



# Grooming Behavior in Naturally *Varroa*-Resistant *Apis mellifera* Colonies From North-Central Argentina

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The Western honey bee, *Apis mellifera*, is an important species in providing honey and pollination services globally. The mite *Varroa destructor* is the major threat to *A. mellifera*, and it is associated with the severe colony winter mortality reported in recent decades. However, *Varroa* mite tolerant or resistant populations of *A. mellifera* have been detected around the world. A proposed mechanism responsible for limiting mite population growth in the colonies is grooming behavior, the physical removal and injury of mites from the adult bee bodies by individual workers or by their nest-mates. This behavioral strategy has been poorly studied in *V. destructor*-resistant colonies worldwide, especially in honey bee populations of European origin. In Argentina, honey bee stocks showing survival without mite treatment have been reported. In the present study, European-derived *A. mellifera* populations established in the Transition Chaco eco-region (Santa Fe province), with a subtropical climate, were characterized at the colony level. A honey bee stock showing natural *Varroa*-resistance (M) was compared to a *Varroa*-susceptible stock (C) for parameters of colony status (colony strength, percentage of *Varroa* infestation in adults and brood, hygienic behavior) and for indirect measures of grooming (percentage of fallen mites and damaged mites). M colonies showed lower phoretic and brood infestation and higher hygienic behavior in early autumn, and higher survival and population strength after wintering, in comparison with C colonies. The mean percentages of fallen mites and of damaged mites, and the injury to mites were higher in M than in C colonies. Our results suggest that, by modulating the parasitization dynamics in colonies, grooming behavior would be associated with the higher survival of *Varroa*-resistant stock. This study sheds light on how honey bee colonies can adaptively respond to mite pressure by modeling their behavior to resist Varroosis and provides evidence for grooming as an emerging factor evolving by natural

selection. Percentage of damaged mites appears to be a reliable measure to enhance this behavior in honey bee colonies by selective breeding. Finally, the importance of improving and protecting locally adapted honey bee populations with natural *Varroa* resistance for regional apiculture is discussed.

**Keywords:** grooming behavior, honey bee health, *Varroa*-resistance, hygienic behavior, natural selection, breeding programs

## INTRODUCTION

The Western honey bee, *Apis mellifera* (Linnaeus), is one of the most valuable pollinators worldwide (Aizen and Harder, 2009; Gallai et al., 2009; Hung et al., 2018), providing essential pollination services to agroecosystems as well as profitable hive products for the apicultural sector (Morse and Calderone, 2000; Klein et al., 2007). Over the last few decades, honey bee colony losses have increased dramatically, as reported mainly in the Northern Hemisphere (Neumann and Carreck, 2010; Potts et al., 2010b; vanEngelsdorp et al., 2011), but also in South Africa (Pirk et al., 2014), Oceania (Brown et al., 2018), and South America (Vandame and Palacio, 2010; Maggi et al., 2016; Antúnez et al., 2017; Requier et al., 2018). The possible driving factors of these losses include a growing number of interacting threats, such as environment and climate change, nutritional deficiencies, pesticides, parasites, and pathogens (reviewed by Le Conte and Navajas, 2008; Potts et al., 2010a; Goulson et al., 2015).

