiments

P:L ratio	Protein concentration (μg mg^{-1})	Lipid concentration (μg mg^{-1})
Altered diet		
1.5:1	155.71	102.59
5.5:1	155.71	27.86
8.7:1	243.84	27.86
19.7:1	549.24	27.86
Natural diet		
1.27:1	44.02	34.53
3.17:1	183.07	57.58
4.77:1	234.77	49.19
6.63:1	158.44	23.88
8.41:1	301.16	35.78
13.32:1	184.00	13.80

P:L ratio, protein to lipid ratio.

In the altered diet trials, diets had P:L ratios of 19.7:1, 8.7:1, 1.5:1, unaltered pollen (5.5:1), sucrose only and a control group (5.5:1, unaltered) that was not exposed to insecticide. Mortality of each cage was recorded daily and dead bees were removed from cages. The experiment ran for 12 days in total. Five trials were conducted for this experiment, with different source colonies of honey bees used for each trial. Two of the colonies were naturally mated, while the other three were from single-drone-inseminated queens. There were 10 bees per cage and 6 cages per treatment group, with a total of 2160 bees observed.

In the natural diet trials, diets had P:L ratios of 1.3:1, 3.2:1, 4.8:1, 6.6:1, 8.4:1, 13.3:1 and a control group (8.4:1) that was not exposed to insecticides. Mortality was again recorded daily and dead bees were removed from cages. Three trials were conducted, and bees were sourced from a different single-drone-inseminated colony each time. There were 10 bees per cage and 4 cages per treatment group, with a total of 1120 bees observed. Natural diet trials were only conducted for 11 days because of restrictions related to Covid-19, and in trial 3 there were no mortality data for day 10.

Effects of diet on pollen consumption and mortality

The amount of pollen eaten by honey bees in each treatment group and the level of mortality in the absence of insecticide treatment were assessed. This experiment was conducted separately from insecticide bioassays. Diets were altered to have ratios of 19.4:1, 10.8:1 or 2.6:1 from a base pollen of 8.4:1. An additional control diet was generated, in which the base pollen was modified by the addition of protein and lipid to keep the same ratio (8.4:1) at an increased concentration. Pollen consumption was measured in two trials from the same single-drone-inseminated colony, with and without insecticide exposure. Pollen dishes were weighed daily to measure consumption and pollen was replaced every 2 days. Each day, the amount of pollen consumed was divided by the number of bees alive that day to determine how much was eaten per individual. At the end of each trial, the amount of pollen consumed over the 12 day period was averaged and compared between treatment groups. Trials with and without insecticide exposure were analyzed separately. Minimal mortality occurred when bees were fed different diets without the presence of insecticides and there were no significant differences between survival of treatment groups (≤8.3% death in all treatments over a 12 day period; see Fig. S1). When not exposed to insecticide, bees did not consume significantly different amounts of each diet (P=0.18, F=1.59; Fig. S2); similar results were observed when bees were fed 11.6 ppm chlorpyrifos dissolved in sucrose (P=0.60, F=0.73; Fig. S3).

Insecticide bioassay data analysis

All data analysis was completed in R (version 4.0.2; http://www.R-project.org/). Kaplan–Meier survival analysis was carried out by using the Survival package (Therneau et al., 2020). Five trials were conducted in the summer of 2019 for the altered diet bioassay, and three trials were conducted in the summer of 2020 for the natural diet bioassay. Because two of the five altered diet trials did not follow the typical trend of dietary rankings, a Cox proportional hazards model was used (see Figs S4 and S5) to determine which colonies had a higher probability of perishing during the study (Therneau and Grambsch, 2000). A forest plot was used to visualize this model.

A linear regression model was used for altered and natural diet trials to evaluate whether protein concentration, lipid concentration or overall ratio explained the changes in mean survival time between treatments. The mean survival time for each treatment group (when data from all trials were combined) was used to fit both models and create scatter plots with lines of best fit. The average for each treatment group across trials was used owing to variation in colony response. Models for protein concentration, lipid concentration and overall ratio were compared and the model with the highest adjusted R^2 value was selected for both altered and natural diets.

RNA extraction and qPCR

To begin to evaluate why different diets led to different health outcomes when bees were exposed to insecticide, we examined the expression of three detoxification genes (*CYP9Q3*, *CYP9S1* and *CYP305D1*), two immune genes (*Toll* and *Defensin-1*) and two genes whose expression has been shown to vary with diet (*Vg* and *SODH-2*) in previous studies, as well as levels of deformed wing virus (DWV) (see Table 2 for primers) (Schmehl et al., 2014; Annoscia et al., 2017). These experiments were conducted using honey bees fed altered diets, and bees were collected after 5 days, prior to exposure to chlorpyrifos. A total of 30 honey bees from each of the altered diet treatments (1.5:1, 5.5:1, 8.7:1, 19.7:1 and sucrose only) from six colonies were analyzed. Two housekeeping genes, *eIF3-S8* and *Rp49*, were also used to normalize the expression of candidate genes, as per Grozinger et al. (2003).

