Journal of Invertebrate Pathology 207 (2024) 108215

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Honey bee viruses in the yellow-legged hornet *Vespa velutina* (Lepelieter 1836): Prevalence, loads, and detection of replicative DWV and LSV forms

M. Shantal Rodríguez-Flores [a,*], Ana R. Lopes [b,c], Ana Diéguez-Antón [a], M Carmen Seijo [a], M. Alice Pinto [b,c]

[a] Department of Plant Biology and Soil Sciences, University of Vigo, Campus As Lagoas, 32004 Ourense, Spain
[b] Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal
[c] Laboratório Associado para a Sustentabilidade e Tecnologia em Regiões de Montanha (SusTEC), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal

ARTICLE INFO

Keywords:
Vespa velutina
Apis mellifera
IAS
Bee viruses
DWV
LSV

ABSTRACT

Apiaries in Galicia, northwestern Spain, are currently facing the invasive alien species *Vespa velutina,* which is well established in the region. The pressure on honey bee colonies is high, resulting in both economic and ecological losses. Honey bee colonies also face the challenge of viruses, which are becoming increasingly diverse. In recent years, honey bee viruses have been spreading across taxonomic groups beyond Apoidea, infecting the Vespoidea superfamily. This cross-species spillover has raised concerns in the scientific community due to the potential risk of viruses spreading in ecosystems. Currently, there is a lack of knowledge on this topic, and further research is needed to address this issue. This study employed qPCR and sequencing to investigate the prevalence, loads, and presence of replicative forms of important honey bee viruses in *V. velutina* individuals collected from 11 apiaries in Galicia. All *V. velutina* individuals tested positive for DWV, BQCV, AKI complex (ABPV, KBV, and IAPV), or LSV but not for CBPV. DWV showed the highest prevalence (97.0 %) and loads, with both DWV-A (67.4 %) and DWV-B (32.6 %) being detected. The AKI complex (46.3 %) and LSV (43.3 %) were also common, whereas BQCV (11.9 %) was rarer. LSV is detected for the first time in *V. velutina*. LSV-2 was the dominant strain (82.1 %), and two less frequent (17.9 %) unknown strains were also detected. All 44 screened *V. velutina* samples carried the replicative form of DWV, and six of these also carried the replicative form of LSV, raising for the first time the possibility of co-infection in the hornet. The detection of honey bee viruses in *V. velutina*, and the ability of these viruses to spread to other species, may indicate a potential risk of spillover in the apiaries.

1. Introduction

Vespa velutina nigritorax (hereafter referred to as *V. velutina* or yellow-legged hornet) is an invasive predator originating from Southeast Asia that successfully established itself in Europe nearly two decades ago (Otis et al., 2023). The impact of this hornet is felt at multiple levels, including human health, economics, and ecosystem functioning (Laurino et al., 2019; Lioy et al., 2022; Monceau et al., 2014). This species causes economic losses in numerous sectors, such as beekeeping (Diéguez-Antón et al., 2022) and wine production (Lueje et al., 2024). Additionally, it can be fatal to individuals with allergies (Monceau et al., 2014). Within ecosystems, the species leads to a loss of biodiversity as it preys upon an increasing number of pollinator insects; thus, this predatory activity is adversely affecting pollination services (Rojas-Nossa et al., 2023; Rojas-Nossa and Calviño-Cancela, 2020).

In Europe, *V. velutina* exerts significant pressure, particularly on honey bees (*Apis mellifera*), having the potential to cause enormous colony losses in apiaries (Laurino et al., 2019; Leza et al., 2019; Requier et al., 2019). The duration of this pressure varies in length, depending on the environmental conditions of the region, but it can span several months (Diéguez-Antón et al., 2022) and is more pronounced during the summer and early fall. During this active predatory period, honey bees are deterred from going out to forage, and therefore they consume the food resources they have accumulated during spring and early summer. Unfortunately, honey bees display minimal defenses against *V. velutina* (Diéguez-Antón et al., 2022). Some colonies may adopt an offensive strategy similar to that of their counterparts in Asia, such as balling behavior (Arca et al., 2014; Monceau et al., 2018). However, this

* Corresponding author.
E-mail address: mariasharodriguez@uvigo.es (M. Shantal Rodríguez-Flores).

https://doi.org/10.1016/j.jip.2024.108215
Received 4 June 2024; Received in revised form 24 September 2024; Accepted 7 October 2024
Available online 9 October 2024

strategy only applies to a limited number of situations (Arca et al., 2014; Monceau et al., 2018).

V. velutina is not the only threat that honey bees face. An array of diverse pathogens can also seriously harm colonies, causing a multitude of illnesses. Among these, viruses play a major role in colony loss (Ullah et al., 2021). They can impact honey bees at any developmental stage without necessarily causing immediate clinical signs (de Miranda, 2008). Although around 72 viruses have been detected in honey bees (Beaurepaire et al., 2020), the most common ones are Deformed wing virus (DWV – *Iflavirus aladeformis*), Black queen cell virus (BQCV – *Triatovirus nigereginacellulae*), Sacbrood virus (SBV – *Iflavirus sacbroodi*), Kashmir bee virus (KBV – *Aparavirus kashmirense*), Acute bee paralysis virus (ABPV – *Aparavirus apisacutum*), Chronic bee paralysis virus (CBPV), and the recently described multi-strain Lake Sinai virus (LSV) (Bigot et al., 2017; **https://ictv.global/**). ABPV, KBV, and Israeli acute paralysis virus (IAPV) constitute the AKI complex, representing closely related viruses within the Dicistroviridae family (de Miranda et al. 2010). Following the first discovery through a metatranscriptomic survey in the US (Runckel et al., 2011), variants of LSV have been detected across the world, including Europe, the Middle East, West and South Africa, South America, Asia, Australia, and the Pacific Islands (Hou et al., 2023).

