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ORIGINAL ARTICLE

# Reduced deformed wing virus of *Apis mellifera* L. nurses by high fat diets under laboratory conditions

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**Abstract**

Deformed wing virus (DWV) is one of the most widespread viral infections of European honey bee *Apis mellifera* L. worldwide. So far, this is the first study which tested the effect of different ratios of synthetic protein to fat (P : F) diets on the health of broodless nurse-aged honey bees in the laboratory. The aim of the current study was to determine the load of DWV in the whole body of *A. mellifera* that were fed different ratios of P : F diets (25 : 1, 10 : 1, 5 : 1, 1 : 1, 1 : 5, 1 : 10, 1 : 12.5 and 1 : 0 as a control). The methods involved feeding bees the tested diets for 10 days and then measuring the virus titre using qPCR technique. The results showed that DWV concentration decreased as the fat content of diets consumed increased. The copy number of viral genomes declined from $7.5 \times 10^5$ in the zero-fat diet (1 : 0) to $1.6 \times 10^2$ virus genomes in 1 : 12.5 (P : F). We can conclude that there is a positive relationship between fat diets and bee immunity and overall results suggest a connection between fat diet and bee health, indicating that colony losses can be reduced by providing a certain protein and fat supplemental feeding.

**Keywords**: deformed wing virus, fat diets, honey bee, nutrition, protein

## Introduction

Honey bee (*Apis mellifera* L.) nurse roles in beehives are largely responsible for preparing nutrients from pollen and distributing the nutritionally valuable protein produced by their hypopharyngeal glands to all hive members (Crailsheim 1991). Poor nutrition is one of the most significant current discussions in honey bee colony collapse disorder (CCD) phenomenon globally (Goulson *et al.* 2015). It has been shown that pollen nutrition affected bee lifespan (Di Pasquale *et al.* 2013), their immunocompetence (Alaux *et al.* 2010), and their resistance to the pathogen (DeGrandi-Hoffman *et al.* 2010; Alaux *et al.* 2011; Basualdo *et al.* 2013). On the other hand, Branchiccela *et al.* (2019) revealed that honey bee colonies restricted to foraging mainly on one crop such as *Eucalyptus grandis* (which has a low crude protein percentage, low lipid content and is deficient in isoleucine), have been highly infected with *Nosema* spp. Much of the available literature on the growth and development of honey bee colonies deal with the study of the relationship between nutrition and immune defense, in particular resistance to pathogens affecting the health of individual organisms (Ponton *et al.* 2011). The health of honey bees is not only limited by the absence of diseases, but also by the presence of well-fed individuals that resist parasites, infections, insecticides and periods of food shortage (Brodschneider and Crailsheim 2010). There is a consensus among researchers that there is a relationship between insect diet and immune function (Ponton *et al.* 2013; Smilanich *et al.* 2014). While the specific role of certain fat levels in the activation of the immune system has been well documented in humans and other animals (Goodman and Cusson 2012), comparatively little is known in honey bees. Several studies have reported that more than 20 viruses infect honey bee colonies (DeGrandi-

-Hoffman and Chen 2015). Among these viruses, the most common are: deformed wing virus (DWV), black queen cell virus and Israeli acute paralysis virus (Tantillo *et al.* 2015). Deformed wing virus is one of the most common viral infections in honey bee colonies worldwide (Martin and Brettell 2019). In asymptomatic bees, covert DWV infections in adult honey bee workers seriously impact long-term foraging and survival (Benaets *et al.* 2017). In many studies, DWV has been associated with both winter mortality and collapsing of bee colonies (Highfield *et al.* 2009; Dainat *et al.* 2012).

In the current study, queen-less honey bee nurses were fed different ratios of protein to fat (P : F) diets to evaluate their impact on bee resistance to DWV. The aim of this study was to measure the possible effect of fat nutrition on honey bee health by potentially decreasing the level of the DWV load. To our knowledge although this is only a limited view of the immune system, such a measure is a reasonable first attempt to investigate whether and how bee responses vary with the fat diet consumed.

