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Presence of *Apis mellifera* pathogens in different developmental stages of wild Hymenoptera species

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Abstract

Pollinators are vitally important for the maintenance of ecosystems and the reproduction of most spontaneous and cultivated plants. However, in the last decades, they are suffering from an alarming decline, with the spread of pathogens and parasites being one of the main causes. *Apis mellifera* L. is the best-studied pollinator, and several studies have highlighted the presence of some of its pathogens in many other insect species. However, until now we have little or no indication of the effects these pathogens may have on other species. Here, we analysed the prevalence and load of several honey bee pathogens in different developmental stages of the non native species, giant resin bee, *Megachile sculpturalis* Smith. Also, newly emerged adults of three wild hymenopteran species, *M. sculpturalis*, *Heriades truncorum* (L.) and *Ancistrocerus* sp., nesting in a bee hotel were analysed. We found *Nosema ceranae* and CBPV in all three species analysed, and in *M. sculpturalis* with an increasing trend in parasite and virus levels from early to late stages. In addition, in free-ranging *M. sculpturalis* adults, we also found DWV, BQCV and *Am*FV. All viruses found were replicative. Our results indicate that honey bee viruses and parasites can replicate in wild Hymenoptera and their pre-imaginal developmental stages. Therefore, the considered viruses and parasites can be considered multi-host viruses and parasites, and wild pollinators could act not only as a reservoir but also as amplification hosts, highlighting an important threat not only for wild but also for managed bees, because they may result in spillback infections.

Key words: wild pollinators, *Megachile sculpturalis*, developmental stages, *Nosema ceranae*, CBPV.

Introduction

Pollinators play a pivotal role in terrestrial ecosystems, in that they allow the reproduction of both wild plants and crops. It is estimated that 87.5% of entomophile angiosperms depend on them (Ollerton *et al*., 2011), and more than three-quarters of the major types of global food crops benefit from animal pollination (Klein *et al*., 2007; Rader *et al*., 2016; Rhodes, 2018). The recent decades witnessed a pollinator decline due to different factors contributing to the reduction of their populations, biodiversity, abundance, and distribution (Szabo *et al*., 2012; Ollerton *et al*., 2014; Powney *et al*., 2019). Together with pesticides, land-use change and global warming, pests and pathogens are deemed major causes of pollinator decline (Goulson *et al*., 2015).

Apis mellifera L. is the most studied and known pollinator, mainly due to its commercial value. The same also applies to the pathogens and pests affecting this species. It is well-known that *A. mellifera* is exposed to several viruses, bacteria, fungi, protists, and mites, which are responsible for severe effects at the individual and social levels. The negative effects of these pathogens may include altered homeostasis and renewal of intestinal tissues, consequent malnutrition, impaired behaviour and orientation, suppressed immune function, problems collecting nectar and pollen and, finally, colony losses (Gisder and Genersch, 2017; McMahon *et al*., 2018; Beaurepaire *et al*., 2020; Boncristiani *et al*., 2020).

Those pathogens do not threaten honey bees only but are reported to spread in the environment and infect wild bees also (Martínez-López *et al*., 2021; Nanetti *et al*., 2021a; Piot *et al*., 2022). The health of managed honey bees, wild bees and other arthropods is interconnected, which implies the implementation of a One Health approach to honey bee health (Mahefarisoa *et al*., 2021; Wilfert *et al*., 2021). This approach is essential to generate suitable ecosystems for pollinators and other arthropods contributing to human livelihoods, promoting environmental health, and preventing the transmission of pathogens and pests to protect managed and wild insect populations (Manley *et al*., 2015).

So far, spillover of honey bee parasites and viruses were documented more frequently for deformed wing virus (DWV), Kashmir bee virus (KBV), Israeli acute paralysis virus (IAPV), sac brood virus (SBV), and *N. ceranae* (Dalmon *et al*., 2021; Nanetti *et al*., 2021a). In addition, the majority of the studies regarded mostly bumblebees, mason bees and leafcutter bees as host species, probably as for honey bees, due to their economic value (Tehel *et al*., 2016; Ngor *et al*., 2020).