After collection with liquid nitrogen, bees were stored at −80°C until RNA extraction. RNA was extracted from whole bee abdomens using the RNeasy mini-kit (Qiagen, Hilden, Germany). cDNA synthesis and PCR were conducted similarly to previous studies (Ray et al., 2020). We annealed cDNA (Applied Biosystems, Foster City, CA, USA) using a Mastercycler nexus eco (Eppendorf, Hamburg, Germany) with the following conditions: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min, and then held at 4°C. qPCR was conducted using a 7900 HT Fast real time qPCR machine (Applied Biosystems) under the following conditions: 50°C for 2 min, 95°C for 10 min, then cycle 40×95°C for 15 s and 59°C for 1 min; a dissociation curve was used to verify the presence of a single product.

qPCR data analysis

Raw C_T data reads were averaged across wells (two PCR wells per sample) and transformed by subtracting the average expression of control genes, *Rp49* and *eIf3-S8* (Grozinger and Robinson, 2007). We then used the $2^{-\Delta\Delta C_T}$ method for relative quantification (Deng et al., 2020). Factorial ANOVA were employed to determine whether a colony by treatment effect was present. One way ANOVA were completed for each gene, followed by Tukey's multiple comparison test to view any significant differences between dietary treatments. *CYP9Q3*, *CYP305D1*, *CYP9S1* and *Defensin-1* expression were analyzed by combining bees from all six colonies as there was not an interaction between colony and treatment. However, *Vg*, *SODH-2* and *Toll* expression and DWV levels did show an interaction between colony and treatment, so colonies were analyzed separately. Graphs are displayed as mean fold-change data and organized by colony and treatment group.

RESULTS

Altered diet insecticide bioassay

There was no difference in mortality (Fig. S1) or the amount of pollen consumed (Fig. S2) in bees fed diets with different P:L ratios in the absence of chlorpyrifos. Honey bees fed low P:L ratio diets (1.5:1 and 5.5:1) lived longer than those fed high P:L ratio diets (8.7:1 and 19.7:1) when subjected to chronic insecticide exposure (Fig. 1). Bees fed the lipid-rich diet with a ratio of 1.5:1 (n=290, 10.72±0.11 days) did not live significantly longer than those fed the unaltered diet of 5.5:1 (n=290, 10.89±0.11 days, P=0.2670), but did have significantly higher survival than bees on the protein-rich 8.7:1 diet (n=301, 10.60±0.10 days, P=0.0312) and 19.7:1 diet (n=300, 10.24±0.12 days, P<0.0001). Bees fed the 5.5:1 diet also lived significantly longer than those fed the 8.7:1 diet (P=0.0011) and the 19.7:1 diet (P<0.0001). Bees fed the 8.7:1 diet lived significantly longer than those fed the 19.7:1 diet (P=0.0312). The control group that was not fed insecticides lived significantly longer than all other treatments (n=300, 11.84±0.06 days, P<0.0001), and bees from all treatments lived significantly longer than those fed only sucrose and insecticide (n=301, 9.12±0.10 days, P<0.0001). See Table 3 for a list of all comparisons between treatments.

Natural diet insecticide bioassay

As in altered diet trials, honey bees fed lower P:L ratio diets derived from natural pollen blends lived longer than those fed higher P:L ratio diets when subjected to chronic insecticide exposure, though bees fed mid-range P:L ratio diets lived the same amount of time (Fig. 2). Bees fed the 1.3:1 diet (n=120, 9.40±0.19 days) and those fed the 3.2:1 diet (n=121, 9.38±0.20 days) lived significantly longer than those fed the 13.3:1 diet (n=121, 9±0.17 days; P=0.0065 and P=0.0498, respectively). Survival of bees fed the 4.8:1 diet (n=120, 9.72±0.17 days), 6.6:1 diet (n=119, 9.56±0.18 days) and 8.4:1 diet (n=120, 9.38±0.18 days) did not significantly differ from each other and these bees did not live significantly longer than those fed the 13.3:1 diet. The control group that was not fed insecticides lived significantly longer than all other treatments (n=120, 10.95±0.05 days, P<0.0001). See Table 4 for a list of all comparisons between treatments.

Fitting regression models to predict ideal diet

For bees fed altered pollen diets and insecticide-laden sucrose, protein concentration was found to be the best predictor of mean

Table 2. List of primers used for qPCR

Target sequence	Forward primer 5′–3′	Reverse primer 5′–3′	Reference
Rp49	AAGTTCATTCGTCACCAGAG	CTTCCAGTTCCTTGACATTATG	Grozinger et al., 2003
eIF3-S8	TGAGTGTCTGCTATGGATTGCAA	TCGCGGCTCGTGGTAAA	Grozinger and Robinson, 2007
Vg	TTGACCAAGACAAGCGGAACT	AAGGTTCGAATTAACGATGAAAGC	Aronstein et al., 2010
SODH-2	CAGTGCATGGTAGCCTGAGA	ACAGTGCTCCTTCAGCCAAT	Schmehl et al., 2014
CYP9Q3	GTTCCGGGAAAATGACTAC	GGTCAAAATGGTGGTGAC	Schmehl et al., 2014
CYP9S1	CTAATTTTCGCGTTCCCAAA	CTCCCGTTACGTTTGTCGAT	Schmehl et al., 2014
CYP305D1	TCGATCTTTTTCTCGCTGGT	TTGCTTTGTCCTCCATGTTG	Schmehl et al., 2014
Defensin-1	GTTGAGGATGAATTCGAGCC	TTAACCGAAACGTTTGTCCC	Aronstein et al., 2010
Toll	TAGAGTGGCGCATTGTCAAG	ATCGCAATTTGTCCCAAAAC	Evans et al., 2006
DWV	GCGCTTAGTGGAGGAAATGAA	GCACCTACGCGATGTAAATCTG	Prisco et al., 2016

