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Symptomatic chronic bee paralysis virus (CBPV) infection in an *Apis mellifera ligustica* queen

Giovanni CILIA, Antonio NANETTI
CREA Research Centre for Agriculture and Environment, Bologna, Italy

Abstract

Chronic bee paralysis virus (CBPV) affects various insect species, including *Apis mellifera*. In honey bee colonies, the infection is associated with easily recognizable symptoms in adult workers. However, CBPV spreads within the colony, so impacting the health conditions of all bee stages. This work focuses on a honey bee queen found affected by CBPV. Similarly, to infected workers from the same colony, the queen exhibited tremors, ataxia and impaired movements. CBPV infection and replication were confirmed in both queen and workers by qPCR and RNA sequencing. This infrequent finding aligns with the increasing incidence of CBPV infections detected across various countries.

Key words: viral infection, CBPV, infectious disease, clinic symptoms, strand-specific RT-PCR.

Introduction

Chronic bee paralysis virus (CBPV) is an unclassified pathogenic RNA virus of honey bees (*Apis mellifera* L.) (Celle *et al*., 2008; Olivier *et al*., 2008). Overt viral infections are associated with symptomatic affections leading the worker bees to die within a week (Bailey, 1975; Bailey *et al*., 1963; 1983). The disease is usually easily recognizable in adult workers, which show ataxia, trembling wings, uncoordinated movements, and hairless and dark abdomen (Bailey, 1975; Olivier *et al*., 2008; Budge *et al*., 2020). In the colony, the disease may linger latent but tends to appear in the spring, when it may be promoted by adverse weather conditions and inappropriate colony management (Ribière *et al.*, 2002; Dittes *et al.*, 2020). CBPV is spread globally, but its incidence is described as increasing in several countries (Traynor *et al*., 2016; Li *et al.*, 2017; Budge *et al.*, 2020). In Italy, the CBPV prevalence was reported to be around 8% in 2009 (Porrini *et al*., 2016), but more recent surveys detected notably increased levels: 48.5% in Emilia-Romagna region (Cilia *et al.*, 2022b), 58.5% in Abruzzo region (Bellucci *et al*., 2019) and from 65.9% to 98.8% in Veneto region (Martinello *et al.*, 2017; Bordin *et al*., 2022). Besides, the virus was detected in wild pollinators (Cilia *et al.*, 2022a; Tiritelli *et al.*, 2024) and in *Vespa velutina* and *Vespa orientalis* individuals (Mazzei *et al*., 2019; Marzoli *et al*., 2021; Power *et al.*, 2023; Zucca *et al*., 2023).

Within a honey bee colony, CBPV may be found in all stages, from eggs to adults (Blanchard *et al*., 2007; Todd *et al.*, 2007; Amiri *et al*., 2014; Seitz *et al.*, 2019; Kohl *et al.*, 2023). However, the adults usually show higher viral loads compared to other developmental stages, (Blanchard *et al.*, 2007; Amiri *et al*., 2014).

This paper reports the observation of a naturally CBPV-infected *Apis mellifera ligustica* queen, which showed blatant symptoms of the associated disease.

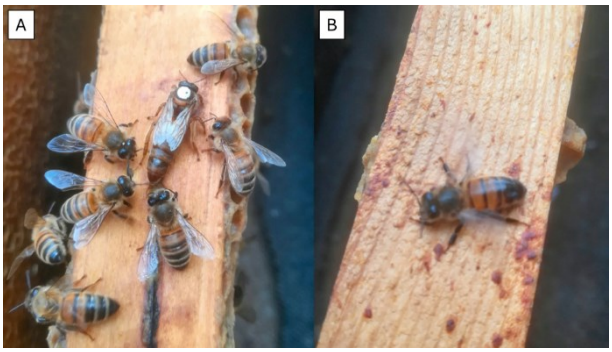

Figure 1. The symptomatic queen surrounded by workers (**A**) and an isolated worker displaying CBPV symptoms (**B**). See also the video, link in the text.