The transmission of honey bee viruses between different hosts is now being demonstrated with greater frequency (Alger et al., 2019; Dalmon et al., 2021; Nanetti et al., 2021). Scientific reviews have highlighted the emerging risk of these viruses spreading through interspecific relationships in ecosystems (Dolezal et al., 2016; Levitt et al., 2013; Nanetti et al., 2021; Rodríguez-Flores et al., 2023; Tehel et al., 2016). Additionally, there is insufficient data on the transmission vehicles from honey bees to other species (Marzoli et al., 2021). However, these viruses have been identified in species that are functionally related to honey bees, such as their predators, including *V. velutina*. Indeed, a wide array of honey bee viruses have been detected in *V. velutina* (some in their replicative form), including the common and epidemiologically important DWV (variants A, B, and C), ABPV, BQCV, IAPV, KBV, SBV, the rarer Acypi-like virus, ALPV, La Jolla virus, Moku, Ifla-like virus, BeeMLV, Menton virus, Mott mill virus, Nora-like virus, Partiti-like virus, Permutotetra-like virus, and Triato-like virus (Dalmon et al., 2019; Garigliany et al., 2017; Marzoli et al., 2021; Yang et al., 2019; and reviewed by Rodríguez-Flores et al., 2023). The detection and occasional replication of some honey bee viruses within *V. velutina* suggest their lack of specificity (Rodríguez-Flores et al., 2022). This, combined with the predatory behavior of the hornet, could potentially pose a risk to honey bees if its capacity to transmit these viruses to colonies and even apiaries is substantiated. In the northwest of the Iberian Peninsula, Galicia, there is limited knowledge on the honey bee viral landscape (Meana et al., 2017), and no information exists on whether honey bee viruses are spilling over to *V. velutina*. Herein, a viral survey was performed on *V. velutina* specimens collected across 11 Galician apiaries to evaluate the presence of important honey bee viruses, such as DWV, BQCV, CBPV, ABPV, KBV, IAPV, and LSV, on *V. velutina*.

2. Material and Methods

2.1. V. velutina sampling

A total of 67 *V. velutina* workers were collected from across 11 apiaries in Galicia, Spain, which were under strong predatory pressure. Each individual was captured manually using a butterfly trap while engaged in the attack of honey bees. The sampling period ranged from summer to the end of autumn, coinciding with the strongest predation pressure in the apiaries.

2.2. Total RNA extraction

Total RNA was extracted from each of the 67 *V. velutina* samples for the purpose of examining seven honey bee viruses (DWV, BQCV, CBPV, AKI Complex (ABPV, KBV, and IAPV), and LSV) (Table 1). To that end, each hornet was transferred into a sterile double strainer bag (BA6040, Seward, Worthing, UK) with 2 mL of nuclease-free water to be homogenized using the MixWell Lab blender (Alliance Bio Expertise, Guipry, France) with 2 cycles (1st: 60 s; 2nd: 30 s) at 380 rpm with 30 s of pause between each cycle. The homogenized samples were subjected to RNA extraction using RNeasy Mini Kit (Qiagen), with slight modifications. Briefly, 300 μL of homogenate, 600 μL of RLT buffer, and two zirconia beads (3 mm) were loaded in a 2 mL tube and placed in the Precellys apparatus (Bertin Instruments, Montigny-le-Bretonneux, France) for mechanical tissue disruption with the following protocol: 6200 rpm; 5 s; 3 times. Next, each sample was centrifuged at maximum speed for 3 min, and the supernatant was used for RNA extraction following the manufacturer's instructions without modifications. The quality and quantity of RNA extracts were evaluated by spectrophotometry in SPECTROstar Nano Microplate Reader (Reader, BMG LABTECH, Germany). The RNA extracts were normalized to a concentration of 250 ng/μL and subsequently stored at −80 °C until the reverse transcription step.

2.3. Reverse transcription and qPCR assays

The cDNA was synthesized from 1 μg of each RNA extract using the iScripts cDNA Synthesis Kit (Bio-Rad, California, USA) following the manufacturer's instructions. The cDNA was diluted in a 1:10 ratio, and 3 μL of the diluted cDNA was used as a template for the qPCR reactions. These reactions were carried out in the QuantStudio™ 5 System (Applied Biosystems, Massachusetts, USA) with the iTaq Universal SYBR® Green Supermix (Bio-Rad, California, USA).

Each qPCR reaction consisted of 1 μL of each primer (5 μM), 5 μL of 2X iTaq Universal SYBR® Green Supermix, and 3 μL of cDNA. All samples were run in duplicate. The thermal cycling conditions used to amplify all the viruses, except DWV, started with an initial activation stage at 95 °C for 30 s, followed by 40 cycles consisting of a denaturation stage at 95 °C for 15 s and an annealing/extension stage at 60 °C for 1 min. The thermal cycling profile for DWV was 95 °C for 30 s, 40 cycles at 95 °C for 15 s, 56 °C for 20 s, and 60 °C for 30 s. This procedure was followed by a melting curve analysis to confirm the specificity of the product (65–95 °C with increments of 0.5 °C s^{-1}). The primers' sequences are shown in Table 1. DWV primers were designed to detect DWV-A, DWV-B, DWV-C, and most likely DWV-D (Lopes et al.. 2024a); the LSV primers were designed to detect at least five of the nine known variants (Daughenbaugh et al., 2015; Lopes et al., 2024b); and the AKI primers were designed to detect simultaneously ABPV, KBV, and IAPV (Mondet et al., 2014). Each plate included two non-template controls (NTC) and a standard curve. Absolute quantification of each virus was performed using a standard curve with seven 10-fold dilutions of a known amount of a viral plasmid. The samples were considered positive if they (i) amplified before the last point of the standard curve, (ii) showed an exponential amplification on the amplification plot, and (iii) had a melting profile concordant with the melting temperature of the positive controls (standard points). The Cq values were converted into copies/μg RNA, considering the various dilution factors during the process. The performance of the qPCR standard curves is shown in Table 1.

2.4. Strand-specific RT-qPCR

The replication forms of the detected viruses DWV and LSV in *V. velutina* were evaluated by the specific identification of negative strand viral RNA using strand-specific RT-PCRs, with the primers shown in Table 2.

The thermal cycling conditions started with an initial activation stage at 95 °C for 30 s, followed by 40 cycles of a denaturation stage at 95 °C for 15 s, an annealing/extension stage at 65 °C for 1 min, followed by a melting curve of 65–95 °C with increments of 0.5 °C s^{-1}.

Table 1
List of primers used to amplify the honey bee viruses in *V. velutina* and statistics for the qPCR standard curve performance.