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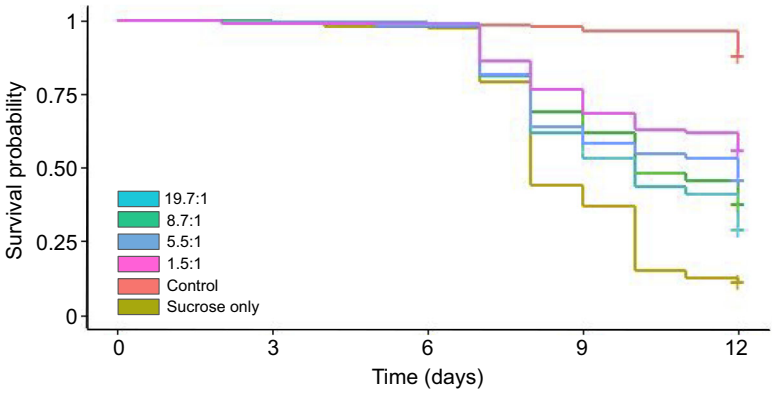

Fig. 1. Pollen diets with altered protein to lipid (P:L) ratios influence survival rate of bees treated with the organophosphate chlorpyrifos. Bees from six different colonies (n=2160 individuals) were fed chlorpyrifos from days 5 to 12 (except the control) and bee diet was altered to give different P:L ratios (1.5:1, 5.5:1, 8.7:1 and 19.7:1). Differences in survival between dietary treatment groups were examined using Kaplan–Meier survival analysis with a log-rank test and Bonferroni correction. This experiment was completed separately for each colony. Mean survival times and significant differences for each dietary treatment group can be found in Table 3.

survival time in a linear regression model ($F_{1,2}$=21.94, adjusted R^2=0.87, P=0.04), with the equation y=11−0.0014x (Fig. 3). This indicates that for every microgram of protein added per milligram of pollen, mean survival time will decrease by 0.0014 days. When extrapolating to full days, this means that for every milligram of protein added per gram of pollen, mean survival time decreases by 1.4 days. Ratio ($F_{1,2}$=7.84, adjusted R^2=0.69, P=0.10) and lipid concentration ($F_{1,2}$=0.14, adjusted R^2=−0.39, P=0.73) were not found to be significant predictors of mean survival time.

When considering natural pollen diets, P:L ratio was found to be the best predictor of mean survival time in a linear regression model ($F_{1,4}$=16.88, adjusted R^2=0.76, P=0.01), with the equation y=10.21−0.05x. We then modeled these data as a polynomial function to include a cut off point for the best ratio and make the model more biologically relevant. The new model also better explained our data when predicting mean survival time from P:L ratio ($F_{2,3}$=35.82, adjusted R^2=0.93, P=0.008), with the equation y=9.88−0.49x_1−0.21x_2 (Fig. 4). With our new model, we then predicted that the P:L ratio 2.8:1 would be the best performing diet for bees experiencing stress from organophosphate exposure, with a mean survival time of 10.04 days.

Differential gene expression of candidate genes and DWV

When analyzing gene expression, we found that three genes and a virus (*Vg*, *Toll*, *SODH-2* and DWV) showed significant colony by treatment group effects. For these genes and DWV, each colony was analyzed separately for significant differences between treatments within each colony. Statistical analysis for genes without significant colony by treatment effects (*CYP9Q3*, *CYP9S1*, *CYP305D1* and *Defensin-1*) was completed by combining data from all colonies. We graphically displayed all genes separated according to colony for clarity. Significant differences for genes with all colonies combined are detailed below.

Table 3. Mean survival time for each treatment group in altered diet trials with *P*-values for comparison between groups

Diet	Mean survival time (days)	*P*-value for each diet group			
		1.5:1	5.5:1	8.7:1	19.7:1
1.5:1	10.72	–	–	–	–
5.5:1	10.89	0.27	–	–	–
8.7:1	10.60	**0.03**	**0.00085**	–	–
19.7:1	10.24	**2.3e−06**	**5.9e−09**	**0.01**	–
Sucrose	9.12	**2e−16**	**2e−16**	**2e−16**	**1.7e−12**
Control	11.84	**2e−16**	**2e−16**	**2e−16**	**2e−16**

See Fig. 1 for survival curves. Bold indicates significance.

Bees fed diets rich in lipids had increased expression of detoxification genes and *Defensin-1* relative to those fed protein-rich diets or those fed sucrose only (without pollen) (Fig. 5). Bees fed the 5.5:1 diet significantly upregulated *CYP9Q3* (F=2.6, d.f.=4, P=0.02) and *CYP9S1* (F=4.06, d.f.=4, P=0.0073) compared with those fed only sucrose. *CYP9S1* was also significantly upregulated in bees fed the 1.5:1 diet compared with those fed sucrose alone (F=4.06, d.f.=4, P=0.01). There were no significant differences between treatments for expression of *CYP305D1*. *Definsin-1* was upregulated significantly more in bees fed the 1.5:1 diet than in those fed sucrose alone (F=4.31, d.f.=4, P=0.0006).