Although parasites and viruses can cause severe damage in apiaries at both individual and colony levels, their effects on other insect hosts remain largely unknown. A notable exception to that is DWV infections in bumblebees, where the presence of the replicative virus was associated with wings deformities in *Bombus terrestris* (L.) and *Bombus pascuorum* (Scopoli) (Genersch *et al*., 2006; Cilia *et al*., 2021) and in wild bee longevity (Fürst *et al*., 2014; Graystock *et al*., 2016; Tehel *et al*., 2022).

Honey bee parasites and viruses can be transmitted by different routes. In arthropods sharing the same environment as the honey bees, some parasites and viruses may occur by orofaecal route (Cilia *et al*., 2018b; de Landa *et*

al., 2020; Nanetti *et al*., 2021b), by ingestion of contaminated pollen, and direct contact (Singh *et al*., 2010). Foraging activity could also promote spillover, as pollen, nectar and floral organs may get contaminated by infected honey bee foragers so spreading the pathogens to other flower visitors (Mazzei *et al*., 2014; Alger *et al*., 2019; Burnham *et al*., 2021). Hornets, wasps and scavengers may get infected as a consequence of honey bee predation and carcass cannibalization (Sébastien *et al*., 2015; Forzan *et al*., 2017; Mazzei *et al*., 2018; 2019)

Honey bee colonies may represent pathogen sources to other pollinator populations and the latter could act as reservoirs (Ravoet *et al*., 2014). However, to the best of our knowledge, the previous studies on the presence of honey bee viruses and parasites in wild insects were limited to adult stages, showing a severe gap in our understanding of the preimaginal susceptibility to infections.

The aim of this work was to study some aspects related to the presence and circulation of honey bee viruses and parasites in wild hymenopterans, in particular: i) we analysed the pathogen presence in different developmental stages of the giant resin bee *Megachile sculpturalis* Smith (Hymenoptera Megachilidae), and ii) we investigated the presence of these viruses and parasites in different newly emerged individuals of three cavity-nesting hymenopterans, including *M. sculpturalis*.

Materials and methods

Sample collection

The study was conducted in April-July 2021 at CREA-AA, Bologna, Italy (44°31'26.8"N 11°21'04.5"E), in a single bee hotel. The bee hotel (artificial nesting site for wild solitary Hymenoptera species) is located in an area of roughly 500 m^2, coexisting with an experimental apiary included approximately forty *Apis mellifera ligustica* Spinola colonies.

The bee hotel consisted of assembled wooden cubes (cavities of 0.2-1.4 cm of diameter, 10-15 cm deep), cut reeds (*Arundo donax* L. of various diameters and lengths), and trunk segments from local wood species (cavities of 0.4-1.0 cm of diameter, 11-12 cm deep). Since its establishment in 2016, the bee hotel was used by *M. sculpturalis* as a nesting site. This species, native to Southeast Asia, was accidentally introduced first to North America in the 1990s, and then to many countries in Europe (Lanner *et al*., 2021). It is a cavity-nesting solitary bee that builds its nests in pre-existing cavities in wooden rods, dead wood, or reed stems, as well as in artificial holes including bee hotels (Guariento *et al*., 2019). In April, *M. sculpturalis* individuals were sampled pre-emergence from nests in reeds, identified based on their peculiar features (i.e., larval cells composed mostly of resin mixed with vegetable debris and the closing plug by a mixture of resin, mud and wood fibres) (Aguado *et al*., 2018). We collected samples belonging to different developmental stages (i.e. prepupae, pupae, and pre-emerged adults). In addition, in the same periods, wooden cubes and trunk segments were placed in mesh cages to allow the subsequent collection of newly emerged individuals of all Hymenoptera species emerging in the same period of study.

