NOTES AND COMMENTS

Occurrence, prevalence and viral load of deformed wing virus variants in *Apis mellifera* colonies in Chile

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Abstract

Deformed wing virus (DWV) is one of the most common viruses in apiaries worldwide, and its presence in Chilean apiaries is no exception. There are three well-defined master variants described as DWV-A, DWV-B (or VDV-1) and DWV-C. We studied the prevalence, load and recombinant genotypes among DWV variants in honey bees from Chilean apiaries. We also compared the viral load in each region in colonies that were treated or untreated against *Varroa destructor*. Using real-time PCR with specific primers enabled us to determine that DWV-A was the most prevalent (71%) throughout Chile, with a higher level than DWV-B (circa 3% prevalence), and almost 60-times more load, especially in northern Chile. The viral load was lower only in treated colonies located in the Metropolitana, Biobío/Ñuble and Los Ríos regions. The DWV-C genotype and recombinants were not detected. This suggests that viral recombinant events are limited since DWV-B is still uncommon in honey bee hives at present. This is the first study involving DWV-B in Chile.

Keywords: Honey bees, DWV variants, DWV-B, DWV-A, viral load, real-time PCR.

Insect pollinators are considered one of the most important components of global biodiversity; the honey bee (*Apis mellifera* L.) is the most predominantly managed species and is responsible for pollinating many crops and wild plants (Potts et al., 2010). However, beekeepers around the world have suffered colony losses in recent years (Neumann & Carreck, 2010). Although published information regarding the mortality of colonies in South America is lacking, several cases of colony losses and colony depopulation have been reported by beekeepers throughout the continent in recent years (Maggi et al., 2016). Previous studies have shown that Deformed Wing Virus (DWV), in the presence of its vector *Varroa destructor*, may be a major factor in the high rate of honey bee colony losses (Highfield et al., 2009; Genersch et al., 2010; Dainat & Neumann, 2013; Kielmanowicz et al., 2015; Wilfert et al., 2016).

Deformed wing virus is a picorna-like, single-stranded, positive-sense, RNA virus (de Miranda & Genersch, 2010). It is present throughout all of the developmental stages and castes of honeybees (Yue & Genersch, 2005; Chen et al., 2005; Tentcheva et al., 2006). Three well-defined variants of this virus have been described: DWV-A, DWV-B (or VDV-1) and DWV-C (Martin et al., 2012; Mordecai et al., 2016). Nevertheless, DWV-B has attracted attention since it is widely spread throughout the landscape and is also considered a more virulent variant than the DWV-A genotype (McMahon et al., 2016). In addition, it has been shown that the prevalence of DWV-B along with *V. destructor* is highly correlated with overwinter colony losses (Natsopoulou et al., 2017). However, recent studies have suggested that DWV-A and DWV-C could also be involved in the collapse of honey bee colonies (Kevill et al., 2017).

Little is known about the prevalence of DWV-B in South America. Recently, DWV-B has been detected in honey bee colonies in the United States (Ryabov et al., 2017), but no previous studies have reported the presence of DWV-B in honey bee colonies in Chile or other

South American countries, with the exception of Brazil (de Souza et al., 2019). In previous studies we have determined the prevalence of DWV in Chilean apiaries (Vargas et al., 2017), but the master variant of this virus is yet unknown. Therefore, the objective of this study was to determine the DWV-variants present in Chilean apiaries and the presence of certain DWV recombinants, as well as the prevalence and viral load in the honey bees analyzed, but also, to compare viral load in colonies that were treated and not treated against *V. destructor* throughout Chile.