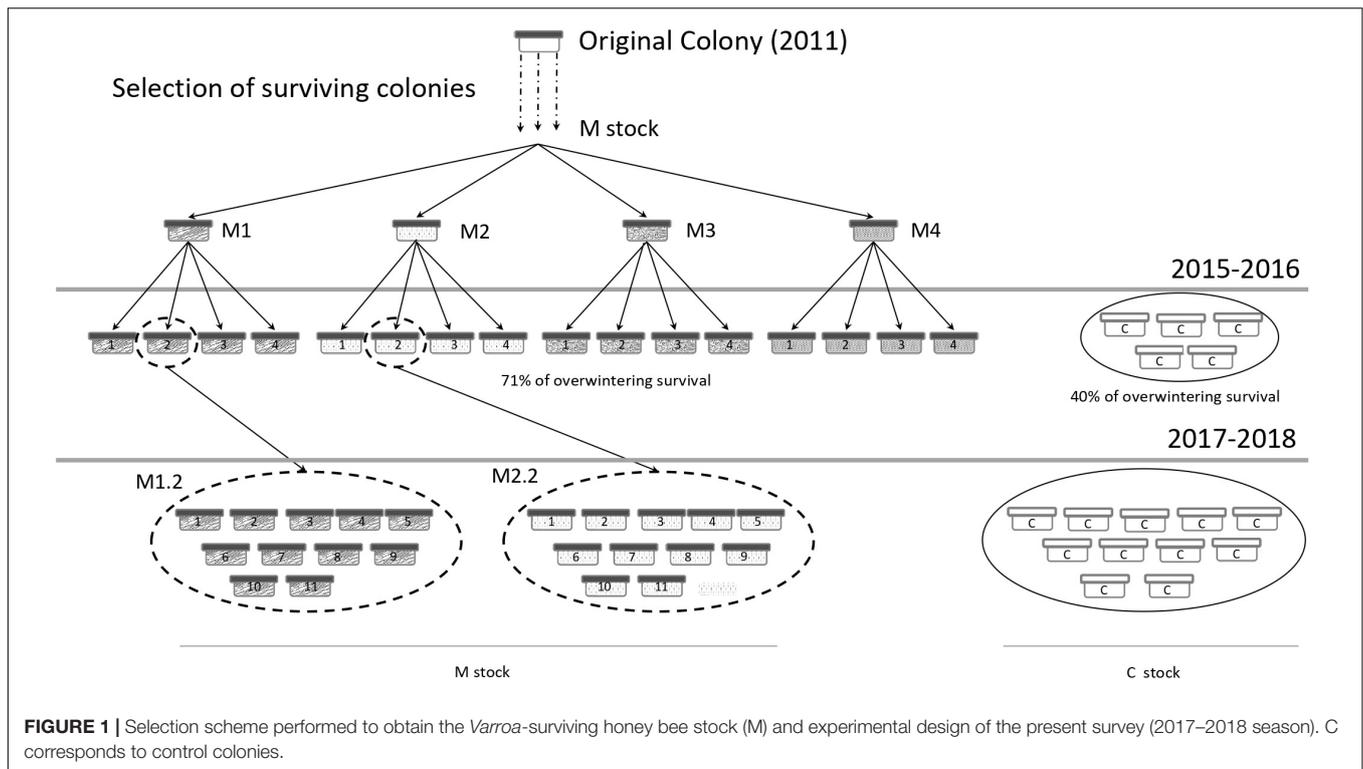
The mite *Varroa destructor* (Anderson and Trueman) is considered the main parasite threatening *A. mellifera* colony survival, mainly in honey bee populations of European origin (Rosenkranz et al., 2010). Although the mite does not directly kill the bees, it has strong effects by weakening brood and adults through feeding on them (Amdam et al., 2004; Zaobidna et al., 2017; Ramsey et al., 2019) and transmitting several honey bee viruses (Dainat and Neumann, 2013; Francis et al., 2013; Mondet et al., 2014; McMenamin and Genersch, 2015). Together, these effects can affect the wing development and shorten the life span of worker bees and generate an epidemic disease within the colony, eventually resulting in colony death (Boecking and Genersch, 2008; Neumann et al., 2012; Van Dooremalen et al., 2012).