Between the end of June and the beginning of July, 69 newly emerged individuals of three different Hymenoptera species (including *M. sculpturalis*) were collected for taxonomic identification and pathogen analyses. At the same time, 100 newly emerged *M. sculpturalis* adults were marked with water-based colours, released open-air and recollected two weeks later (hereafter: "free-ranging adults").

All the samples were stored in individual tubes at −80 °C until analysis.

Extraction of total nucleic acids

Before extraction, all samples were washed with 95% ethanol to remove external microbial contaminations. Wild hymenopterans were analysed individually to clear asses their infections. Each sample was placed in a 2 mL microtube with 500 µL of DNA/RNA Shield (Zymo Research, Irvine, CA, USA) and crushed with a TissueLyser II (Qiagen, Hilden, Germany) for 3 minutes at 30 Hz, as previously reported (Cilia *et al*., 2019; Nanetti *et al*., 2021b). The obtained suspensions were split into two aliquots, from which DNA and RNA were separately extracted.

The above-described procedures were accomplished by using respectively Quick DNA Microprep Plus Kit (Zymo Research) and Quick RNA Microprep Plus Kit (Zymo Research) following the modified manufacturer's instructions for solid tissue processing (Mazzei *et al*., 2019; Nanetti *et al*., 2021c).

The obtained nucleic acids were eluted in 50 µL of DNAase-RNase-free water and the extracts were stored at −80 °C until the qPCR assays.

qPCR assays to detect parasites and viruses

The extracted nucleic acids were analysed using Real-Time PCR to quantify the abundance of detected parasites and viruses in the samples, using the primers reported in table 1. For each target gene, a total reaction volume of 15 µL was prepared as previously described (Cilia *et al*., 2020; 2021) using PowerUp™ SYBR™ Green Master Mix (ThermoFisher, Waltham, MA, USA) for DNA microorganism and Power SYBR™ Green Cells-to-CT™ Kit (ThermoFisher Scientific) for RNA viruses. The real-time PCR assay was performed on an Applied Biosystems® 7500 Real-Time PCR (ThermoFisher Scientific), following the protocols for each gene sequence (Dobbelaere *et al*., 2001; Martin-Hernandez *et al*., 2007; Roetschi *et al*., 2008; Arismendi *et al*., 2016; Cilia *et al*., 2018a; Xu *et al*., 2018). DNA and RNA previously extracted from positive honey bee samples were used as positive controls for each investigated parasite and virus.

For each target gene, a standard curve was generated by amplifying serially diluted recombinant plasmids containing the pathogen-specific DNA and RNA fragments from 1×10^1 to 1×10^9 copies in a qPCR assay on an Applied Biosystems® 7500 Real-Time PCR (ThermoFisher Scientific), as previously reported (Mazzei *et al*., 2019; Cilia *et al*., 2020; 2021; Nanetti et al., 2021c), following the amplification and quantification protocols (Dobbelaere *et al*., 2001; Chantawannakul *et al*., 2006; Martin-Hernandez *et al*., 2007; Roetschi *et al*., 2008; de Miranda *et al*.,

Table 1. List of primers used to detect parasites and viruses.