Groups of 50 worker bees were collected between the spring of 2015-2016 and the summer of 2017-2018 from each of the following regions: Coquimbo (29°54′S - 71°15′W), Valparaiso (33°03′S - 71°38′W), Metropolitana (33°26′S - 70°39′W), O'Higgins (34°10′S - 70°43′W), Maule (35°25′S - 71°39′W), Biobío/Ñuble (36°46′S - 73°03′W), Araucanía (38°44′S - 72°35′W), Los Ríos (39°48′S - 73°14′O) and Los Lagos Regions (41°28′S - 72°56′O). In total, 612 honey bee colonies were sampled. A pooled sample of 10 honey bees per colony was used for RNA isolation and cDNA synthesis, according to the methodology reported by Vargas et al. (2017). In order to detect the prevalence of DWV-A, DWV-B, DWV-C, and the presence of certain DWV-recombinants in Chilean honey bee apiaries, a strand-specific real-time PCR (qPCR) (Stratagene Mx3000P, Agilent Technologies, CA) was conducted according to Vargas et al. (2017), using specific primers reported by Kevill et al. (2017) and McMahon et al. (2016). (Table S1, Supplementary Material). On the other hand, in order to screen for possible recombinants, qPCR amplification in two regions at either end of the genome, which included the leader polypeptide (Lp, 5′ end of genome) and RNA-dependent RNA polymerase (RdRp, 3′ end of genome), were conducted according to Natsopoulou et al. (2017). The PCR reactions were carried out in 15 µL, containing 20 ng of cDNA, 1X of KAPA SYBR FAST Universal 2X qPCR

93 Master Mix (Kapa Biosystems, Wilmington, Massachusetts, USA), 530 nM of each primer, and
94 filled with sterile-filtered molecular grade water. The thermal conditions were achieved with one
95 cycle at 96 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 15 s and 72 °C for
96 30 s. A dissociation analysis was conducted after all of the amplifications were completed in
97 order to detect the primer dimmers and the unspecific amplicons. Primers designed by Yang and
98 Cox-Foster (2005) based on the β-actin gene were used as an internal control (Table S1). A
99 positive control for DWV-A and negative control sample, were included in all of the reactions.
100 Real time PCR assays were performed in a thermocycler Stratagene Mx3000P (Agilent
101 Technologies, Santa Clara, California, USA), and the data were analyzed using MxPro software
102 (Stratagene). Some amplicons that tested positive for DWV-variants (DWV-A (n = 6) and DWV-
103 B (n = 4) were purified and sequenced in both directions by Macrogen (Macrogen, Seoul, South
104 Korea). Analyses of the homologies with other DWV sequences were carried out with the Basic
105 Local Alignment Search Tool (BLAST, NCBI) and were aligned online using Clustal Omega
106 (EBI, Cambridge, UK) with other sequences previously reported as DWV-variant genomes
107 (NC_004830 and AY251269).

108 In order to determine the viral load in positives cases, an absolute quantification of
109 DWV-variants was performed. In brief, we first performed a standard curve using purified PCR
110 product (Wizard® SV gel and PCR clean-up system, Promega, Madison, *WI*, USA) belonging to
111 the viral target sequence. Then, the purified amplicon was quantified (Epoch™ Microplate
112 Spectrophotometer, BioTek, VT, USA) to calculate the copy number according to Wu et al.
113 (2017). Linear standard curves (efficiency 95-100%) were then generated with the serial dilution
114 (10^{-1} to 10^{-9}) of viral copy numbers of purified cDNA; this was followed by the plotting of Ct
115 values against copy number values ($\log_{10}$). Thus, the sample copy numbers were estimated by

using the Ct values and comparing them with the lineal equation of the standard curve and then normalizing values to the housekeeping β-actin gene. Afterwards, data were expressed as the number of copies of DWV per bee by taking into account the dilutions that were performed in the cDNA synthesis and qPCR reaction.

The data regarding positive cases of DWV-A, DWV-B and mixed infections (DWV-A + DWV-B) are shown as percentages of infected colonies in relation to the total number of colonies analyzed (612 honey bee colonies); although, a Chi-square test (X^2) was performed to assess for significant differences ($p < 0.05$) between the observed and expected frequencies of DWV-variants and mixed infection. Then, pairwise multiple comparisons among DWV-A, DWV-B and mixed infections were run; *p*-values were corrected with the Holm-Bonferroni method (Holm, 1979).