Today, most managed *A. mellifera* colonies depend on mite control treatments to survive (Rosenkranz et al., 2010). However, several *Varroa*-surviving honey bee populations have been documented around the world as a result of selective breeding or natural selection (e.g., Locke, 2016; Le Conte and Mondet, 2017). Bees may survive *Varroa* through the expression of resistance or tolerance traits. Resistance involves a reduction in *Varroa* growth, while tolerance reduces parasitic burden despite similar levels of *Varroa* growth (Schneider and Ayres, 2008; Kurze et al., 2016). Resistance or tolerance to *V. destructor* mites is a typical characteristic of Africanized bees from South and Central America (e.g., Martin and Medina, 2004; Mondragón et al., 2005; Rivera-Marchand et al., 2012). There are also accounts of *Varroa* resistant and tolerant European-derived *A. mellifera* populations from North America, Europe, and other parts of the world (e.g., Fries et al., 2006; Le Conte et al., 2007;

Seeley, 2007; Pritchard, 2016). Specific adaptive behaviors have evolved in these honey bee populations, mainly related to resistance mechanisms, such as hygienic behavior specifically targeting *Varroa*-infested capped brood cells (VSH), recapping, and grooming (reviewed by Mondet et al., 2020).

Grooming behavior involves the physical removal, and often injury, of parasitic mites from the body of adult bees by individual workers or by their nest-mates. Through this behavior, the parasitized bees can dislodge mites themselves using their legs and mandibles (autogrooming) or receiving help from other bees (allogrooming) (Boecking and Spivak, 1999). Grooming is one of the main mechanisms of resistance against mite in *A. cerana* (Peng et al., 1987), and it is also observed in *A. mellifera* but expressed at a lower frequency (Boecking and Ritter, 1993; Fries et al., 1996). Despite these observations, several studies have evidenced that grooming behavior confers some degree of resistance against the *Varroa* mite in populations of Africanized bees (Moretto et al., 1993; Guzman-Novoa et al., 1999, 2002; Arechavaleta-Velasco and Guzmán-Novoa, 2001; Martin and Medina, 2004; Mondragón et al., 2005, 2006; Invernizzi et al., 2015). In European races of honey bees, grooming effectiveness against varroa mites is still unclear, although phenotypic variation for this behavior has been documented (Moosbeckhofer, 1997; Currie and Tahmasbi, 2008; Andino and Hunt, 2011; Båk and Wilde, 2015). Guzman-Novoa et al. (2012) compared mite-resistant and susceptible honey bee stocks of different origins (Africanized bees, Russian, and European races) and found that all resistant stocks showed comparatively higher proportions of injured mites falling from colonies and increased intensity of individual grooming actions in laboratory assays, which underscores the importance of this trait in *Varroa* resistance. In fact, higher proportions of mite injuries inflicted by grooming have been associated with decreases in mite infestation levels observed in *A. mellifera* colonies (Moosbeckhofer, 1992; Arechavaleta-Velasco and Guzmán-Novoa, 2001; Mondragón et al., 2005; Guzman-Novoa et al., 2012).

Over the last three decades, numerous breeding programs have been initiated to selectively enhance heritable resistance or tolerance to the mite on specific honey bee populations (reviewed by Guichard et al., 2020). Such developments relied on the identification of specific traits that characterize these populations. This is a critical point since some characteristics that strongly confer mite resistance to some bee populations may not have a great influence on others (Locke, 2016). In Argentina, efforts have been made to identify and select local stocks that survive without mite treatment and characterize the underlying mechanisms. One of the criteria used by local breeding programs is the selection of hygienic behavior. This behavior involves



the workers' detection, uncapping and removal of unhealthy or dead brood (Rothenbuhler, 1964). Based on the pin-killed brood method, Argentinian honey bee populations have been studied and selected (Palacio et al., 2000, 2010). These honey bee stocks were later evaluated in relation to *Varroa* resistance in regions of temperate climate, where the mite has become a serious problem (Merke, 2016; Visintini, 2018). However, the phenotypic variation of grooming behavior and its contribution to colony survival has not been previously addressed in *Varroa*-resistant stocks from the country.

The objectives of this study were to characterize a *Varroa*-surviving honey bee stock located in North-Central Argentina, a region with a subtropical climate, and to evaluate the contribution of grooming behavior to mite-resistance. The integral characterization of this naturally selected honey bee population and the associated varroa mite provides a better understanding of the adaptive ways in which honey bee colonies can respond to mite infestation. Our results contribute to enhancing the management and breeding strategies for regional apiculture.