For *Vg* and *SODH-2*, bees fed any pollen diet had significantly higher expression levels than bees fed sucrose alone for all colonies (Fig. 6). In addition, in colony 6, bees fed diets rich in protein had lower expression of *Vg* and *SODH-2* compared with bees fed less protein. In this colony, bees fed the 19.7:1 diet had significantly lower expression of *Vg* than those fed the 1.5:1 diet (F=17.68, d.f.=4, P=0.0008), the 5.5:1 (control) diet (F=17.68, d.f.=4, P=0.0061) and the 8.7:1 diet (F=17.68, d.f.=4, P=0.0017); these bees also had significantly lower expression levels of *SODH-2* than those fed the 1.5:1 diet (F=12.29, d.f.=4, P=0.0021), the 5.5:1 diet (F=12.29, d.f.=4, P=0.01) and the 8.7:1 diet (F=12.29, d.f.=4, P=0.0019). There were no significant differences in expression of the *Toll* gene aside from one outlying colony; bees from colony 4 fed the 19.7:1 diet showed significant upregulation of *Toll* compared with those fed only sucrose (F=3.9, d.f.=4, P=0.02).

DWV levels were the same for all treatments and all colonies except for a few outlying groups. In colony 1, bees fed the 8.7:1 diet had significantly higher levels of virus than all other treatments (F=24.21, d.f.=4, P<0.0001), and bees fed the 5.5:1 diet also had significantly higher levels of virus compared with those fed only sucrose (F=24.21, d.f.=4, P=0.0479). In colony 2, bees fed the 8.7:1 diet had significantly higher levels of virus compared with those fed only sucrose (F=2.97, d.f.=4, P=0.04). Finally, bees from colony 5 fed the 8.7:1 diet had significantly higher levels of virus compared with those fed the 1.5:1 diet (F=4.45, d.f.=3, P=0.01).

DISCUSSION

Our results support our hypothesis that bees fed diets with higher levels of lipids and lower amounts of protein (lower P:L ratios) are more resilient to insecticide stress. Our model fit from the natural pollen diet survival data predicts that the best pollen P:L ratio to improve survival of bees exposed to insecticide stress is ∼2.8:1. While previous studies in other insects suggested a link between insecticides that interfered with acetylcholine signaling and lipid metabolism, this is the first study to show that low P:L ratios improve resilience to insecticides. Interestingly, diets with increased

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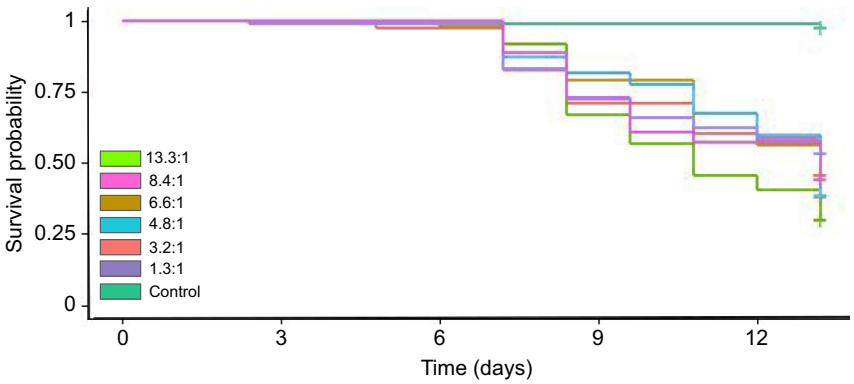

Fig. 2. Natural pollen diets with variable P:L ratios influence survival rate of bees treated with chlorpyrifos. Bees from three different colonies (*n*=1120 individuals) were fed chlorpyrifos from days 5 to 11 (except the control) and pollen diets of naturally different P:L ratios (1.3:1, 3.2:1, 4.8:1, 6.6:1, 8.4:1 and 13.3:1). Differences in survival between dietary treatment groups were examined using Kaplan–Meier survival analysis with a log-rank test and Bonferroni correction. This experiment was completed separately for each colony. Mean survival times and significant differences for each dietary treatment group can be found in Table 4.

levels of protein were associated with reduced resilience. Our model indicates that for every milligram of protein added per gram of pollen, mean survival time decreases by 1.4 days, although we were unable to determine the ideal cut off point for minimum protein concentration because of the range of diets we tested. Overall, these results highlight the importance of lipids in bees' diets, and the importance of balancing P:L ratios rather than simply increasing concentrations of macronutrients.

However, our data do not support our hypothesis that insecticide mortality could be mitigated by altering diets with casein powder or canola oil, as survival of bees fed the unaltered (5.5:1) diet was not significantly different from that of bees fed the diet with canola oil added (1.5:1), and adding casein powder lowered survival. Adding canola oil may improve survival if the base pollen has a higher P:L ratio, but further research is needed to evaluate this. Our consumption data also confirm that added casein powder and canola oil do not directly increase mortality, as total mortality was ∼8% in the absence of insecticides across these diets. Future studies are needed to understand how pollen should be altered to reach the best ratio for honey bees in colonies in the field.