## Materials and Methods

### Experimental animals

Nearly hatched frames of honey bee workers were collected from colonies of *A. mellifera* "Buckfast" hybrid strain, which were kept on a building rooftop at Newcastle University campus in the summer of 2017. Brood frames were placed in a wooden box inside the ventilated incubator (Sanyo MIR-553) set at 34°C in the dark to mimic natural field conditions (Winston 1991). Thirty asymptomatic (naturally infected) newly emerged bees (NEB) were taken each day for each cohort with 10 cohorts/treatment. Bees were reared in a perspex box (11 × 6 × 20 cm, Fig. 1) supplied with four, 2 ml Eppendorf tubes with four holes (3 mm diameter) for access as feeding tubes. Two feeding tubes were filled with a treatment solution and two with 1 M sucrose. A piece of paper was added to the hoarding box, covering the base.

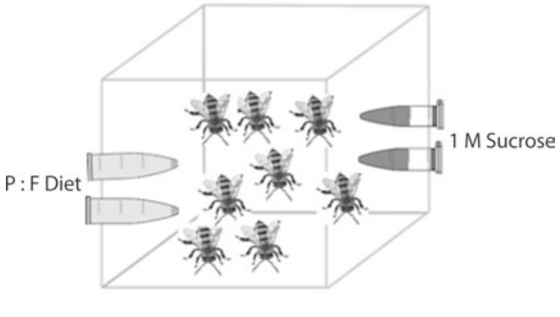


**Fig. 1.** Hoarding cage of honey bee (*Apis mellifera*)

### Experimental diets

Each protein part of the treatments was composed of a mixture of 10 essential amino acids (eAAs) required by honey bees (deGroot 1953): methionine, tryptophan, arginine, lysine, histidine, phenylalanine, isoleucine, threonine, leucine and valine (Table 1).

This mixture was added to 1 M sucrose solution, and 6 mg · ml$^{-1}$ from this mixture was chosen to be added to 342.3 mg · ml$^{-1}$ sucrose to get 1 : 56 w/w protein to carbohydrate (P : C) ratio (Vaudo *et al.* 2016). The fat source used in this study was lecithin (Optima® Bradford, UK). Lecithin was chosen as the fat source because it is an emulsifier and can be used for liquid diets. Ratios of eAAs/protein to fat (P : F) used in this study were calculated on a weight to weight (w/w) basis as the following: 25 : 1, 10 : 1 and 5 : 1 (low-fat diet, LFD); 1 : 1 (equal-fat diet, EFD); 1 : 5, 1 : 10, 1 : 12.5 (high-fat diet, HFD) and 1 : 0 (zero-fat diet) as a control (Table 2).

The particular P : F ratios used in this study were chosen to cover possible ranges of P : F ratios in natural pollen (Roulston *et al.* 2000; Vaudo *et al.* 2016a), as well as values outside of the reported range of P : F ratios

**Table 1.** Essential amino acids required by honey bees (de Groot 1953)

| Amino acid | g/16 g N |
|---|---|
| Leucine | 4.5 |
| Isoleucine | 4.0 |
| Valine | 4.0 |
| Threonine | 3.0 |
| Lysine | 3.0 |
| Arginine | 3.0 |
| Phenylalanine | 2.5 |
| Methionine | 1.5 |
| Histidine | 1.5 |
| Tryptophan | 1.0 |

**Table 2.** Nutrient proportions in each dietary treatment

| Treatment solution | Essential amino acids [mg · ml$^{-1}$] | Carbohydrate [mg · ml$^{-1}$] | Fat [mg · ml$^{-1}$] |
|---|---|---|---|
| 25 : 1 | 6.113 | 342.3 | 0.24452 |
| 10 : 1 | 6.113 | 342.3 | 0.6113 |
| 5 : 1 | 6.113 | 342.3 | 1.2226 |
| 1 : 1 | 6.113 | 342.3 | 6.113 |
| 1 : 5 | 6.113 | 342.3 | 30.565 |
| 1 : 10 | 6.113 | 342.3 | 61.13 |
| 1 : 12.5 | 6.113 | 342.3 | 76.4125 |
| 1 : 0 | 6.113 | 342.3 | 0 |
| 1 M sucrose | 0 | 342.3 | 0 |

in pollen. Diets were used in the form of a liquid because this makes them easier for the bees to ingest and it gives an accurate measurement of consumption.

Newly emerged bees were given access to food tubes containing 1 M sucrose solution and to tubes with one of the specific P : F ratios. The sucrose-only food source was provided in all treatments as it was necessary to allow bees to reach their high carbohydrate requirements. It also needed to be separate so that bees could freely consume without any enforcing consumption of proteins and fat mixture. This also provided an imitation of what honey bees actually experience by feeding on a carbohydrate-only source as 'nectar' and a fixed protein/fat/sugar source as 'pollen'. Protein was kept constant while we adjusted the lipid concentration (Table 2).

