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COMPARISON BETWEEN THE EFFECTIVENESS AND SENSITIVITY OF THE SUGAR SHAKE METHOD VERSUS THE SOAPY WATER WASHING TECHNIQUE TO DETECT PHORETIC MITES OF *Varroa destructor*

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ABSTRACT

Colony infestation caused by *Varroa destructor* is a major concern in the apiculture industry because it often results in production losses and reduced colony survival. Over the years, several diagnostic methods have been developed to estimate Varroa infestation levels in a colony, being *Soapy Water Washing* (SWW) and *Sugar Shake* (SS) the most widely used methods. However, the effectiveness and sensitivity of the latter remain unclear. This represents a potential risk for beekeepers who use SS as it could lead them to underestimate the infestation level of their colonies, potentially delaying treatment application. The objective of this study was to evaluate the effectiveness and sensitivity of SS as compared to the "*gold standard*" SWW, for the detection of *V. destructor* in adult bees. Ninty-nine samples were collected and divided into 3 groups according to infestation rate (IR): low (<3%); medium (3.01-5.0%); and high (≥5.01%). It was found that the SS and SWW methods showed 76.6% and 100% effectiveness of mite removal, respectively ($p < 0.05$). The sensitivity of SS was lower as compared to SWW. In samples with a low IR, 5 of them resulted in false negatives and 23 had a poorly estimated IR. This did not occur in samples with a medium or high IR. Our results suggest that the SS method is less efficient in detecting and removing phoretic mites in adult bee samples, which can underestimate Varroa infestation levels, especially when the number of mites is low.

Key words: Effectiveness, sensitivity, mite, infestation rate.

INTRODUCTION

The *Varroa destructor* mite is an ectoparasite transmitted via direct contact between adult bees due to the movement of infested individuals, as well as the contact with contaminated bees or biologic materials such as infested brood, nucs or queen (OIE, 2017). This mite feeds on hemolymph and fat bodies, causing a huge damage at individual and colony levels (Ramsey et al., 2019) because it reduces the immune response and is a vector of many virus diseases (Riveros et al., 2019). Currently, it constitutes the greatest threat to apiculture. In fact, no other pathogen has had a comparable impact throughout the history of this industry (Rosenkranz et al., 2010). Infestation often results in production losses, lower colony viability for wintering, and higher production expenses (Bounous and Boga, 2005).

In recent years, *Varroa destructor* is considered the main cause of bee colony loss in Chile (Maggi et al., 2016). It has been present in the

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country since 1992, being a widely distributed pathogen (Lesser, 1998; Maggi et al., 2016). As it has not been eradicated, the severity of varroa infestations needs to be kept below damaging thresholds, which corresponds to an infestation rate (IR) not exceeding more than 3 phoretic mites per 100 adult bees (IR <3%) (low infestation rate). Medium and high infestations are those between 3.01% and 5%, and greater than 5.01%, respectively (Aldea et al., 2013).

There are different diagnostic methods for the detection and objective evaluation of *Varroa destructor* presence in a colony. Some of these methods include: (i) Ether roll, (ii) Alcohol wash, (iii) Capped brood examination, (iv) acaricide strips (Ellis and Macedo, 2001), v) natural mite fall, vi) Soapy wash, and vii) Sugar shake (Branco et al., 2006; Pietropaoli et al., 2021). Unfortunately, some of these methods result in bee death due to the nature of the sampling and analysis processes. In addition, there is no clear information on the effectiveness of some methods in determining infestation levels in a colony (Aldea et al., 2013; Oliver 2020a; Oliver 2020b; Pietropaoli et al., 2021). Furthermore, some methods can be expensive for beekeepers.