## MATERIALS AND METHODS

### Colonies for the Present Survey

*A. mellifera* colonies from two stocks were sampled: (1) a *Varroa*-surviving honey bee population (M, 22 colonies), and (2) a susceptible honey bee population (considered a control to our assays, C, 11 colonies) located at the apiary of Reconquista Agricultural Experimental Station (EEA

Reconquista, 29°15'31.8"S 59°44'36.0"W) of the National Institute of Agricultural Technology (INTA). They were surveyed during the 2017–2018 season (**Figure 1**). This region is defined as Transition Chaco and characterized by a subtropical climate with a dry season. The control population was chosen for its geographical sympatry with the surviving population. Control colonies were headed by commercial queens of European origin and were known to require synthetic acaricide treatments against *V. destructor* twice a year (one in early autumn and one in early spring) or else suffer severe losses. A previous study (Russo et al., 2018) evidence 60% of overwinter colony mortality for this stock in absence of mite treatment. Colonies of both stocks received the same beekeeping practices and were not subjected to acaricide treatment during the survey.

### Origin and Selection of the *Varroa*-Surviving Honey Bee Stock

The *Varroa*-surviving stock (M) is a honey bee population that had been kept without mite treatment for 6 years prior to the beginning of the present study (March 2017) (**Figure 1**). This stock was derived from a single colony from an abandoned commercial apiary at Reconquista locality (north of Santa Fe province, Argentina), where most of the colonies had died. The surviving colony was transported to the EEA Reconquista in 2011 and multiplied. Every spring, daughter colonies that survived winter without *Varroa* treatment and showed vitality in terms of colony growth were selected for the new generation. In the early spring of 2014, four colonies of M stock (named M1, M2, M3, and M4; **Figure 1**) were selected as mothers of the next generation and

split into four new colonies each. The resulting sixteen daughter honey bee colonies were firstly monitored during the 2015–2016 season (Figure 1) and they showed higher overwintering survival and a higher proportion of fallen mites than the colonies from a commercial control stock (Russo et al., 2018). For the next generation, two colonies of M stock were selected as mothers and multiplied in 11 daughter colonies each to perform the present survey. During the selection process, queens of all colonies were naturally mated.

## Genetic Characterization of Stocks

The mitochondrial (mt) haplotypes of all surveyed colonies were analyzed. Briefly, adult workers were collected from all colonies of M and C stocks during spring 2017. Total DNA was extracted from the thorax of one worker per colony following a high-salt protocol (Baruffi et al., 1995). DNA samples of honey bee workers were analyzed using a PCR-RFLP-based method. A fragment of 1,001 bp from the mitochondrial COI-COII region was amplified by PCR using the primers and conditions described by Hall and Smith (1991) and Lobo Segura (2000). PCR products were digested with *HinfI* (Promega, Madison, MN, United States) following the manufacturer's recommendations. The restriction fragments were separated on 4% (wt/v) agarose gels, stained with GelRed, and photographed under UV light. The mt haplotypes detected in the restriction analysis using *HinfI* were assigned as previously described by Agra et al. (2018).

## Parameters Measured During the Survey

During the 2017–2018 season, the experimental apiary at EEA Reconquista was visited once in March 2017 (early autumn) and monthly during the active season, from September 2017 (early spring) to February 2018. During the visits, the following measurements were registered in each colony from both stocks (M and C): populations of adult bees and brood, percentage of mite infestation of adult bees, number of naturally fallen mites, and number of damaged mites. Overwintering survival of each stock was also registered. In addition, hygienic behavior and percentage of mite infestation on brood were measured twice, in March 2017 and September 2017.

## Overwintering Colony Survival and Bee Population

The number of colonies that survived the winter was registered in spring (October 2017) for both stocks. Adult and brood populations were assessed in each colony by estimating the total area of comb covered by adult bees and brood according to DeGrandi-Hoffman et al. (2008). Briefly, once each hive was opened, frames were sequentially removed, and the percentage of the comb surface covered by adults and brood on both sides were registered. Then, the number of total frames fully occupied by adults and brood was estimated for each colony. The total number of bees per colony (total worker population) was estimated according to Delaplane et al. (2013).