Target	Primer Name	Sequence (5′−3′)	Amplicon Length (bp)	qPCR performance	Reference
DWV	DWV-ABC-F	TACTAGTGCTGGTTTTCCTTT	210	E = 90.8 %	Lopes et al., 2024a
	DWV-ABC-R	CAAGTATCCTTCAAACAATC		R^2 = 0.98	
BQCV	BQCV-qF7893-F	AGTGGCGGAGATGTATGC	294	E = 93.6 %	Locke et al., 2012
	BQCV-qB8150-R	GGAGGTGAAGTGGCTATATC		R^2 = 0.99	
AKI	KIABPV-F6648-F	CCTTTCATGATGTGGAAAC	98		Mondet et al., 2014
	KIABPV-B6707-R	CTGAATAATACTGTGCGTATC			
LSV	LSV1-4-F	CGTGCGGACCTCATTTCTTCATGT	152	E = 111 %	Daughenbaugh et al., 2015
	LSV1-4-R	CTGCGAAGCACTAAAGCGTT		R^2 = 0.99	
CBPV	CBPV1-qF1818-F	CAACCTGCCTCAACACAG	296	E = 107 %	Locke et al., 2012
	CBPV1-qB2077	AATCTGGCAAGGTTGACTGG		R^2 = 0.99	

Table 2
List of primers used to reveal the replicative form of detected viruses in *V. velutina*.

Target	Primer Name	Sequence (5′−3′)	Amplicon Length (bp)	References
DWV	DWV_8450	TGGCATGCCTT GTTCACCGT	504	Marzoli et al., 2021
	DWV_8953	CGTGCAGCTCGATAGGATGCCA		
LSV	LSVU-R-1717	CCATATCATAAGTTGGCAAGTG	234	Runckel et al., 2011
	LSVU-F-1483	GACTTCATCATCCATCTGTGCGA		

2.5. Sample sequencing and phylogenetic analysis

The 210-bp amplicons of the non-replicative forms of a subset of DWV-positive samples, selected to represent all the apiaries, and all LSV-positive samples were sequenced by the Sanger method in "A Unit of Xenomics of CACTI", University of Vigo, Spain, using the ABI3130 capillary sequencer (Applied Biosystems, Massachusetts, USA). Sequencing quality control and edition were conducted on BIOEDIT software (Hall et al., 2011). DWV and LSV reference sequences were retrieved from GenBank (NCBI, National Center for Biotechnology Information) and incorporated in the phylogenetic trees, with the corresponding accession numbers. The DWV and LSV sequences generated here were aligned with the reference sequences in Mega X (Kumar et al., 2018) using the ClustalW algorithm. The best nucleotide substitution model was selected using the Akaike Information Criteria and then employed to construct the phylogenetic trees using the Maximum Likelihood method in Mega X. Tamura 3-parameter (Tamura, 1992) was the selected model for DWV and Kimura 2-parameter (Kimura, 1980) for LSV. Node support in the phylogenetic trees was evaluated through 1000 bootstrap replicates. The phylogenetic trees were further annotated and edited on the Interactive Tree of Life (iTOL) web-based tool (Letunic and Bork, 2021).

2.6. Statistics analysis

The data analysis was conducted using STATGRAPHICS Centurión 19® (Statgraphics Technologies, Inc., Virginia, USA). The differences in the prevalence and load among the viruses (DWV, BQCV, LSV, and the AKI complex) were evaluated by an ANOVA test with a 5 % level of significance. A Fisher's Least Significant Difference (LSD) multiple range test was used to determine which means were significantly different from others. This was used to detect differences in virus prevalence and loads among apiaries. All tests were checked for and complied with the required assumptions. Statistical significance was defined as a p-value < 0.05.

3. Results

3.1. Viral prevalence in V. velutina

Of the 67 samples of *V. velutina* analyzed in this study, 65 (97.0 %) tested positive for at least one virus. DWV, BQCV, LSV, and the AKI complex were detected in 65 (97.0 %) samples, whereas CBPV was not detected in any (Table 3). All samples collected in the apiaries of San Sadurniño, Ribeira, Sergude, and Toén tested positive for the four viruses and the AKI complex. DWV was detected in every single apiary with a total prevalence of 97.0 % (65 samples), ranging from 83.3 % in Luíntra to 100 % in 9 of the 11 apiaries (Table 3). In contrast, BQCV was detected in only four apiaries, with a total prevalence of 11.9 % (8 samples), with the highest prevalence of 37.5 % found in both Ribeira and Sergude. The AKI complex was detected in 9 apiaries, with a total prevalence of 46.3 % (31 samples), ranging from 12.5 % in San Sadurniño to 100 % in Culleredo. Finally, LSV showed a total prevalence of 43.3 % (29 samples) and was detected in *V. velutina* samples from eight apiaries, with Sergude showing the highest prevalence (87.5 %). The total prevalence of DWV was significantly higher (p-*value* < 0.05; LSD test) than that of any other viruses, whereas the total prevalence of BQCV was significantly lower (p-*value* <0.05) than that of any other viruses. Of the 67 *V. velutina* samples, 46 harbored at least two viruses, with the most frequent combination being DWV and AKI complex (31 samples).

3.2. Viral loads in V. velutina

The descriptive statistics of DWV, BQCV, and LSV loads are depicted in Table 4. There was no statistically significant difference between the

Table 3
Prevalence statistics of the honey bee viruses detected in *V. velutina* sampled in each apiary. N refers to the sample size. The different letters indicate the means that are significantly (p-value < 0.05) different from each other by the Fisher's least significant difference (LSD) procedure. (−) Negative sample.

Apiary	N	DWV %	BQCV %	AKI %	LSV %	CBPV %
Toen	11	90.9	9.1	81.8	9.1	–
Culleredo	4	100	0.0	100	75.0	–
San Sadurniño	8	100	12.5	12.5	37.5	–
Luíntra	6	83.3	0.0	16.7	0.0	–
Gondomar	8	100	0.0	25.0	50.0	–
A Cañiza	3	100	0.0	0.0	66.7	–
Castrelo do Miño	3	100	0.0	0.0	0.0	–
Ribadumia	1	100	0.0	100	0.0	–
Ribeira	8	100	37.5	25.0	50.0	–
Fonsagrada	7	100	0.0	85.7	71.4	–
Sergude	8	100	37.5	62.5	87.5	–
Total	**67**	**97.0$_c$**	**11.9$_a$**	**46.3$_b$**	**43.3$_b$**	–

Table 4
Descriptive statistics for the DWV, BQCV and LSV loads in each of the apiaries classified according to geographical location (coast or inland). N refers to sample size. The different letters indicate the means that are significantly (p-value < 0.05) different from each other by the Fisher's least significant difference (LSD) procedure.