There is a low-cost technique called *Soapy Water Washing* (SWW), also known as the "De Jong Method," which uses soapy water to remove mites (Goodwin and Cliffvan, 2001). Another method to control varroa infestation is called *Sugar Shake* (SS), which consists of covering a sample of bees with powdered sugar in a container. The container is then shaken for a few minutes, causing the mites to lose their grip and fall. This method stimulates the grooming behavior of bees, favoring natural mite removal (Dietemann et al., 2013), and allowing for determining the number of mites present in a sample (Yates, 2013). Once sampling is performed, the sampled bees can be returned to the colony because powdered sugar does not represent a risk to the other bees as it does not enter or accumulate in bee spiracles (Fakhimzadeh, 2001).

In recent years, SS has gained popularity, being even used as a reference for selecting colonies showing tolerance to *Varroa destructor* (Büchler et al., 2013; Fan et al., 2017). However, there are few studies that have comparatively evaluated the effectiveness and sensitivity of SS versus SWW to detect varroa, particularly because SWW is now considered the "*gold standard"* method. In this sense, it is important to determine if SS differs from SWW in terms of effectiveness or sensitivity as this would allow for better informed decisions by beekeepers. According to Bak et al. (2009), the effectiveness of mite removal by SS is similar to that of SWW, while its sensitivity reaches 93%. However, the procedure for sample analysis used by the authors partly differs from that recommended for SS by Dietemann et al. (2013), and for SWW by the International Office of Epizootics (OIE).

Dietemann et al. (2013) stated that it is essential to obtain all mites in a standardized and safe way to be able to compare infestation levels among samples. In this way, it is easier to compare the results obtained and, therefore, calculate the effectiveness of the diagnostic method used. In this sense, it is critical to find an easy-to-conduct, effective and reliable method of sampling regardless of the level of infestation. Finally, a good diagnostic method should allow for a timely treatment of the disease (Lee et al., 2010), or the selection of bee colonies with greater resistance to mites. If this could be done on-site (in the apiary) and as a control technique in beekeeping (Büchler et al., 2013), varroa control would be faster and more effective.

As beekeepers are increasingly using SS, it is important to know if this method is reliable, particularly for the exports of bees and been queens. In this sense, international standards allow a mite infestation rate of less than 1.0% in adult bees as determined by SSW (SAG, 2018). Therefore, the objective of this study was to evaluate the effectiveness and sensitivity of *Sugar Shake* as compared to *Soapy Water Washing,* for the detection of *V. destructor* in adult bees. The following specific objectives were proposed: 1) to determine the effectiveness of SS to remove phoretic mites compared to the standard method; 2) to determine the sensitivity of SS in mite detection; and 3) to compare the effectiveness and sensitivity in mite detection by SS using samples with different infestation rates (low, medium, and high).

MATERIALS AND METHODS

Sampling was performed in 25 apiaries located in the O'Higgins, Metropolitan and Valparaíso regions of Chile, between 32º 02' and 34° 45' south latitude and between 70° and 72° W longitude.

For SS, sampling to detect *V. destructor* was performed *in situ* in the apiaries. Once the procedure was completed, the bee samples were stored in hermetic bags and taken to the laboratory where the SWW method was applied. Both procedures were performed according to the protocols recommended by COLOSS (Dietemann et al., 2013; Fan et al., 2017).

Based on previous studies conducted by De Jong et al. (1982), Macedo et al. (2002) and Durán and Calzadilla (2011), 99 samples were used from Langstroth-type colonies of the species

Apis mellifera (n=99), which are used for various commercial purposes. Colonies with at least 8 frames covered by bees were used and one sample was obtained for each colony. The samples were grouped into sets of 33 samples according to their level of infestation (low, medium, or high). The bee samples were included in the statistical analyses.

Sampling was conducted by beekeepers, excluding weakened colonies or those whose queen could not be found at the time of sampling. Each sample was taken using a jar of 90 mL, collecting approximately 200 bees that were extracted from brood frames in the breeding chamber. Once SS was performed, the same bee samples were analyzed using SWW to determine if SS was capable of removing all the mites from the sample. To analyze the results, the samples were grouped according to the infestation rate (IR) obtained using SWW.