Provided that the data regarding DWV-variants load in infected colonies were not normally distributed (Shapiro–Wilk test, $p < 0.05$), a Mann-Whitney U test ($p < 0.05$) was performed to compare DWV-variants load per bee in the positive colonies with single and mixed-DWV infection. Also, DWV-variants load (DWV-A or DWV-B copy number per bee) in each region of Chile was compared using a Kruskal-Wallis H test ($p < 0.05$) followed by *post-hoc* multiple comparisons of mean ranks for all groups.

To compare viral load in colonies that were either treated or not treated against *V. destructor* in different regions of Chile, we used information provided by beekeepers, in which case, only 312 colonies were considered for this analysis. Thus, a Mann-Whitney U test was performed to compare viral load in colonies that were treated (n = 255) and not treated (n = 57) against *V. destructor*. All statistical analyses were performed with STATISTICA 7.0 software (StatSoft, Tulsa, OK, USA).

Real-time PCR using specific primers allowed us to detect variants of DWV in honey bee samples from apiaries distributed throughout Chile (Figure 1A). The DWV-A variant (71%) was significantly (X^2 = 618.76; $p < 0.001$; df = 1) more prevalent than the DWV-B variant (circa 3%), while the DWV-C genotype was not detected in the honey bee samples analyzed in this study. Colonies infected with the DWV-A variant contained higher levels (copy number per bee) of this genotype compared to the DWV-B variant (Mann-Whitney test, U = 935.00, $p < 0.001$) (Figure 1B). Similarly, levels of DWV-A varied among regions and were significantly higher (Kruskal-Wallis test, $H_{(8, N = 436)}$ = 118.69; $p < 0.001$) in regions located in northern rather than southern Chile (Figure 1C). Mixed infections (DWV-A + DWV-B) were also detected, showing significant differences (Mann-Whitney test, U = 16.00, $p = 0.003$) in DWV-variant loads in bees with these DWV-genotypes (DWV-A median 5.62 x 10^5 copy number per bee (min 2.69 x 10^4 – max 5.89 x 10^9 copy number per bee); DWV-B median 1.86 x 10^4 copy number per bee (min 5.37 x 10^3 – max 1.00 x 10^6 copy number per bee)). However, mixed infections had a very low prevalence (< 2%) compared to DWV-A (X^2 = 636.55; $p < 0.001$; df = 1), but not significantly different than DWV-B (X^2 = 0.95; p = 0.330; df = 1). On the other hand, DWV-A/B recombinants were not detected in the analyzed samples. These results may be explained by the fact that we detected a low prevalence of colonies with mixed infections (< 2%), considering that the opportunity for emergent DWV recombinant types is provided when different genotypes infect and remain in the same host (Dalmon et al., 2017). However, deep sequencing analyses are still required to definitively discard the presence of DWV recombinant types in Chilean apiaries.

In order to verify the DWV-variants detected by qPCR, positive samples were subjected to sequencing, in which case, sequencing data confirmed our findings, identifying DWV-variants such as DWV-A and DWV-B, according to reference genome sequences NC_004830 and

AY251269, respectively (Fig. S1, Supplementary Material). To the best of our knowledge, this is the first report involving DWV-B in Chile. Recently, DWV-B has been reported in Brazil in stingless bees and Africanized honey bees (de Souza et al., 2019), but not in European honey bees in the neighboring South American countries. Nonetheless, DWV-B has only recently increased in importance, since new information has reported its possible negative impacts on honey bee health (McMahon et al., 2016; Natsopoulou et al., 2017; Ryabov et al., 2017). Field surveys and laboratory assays have demonstrated that the emergent DWV-B genotype is more virulent than the established DWV-A (McMahon et al., 2016; Natsopoulou et al., 2017). In fact, honey bees infected with DWV-B have proven to survive less than those with DWV-A, driving colonies to collapse sooner than those infected with the DWV-A genotype (McMahon et al., 2016). Fortunately, we found a low prevalence and low loads of the DWV-B genotype in Chilean honey bees (median 1.80×10^4 and mean 2.18×10^5 copy number per bee, Fig. 1C), relative to those found in VDV-1 positive colonies in the United States (mean 7.45×10^{12} copy number per bee) (Ryabov et al., 2017) and in England and Wales (mean 1.14×10^{12} genome equivalent per bee) (Kevill et al., 2019). On the other hand, Natsopoulou et al. (2017) found that both the loads and prevalence of DWV-B were significantly reduced in honey bee samples taken in the spring, compared to those taken in autumn. Since we only have data from samples collected in the spring-summer and no information from Chilean colonies in autumn-winter, we can make no comparisons among seasons regarding the prevalence of DWV-variants. Therefore, additional samples are required in the autumn and winter in different years in order to compare among seasons, and thus avoid a possible underestimation of the prevalence and load of DWV-B, considering that this variant is more frequent in colder seasons in temperate climates (Natsopoulou et al., 2017). In this study, we found that 50% of the samples had almost 60-times