## Phoretic and Brood Infestation

The percentage of phoretic *Varroa* was determined by collecting and examining samples of approximately 300 workers from each colony. The samples were taken from the three central frames of

each hive, by collecting the bees in plastic flasks previously filled with 70% ethanol. The number of mites detected in each sample was divided by the number of bees in the sample and multiplied by 100 to obtain the percentage of phoretic *Varroa* (De Jong et al., 1982). The total phoretic mite population was estimated for each colony using the percentage of phoretic *Varroa* and the estimated total worker population.

In addition, the percentage of mite infestation on brood was assessed once in autumn (March) and once in spring (October) 2017. Briefly, in each colony from both stocks, a frame with recently sealed brood (pupae not older than the purple- to dark-purple-eye stage) was identified. Fifty sealed brood cells from each side of the frame (a total of 100 cells per colony) were randomly selected and examined for the presence of adult female mites (Branco et al., 2006). The percentage of mite infestation on brood was the number of mite-infested cells.

## Grooming Behavior

Grooming behavior was estimated by registering the mite fall and the damaged mites (indirect measures of grooming) (Boecking and Spivak, 1999). To this end, the screen bottom board method described by Pettis and Shimanuki (1999) was used. The original bottom board of each colony was replaced with a screened bottom board, allowing only the mites to fall through it and onto the slide-out inspection board. Before each measurement, the slide board of each colony was removed, cleaned, and reintroduced. Forty-eight hours later, the sliding boards were pulled out and the fallen mites were collected from the debris using a fine hairbrush. All fallen mites from each colony were counted and examined under a stereoscopic magnifying glass. Each mite received a binary score of “undamaged” or “damaged” for the analysis. In these cases, damage to the dorsal shield, gnathosoma, and legs was identified according to Rosenkranz et al. (1997) and Corrêa-Marques et al. (2000). The proportion of damaged mites in each colony was obtained by dividing the number of damaged mites by the total number of fallen mites collected at the end of the collection period (48 h). The proportion of fallen mites was obtained by dividing the number of fallen mites by the estimated total *Varroa* population of each colony, which represents the fraction of the mite removed by honey bees off their bodies relative to the total mite population present in the colonies.

## Hygienic Behavior

Hygienic behavior was measured using the pin-killed brood assay (Newton and Ostasiewski, 1986; Palacio et al., 2000). Briefly, one frame of each colony containing a uniform capped brood was selected. On each frame, capped brood cells contained in a 10 × 5 cm comb section were perforated using an entomological pin (No. 1) to kill the brood. The frames were reintroduced in the original colony and inspected 24 h later to count the number of cells that had been cleaned by the bees. The hygienic activity of the colony was determined using the following equation:

$$HB\% = \left( \frac{\begin{array}{l} \text{Total pin killed capped cells} \\ - \text{remaining capped cells} \\ - \text{uncapped cells with dead brood inside} \end{array}}{\text{Total pin killed sealed cells}} \right) \times 100$$

## Statistical Analysis

Overwintering colony survival was compared between stocks using a contingency-table analysis. To investigate whether adult bee population (number of frames fully occupied by bees), brood population (number of frames fully occupied by brood), and the percentage of phoretic *Varroa* differed between stocks and months across the season, separate generalized linear models (GLM) were performed including stock (M, C) and months of the active season (March, September, October, December, January, February) as fixed factors, and colonies as random factors. Logit transformation (ln) was applied to phoretic *Varroa* data. Similarly, the percentage of hygienic behavior was compared between stocks (M, C) and seasons (early autumn, early spring) by using GLM. Multiple comparisons were performed using Fisher LSD ( $\alpha = 0.05$ ) in all cases.