It is surprising that adding crude dietary protein did not increase honey bee resilience to insecticide stress, as dietary protein has been found to be an important factor contributing to overwintering survival, immune response and brood production (DeGrandi-Hoffman et al., 2016; Tritschler et al., 2017; Schmickl and Crailsheim, 2001). Bumble bees readily consumed diets containing casein-modified pollen in a previous study (Vaudo et al., 2016). Bumble bees were also more attracted to diets that were modified to match pollen ratios in their ideal range (5–10:1) (Vaudo et al., 2016). This suggests that if pollen were at a very low starting P:L ratio in our study, casein could be used to raise the pollen P:L ratio to the ideal intake range. However, additional studies will still be needed to understand how diets can best be modified for bees in field settings.

Lower P:L ratios may improve resilience as a result of improved detoxification of acetylcholinesterase antagonists by increased lipid metabolism, which could also provide a source of energy to fuel the stress response, or mitigate the effects of excess ROS (Rand et al., 2015; Derecka et al., 2013; Martelli et al., 2020). We found that honey bees fed lower P:L ratio diets with higher amounts of lipids had longer mean survival times in both the altered diet and natural diet experiments, which is contrary to evidence from a previous study indicating that a high amount of lipids leads to higher mortality in bumble bees, and that bumble bees use lipid concentration as a cue for overall diet quality to maximize fitness (Ruedenauer et al., 2020). This may not be the case with honey bees because of differences in life history traits, such as bee body size, physiology and colony life cycle. Honey bees could also require higher amounts of lipids when exposed to stressors, as insects have been shown to need different levels of nutrients for different contexts (Boggs, 2009). Finally, experimental conditions could be responsible for changes in macronutrient requirements.

While these results clearly demonstrate an association between dietary pollen P:L ratios and insecticide resilience, further work is needed to determine whether and how these results can be translated to honey bee colonies in the field. There are many factors which might influence bees' nutritional requirements and response to insecticide exposure. For example, in normal colonies, honey bee workers are exposed to pheromones produced by the queen, which influence many aspects of worker physiology and behavior (Kocher and Grozinger, 2011). Exposure to queen mandibular pheromone (QMP), for example, slows the transition from nursing to foraging behavior, increases lipid storage levels in the fat bodies and decreases the risk of starvation (Fischer and Grozinger, 2008). Nurse bees consume pollen, while foragers do not (Wright et al., 2018). Exposing bees to QMP may increase the consumption of pollen and the duration of pollen consumption as bees exposed to QMP stay in the nursing state longer and thus can continue to digest proteins for longer (Paoli et al., 2014). Bees in our study were also not exposed to brood pheromone, which could trigger bees to consume particular macronutrient levels to feed developing brood,

Table 4. Mean survival time (days) for each treatment group in natural diet trials with *P*-values for comparison between groups

		P-value for each diet group					
Diet	Mean survival time (days)	1.3:1	3.2:1	4.8:1	6.6:1	8.4:1	13.3:1
1.3:1	9.40	–	–	–	–	–	–
3.2:1	9.95	0.51	–	–	–	–	–
4.8:1	10.04	0.27	0.74	–	–	–	–
6.6:1	10	0.20	0.59	0.74	–	–	–
8.4:1	9.86	0.46	0.88	0.84	0.74	–	–
13.3:1	9.41	**0.0065**	**0.04**	0.06	**0.0065**	**0.06**	–
Control	10.95	**2.4e−15**	**2e−16**	**2e−16**	**2e−16**	**2e−16**	**2e−16**

See Fig. 2 for survival curves. Bold indicates significance.

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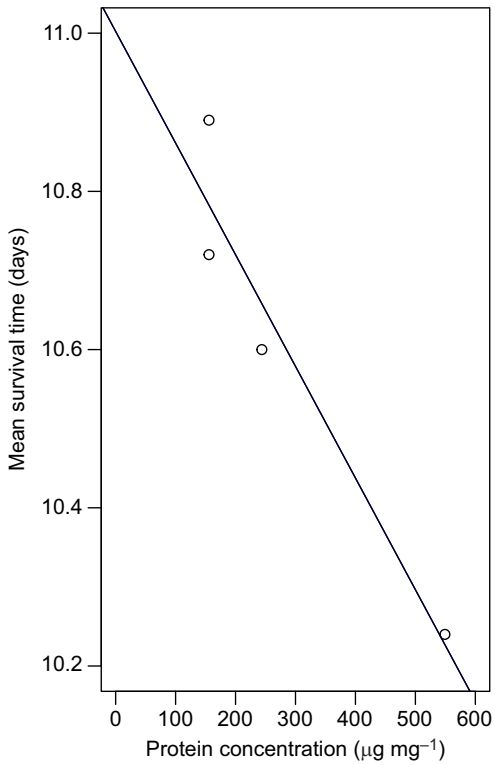

Fig. 3. Increased dietary protein is associated with lower mean survival times. A scatter plot of protein concentration and mean survival time for bees fed altered pollen diet with a regression line of best fit shows that mean survival decreases by 1.4 days as protein concentration increases by 1 mg per gram of pollen (y=11−0.0014x, $F_{1,2}$=21.94, adjusted R^2=0.87, P=0.04). Mean survival times were averaged across 5 trials, each conducted with separate colonies (n=2160 individual bees).