Materials and methods

On March 31, 2023, a colony of *A. mellifera ligustica* from the experimental apiary of CREA-AA in Bologna, Italy (44°31'26.5"N 11°21'03.5"E) was found to be on the verge of collapse. The colony's two-year-old queen was surrounded by a few workers and displayed evident tremors, ataxia, impaired movements, hairless and dark body (figure 1A). Despite the queen's swollen abdomen, no brood stages were detected in the comb cells. Additionally, some workers from the same colony exhibited typical symptoms of CBPV infection (figure 1B), raising suspicion of infection in both the workers and the queen. Consequently, the queen and 10 symptomatic workers were separately sampled into two tubes. Video footage illustrating the behaviour of both the queen and workers is available at the following link (https://www.youtube.com/watch?v=Z7tJCBip3jQ).

The queen and the ten workers were analysed respectively individually and as a pool. Each sample was homogenized with 300 µL of DNA/RNA Shield (Zymo Research, Irvine, CA, USA) and total RNA was extracted using the Quick RNA Microprep Plus Kit (Zymo Research), as previously reported (Cilia *et al.*, 2022a; 2022b). The extracted RNAs were used in a quantitative Real-Time PCR (qPCR) using the Power SYBR™ Green

Cells-to-CT™ Kit (ThermoFisher Scientific, Waltham, MS, USA) amplifying the CPV304 and CPV371 primers (Chantawannakul *et al.*, 2006). For the target gene, a standard curve was generated by amplifying serially diluted recombinant plasmids containing the CBPV RNA fragment from 10^1 to 10^9 copies, as previously reported (Mazzei *et al*., 2019; Cilia *et al.*, 2020; 2021; Nanetti *et al*., 2021b), following the amplification and quantification protocols (Chantawannakul *et al*., 2006). All the analyses were conducted in three technical replicates. For each sample, the average viral load was determined from the three replicates (Cilia *et al*., 2022a; 2022b). For the workers, this value was divided by the number of bees present in the sample to obtain the individual viral load.

To assess the viral replication, a strand-specific RT-PCRs using QuantiTect Reverse Transcription Kit (Qiagen) for the *RNA-dependent RNA-polymerase* (*RdRp*) gene was performed, as previously described (Mazzei *et al*., 2018; Cilia *et al*., 2022a; 2023). The amplicons were sequenced throughout the SeqStudio™ (ThermoFisher Scientific) using standard Sanger methodology and analysed using BLAST (Altschul *et al*., 1990). A phylogenetical analysis was performed on each CBPV *RdRp gene* sequence deposited in GenBank using the Neighbor-Joining method and Tamura-Nei model (Saitou and Nei, 1987; Tamura *et al*., 2004) associating taxa clustered together in the bootstrap test (500 replicates) (Felsenstein, 1985). Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

Results

Both samples were positive for CBPV. The resulting viral titre was 3.56×10^7 copies in the queen and 5.91×10^8 in the symptomatic workers. In the individuals of both casts, the CBPV was found to occur in the replicative form. A phylogenetic analysis was conducted, which resulted in close similarity with CBPV sequences isolated from *A. mellifera* and other bees in the Emilia-Romagna region (figure 2).

Discussion

The results of this investigation highlighted the presence of CBPV in a symptomatic honey bee queen. While all CBPV symptoms (ataxia, trembling wings, uncoordinated movements, hairless and dark abdomen) are well-documented in adult worker bees (Ribière *et al*., 2010), to the best of the authors' knowledge, this is the first reported instance of a natural symptomatic infection in a honey bee queen.

Honey bee queens that were artificially infected with 2×10^9 viral copies, showed trembling legs and wing misalignment at day 6 post-infection and 100% mortality at day 14 (Amiri *et al*., 2014). Those symptomatic queens showed a viral titre ranging between 10^4 and 10^7 (Amiri *et al*., 2014), which is in line with the natural infection observed in this study. The emergence of CBPV symptoms in two year old queens may indicate a different route compared to worker bees, which show the first signs of infection at the age of 5-8 days (Bailey *et al*., 1983; Ribière *et al*., 2007). The CBPV outbreak may be linked to unfavourable weather conditions, and the absence of brood cells in the dwindling colony is likely due to the disease, as the virus may be transmitted vertically to eggs and larvae (Chen *et al*., 2006; Ryba *et al*., 2012; Seitz *et al*., 2019). Besides, a CBPV-infected queen may transmit the virus to nurse bees by direct contact (via the epidermal cytoplasm and/or trophallaxis), as shown by previous experiments (Amiri *et al*., 2014). The CBPV circulation may be associated also with swarm and queen trading, increasing the circulation of the virus within specific environments (Pentikäinen *et al*., 2009; Wilfert *et al*., 2016; Budge *et al*., 2020; Gray *et al*., 2022; Aguado-López *et al*., 2023).