Fallen mites and damaged mites were analyzed separately by using the general linear mixed model (GLMM) with a binomial distribution and logit link function (fallen vs. not fallen mites and damaged vs. undamaged mites, respectively) considering stocks and months, as fixed factors, and colonies, as a random factor. In the case of damaged mites, the comparisons between months were performed separately for each stock to obtain a better adjustment to the model. Multiple comparisons were performed using Fisher LSD ( $\alpha = 0.05$ ). In all cases (GLMs and GLMMs), the Shapiro-Wilks and Levene tests and the residue normality were analyzed. To obtain the most appropriate structure of variance, the Akaike information criterion was used.

In addition, to find relationships between the measurements of grooming behavior and phoretic infestation of adults, the percentages of fallen mites, damaged mites, and phoretic mites were subjected to Spearman Rank Correlation analysis for each stock.

Possible differences in the types of damage on fallen mites from C and M colonies were analyzed with contingency tables. Specifically, the frequency of different categories of damage described above (legs, dorsal shield, gnathosoma) and the frequencies of multiple (legs + body) vs. simple (legs or body) damage were considered.

The frequencies of mite infestation on brood were compared between stocks by using contingency-table analysis. All statistical analyses were performed using InfoStat (Di Rienzo and Montiglio, 2016).

## RESULTS

The genetic characterization of honey bee colonies used in the present study showed the presence of 100% of European haplotypes (C1) in both M and C colonies.

The percentage of overwintering survival (March to September 2017) was higher for M (81.8%) than for C (45.4%) stock [ $\chi^2_{(1)} = 4.59$ ,  $P = 0.032$ ]. The adult bee population across the active season was similar between C and M stocks with a significant difference only in early spring [GLMM results:  $F_{(1,31)} = 0.01$ ,  $P = 0.92$  for stock;  $F_{(5,105)} = 24.45$ ,  $P < 0.001$  for month;  $F_{(5,105)} = 2.59$ ,  $P = 0.03$  for interaction stock  $\times$  month; *post hoc* comparisons in **Figure 2A**]. Specifically in September,

the mean number of frames completely covered by bees was higher in M ( $6.76 \pm 0.55$ ) than in C ( $5.05 \pm 0.98$ ) (**Figure 2A**). Within C stock, the adult bee population was significantly lower in spring (September:  $5.5 \pm 0.9$ , and October:  $5.8 \pm 0.9$ ) than in the other months evaluated (mean value:  $8.6 \pm 0.3$ ), while no significant differences in this variable was detected across the season for M colonies (mean value:  $7.4 \pm 0.4$ ) (**Figure 2A**).

Regarding the estimated brood population, variation in the number of frames occupied by brood across the season was detected for both stocks [GLMM results:  $F_{(1,52)} = 2.91$ ,  $P = 0.09$  for stock;  $F_{(5,76)} = 20.01$ ,  $P < 0.001$  for month;  $F_{(5,76)} = 4.47$ ,  $P < 0.01$  for interaction stock  $\times$  month; **Figure 2B**]. Though similar brood patterns were observed between stocks in most monitored months, a significant difference was detected between M and C colonies for the mean number of frames with brood in early spring (September: M =  $4.35 \pm 0.23$ ; C =  $3.20 \pm 0.45$ ; **Figure 2B**).