185 more copy numbers per bee of DWV-A (median 1.03 x 10^6 copy number per bee) than DWV-B
186 (median 1.80 x 10^4 copy number per bee). Also, the highest DWV-A levels were detected in
187 northern Chile (Kruskal-Wallis test, H (8, N = 436) = 116.16 $p < 0.001$), in the Coquimbo
188 region. This viral level may be related to a *V. destructor* infestation, considering that when the
189 varroa mite has been found in high levels in honey bee colonies, high levels of DWV have also
190 been detected with low strain diversity, generating a dominance of certain variants (Di Prisco et
191 al., 2011; Martin et al., 2012). However, we do not know the prevalence of *V. destructor* in the
192 samples tested from this region and thus, cannot speculate regarding the possible relationship
193 between DWV-A and this ectoparasite. Nevertheless, we expected analyzed colonies exposed to
194 control measures for mites to show reduced DWV-A loads compared to untreated colonies. This
195 hypothesis was partially true in some Chilean regions, wherein the viral load (DWV-A) was
196 lower in treated colonies located in the Metropolitana (Mann-Whitney test, U = 7.00, $p = 0.021$),
197 Biobío/Ñuble (U = 247, $p < 0.001$) and Los Ríos Regions (U = 13.00, $p = 0.009$) (Fig. 2). In
198 fact, there were no statistical differences in viral levels in *Varroa*-treated and untreated colonies
199 in the remaining studied regions, especially in northern Chile (Fig. 2). However, we reported
200 only the available information provided by beekeepers regarding colonies that were treated and
201 untreated against *V. destructor*, in which case, 312 out of 437 colonies (DWV-A infected
202 colonies) were evaluated in this parameter (Table S2, Supplementary Material). Therefore, these
203 data could underestimate the potential viral loads as well as their relationship with mite control
204 measures. However, DWV-A infected colonies in each region (Fig. 1C) were compared in terms
205 of their *V. destructor* treatment status (treated and untreated) (Fig. 2), and they proved to show
206 similar trends in viral levels, which a *priori*, rules out the aforementioned assumption. Over 90%
207 of the total treated colonies were treated with synthetic acaricides based on flumethrin and

208 formamidine (amitraz), e.g. Verostop (Primavet, Sofia, Bulgaria) and Amivar 500 (Apilab SLR,
209 Tandil, Argentina). There is evidence that DWV load can increase when colonies are treated with
210 chemical compounds against *V. destructor* (Locke et al., 2012), in which case, the acaricide
211 could have negative impacts in physiology and/or immune system responses of honey bees,
212 causing increased host susceptibility to DWV infection (Locke et al., 2012; Tihelka, 2018). This
213 may explain the lack of differences in DWV load in *Varroa*-treated and untreated colonies in
214 northern Chile, considering that DWV loads may remain high for weeks after *V. destructor*-
215 infested colonies were treated (Locke et al., 2012; Locke et al., 2017), a period that could have
216 coincided with the bees sampling for this study. On the other hand, the lack of differences in the
217 viral load of *Varroa*-treated and untreated colonies might also be associated with a resistance to
218 synthetic acaricides by *V. destructor,* provided that the active ingredients (flumethrin and
219 formamidine) of the acaricides used in Chile have proven to induce resistance by *V. destructor*
220 populations (Rodríguez-Dehaibes et al., 2005). This resistance would therefore reduce these
221 treatments efficacy to control varroa mite, consequently resulting in non-significant changes in
222 DWV loads in colonies that were treated and untreated against this parasite.