Apiary	Viral load (copies/µg RNA)				
	Virus	Average	Maximum	Minimum	SD
Culleredo	DWV**bc**	$1.27x10^{8}$	$3.85x10^{8}$	$1.22x10^{7}$	$1.76x10^{8}$
	BQCV	–	–	–	–
	LSV**b**	$1.59x10^{6}$	$4.55x10^{6}$	$1.32x10^{3}$	$2.57x10^{6}$
San Sadurniño	DWV**c**	$1.33x10^{8}$	$5.43x10^{8}$	$7.75x10^{2}$	$2.42x10^{8}$
	BQCV	$5.30x10^{3}$	–	–	–
	LSV**ab**	$8.89x10^{5}$	$1.78x10^{6}$	$2.66x10^{2}$	$1.26x10^{6}$
Gondomar	DWV**ab**	$1.91x10^{7}$	$7.12x10^{7}$	$2.34x10^{6}$	$2.40x10^{7}$
	BQCV	–	–	–	–
	LSV**a**	$3.43x10^{4}$	$1.71x10^{5}$	$4.83x10^{1}$	$7.63x10^{4}$
Ribadumia	DWV**abc**	$9.99x10^{4}$	–	–	–
	BQCV	–	–	–	–
	LSV**a**	–	–	–	–
Ribeira	DWV**a**	$1.19x10^{5}$	$2.70x10^{5}$	$3.01x10^{3}$	$1.14x10^{5}$
	BQCV	$5.60x10^{3}$	$1.29x10^{4}$	$1.67x10^{3}$	$6.35x10^{3}$
	LSV**a**	$2.89x10^{3}$	$8.46x10^{3}$	$5.90x10^{2}$	$3.73x10^{3}$
Sergude	DWV**ab**	$1.11x10^{7}$	$7.94x10^{7}$	$8.79x10^{2}$	$2.76x10^{7}$
	BQCV	$3.06x10^{5}$	$8.92x10^{5}$	$6.84x10^{2}$	$5.08x10^{5}$
	LSV**a**	$8.15x10^{4}$	$5.41x10^{5}$	$4.29x10^{1}$	$2.03x10^{5}$
Toen	DWV**a**	$5.90x10^{5}$	$3.68x10^{6}$	$2.61x10^{3}$	$1.24x10^{6}$
	BQCV	$7.21x10^{5}$	–	–	–
	LSV**ab**	$2.55x10^{3}$	–	–	–
Fonsagrada	DWV**ab**	$4.19x10^{4}$	$8.75x10^{4}$	$1.82x10^{4}$	$2.65x10^{4}$
	BQCV	–	–	–	–
	LSV**a**	$5.88x10^{3}$	$1.71x10^{4}$	$1.73x10^{3}$	$6.37x10^{3}$
Luíntra	DWV**ab**	$1.69x10^{5}$	$7.96x10^{5}$	$1.36x10^{3}$	$3.51x10^{5}$
	BQCV	–	–	–	–
	LSV**a**	–	–	–	–
A Cañiza	DWVabc	$1.29x10^{8}$	$2.88x10^{8}$	$1.32x10^{5}$	$1.46x10^{8}$
	BQCV	–	–	–	–
	LSV**ab**	$6.84x10^{3}$	$1.01x10^{4}$	$3.60x10^{3}$	$4.57x10^{3}$
Castrelo do Miño	DWV**abc**	$7.60x10^{4}$	$1.97x10^{5}$	$1.46x10^{4}$	$1.05x10^{5}$
	BQCV	–	–	–	–
	LSV	–	–	–	–
Total	**DWV**	**$3.40x10^{7}$**	**$5.43x10^{8}$**	**$7.75x10^{2}$**	**$1.07x10^{8}$**
	BQCV	**$2.08x10^{5}$**	**$8.92x10^{5}$**	**$6.84x10^{2}$**	**$3.73x10^{5}$**
	LSV	**$2.53x10^{5}$**	**$4.55x10^{6}$**	**$4.29x10^{1}$**	**$8.94x10^{5}$**

mean loads of the honey bee viruses; however, DWV exhibited the highest mean load ($3.40 \times 10^{7} \pm 1.07 \times 10^{8}$ copies/µg RNA), followed by LSV ($2.53 \times 10^{5} \pm 8.94 \times 10^{5}$ copies/µg RNA) and BQCV ($2.08x10^{5} \pm 3.73 \times 10^{5}$ copies/µg RNA) (Fig. 1).

DWV mean loads exhibited substantial variation across the apiaries, spanning from $4.19 \times 10^{4} \pm 2.65 \times 10^{4}$ copies/µg RNA in Fonsagrada to $1.33 \times 10^{8} \pm 2.42 \times 10^{8}$ copies/µg RNA in San Sadurniño. A number of significant differences (p-value < 0.05; LSD test) were identified between the samples, with the load virus in Sadurniño exhibiting a higher load than the other samples, with the exception of the mean load of Culleredo, A Cañiza and Ribadumia (Fig. 2). BQCV was detected in only four apiaries, and in two of them there was only a single positive *V. velutina* sample with a load of 5.30×10^{3} copies/µg RNA in San Sadurniño and 7.21×10^{5} copies/µg RNA in Toen. In the other two apiaries, the mean load varied between $5.60 \times 10^{3} \pm 6.35 \times 10^{3}$ copies/µg RNA in Ribeira and $3.06 \times 10^{5} \pm 5.08 \times 10^{8}$ copies/µg RNA in Sergude. Similar to BQCV, in the apiary of Toen, there was a single *V. velutina* sample that tested positive for LSV and this had a load of 2.55×10^{3} copies/µg RNA. In the remaining apiaries, LSV mean load ranged from $2.89 \pm 3.73 \times 10^{3}$ in Ribeira to $1.59 \times 10^{6} \pm 2.57 \times 10^{6}$ copies/µg RNA in Culleredo. This last was significant higher (p-value < 0.05; LSD test) than the rest apiaries with the exception of Toen, A Cañiza and San Sadurniño.