The sequence analysis showed similarity with a CPBV variant previously detected in Italy. Nevertheless, the natural occurrence of CBPV in a honey bee queen aligns with the increased incidence that is presently detected in several areas of the world (Ai *et al*., 2012; Chauzat *et al*., 2016; Porrini *et al*., 2016; Traynor *et al*., 2016; Li *et al*., 2017; Martinello *et al*., 2017; Budge *et al*., 2020; Bordin

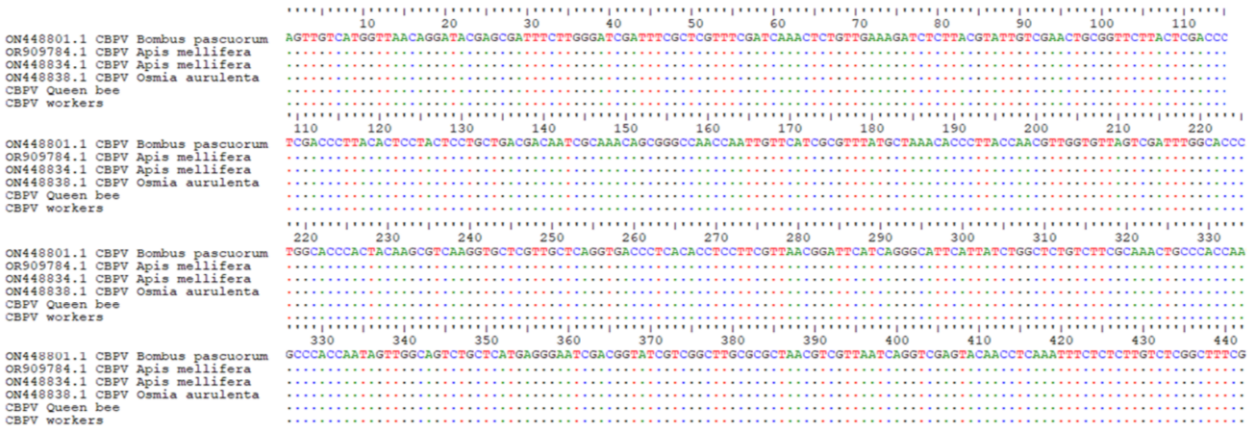

Figure 2. Alignment of the detected CBPV sequences with deposited sequences isolated from *Apis mellifera*, *Bombus pascuorum* and *Osmia aurulenta* from the Emilia-Romagna region.

et al., 2022; Cilia *et al*., 2022b), which may be epiphenomenal to the enhanced transmission due to the high environmental circulation of the virus in wild pollinators (Celle *et al*., 2008; Fernandez de Landa *et al*., 2020; Dalmon *et al*., 2021; Nanetti *et al*., 2021a; Cilia *et al*., 2022a; Pislak Ocepek *et al*., 2022; Power *et al*., 2023).

The finding of infected queens is probably not a sporadic occurrence, but the lack of recognition could decrease sightings of them, especially by inexperienced beekeepers. Improving knowledge and diagnosis of CBPV, even in queens, could increase the management of good beekeeping practices to control and monitor the virus.

In conclusion, this study confirmed a natural CBPV infection in a honey bee queen, displaying symptoms similar to those normally observed in affected adult workers. Furthermore, the consequences of the disease may have disrupted her ability to lay eggs. Additional research is necessary to elucidate the effects of CBPV infections at both individual queen bee and colony levels, as well as to assess their broader environmental impact.

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Authors' addresses: Giovanni CILIA (corresponding author: giovanni.cilia@crea.gov.it), Antonio NANETTI, CREA Research Centre for Agriculture and Environment, via di Corticella 133, 40128 Bologna, Italy.

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