Target	Primer Name	Sequence (5′-3′)	Reference
Nosema ceranae	Hsp70_F Hsp70_R	GGGATTACAAGTGCTTAGAGTGATT TGTCAAGCCCATAAGCAAGTG	Cilia *et al*., 2018a
Nosema apis	321APIS_F 321APIS_R	GGGGGCATGTCTTTGACGTACTATGTA GGGGGGCGTTTAAAATGTGAAACAACTATG	Martin-Hernandez *et al*., 2007
Paenibacillus larvae	AFB-F AFB-R	CTTGTGTTTCTTTCGGGAGACGCCA TCTTAGAGTGCCCACCTCTGCG	Dobbelaere *et al*., 2001
Melissococcus plutonius	MelissoF MelissoR	CAGCTAGTCGGTTTGGTTCC TTGGCTGTAGATAGAATTGACAAT	Roetschi *et al*., 2008
Crithida mellificae	Cyt_b_F Cyt_b_R	TAAATTCACTACCTCAAATTCAATAACATAATCAT ATTTATTGTTGTAATCGGTTTTATTGGATATGT	Xu *et al*., 2018
Lotmaria passim	Lp2F 459 Lp2R 459	AGGGATATTTAAACCCATCGAA ACCACAAGAGTACGGAATGC	Arismendi *et al*., 2016
KBV	KBV 83F KBV 161R	ACCAGGAAGTATTCCCATGGTAAG TGGAGCTATGGTTCCGTTCAG	Chantawannakul *et al*., 2006
DWV	DWV Fw 8450 DWV Rev 8953	TGGCATGCCTTGTTCACCGT CGTGCAGCTCGATAGGATGCCA	Mazzei et al., 2018
ABPV	APV 95F APV 159R	TCCTATATCGACGACGAAAGACAA GCGCTTTAATTCCATCCAATTGA	Chantawannakul *et al*., 2006
IAPV	IAPV B4S0427_R130M IAPV B4S0427_L17M	RCRTCAGTCGTCTTCCAGGT CGAACTTGGTGACTTGARGG	Kajobe *et al*., 2010
BQCV	BQCV 9195F BQCV 8265R	GGTGCGGGAGATGATATGGA GCCGTCTGAGATGCATGAATAC	Chantawannakul *et al*., 2006
SBV	SBV 311F 79 SBV 380R	AAGTTGGAGGCGCGyAATTG CAAATGTCTTCTTACdAGAGGyAAGGATTG	Chantawannakul *et al*., 2006
CBPV	CPV 304F 79 CPV 371R	TCTGGCTCTGTCTTCGCAAA GATACCGTCGTCACCCTCATG	Chantawannakul *et al*., 2006
SBPV major	SPV 8383F 81 SPV 8456R	TGATTGGACTCGGCTTGCTA CAAAATTTGCATAATCCCCAGTT	de Miranda *et al*., 2010
SBPV minor	SPV Minor F1 SPV Minor R1	ATAGCGCTTTAGTTCAATTGCCAT CTGGAATATGACCATCACGCAT	Martin *et al*., 2012
*Am*FV	AmFV2-F AmFV2-R	ACCCAACCTTTTGCGAAGCGTT ATGGGGCGTCTCGGGTAACCA	Hartmann *et al*., 2015

KBV: Kashmir bee virus; DWV: deformed wing virus; ABPV: acute bee paralysis virus; IAPV: Israeli acute paralysis virus; BQCV: black queen cell virus; SBV: sac brood virus; CBPV: chronic bee paralysis virus; SBPV: slow bee paralysis virus; *Am*FV: *Apis mellifera* filamentous virus.

2010; Kajobe *et al*., 2010; Martin *et al*., 2012; Hartmann *et al*., 2015; Arismendi *et al*., 2016; Cilia *et al*., 2018a; Mazzei *et al*., 2018; Xu *et al*., 2018).

Strand-specific RT-PCR

The active replication of viruses was evaluated through strand-specific RT-PCRs using specific primers to detect positive and negative RNA strands, as previously described (Mazzei *et al*., 2018; Nanetti *et al*., 2021b). Reactions were performed for all RNAs extracted using QuantiTect Reverse Transcription Kit (Qiagen). For DWV, CBPV and BQCV, the obtained cDNAs were amplified by PCR for the related viral targets, and the amplicons were visualized on a 2% agarose gel. On the other hand, to assess the replicative activity of *Am*FV, an aliquot of 10 µl from extracted RNA was treated with DNase and, after, the obtained cDNA was amplified by PCR and visualized as reported above. Replicative viruses previously extracted from positive honey bees were used as the positive control for each investigated virus. Subsequently, the amplicons were sequenced (BMR Genomics, Padua, Italy) and analysed using BLAST (Altschul *et al*., 1990).