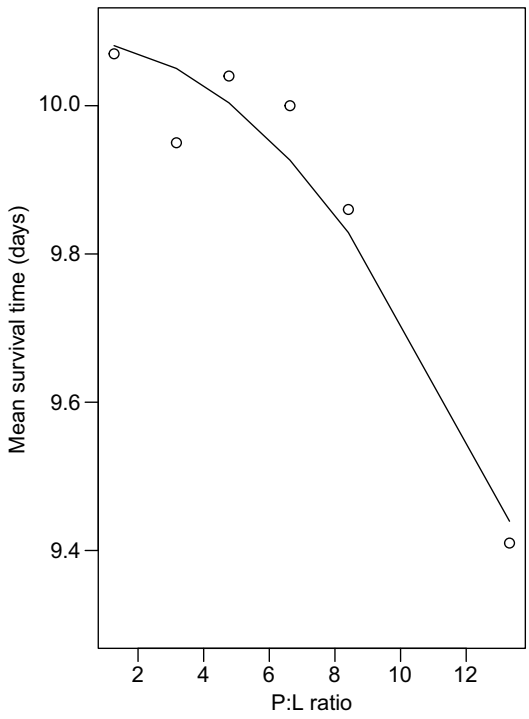

Fig. 4. Lower P:L ratios are associated with higher mean survival times. A scatter plot of pollen P:L ratio and mean survival time for bees fed natural pollen diet with a polynomial regression line of best fit, showing that mean survival time decreases as P:L ratio increases. Mean survival times were averaged across three trials, each conducted with separate colonies (n=1120 individual bees). This model predicts that the best-performing P:L ratio for bees exposed to organophosphates is 2.8:1 with a mean survival time of 10.04 days (y=9.88−0.49x_1−0.21x_2, $F_{2,3}$=35.82, adjusted R^2=0.93, P=0.008).

as brood pheromone stimulates development of the hypopharyngeal glands (Le Conte et al., 2001). It would be beneficial for future studies to address these additional variables in nutritional choice or requirements. It is also necessary to understand whether the best performing ratios in our study are also the best performing ratios when bees are exposed to other stressors, and whether low P:L ratios are the preferred intake target for nurse bees that are not exposed to stressors. We still have much to learn about bee nutrition, and this area is rich in possibilities for future studies.

Intriguingly, honey bees that ate mid-range (4.8–8.1:1) diets from the natural diet bioassays did not perform significantly differently from bees fed the highest (13.3:1) and lowest (1.3:1 and 3.2:1) diets, and adding lipids to altered pollen diets did not increase resilience. This raises the intriguing question of why there is so much stability in the mid-range diets. Our data did not show differences in consumption amounts or mortality when bees were fed altered diets at a range of 2.6–19.4:1 without insecticides, so the consequences of unregulated diets are probably not apparent in the absence of additional stressors. Furthermore, our pollen collection data showed that field colonies collected pools of pollen ranging from 1.3:1 to 13:1 P:L ratio throughout the summer. This is consistent with other studies that showed large collection ranges in protein concentration (11.7–31.3%) and P:L ratio (~1:1–25:1) (Quinlan, 2020; Jones, 2020). Honey bees may have evolved to tolerate a wide range of P:L ratios in the absence of stress because of the wide range in P:L ratios of available pollen forage. It is also possible that collected P:L ratios average out over a season. Additional nutritional variables that differ across pollen types and could 'buffer' mid-range diets are omega-3 and omega-6 fatty acids, levels of essential amino acids and concentrations of micronutrients (Arien et al., 2015; Hendriksma et al., 2019; Bonoan et al., 2018). Though the same base multifloral pollen was used for the altered diet experiments, the natural diets were multifloral compositions of different botanical origin that were collected throughout the spring and summer of 2020. Without identifying pollen via microscopy or meta-barcoding, it is not possible to know what bees in our natural diet experiments were consuming. Pollen has been found to contain many plant secondary compounds that can be harmful to pollinators, which could have impacted bee survival in our study (Rivest and Forrest, 2020). However, results between our altered and natural diet survival experiments were fairly consistent, with survival in bees fed the varying natural diets held to a less ratio-strict standard. This increases our confidence that a toxic secondary compound from one of the diets used did not drastically impact our results. In future studies, it may be beneficial to use pollen from single plant species of different ratios to truly untangle whether differences in survival are due to P:L ratio or plant origin.

Data from the qPCR part of our study revealed how pollen diets and different P:L ratios impacted bee physiology. *Vg* and *SODH-2* genes were regulated by the presence of any pollen diet compared with sucrose alone, and were not affected by different P:L ratios. Lower expression in bees fed only sucrose is indicative of poor diet, as expression of *Vg*, which is responsible for lipid transport and an egg-yolk precursor in insects, is typically upregulated with rich diets that are better for honey bee health (Havukainen et al., 2013; Azzouz-Olden et al., 2018). *Vg* levels have also been found to be