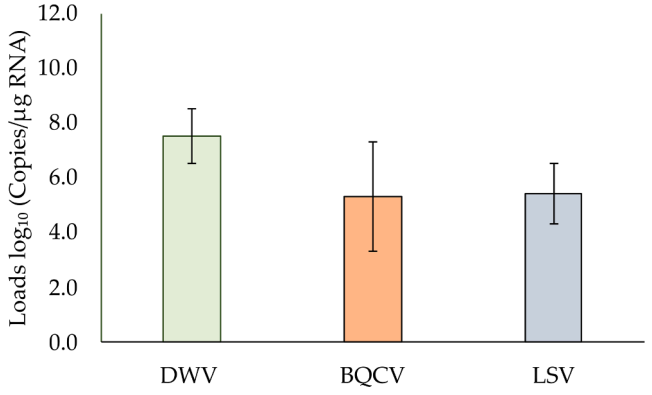

Fig. 1. Bar plot for DWV, BQCV, and LSV mean loads and the error bars: SE. Log_{10} transformed data is presented.

3.3. Replicative forms of DWV and LSV

The replicative forms were analyzed for the viruses with the highest loads: DWV and LSV. The strand-specific RT-qPCR assay revealed the presence of replicative forms in *V. velutina* samples for both viruses (Table 5). Negative-strand RNA was identified in all 44 samples that were screened for DWV. In contrast, only six of the 29 (20.7 %) samples that were screened for the negative-strand RNA of LSV were positive. These six samples were spread across four apiaries and co-occurred with the replicative form of DWV.

3.4. Phylogenetic analysis

The phylogenetic tree reconstructed from DWV sequences obtained from 43 *V. velutina* samples is depicted in Fig. 3. All chromatograms exhibited clean peaks, consistent with the presence of a single or a highly dominant strain in each sample. The DWV sequences were assigned to either DWV-A (29; 67.4 %) or DWV-B (14; 32.6 %). The rare DWV-C and DWV-D variants were not detected in any of the 43 sequenced samples, suggesting that they were absent or present at levels below the detection threshold of the Sanger method. DWV-A was exclusive in 7 apiaries (A Cañiza, Castrelo do Miño, Culleredo, Fonsagrada, Gondomar, Ribeira, and Toén) and DWV-B in 3 apiaries (Ribadumia, Sergude, and Luíntra). The two variants co-occurred in only one apiary (San Sadurniño) (Fig. 3).

The phylogenetic tree reconstructed from LSV sequences obtained from 28 *V. velutina* samples is depicted in Fig. 4. The phylogenetic tree revealed that most hornets (23; 82.1 %) carried LSV-2 as the dominant variant. However, five sequences (17.9 %), two from Ribeira, one from Toen, and two from Sergude, formed two clusters apart from the known LSV variants, suggesting two putatively novel variants, which we designated LSV-10 and LSV-11.

4. Discussion

Honey bee colony losses are caused by a combination of factors, including various parasites, pathogens, and predators such as the invasive hornet *V. velutina* (Diéguez-Antón et al., 2022; Hristov et al., 2021; Laurino et al., 2019; Meana et al., 2017; Rojas-Nossa et al., 2022). Among the pathogens, several honey bee viruses, often associated with the presence of the ectoparasitic mite *Varroa destructor*, play a major role in honey bee health (Traynor et al., 2020). While 13 major families of viruses have been identified in honey bees, the most important ones are *Dicistroviridae* and *Iflaviridae*, both belonging to the order Picornavirales (Beaurepaire et al., 2020). Several viruses of these families have been identified in Spanish apiaries, including DWV, IAPV, BQCV, SBV, and KBV (Barroso-Arévalo et al., 2019; Higes et al., 2010). However, few studies have been carried out in Galicia, northwestern Spain, and these identified DWV and BQCV (Kukielka et al., 2008; Meana et al., 2017) as the most prevalent, whereas KBV and SBV (Meana et al., 2017) were rarely found.