## Molecular quantification of DWV

### RNA extraction and cDNA synthesis

The DWV genome consists of a 10-kb positive single-stranded RNA (Forzan *et al.* 2017). The total RNA was isolated from the whole body of honey bees using a TRIzol® Plus RNA Purification Kit (Ambion, cat.# 15596-018), following the manufacturer's protocol. DNA contamination was removed by using RQ1 RNase-Free DNase (Promega, cat.# M6101). RNA was quantified on a NanoDrop spectrophotometer (Nanodrop 2000c, Thermo Fisher Scientific, Rockford, Illinois)). One μg of RNA was used to prepare cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad, Montreal, Quebec, Canada) according to the manufacturer's instructions. cDNA was used as a template to RT-qPCR and PCR reactions.

### PCR product

The cDNA synthesized in the described method served as a template for the PCR product. The primer sequence for β-actin and DWV has been given elsewhere (Di Prisco *et al.* 2016). All primers (Table 3) were used at a final concentration of 1.5 μM which was chosen after preliminary trials. The PCR reaction involved: 25 μl of PCR Master Mix, 1 μl each of forward and reverse primers, 1 μl of template cDNA, and finally, the total volume was made up to 50 μl by adding Ambion® Nuclease-Free Water. After gentle vortexing, the samples were amplified by thermal cycler (BIO-RAD® PCR system T100™). PCR conditions were as follows: 1 cycle at 95°C for 3 min for initial denaturation followed by 35 cycles at 95°C for 30 sec denaturation, then annealing at 60°C for 30 sec, next, the extension step at 72°C for 6 sec, and finally an additional polymerization step at 72°C for 10 min. Following electrophoresis (BIO-RAD, cat.#1640304), bands in the gel were purified using a QIAquick MinElute Gel Extraction Kit (QIAGEN GmbH, Germany), according to the manufacturer's recommended protocol and were then cloned into StrataClone vector pSC-A-amp/kan (Stratagene CA, USA) following the manufacturer's protocol. The QIAprep Spin Miniprep Kit (QIAGEN GmbH, Germany) protocol was used to purify the plasmid DNA. These plasmids were sent for sequencing to confirm the cloned insert.

**Table 3.** Sequences of primers used for RT-qPCR and PCR analysis in *Apis mellifera* nurse bees for deformed wing virus (DWV) quantification

| Application | Primer ID | Sequence of primer |
|---|---|---|
| qPCR. DWV | FDWV1 | GCGCTTAGTGGAGGAAATGAA |
| | RDWV1 | GCACCTACGCGATGTAAATCTG |
| qPCR.β-actin (Reference gene) | FB2 | GATTTGTATGCCAACACTGTCTT |
| | RB2 | TTGCATTCTATCTGCGATTCCA |

### Reverse transcription-quantitative PCR (RT-qPCR)

The transcription levels of DWV genome copies in adult honey bees (Fig. 2) were determined by SYBR Green RT-qPCR using the following conditions: 95°C for 3 min, followed by 35 cycles at 95°C for 30 sec, 60°C for 30 sec, and 72°C for 0.06 sec. β-actin was used as a reference gene. The standard curve was established by plotting the logarithm of four 10-fold dilutions of a starting solution with 20 fg plasmid DNA using