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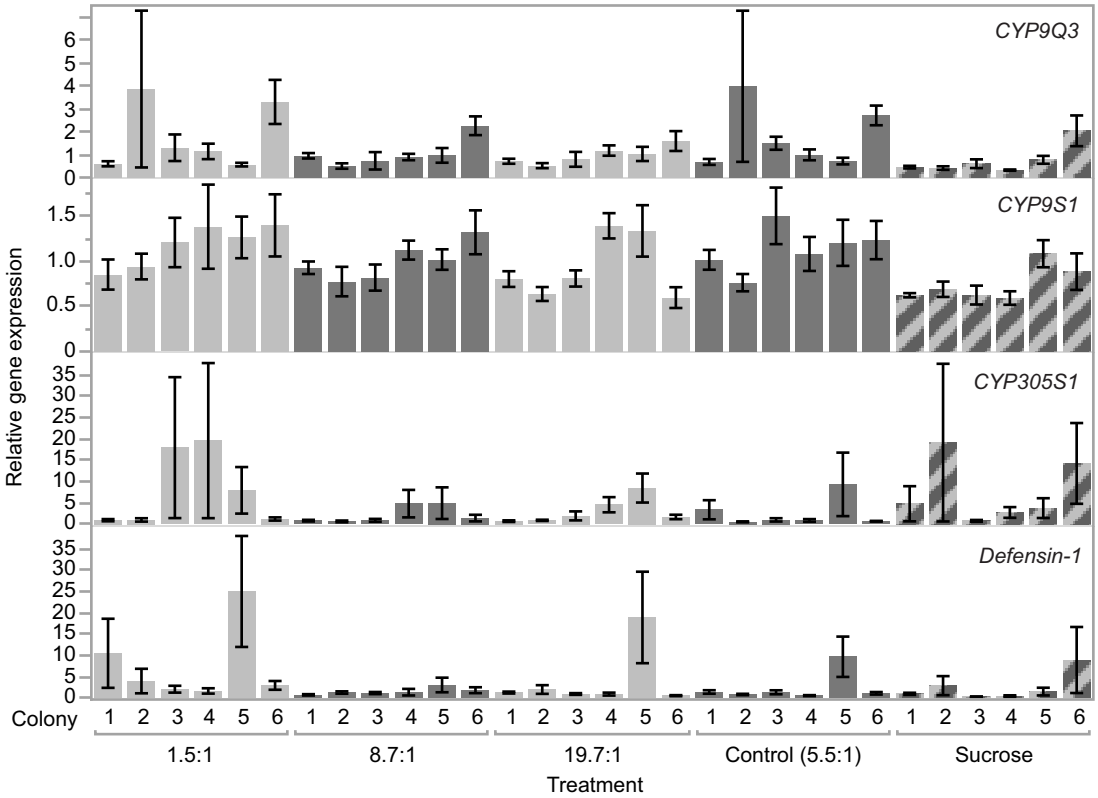

Fig. 5. Diets with lower P:L ratios are associated with upregulation of detoxification and immune genes. Bees fed diets rich in lipids showed increased expression of *CYP9Q3*, *CYP9S1* and *Defensin-1* relative to those fed protein-rich diets or no pollen. There were no significant differences in *CYP305D1* expression between treatments. Bees fed altered diets were collected on day 5 of feeding and were not exposed to insecticides. Colony gene expression data were combined as there was no treatment by colony effect for these genes. An ANOVA and Tukey's multiple comparison test were used to compare expression levels between treatment groups. See Materials and Methods for details (n=30 per treatment group).

positively correlated with oxidative stress tolerance in honey bees (Seehuus et al., 2006). *SODH-2*, a gene involved in antioxidant activity in response to pesticide and secondary metabolite stress, has also previously been shown to be upregulated when bees were fed honey or pollen versus sucrose alone (Johnson et al., 2012; Ament et al., 2011). A previous study also found significantly higher expression of *SODH-2* in bees fed pollen or a soy protein supplement that lacked a lipid component (Schmehl et al., 2014), which may indicate that this gene responds to the presence of protein in pollen. Expression of *CYP9Q3*, *CYP9S1* and *Defensin-1* was more sensitive to the particular P:L ratio and was generally upregulated when bees were fed diets richer in lipids (1.5:1 or 5.5:1). Upregulation, and therefore increased activity, of detoxification genes could be why bees that ate the 5.5:1 diet exhibited the longest mean survival times in the altered diet studies. DWV levels and *Toll* expression were statistically similar for all treatments and all colonies except a few outlying groups. We expected that DWV rankings for each colony would align with the Cox model rankings, but this was not the case. This suggests that there are other factors in colony health leading to differences in hazard ratio, such as larval nutrition, genetic variation and predisposition to different diets, or a lower baseline level of health from other diseases.

Although it is well established that there are negative outcomes for bees with induced nutritional stress, or a combination of nutritional stress and other stressors, little is known about the underlying molecular and physiological mechanisms (Grozinger and Zayed, 2020). Future research should examine the mechanisms by which lipids improve insecticide tolerance or resistance in honey bees, as well as how nutrition and metabolic processes influence responses to other abiotic stressors. Using dietary nutrition to improve resilience to insecticides is a valuable management strategy: although it is desirable to reduce insecticide use through integrated pest management approaches, this is not always feasible and thus using dietary nutrition to improve bee tolerance or resistance to insecticides could be a valuable management tool for growers and beekeepers. An understanding of these mechanisms and how they influence other levels of biological organization (including individual bee or colony-level behavior) could also be useful for the development of 'adverse outcome pathways' for evaluating the interaction between poor nutrition and insecticide stress (Ankley et al., 2010; Grozinger and Zayed, 2020; LaLone et al., 2017). The identification of these molecular mechanisms and associated adverse outcome pathways could facilitate studies of stressor impacts on bees, and the development of diets that can mitigate the impacts of these stressors. The development of adverse outcome pathways would also indicate which management and environmental conditions, such as land use and seasonal changes in pollen availability, will lead to different outcomes for bees.