223 Our findings suggest that DWV-B is a new emergent genotype in Chile and could be a
224 significant problem, especially in the winter season due to its putatively higher virulence, even in
225 the absence of its vector, the varroa mite (McMahon et al., 2016). In this sense, Ryabov et al.
226 (2017) reported the recent spread of VDV-1 (DWV-B) in the United States; when they analyzed
227 75 colonies sampled in 2010, they found that circa 3% tested positive for DWV-B, but when they
228 analyzed 603 apiaries sampled in 2016 they found that DWV-B was present in 66% of them.
229 Therefore, it is imperative to monitor the DWV-variants, especially DWV-B and their

230 recombinants in order to establish their possible impacts on Chilean apiaries or their association
231 with *V. destructor* and other pathogens present in the country.
232
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236
237 **References**
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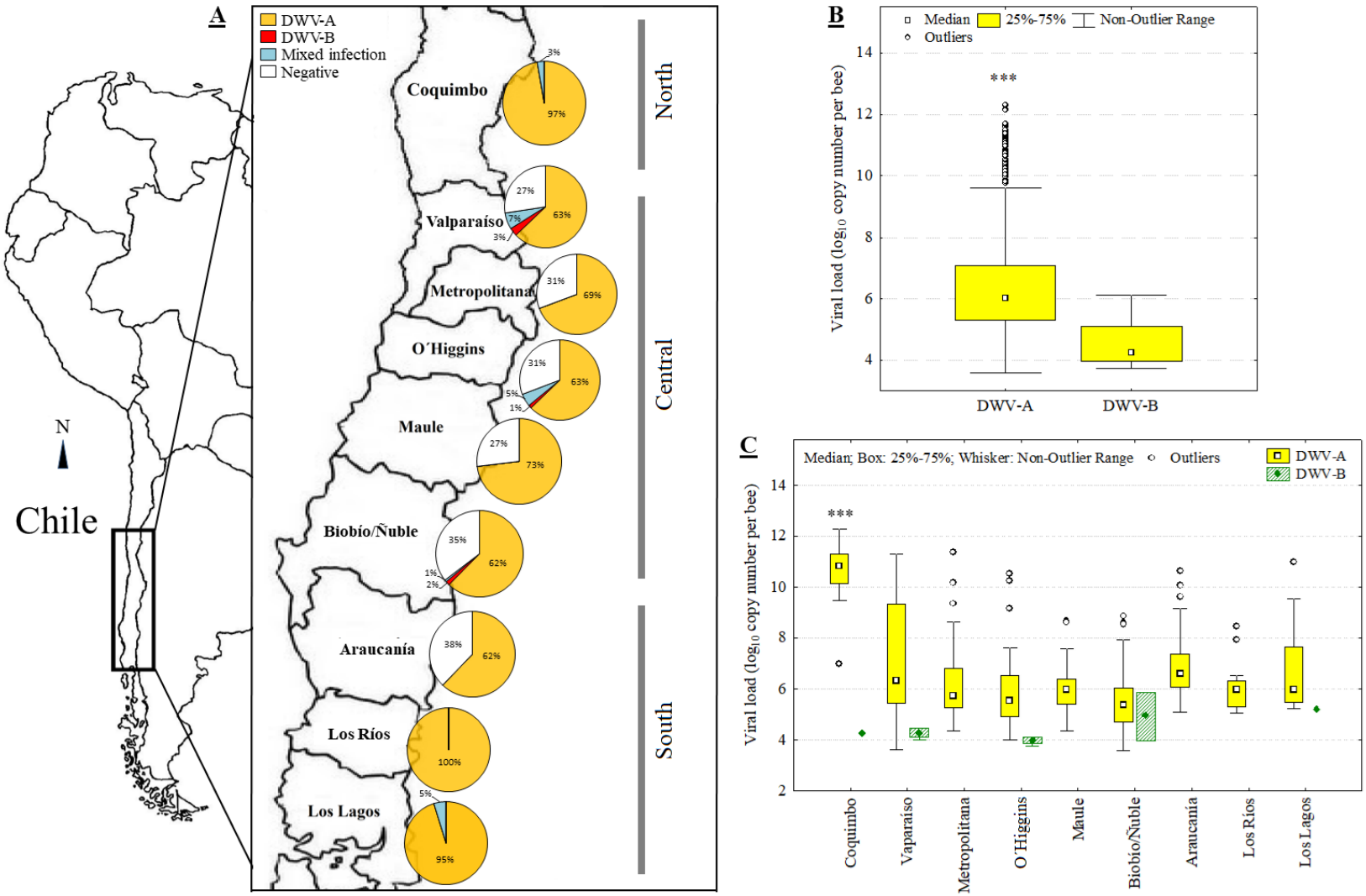