For SS, roughly 200 adult bees were collected from breeding frames using 83-ml containers. Once the absence of the queen was confirmed, the samples were placed into a special container with a mesh lid. This allowed for mite detachment during agitation, while it retained the bees. Before adding the bees to the sampling bottle, two tablespoons of powdered sugar were added. Once the bees were introduced, the bottle was gently rolled to ensure that they were covered in sugar, and then let to rest for at least a minute (Dietemann et al., 2013). The bottle was then manually shaken for a period of one minute, and the powdered sugar was then removed and placed on a white surface that contrasted with the mites that had fallen (Fan et al., 2017). The mites obtained were then counted. Subsequently, the IR obtained by SS was determined. The bee samples were placed in hermetic bags and maintained in alcohol diluted to 75% to avoid their decomposition during transport. In addition, each sample was labeled with the name of the beekeeper, the colony number, and the number of mites obtained using SS. In the laboratory, the samples were immediately analyzed using SWW.

For SWW, the same samples (where SS was applied) were used, following the protocol recommended by the OIE. Each sample of bees was transferred to a bottle and a 2% soapy water solution was added until completely covered. Then, the bottles were covered and stirred for one minute and a half. Once the process was completed, the contents were drained using a double sieve: a 3 mm sieve for retaining the bees, and another 0.5 mm mesh for retaining the mites (Bak et al., 2009). Subsequently, the sample (while still in the double sieve) was rinsed under a jet of water to ensure to wash off the last hidden mites. Then, the sieve that contained the mites was checked to confirm their presence. The whole process was repeated three times, ensuring that the method removed all the mites that the sample could possibly contain, and those that SS might not have removed. The parasites obtained by using SWW were counted. This allowed comparing the number of mites and IRs obtained with each method. Likewise, the bees of each sample were also counted. The infestation rate of each colony was calculated based on the following formula:

$$Infestation\ rate\ (IR) = \frac{N°\ of\ mites\ obtained}{N°\ of\ total\ bees\ in\ the\ sample} * 100$$

The samples were grouped according to the IR obtained by SWW. IR values were classified as follows: low (IR ≤ 3%), medium (IR= 3.01 to 5%) and high (IR ≥ 5.01%) (Aldea et al., 2013; Honey Bee Health Coalition, 2015).

The effectiveness (% of mites removed) of SS (Objective 1) was calculated by applying the following formula to each of the 99 samples collected. Subsequently, the average was calculated along with its standard deviation.

$$SS\ effectiveness = \frac{N°\ of\ mites\ removed}{N°\ of\ total\ mites\ in\ the\ sample} * 100$$

The sensitivity (% of samples in which at least 1 mite was detected) of SS (Objective 2) was calculated using the following formula (applied to the whole sample set):

$$SS\ sensibility = \frac{N°\ positive\ detections}{N°\ of\ positive\ detections + N°\ false\ negatives} * 100$$

Where "N° of positive detections" are the mites recovered by SS and the "N° of false negatives" are the mites recovered by SWW.

The effectiveness of SS was determined in populations with low, medium, and high infestation rates (Objective 3) for a total of 99 samples (33 for each group). Effectiveness among the three populations was calculated with the same formula used in Objective 1. To determine and compare the sensitivity of the method among populations according to their IRs, the same formula applied to Objective 2 was used.

Statistical analysis

The average effectiveness of mite removal obtained with the SS and SWW methods were compared, considering samples with low, medium, and high infestation rates. A statistical analysis based on the non-parametric Wilcoxon test for related samples was performed.

To determine if there was an association between the capacity of SS to discriminate between positive and negative samples, a Mann-Whitney-Wilcoxon test was performed because the Levene test yielded a value of p=0.04 when the equality of variances was determined. This led us to conclude that variances were not equal between both groups. To determine if the sensitivity of SS was affected by IR, the Fisher-Freeman-Halton exact test was used with one degree of freedom (Freeman et al., 1951; IBM, 2017; NCSS, 2017).

The statistical software used in the analyses was IBM SPSS Statistics 23 and a 95% confidence interval was used. The graphics were made using SigmaPlot 11.0 software.