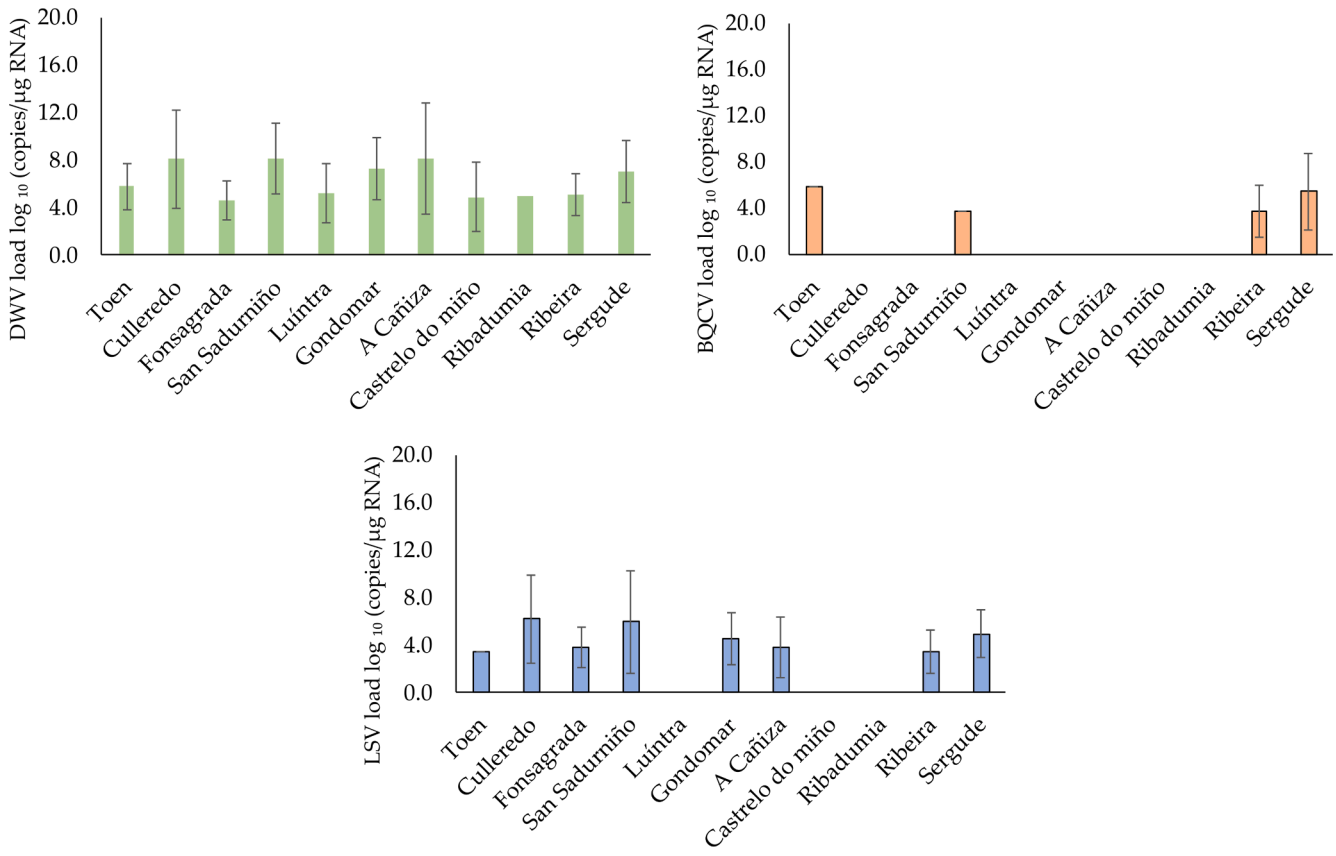

Fig. 2. Box and whisker diagrams for DWV, BQCV and LSV loads by apiary. The scales for the BQCV and LSV loads have been adjusted to the exponent $\times 10^6$ copies/µg RNA for visualization, in contrast to the DWV load which is exponentiated to $\times 10^8$ copies/µg RNA.

Table 5
Detection of DWV and LSV negative strands by strand specific RT-PCR.

Apiary	# Samples evaluated for DWV	# Positive samples for DWV (%)	# Samples evaluated for LSV	# Positive samples for LSV (%)
Toen	10	8 (80.0)	1	0.0
Culleredo	4	4 (100.0)	3	2 (66.7)
Fonsagrada	7	3 (42.9)	5	0.0
San Sadurniño	8	4 (50.0)	2	0.0
Luíntra	5	0.0		
Gondomar	8	8 (100.0)	5	0.0
A Cañiza	3	2 (66.7)	2	2 (100.0)
Castrelo do Miño	3	2 (66.7)		
Ribadumia	1	1 (100.0)		
Ribeira	8	7 (87.5)	4	1 (25.0)
Sergude	8	5 (62.5)	7	1 (14.3)
Total	**65**	**44 (67.7)**	**29**	**6 (20.7)**

Herein, three viruses (DWV, BQCV, and LSV) along with the AKI complex (ABPV, KBV, and IAPV) were identified in *V. velutina* specimens collected from 11 apiaries in Galicia. This is the first study detecting and quantifying these viruses in *V. velutina* in northwestern Spain and one of the few in Europe (Chauzat et al., 2015; Dalmon et al., 2019; Marzoli et al., 2021; Mazzei et al., 2018b, Mazzei et al., 2018a). The most prevalent virus, with the highest load detected in the *V. velutina* samples, was DWV. In southern Spain, DWV plays a significant role in weakening colonies (Barroso-Arévalo et al., 2019), although this honey bee virus should also be considered a serious threat in northern Spain (Cepero et al., 2014; Meana et al., 2017). The high frequency of DWV observed herein in individual hornets may have originated from its high occurrence in honey bee colonies. This could explain why the viral load presented by the hornets was similar to that quantified in other studies with *A. mellifera* (Tentcheva et al., 2006; Šimenc et al., 2021). DWV has been identified in *V. velutina*, with up to three variants detected: DWV-A (Mazzei et al., 2018a; Yang et al., 2019), DWV-B (Dalmon et al., 2019; Marzoli et al., 2021), and DWV-C (Dalmon et al., 2019). This study found only DWV-A and DWV-B in the Galician apiaries, and these are the two most widespread variants in the world (Paxton et al., 2022). In the last decade, DWV-B has rapidly replaced DWV-A in many parts of the world, and both are now found in species of wild bees and other wild pollinators (Paxton et al., 2022). However, assuming that *V. velutina* can be used as a proxy for honey bee epidemiology, this does not seem to be the case for the Galician honey bees, as 67 % of the *V. velutina* individuals harbored DWV-A, while the remaining 23 % had DWV-B.

BQCV is a widespread virus in honey bees (Beaurepaire et al., 2020), which causes the death of queens during their larval or prepupal stages. It is a covert disease in adult honey bees, but it may contribute to honey bee losses during the winter (Faucon et al., 2002; Higes et al., 2008). BQCV has been detected in Araneae and Opiliones (Levitt et al., 2013) as well as in *V. velutina* (Dalmon et al., 2019; Mazzei et al., 2019). Some

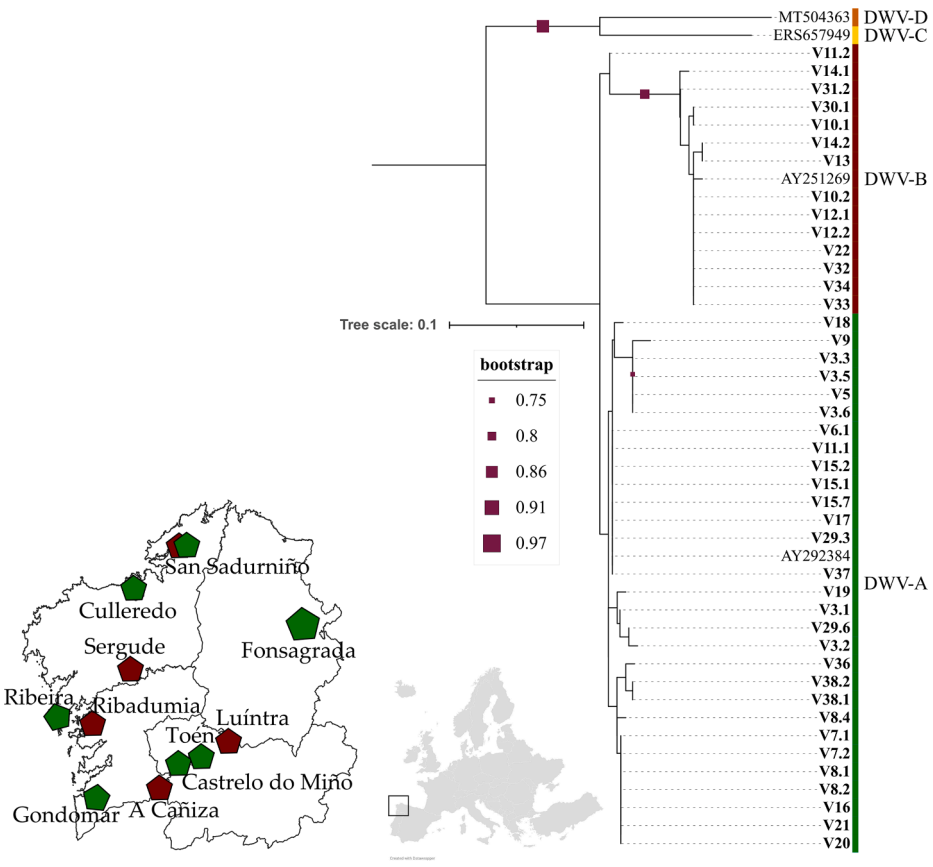

Fig. 3. DWV phylogenetic tree constructed from 43 sequences from Galicia, northwestern Spain, with reference variants obtained from GenBank. The geographical distribution of DWV-A and DWV-B and their corresponding frequencies are shown in the map of Galicia. Mapa Europe from Datawrapper.