Statistical analysis

Pathogen prevalence among the three wild Hymenoptera species and the different developmental stages of *M. sculpturalis* were analysed by chi-square test with Bonferroni correction. Data on pathogen abundance were first tested for normality using Shapiro-Wilk test. The abundance of the different viruses and parasites in positive samples calculated by averaging each of the two replicates of qPCR was analysed by Kruskal-Wallis H-test, and Mann-Whitney U-test for the investigated viruses and parasites. The significance threshold was set at 0.05.

All data on pathogen abundance were presented as mean ± SE. Analyses were performed and figures were drawn in R version 4.1.2 using *ggplot2* package (Wickham, 2009; R Core Team, 2021).

Results

Raw data on pathogen abundance, sample sizes of individuals collected and samples positive to viruses and parasites are reported in supplemental material table S1.

M. sculpturalis developmental stages

We analysed a total of 79 individuals of *M. sculpturalis* belonging to five different developmental stages: prepupae (N = 12), pupae (N = 15), pre-emerged adults (N = 17), newly emerged adults (N = 23), and free-ranging adults (N = 12). We found that 49.4% of all analysed samples were positive to *N. ceranae* and 31.7% to CBPV (figure 1) and we did not find statistical differences in pathogen prevalence among stages (*N. ceranae*: $\chi^2_4 = 1.70$, p = 0.790;

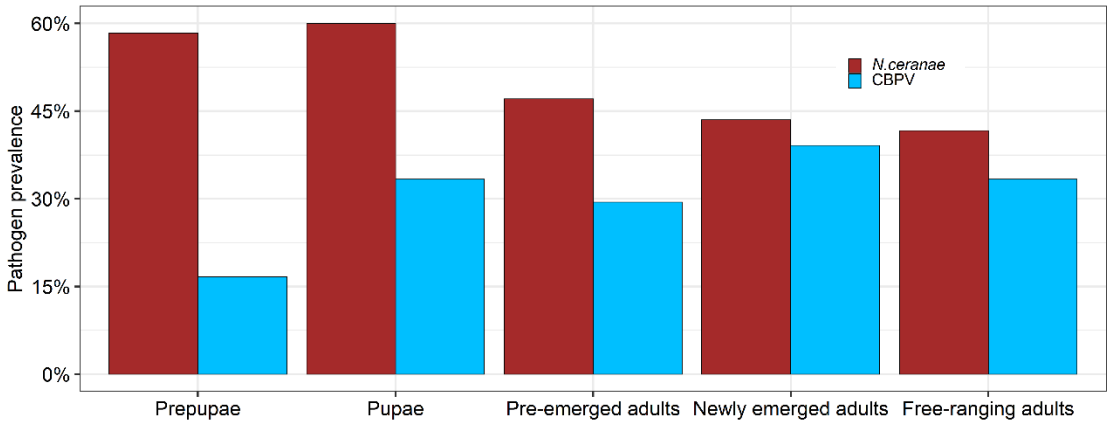

Figure 1. Percentage of *M. sculpturalis* samples at different developmental stages that were positive to *N. ceranae* and CBPV.

CBPV: $\chi^2_4 = 1.92$, p = 0.751). In addition, although non-significant (*N. ceranae*: H = 3.16, p = 0.531; CBPV: H = 4.00, p = 0.405), there was an increasing trend in the abundance and range variability from pre-imaginal to newly emerged stages, especially as regards CBPV (figure 2). The strand-specific PCR demonstrated active viral replication of CBPV in all PCR-positive samples. Among free-ranging *M. sculpturalis* adults, we found DWV in 50% of the samples, and *Am*FV and BQCV in one sample each. All these three viruses were replicative in all samples. Concerning viral sequences, the analysis highlighted the complete homology of CBPV (supplemental material figure S1) among all stages of *M. sculpturalis.*

Finally, considering all the developmental stages, we found nine individuals presenting a co-infection (supplemental material table S2). Of these, five individuals simultaneously presented *N. ceranae* and CBPV, two *N. ceranae* and DWV, one CBPV and DWV, and one *N. ceranae* and BQCV.