RESULTS

The average IR obtained with SS was 4.16 ± 4.58%, with a minimum value of 0.0% and a maximum value of 22.93%, whereas the average IR obtained with SWW was 5.24 ± 5.30%, with a minimum value of 0.33% and a maximum value of 28.66%. Therefore, there were difference between the methods, resulting in different IRs according to the method used. SS showed an average removal effectiveness of 76.60% with respect to SWW (Table 1); there were 5 cases in which no mites were removed, 34 cases in which fewer mites were removed with respect to SWW, and 60 cases in which all mites were removed by SWW (Fig. 1a, b and c).

When comparing the average effectiveness of SS and SWW in all the samples, significant differences were found between the methods in terms of mite removal capacity ($Z=6.69$, $p<0.05$). The results are shown in Table 1.

Differences were also found in the amount of phoretic mites that were detached by SS in samples with low, medium, and high IRs. Thus, in those samples with a low IR, there were 5 samples with an effectiveness (removal rate) of 0%, 11 samples with an effectiveness that varied between 17% and 88%, and 17 samples with an effectiveness of 100%. In this same group of results, SS obtained an IR of less than 1% in 4 samples, while this value was much higher when SWW was used (Fig. 1a). In samples with medium IR values, SS showed greater effectiveness as there were no samples where no mites were removed even when present, 25 samples where effectiveness fluctuated between 15% to 93%, and 8 samples where effectiveness was 100%. It is important to point out that, in 16 samples, SS resulted in an IR of less than 3%, which corresponds to a low infestation level. However, when using SWW, the resulting IR was greater than 3.01%, which corresponds to a medium infestation level (Fig. 1b). Finally, in those samples with a high IR, there were no samples where no mites were removed, 24 samples where effectiveness was between 17% and 97%, and 9 samples where it was 100% (Fig. 1c). As in the previous group, when using the SS method, 3 samples with an IR of less than 3% (low IR) and 4 samples with an IR between 3.01-5.00% (average IR) were classified erroneously, unlike the IR determined by SWW, which was higher (Table 2).

Table 2 summarizes the IR values obtained when using SS and SWW. The average effectiveness of mite removal for the groups of samples with low, medium, and high IRs was 73.80%, 76.2% and 79.8%, respectively. According to the Wilcoxon Test, significant differences were found in terms of infestation rate and effectiveness of mite removal by SS when the IR is low ($Z= -5.03$, $p<0.05$), medium ($Z = -5.11$, $p<0.05$) or high ($Z = -5.15$, $p<0.05$), compared with SWW.

The sensitivity of SS was 94.95% for the total number of samples (Table 3). When comparing the sensitivity of the method in relation to the IR of the sample, it was found that this difference was statistically significant ($t= -9.22$, $p<0.05$), indicating that there is a difference in the sensitivity of the test. The mean difference was -0.051 with a 95% confidence interval, ranging between -0.062 and -0.039 (Table 3).

When comparing the sensitivity of SS according to the infestation rate of the samples, it was found that sensitivity was 84.85% for the samples with a low IR and 100% for the samples with medium and high IRs. Therefore, in the samples with a

Table 1. Comparison between the effectiveness of phoretic mite removal using the *Sugar Shake* (SS) and *Soapy Water Washing* (SWW) methods for the total number of samples.

Diagnostic method	Infestation rate (%) Mean ± 1 D.E.	Minimum value	Maximum Value	Average effectiveness (%)	Wilcoxon test
Sugar Shake (SS)	4.16 ± 4.58	0.0	22.93	76.60	Z = 6.69
Soapy Water (SWW) Washing	5.24 ± 5.30	0.33	28.66		p < 0.05