studies (e.g., Dalmon et al., 2019) have detected the replicative strand in the gut and muscle samples of *V. velutina* collected at the same time as honey bees, suggesting predator–prey transmission. BQCV has been identified throughout Spain (Antúnez et al., 2012; Barroso-Arévalo et al., 2019; Buendía et al., 2018; Higes et al., 2007; Kukielka et al., 2008), including in apiaries located in the north (Cepero et al., 2014; Meana et al., 2017). Interestingly, *V. velutina* samples collected in this study showed a low BQCV prevalence and viral load, suggesting that Galician apiaries were not highly infected by this detrimental virus.

ABPV, KBV, and IAPV are often analyzed as a complex of related species (Ribière et al., 2008). These viruses have been associated with honey bee colony losses, especially when colonies are co-infected with *V. destructor* (Beaurepaire et al., 2020). In apiaries, the AKI complex has been detected in high quantities along with DWV and can cause colony losses during the winter (Francis et al., 2013). The AKI complex could not be limited to honey bees, but, as BQCV, it is a multi-host pathogen that can infect numerous species (Levitt et al., 2013; Yañez et al., 2020). The three viruses of the AKI complex have been identified in *V. velutina*: ABPV (Dalmon et al., 2019), KBV (Dalmon et al., 2019; Mazzei et al., 2019), and IAPV (Yañez et al., 2012; Yang et al., 2019). Unfortunately, this study did not allow us to identify which viruses of the complex were present in the *V. velutina* samples, and a better understanding of their distribution in Galician apiaries requires their individual analysis. However, ABPV, KBV, and IAPV have been detected in many apiaries in Spain (Antúnez et al., 2012; Kukielka and Sanchez-Vizcaíno, 2010), and our detection of the complex in 31 *V. velutina* samples suggests that at least one of these viruses is present in Galicia.

LSV is a recently discovered honey bee virus, and since its first detection in colonies near Lake Sinai, South Dakota, USA (Runckel et al., 2011), it has been identified worldwide, including in apiaries throughout Spain (Alonso-Prados et al., 2020; Cepero et al., 2014; Granberg et al., 2013). LSV is a highly diverse virus, with multiple strains coexisting globally and locally (Hou et al., 2023). In this study, most *V. velutina* samples harbored LSV-2, one of the most widespread variants found in honey bees (Faurot-Daniels et al., 2020; Hou et al., 2023), but there were also samples harboring LSV sequences that did not cluster with any of the known strains, suggesting that they might correspond to a novel strain. In addition to honey bees, LSV-2 was also detected in other species such as *Vespa bicolor* (Yang et al., 2019), *Polistes rothneyi* (Yang et al., 2019), harvester ants (Bigot et al., 2017), solitary bees (Ravoet et al., 2014), and *Bombus* spp. (Dolezal et al., 2016; Gamboa et al., 2015; Parmentier et al., 2016). This study adds, for the first time, *V. velutina* to the increasing list of species that are putative hosts for LSV. In addition, as in the case of DWV, there was significant variation in viral load among the colonies where it was detected.

In this study, no clinical signs were observed in the *V. velutina* samples that tested positive for DWV and LSV negative-strand RNA. DWV has been shown to infect and cause wing deformities in vespids such as *V. crabro* (Forzan et al., 2017), and these symptoms have also been observed in *V. velutina* from France (observations by Antoni Armengol, 2019) and Italy (Mazzei et al., 2018b). In contrast, LSV pathology is not well understood in honey bee individuals, although it has been associated with colony weakness and reduced size (Faurot-Daniels et al., 2020). The presence of DWV and LSV replicative forms in asymptomatic *V. velutina* samples from Galicia can confirm that there was no relationship between their detection and the appearance of symptoms. This lack of correlation has been observed in other studies (Chauzat et al., 2015).

All screened *V. velutina* samples carried the DWV replicative strand, indicating that this virus is present in honey bees from apiaries in Galicia. This could be due to the replication of DWV in *V. velutina* itself or to the presence of the replicative form in infected honey bees preyed