Newly emerged hymenopterans

Collected newly emerged individuals were identified as the individuals *Heriades truncorum* (L.) (N = 19), *M. sculpturalis* (N = 23), and *Ancistrocerus* sp. (N = 27). The first two species belong to the family Megachilidae, while the latter belongs to the family Vespidae (subfamily Eumeninae). Concerning the newly emerged individuals of the three Hymenoptera species, we obtained similar results to those of the different developmental stages of *M. sculpturalis*: 53.6% of individuals were positive to *N. ceranae* and 27.5% to CBPV and the prevalence did not statistically differ depending on the species (*N. ceranae*: $\chi^2_{22} = 0.068$, p = 0.967; CBPV: $\chi^2_{22} = 0.146$, p = 0.930; figure 3). All individuals were negative to the other investigated viruses and parasites.

We did not find a significant interspecific difference in the abundance of *N. ceranae* ($F_{3,43} = 8.20$, p < 0.001; figure 4a), and in the abundance of CBPV ($F_{3,25} = 7.12$, p = 0.001; figure 4b).

A BLAST analysis highlighted the complete homology of the CBPV viral sequences found in the three species (supplemental material figure S2). The strand-specific PCR demonstrated active viral replication of CBPV in all PCR-positive samples.

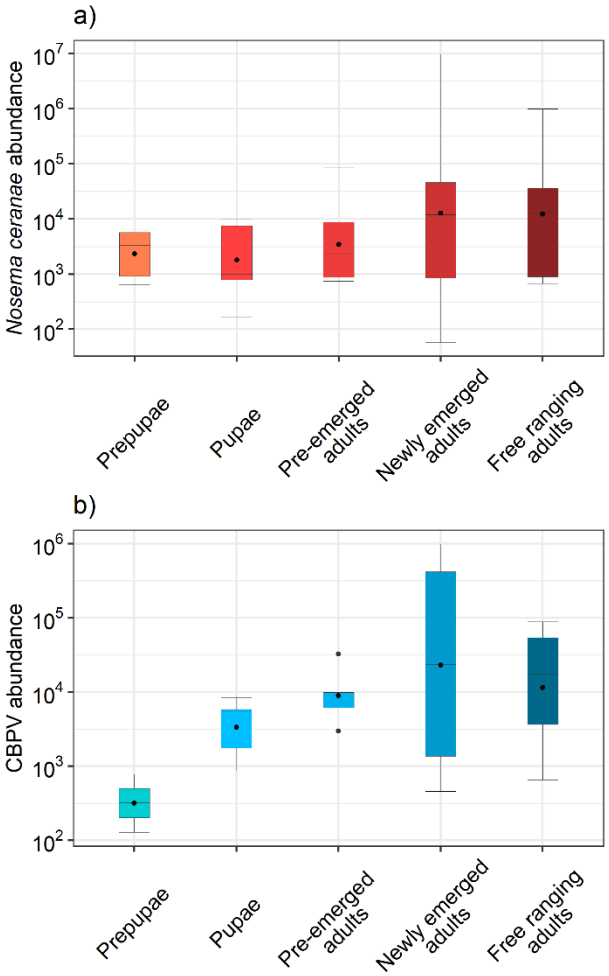

Figure 2. The abundance of *N ceranae* (**a**) and CBPV (**b**) samples of *M. sculpturalis* at different developmental stages. Means are showed as black dots, medians, interquartiles and ranges (or 95% CIs) are shown.

Finally, we found six samples (8.7%) co-infected by *N. ceranae* and CBPV (supplemental material table S1). One of those samples belonged to *H. truncorum*, two to *M. sculpturalis* (already mentioned in the analysis among different developmental stages), and three to *Ancistrocerus* sp.