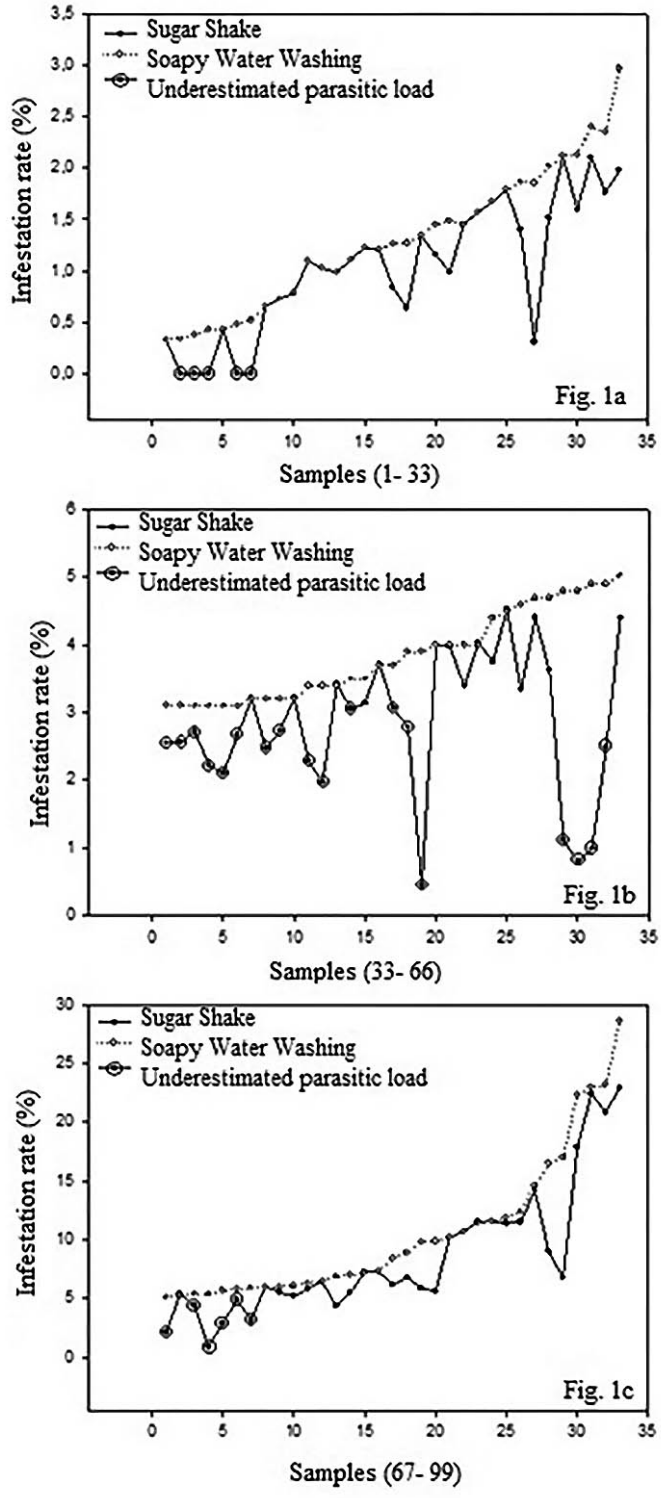

Fig. 1. Effectiveness of the *Sugar Shake* (SS) method compared with Soapy Washing Water (SWW) in the removal of phoretic mites according to samples with low (1a), medium (1b) and high (1c) infestation rates. Results where the mite load was underestimated are identified.

Table 2. Comparison between the effectiveness of mite removal using the *Sugar Shake* (SS) and *Soapy Water Washing* (SWW) methods for groups with low, medium, and high infestation rates.