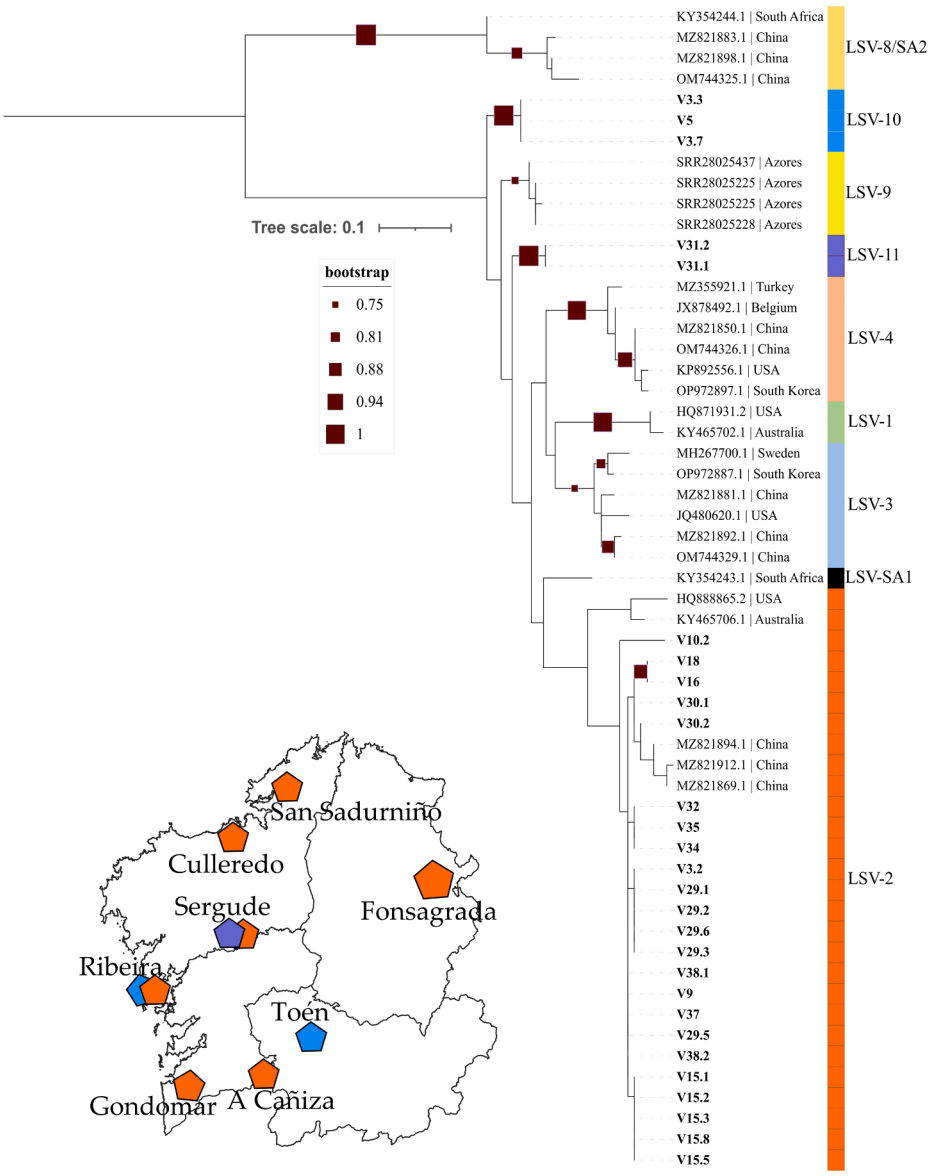

Fig. 4. LSV phylogenetic tree constructed from 28 sequences from Galicia, northwestern Spain, with reference variants obtained from GenBank. LSV-other refer to unnamed variants.

by the vespid. While further research would be needed to confirm the former, there is a high probability of finding DWV replication in *V. velutina*, as the replication of both DWV-A and DWV-B variants has been observed in other studies ((Dalmon et al., 2019; Mazzei et al., 2018a). In contrast with DWV, the analysis of the negative-strand of LSV only revealed six (20.7 %) positive *V. velutina* samples. In these samples, the replicative form of LSV-2 was detected along with the replicative form of DWV-A, except for one *V. velutina* sample, which carried DWV-B. This suggests a potential co-infection of both viruses in the same host. Replicative forms of other honey bee viruses have been detected in *V. velutina*, including Acypi-like virus (Dalmon et al., 2019), ALPV (Marzoli et al., 2021; Yang et al., 2019), BQCV (Dalmon et al., 2019; Mazzei et al., 2019), KBV (Mazzei et al., 2019), and Triato-like virus (Dalmon et al., 2019).

The presence of negative-strand RNA in *V. velutina* can be explained by two non-mutually exclusive hypotheses. While the detection of negative-strand RNA for both DWV and LSV could be a result of virus replication within *V. velutina*, an alternative hypothesis is that negative-strand RNA is acquired by the hornet when it preys on infected honey bees. A comparable mechanism was previously described by Posada-Florez et al. (2019) concerning the replication of DWV-A in *V. destructor*. Further research is needed to confirm whether both viruses are truly replicating in *V. velutina*.

A comprehensive understanding of the mechanisms underlying the transmission of the majority of honey bee viruses remains elusive. Among these, DWV is one of several insect RNA viral pathogens that have been identified in a diverse range of invertebrate species, including *V. velutina*. The vectoring capacity of DWV in the colonies of *A. mellifera* is high, with detection in all castes and at all stages of development (de Miranda and Genersch, 2010; Tentcheva et al. 2006). The ability of *V. velutina* to prey on honey bees over extended periods raises concerns that it may act as a transmission vector (e.g., via oral-faecal route or during the removal of occasionally killed hornets), exacerbating the health issues observed in apiaries. However, further research is required to elucidate the transmission dynamics of viruses from *V. velutina* to *A. mellifera*.

5. Conclusion

This study shows the presence of honey bee viruses in *V. velutina* collected in apiaries in Galicia, Spain. The most prevalent honey bee virus found in the *V. velutina* samples was DWV, with a phylogeny revealing the presence of both DWV-A and DWV-B variants. BQCV, AKI, and LSV were also detected, but at a much lower frequency. Furthermore, this study shows evidence of the presence and possible replication of LSV-2 in *V. velutina* for the first time. In recent years, honey bee viruses have been detected in divergent taxonomic groups, which could pose a risk to the ecosystems where they are found. This highlights the need for further research on honey bee-*V. velutina* virus transmission and its impacts on apiaries and ecosystems.

CRediT authorship contribution statement

M. Shantal Rodríguez-Flores: Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ana R. Lopes:** Writing – review & editing, Writing – original draft, Methodology, Funding acquisition, Formal analysis, Data curation. **Ana Diéguez-Antón:** Writing – review & editing, Writing – original draft, Methodology. **M Carmen Seijo:** Writing – review & editing, Writing – original draft, Supervision, Data curation, Conceptualization. **M. Alice Pinto:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors express their gratitude to the beekeepers who participated in collecting the samples for their collaboration. A special acknowledgment is due to the Agrupación Apícola de Galicia for their support in this research. M.S. Rodríguez-Flores expresses her gratitude for the support of Postdoctoral Financial Aid of Xunta de Galicia and the University of Vigo, grant number ED481D-2022-021, founded by Consellería de Cultura, Educación e Ordenación Universitaria, and Vicepresidencia segunda e Consellería de Economía, Empresa e Innovación (Government of Galicia, Spain). A.R. Lopes expresses her gratitude to Fundação para a Ciência e a Tecnologia (FCT) for the individual research grant (SFRH/BD/143627/2019). FCT provided financial support from national funds (FCT/MCTES) to CIMO (UIDB/00690/2020 and UIDP/00690/2020) and SusTEC (LA/P/0007/2021). Funding for open access charge: Universidade de Vigo/CISUG.

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