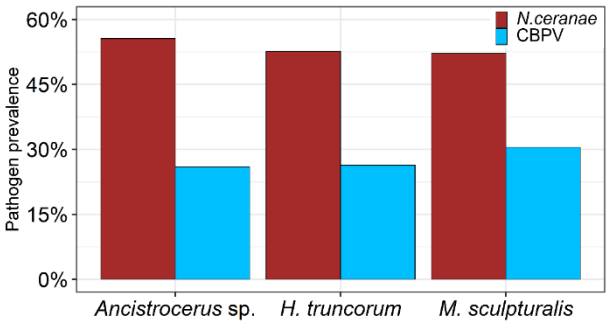

Figure 3. Prevalence of *N. ceranae* and CBPV in newly emerged individuals of the three wild Hymenoptera species.

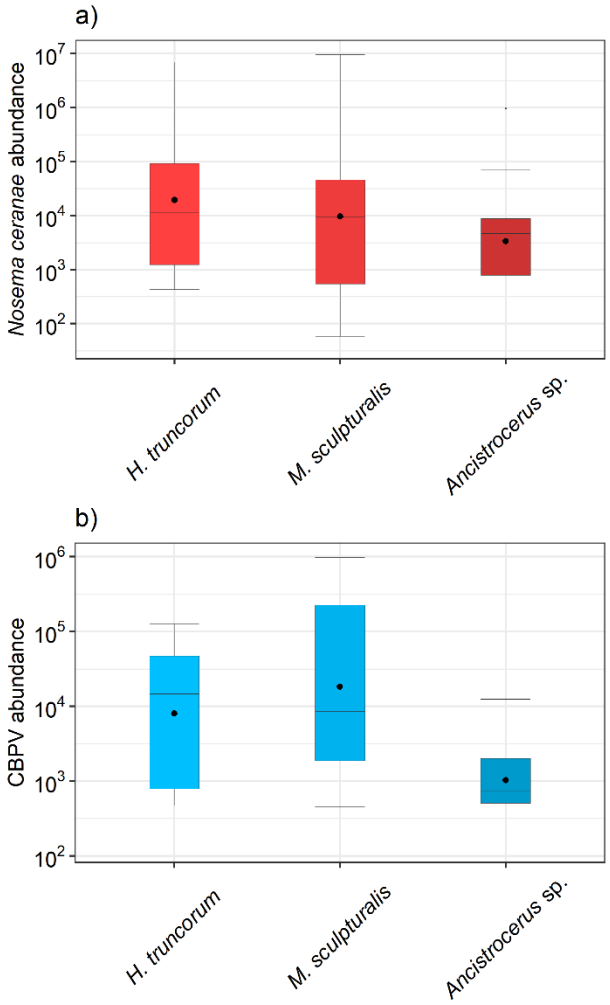

Figure 4. The individual abundance of *N. ceranae* (**a**) and CBPV (**b**) in positive samples of the three hymenopteran species in the bee hotel. Means are showed as black dots, medians, interquartiles and ranges (or 95% CIs) are shown.

Discussion

We found *N. ceranae* and CBPV in all the developmental stages of *M. sculpturalis* and also in the other two wild Hymenoptera species nesting in the same bee hotel. In addition, in *M. sculpturalis* free-ranging adults we also found pathogens absent in the bee hotel (i.e. DWV, *Am*FV, and BQCV). Although honey bee viruses and parasites had already been found in wild pollinators (Graystock *et al*., 2013; Ngor *et al*., 2020; Pritchard *et al*., 2021; Cilia *et al*., 2022a), our results showed that the viruses found were all replicative, indicating that they can be considered multi-host viruses and parasites.

The abundance of the viruses and parasites identified in the investigated species were lower than the threshold value measured in symptomatic honey bee (> 1×10^6 copies) in previous studies (Chen *et al*., 2006; Mazzei *et al*., 2014; Martín-Hernández *et al*., 2018). This is the only kind of comparison that can be made, because unfortunately no threshold values for these infections have yet been defined for wild bees and, especially, no correlation between copy number and disease symptoms has been seen. This is one of the most important gaps because possibly even low levels of infection of honey bee viruses and parasites can be harmful to wild bees and other hymenopterans (Dolezal *et al*., 2016).