To our knowledge, this is the first study to examine physiological responses of honey bees to altered and natural pollen diets with different P:L ratios, as well as to predict the best diet to improve honey bee resilience to insecticides using regression models. These results demonstrate the importance of lipids and P:L ratio in mitigating the effects of insecticide stressors. Our study lays the groundwork for future research to understand the molecular mechanisms by which diet influences physiological responses to

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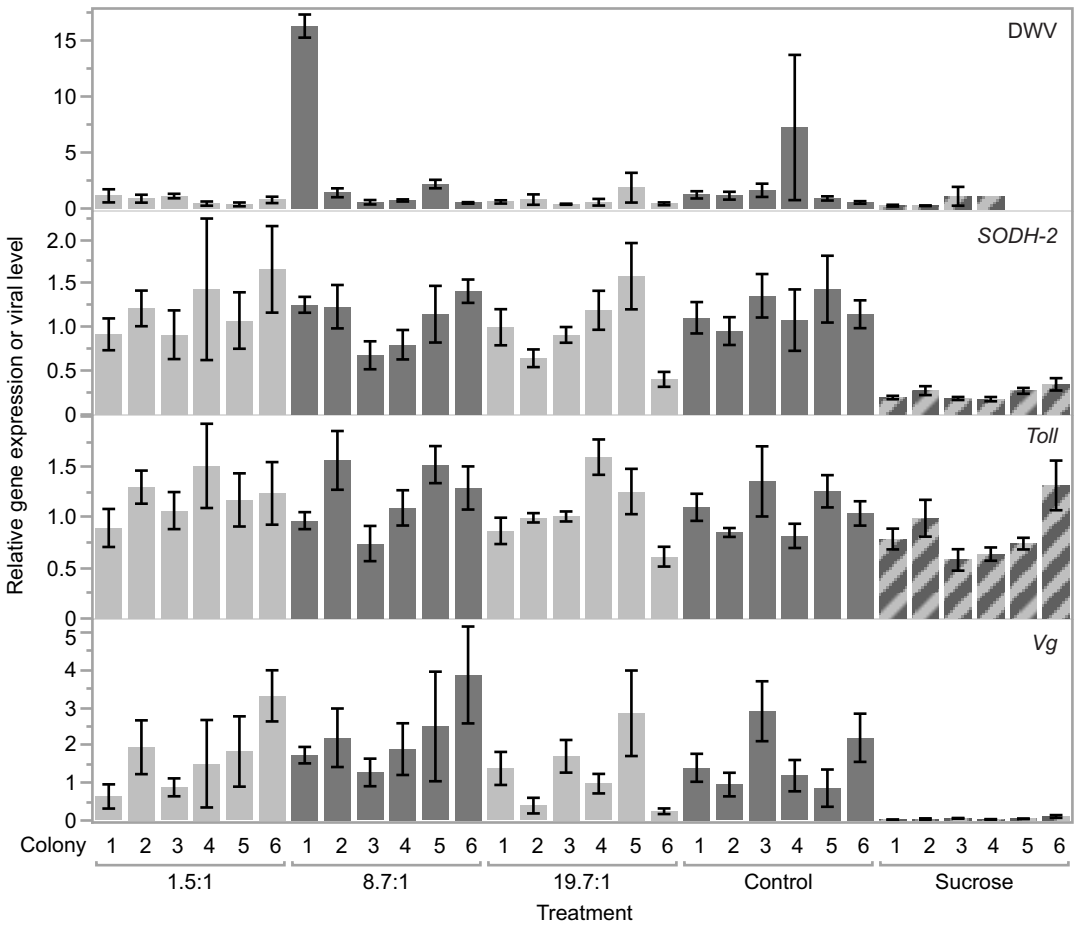

Fig. 6. P:L ratio is generally not associated with changes in DWV levels, or *Vg*, *SODH* and *Toll* expression. High pollen diets were associated with upregulation of *Vg* and *SODH-2* expression, but P:L ratio did not impact expression levels between dietary treatment groups. *Toll* expression and DWV (deformed wing virus) levels were generally not impacted by P:L ratio except for a few outlying colonies. Bees were collected on day 5 of feeding without exposure to insecticides. There was a treatment by colony effect for these genes, so statistical analysis of each colony was carried out separately. An ANOVA and Tukey's multiple comparison test were used to compare expression levels between treatment groups and colonies. See Materials and Methods for details (n=30 per treatment group).

diverse stressors in honey bees and other insects. Our nutritional models may also be useful for informing selection of plant species for the development of floral provisioning areas and the improvement of artificial pollen supplements that are given to honey bee colonies during the summer dearth, during travel to agricultural areas for pollination services, or over the winter when resources are scarce.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: C.G.; Methodology: M.K.C., C.G.; Validation: M.K.C.; Formal analysis: M.K.C.; Investigation: M.K.C.; Resources: M.K.C., C.G.; Writing - original draft: M.K.C.; Writing - review & editing: M.K.C., C.G.; Visualization: M.K.C.; Supervision: C.G.; Project administration: M.K.C., C.G.; Funding acquisition: M.K.C., C.G.

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Supplementary information
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