	Low infestation rate (%)					
Diagnostic method	**Mean ± 1 D.E.**	**Minimum value**	**Maximum value**	**N° of wrongly classified samples**	**Average effectiveness (%)**	**Wilcoxon test**
Sugar Shake (SS)	1.02 ± 0.64	0.0	2.12	5	73.80	Z = -5.03 p < 0.05
Soapy Water Washing (SWW)	1.29 ± 0.68	0.33	2.97			
	Medium infestation rate (%)					
Diagnostic method	**Mean ± 1 D.E.**	**Minimum value**	**Maximum value**	**N° of wrongly classified samples**	**Average effectiveness (%)**	**Wilcoxon test**
Sugar Shake (SS)	2.88 ± 1.03	0.45	Z = -5.03 p < 0.05	16	76.2	Z = -5.11 p < 0.05
Soapy Water Washing (SWW)	3.84 ± 0.69	0.33	5.00			
	High infestation rate (%)					
Diagnostic method	**Mean ± 1 D.E.**	**Minimum value**	**Maximum value**	**N° of wrongly classified samples**	**Average effectiveness (%)**	**Wilcoxon test**
Sugar Shake (SS)	8.57 ± 5.61	0.91	Z = -5.03 p < 0.05	7	79.80	Z = -5.15 p < 0.05
Soapy Water Washing (SWW)	10.58 ± 6.14	5.16	28.66			

Table 3. Sensitivity of the *Sugar Shake* (SS) and *Soapy Water Washing* (SWW) methods in the detection of at least one phoretic mite in the total number of samples.

	Levene's Test for Equality of Variances		**t-test for the means equality**							
Infestation rate	**F**	**p**	**t**	**F.D.**	**Sig. (2-tails)**	**Differences between the means**	**Difference between the standard error**	**95% i.c. between the means**		**Sensibility of SS (%)**
								Lower	**Higher**	
Assumed equality of variances	3.97	0.04	-2.12	97	0.04	-0.0507	0,0239	-0.098	-0.003	94.95
Not assumed equality of variances			-9.22	93.82	0.05	-0.051	0,006	-0.062	-0.039	

low IR, 15.15% of samples were found to be false negatives when SS was used (Table 4). According to the Fisher-Freeman-Halton exact test, this difference was significant (F = 7.921, p <0.05).

DISCUSSION

There are few studies that examine the effectiveness of the different methods used to determine Varroa infestation rates. Branco et al. (2006) and Lee et al. (2010) evaluated the capacity to estimate the parasitic load in a colony by comparing the results of the most widely used methods such as natural mite fall, *Soapy Water Washing* or capped brood estimation (Fan et al., 2017) compared to SWW with a new agitation method, which proved to be highly effective in collecting small mites, and equally effective in collecting mites in general. However, none of these studies used the same sample of adult bees to evaluate if the method used resulted in the complete removal of mites (Branco et al., 2006; Fan et al., 2017; Lee et al., 2010). Because of this, the present study evaluated the potential false negatives or errors when evaluating infestation rates to determine the differences between SS and SWW in terms of accuracy, sensitivity, and effectiveness. A relatively similar study was conducted by Bak et al. (2009), but their results differed from those obtained herein. The authors found that there were no statistically significant differences between IRs determined by SS or SWW (10.43 and 11.09, respectively). Conversely, our results indicate that the effectiveness of SS was significantly lower than that of SWW, which is considered the reference method by the OIE (p <0.05). This discrepancy could be explained by the fact that the authors made three measurements in two samples of bees from the same colony. Firstly, they measured the effectiveness of SS in a sample of bees, then the effectiveness of SWW in a second sample, and finally evaluated the effectiveness of SS by applying SWW to the first sample. In the present study, effectiveness was determined by using a single sample and applying SS followed by SWW to verify the effectiveness of the former in relation to the "*gold standard*" method. Furthermore, when verifying the sample of bees that had been subjected to SS followed by SSW, Bak et al. (2009) used only one wash. In contrast, the present study used three washes with one rinse under a water jet between each wash. This method ensured that all the mites that could have remained in the sample were effectively removed, obtaining the same effectiveness as De Jong (1982), i.e., 100% effectiveness by continuous shaking with a mechanical shaker for 30 minutes.

The results of the present study showed that total IR differs depending on the diagnostic method used (Fig. 1). SS was found to be less effective in removing mites as there were cases where it did not remove any mites or removed few mites, resulting in different IR values than those obtained using SWW. This was even more evident given the lower IR values or parasitic load of the sample as an IR of less than 1% corresponded to just 5 samples where mites were not removed. Of the 99 samples collected, SS resulted in an IR that was lower than the actual value in 28 samples (5, 16 and 7 samples with low, medium, or high IR, respectively). This tendency to underestimate could be used in integrated beekeeping management (Lee et al., 2010) by helping determine the appropriate treatment of an infested colony (Dietemann et al., 2013), or the commercialization of biological material or bee families exhibiting mite resistance (Büchler et al., 2013), particularly if this method were the only method used to determine the infestation rate.