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341 **Fig. 1**. Prevalence of DWV-variants and mixed infections (A), total viral load in the country (B) and per region (C) detected in

342 Chilean honey bee colonies. Asterisks in the boxplot indicate significant differences (*** = $p < 0.001$) between DWV-A and DVW-

B according to the Mann-Whitney U test (B) and Kruskal-Wallis H test (C) followed by *post-hoc* multiple comparisons of mean ranks for all regions.

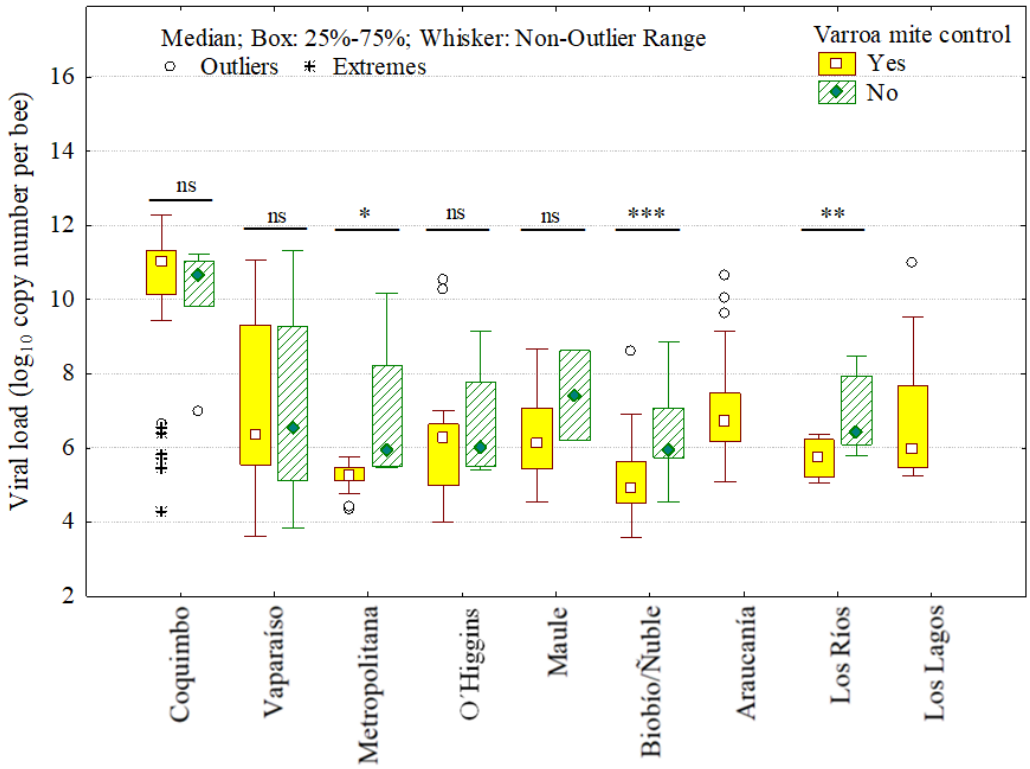

Fig. 2. DWV-A loads detected in Chilean honey bee colonies that were treated and not treated against *Varroa destructor*. Asterisks in the boxplots indicate significant differences between treated (n = 255) and untreated (n = 57) colonies according to the Mann-Whitney U test (*** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$; ns = no significant differences). There were no data for untreated colonies in the Araucanía and Los Lagos regions.