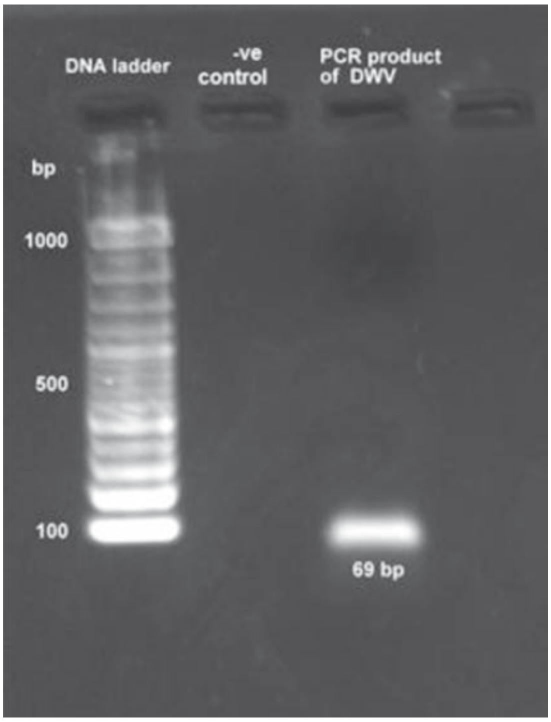


**Fig. 2.** PCR products of deformed wing virus (DWV; 69 bp), visualized on 2% agarose

a Strata Clone PCR cloning kit (Agilent Technologies Inc., Santa Clara, CA, USA) with a DWV insert (from 20 fg to 0.02 fg), against the corresponding $C_T$ values as three biological replicates of cDNA containing nine pooled insects for each were used, and they were normalized against the reference gene (Di Prisco *et al.* 2016). The relative abundance of the DWV in different P : F diets was examined using RT-qPCR to ensure that subsequent studies were applied to the appropriate treatment. The relative transcript quantity of the DWV gene from honey bees fed different ratios of P : F diets was calculated by plotting $C_T$ values on the standard curve mentioned above to obtain the amount of DWV copy, according to the following equation (Staroscik 2004):

$$\text{Number of copies} = (\text{amount} * 6.022 \times 10^{23}) / (\text{length} * 1 \times 10^{9} * 650),$$

where: the amount of DNA in nanograms, $6.022 \times 10^{23}$ = Avogadro's number, the length of DNA fragment in base pairs. We multiplied by $1 \times 10^9$ to convert to nanograms. The average weight of a single DNA base pair is 650 Daltons. This can also be written as $650 \text{ g} \cdot \text{mol}^{-1}$.

### Statistical analysis

Most analyses were conducted using SPSS (IBM SPSS Statistics v.23) with diet as the main effect. *Post hoc* comparisons were made using the least-squares difference (LSD) analysis. Data were analyzed using a generalized linear model ANOVA. When the ANOVA was statistically significant ($p \le 0.05$), $H_0$ was rejected. Univariate ANOVAs were performed for each response variable using sums of squares adjusted for the other dependent variables in the model. Data from each diet group were also subjected to a multiple comparisons analysis with LSD to examine possible individual significant differences (Minitab, State College, PA, USA).

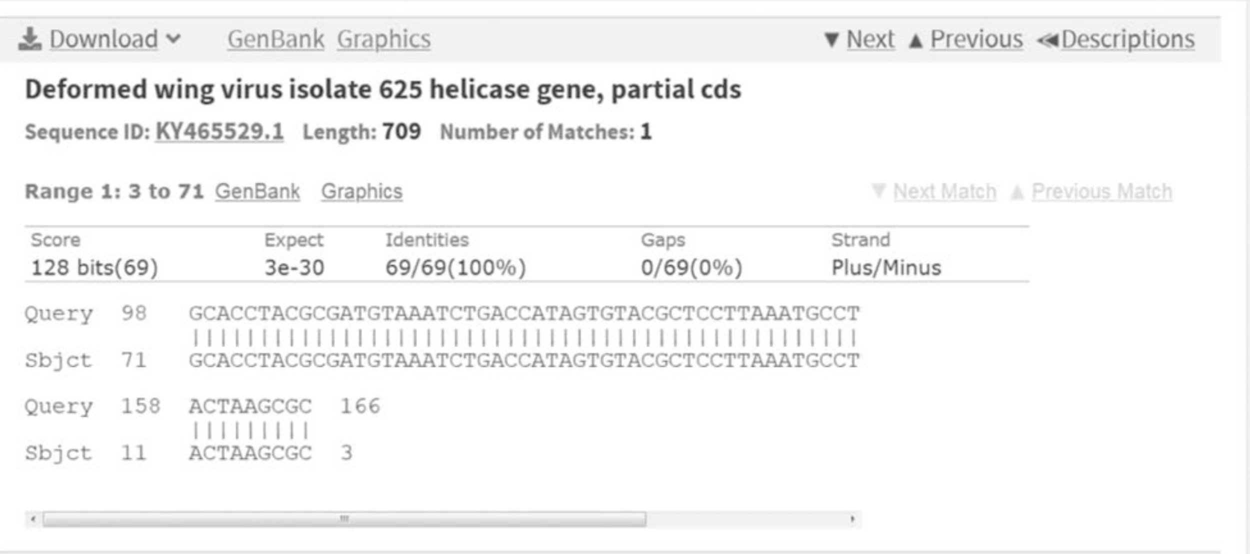
## Results and Discussion

### Clone of DWV fragment

A 69 bp fragment of the DWV was cloned in a StrataClone vector pSC-A-amp/kan and then sent for sequencing to confirm the identity of the inserted piece. Using NCBI BLAST (www.ncbi.nlm.nih.gov/BLAST), a sequence alignment was applied which confirmed 100% homology between the insert and deformed wing virus (taxid:198112) (Fig. 3).