The sensitivity of SS reached 94.9% in the present study, which agrees with that (93%) reported by Bak et al. (2009). Furthermore, SS

Table 4. Sensitivity of the *Sugar Shake* (SS) method in the detection of positive and false negative samples according to infestation rate.

Infestación rate		Sensitivity		Total	Fisher's exact test
		Yes	No		
Low	Quantity	28	5	33	F = 7.921
	% inside Infestation Rate	84.85%	15.15%	100.0%	p < 0.05
Medium	Quantity	33	0	33	
	% inside Infestation Rate	100.0%	0.0%	100.0%	
High	Quantity	33	0	33	
	% inside Infestation Rate	100.0%	0.0%	100.0%	
Total	Quantity	94	5	99	
	% inside Infestation Rate	94.95%	5.05%	100.0%	

resulted in 5.05% of false negative samples of the total number of samples. In this sense, SS was clearly superior in samples with medium and high IRs, where the sensitivity was the same as with SWW (100%). In the samples with low IRs, the sensitivity was 84.85%, which agrees with previous studies (Bak et al., 2009; Macedo et al., 2002). This indicates that IR is an important factor to determine the sensitivity of the method. In this group of samples, 15.15% of false negative samples were obtained, with significant differences according to the Fisher-Freeman-Halton exact test ($F= 7.921$, $p <0.05$).

Although SS allows estimating the level of infestation of a colony, the underestimation of parasitic loads resulting from the use of this method leads to a significant margin of error with respect to SWW. This was more evident in those samples with low IR values, where SS showed decreased effectiveness and sensitivity (Tables 2 and 4). Therefore, the use of SS could represent a risk for those beekeepers whose colonies have IR values fluctuating between low and medium values, particularly when the number of samples taken in the apiary is 10% of the colonies or less. This can impact on the decision of not using an acaricidal treatment, preventing a timely treatment of infestations or the implementation of measures towards an integrated management of the disease (Aldea et al., 2013; Lee et al., 2010). Consequently, parasites would spread to other colonies or apiaries, eventually resulting in increased maintenance costs, production losses, and lower colony viability (Bounous and Boga, 2005; Rosenkranz, 2010; Sanabria, 2015). In addition, breeders of queens for export, who use SS for estimating IR, can have considerable losses because of loads rejected owing to levels of infestation exceeding the acceptable limits (less than 1% in adult bees) required by the Chilean Agricultural and Livestock Service (SAG, 2018).

Further research is required to standardize SS since it could become a method of choice for the diagnosis of *Varroa destructor* since it is faster and easier to perform, can be conducted on-site (in the same apiary), has a low level of bee mortality during sampling, and is more environmentally friendly (Dietemann et al., 2013). However, the effectiveness and sensitivity of this method is lower compared with the diagnostic method of reference *Soapy Water Washing* (OIE, 2015; SAG, 2018).

CONCLUSIONS

The effectiveness of the *Sugar Shake* method in estimating the infestation rate in honey bee colonies was significantly lower compared with the Soapy Water Washing method. Similarly, the sensitivity of SS was lower with respect to SWW. Furthermore, the effectiveness of mite removal when using SS for samples with low, medium, and high infestation rates was found to be significantly lower than that obtained by using SWW. Moreover, when the infestation rate was low, SS had a lower sensitivity, resulting in false negative samples. Conversely, the sensitivity with medium and high infestations rates was like that of SW.

The proposed hypothesis was rejected because *Sugar Shake* proved to be less effective and less sensitive than *Soapy Water Washing* in the detection of *Varroa destructor* mites in *Apis mellifera* colonies.

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