Regarding *N. ceranae*, however, we must point out that this microsporidium parasite could have not truly infected the species analysed. Although some samples resulted infected by more than 1×10^7 microsporidian copies, the other ones showed a lower level of infection presumably not enough to trigger the infection in the midgut (Gisder *et al*., 2020; Higes *et al*., 2020).

M. sculpturalis developmental stages

To the best of our knowledge, this is the first identification of DWV, CBPV, *Am*FV, BQCV and *N. ceranae* in *M. sculpturalis* individuals, and several developmental stages of a wild pollinator. Previously, only SBV were found in *M. sculpturalis* adults (Cilia *et al*., 2022b).

We hypothesise that contaminated pollen generated infections in the larvae (Singh *et al*., 2010; Alger *et al*., 2019), that remained active throughout the subsequent developmental stages.

Although non-significant, the abundance of CBPV in pupae than in prepupae could be linked to the time elapsed since the interruption of defecation, which occurs just before the stage of prepupa and could have slightly decreased the viral load; during the time elapsed between defecation and pupal development, the level of infection has continued to grow, until the complete formation of the individual. Another possible explanation may be a general immune deficiency of pupae. On the other hand, the slightly lower load in free-ranging adults could be linked to the resumption of defecation in the environment, which is the main vehicle for spreading the infection. Moreover, the hatching of already infected individuals could be due to the vertical transmission of these viruses and parasites that could consistently increase individual positive rates within a specific population, improving the disease spreading.

Considering the other viruses found in free-ranging adults, the most plausible cause is contact with contaminated pollen via flowers.

Newly emerged individuals

The viruses DWV and CBPV have never been identified before in *H. truncorum*, while *N. ceranae* has been previously detected in individuals of the same species sampled in Belgium (Ravoet *et al*., 2014). Concerning

eumenine wasps, to date, only ABPV and SBV were detected in *Ancistrocerus auctus* (F.) in Argentina (Alvarez *et al*., 2018). This is not the first case in which honey bee viruses and parasites are shared between individuals using the same nests or living in the same area (Radzevičiūtė *et al*., 2017; Keller *et al*., 2018; Purkiss and Lach, 2019; Macías-Macías *et al*., 2020).

The detected viruses were replicative in the investigated individuals, highlighting their role as "new" hosts for these honey bee viruses and parasites. Furthermore, the occurrence of replicative DWV and CBPV or one replicative virus with *N. ceranae* in the same individuals indicates the possibility of pathogen co-infections in the hymenopteran species, as already reported in *A. mellifera* and other insect species (de Miranda *et al*., 2010; Nanetti *et al*., 2021b). Moreover, the active replication in species other than *A. mellifera* could represent an important ecological threat not only for wild bees but also for managed honey bees, because they may occur in re- or new infection (Tapia-González *et al*., 2019; Piot et al., 2020; Yañez *et al*., 2020; Burnham *et al*., 2021; Morfin *et al*., 2021). This is also demonstrated by the presence of *Am*FV and BQCV in free-ranging *M. sculpturalis* adults. In addition, the fact that this species is rapidly spreading (Lanner *et al*., 2021) may have an impact on bee disease dynamics in the future.

Conclusion

The spillover of viruses and parasites from managed honey bees to wild bees and other hymenopterans represents an important threat to the health of pollinators with a potentially high impact on individual and population fitness (Graystock *et al*., 2013; Gisder and Genersch, 2017; Nanetti *et al*., 2021a).

Unfortunately, there is a lack of information on this topic. Few studies analysed the pathogen replication, thus verifying the occurrence of an effective spillover. In addition, only very few studies analysed the effects of these viruses and parasites on wild insects, focusing mainly on mortality and no other crucial aspects of their fitness as flower-visiting behaviour, mating and reproductive success, nesting, pollen provisioning, and larval development.

Despite the lack of replication of our study (only one bee hotel and one season considered), further studies are needed to evaluate the risk of pathogen mutual transmission between wild and managed bees, which may create a 'vicious infection circle'.

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The authors declare no competing interests.

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