### Virus titre

qRT-PCR analysis revealed that DWV transcript levels in nurses fed different ratios of P : F diets has caused a significant difference between HFD, EFD and control ($p < 0.05$, Table 4). Average honey bee workers harbored a viral genome, with DWV copy numbers declining greatly from $7.5 \times 10^5$ in the control (1 : 0) to $1.6 \times 10^2$ genome equivalent per a symptomatic honey bee in the 1 : 12.5 (P : F) diet (Table 4). It is well-known that the immunocompetence of herbivorous insects is influenced by a variety of nutrients (DeGrandi-Hoffman and Chen 2015). The diets provided in this study through the administration of different ratios of protein to fat diets revealed that high-fat nutrition helped bees to reduce the DWV level in their bodies (Table 4). Because this study was done using caged bees under controlled conditions, we could not calculate the DWV



**Deformed wing virus isolate 625 helicase gene, partial cds**

**Sequence ID:** KY465529.1 **Length:** 709 **Number of Matches:** 1

**Range 1: 3 to 71**

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 128 bits(69) | 3e-30 | 69/69(100%) | 0/69(0%) | Plus/Minus |

```
Query  98   GCACCTACGCGATGTAAATCTGACCATAGTGTACGCTCCTTAAATGCCT
            |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  71   GCACCTACGCGATGTAAATCTGACCATAGTGTACGCTCCTTAAATGCCT

Query  158  ACTAAGCGC  166
            |||||||||
Sbjct  11   ACTAAGCGC  3
```

**Fig. 3.** The output from BLAST alignment tool showing sequences of 100% homology between plasmid insert (Query) and deformed wing virus (DWV) (Sbjct). The line between nucleotides show sequence homology

**Table 4.** DWV copy number in a symptomatic honey bees which consumed different ratios of P : F diets after 10 days feeding

| Treatment (P : F) | Fat level | DWV copy number |
|---|---|---|
| 1 : 0 (control) | zero | $7.5 \times 10^5$ a |
| 25 : 1 | low | $4.7 \times 10^4$ ab |
| 10 : 1 | low | $5.9 \times 10^4$ ab |
| 5 : 1 | low | $1.1 \times 10^4$ b |
| 1 : 1 | equal | $2.3 \times 10^4$ b |
| 1 : 5 | high | $2.2 \times 10^4$ b |
| 1 : 10 | high | $3.3 \times 10^4$ b |
| 1 : 12.5 | high | $1.7 \times 10^2$ b |

Means followed by the same letter in the column do not differ significantly from each other by the Post Hoc test at $\alpha = 0.05$ significance level

genome copy under brood and nestmate interactions that exist normally in a colony level, especially in the presence of *Varroa* mites. Thus, there might be other factors that need to be examined in future work. For example, when *Varroa* mites infect honey bees, DWV replication increased in the newly emerged adult bees (Moore *et al.* 2011). In the current study, it seems that in general the HFD consumption resulted in a positive effect on bee immunity by decreasing the DWV level to reach $1.6 \times 10^2$ virus genomes at 1 : 12.5 (P : F) diet compared with $7.5 \times 10^5$ virus genomes at zero-fat diet, 1 : 0 (P : F). One of the explanations of the positive impact of fat diets on the immunity is that the addition of more lipid to the cell membrane will eventually form a good barrier against chemical or biological attack (Im *et al.* 2011). Another explanation is that the lipids of the membrane bilayer are important in all cell-environment interactions (Jackman and Cho 2020); thus, the regulation of the lipid level is necessary for cell-environment interactions (Im *et al.* 2011).

This research has raised many questions in need of further investigation in order to establish whether fat nutrition has a positive or negative impact on honey bee immunity. To summarize, when honey bee hives are healthy, the virus remains via vertical transmission and exists in a dormant state without affecting host immunity. However, when bees face stressful conditions such as *Varroa* mite invasion and nutritional stress, the virus replicates quickly and becomes more infectious, leading to the death of bees and eventually collapse of the colony. The findings from this study may contribute to current efforts to increase pollinator populations.

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