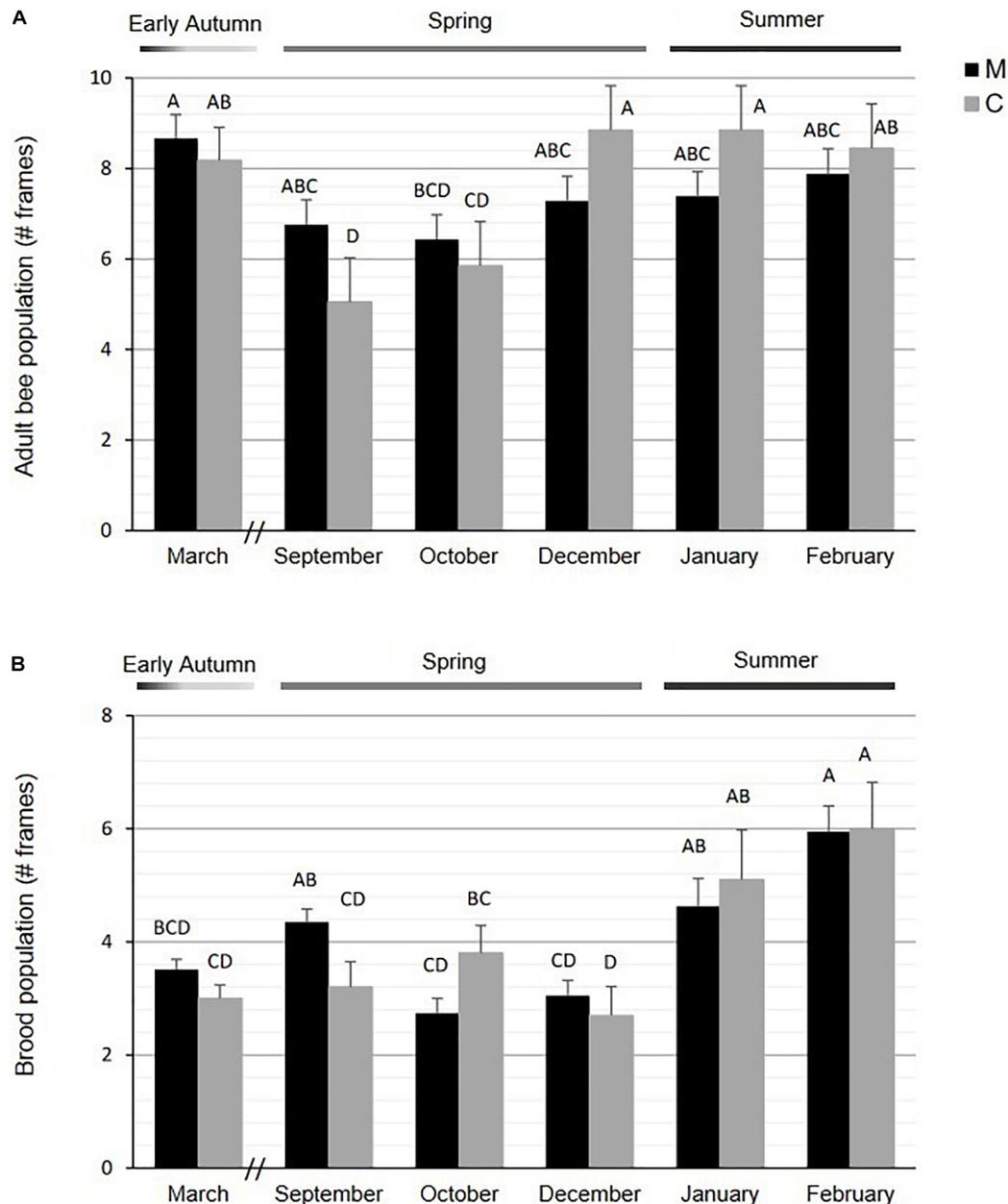
According to the population dynamics of the colonies, the percentage of phoretic *Varroa* varied throughout the active season [GLMM results:  $F_{(1,31)} = 2.66$ ,  $P = 0.11$  for stock;  $F_{(5,73)} = 22.94$ ,  $P < 0.001$  for month;  $F_{(5,73)} = 2.40$ ,  $P = 0.049$  for interaction stock  $\times$  month, **Figure 3A**]. The dynamics of phoretic infestation was similar in M and C colonies, with growing levels from spring to summer, and a peak in early autumn (**Figure 3A**). The increase in the percentage of phoretic *Varroa* observed in March was significantly higher for C ( $5.2 \pm 1.2$ ) than for M colonies ( $11.5 \pm 1.7$ ) (**Figure 3A**).

Consistent with the results of phoretic *Varroa*, the percentage of mite infestation on brood was significantly higher in C (6.64%) than in M (2.57%) in early autumn [ $\chi^2_{(1)} = 31.28$ ,  $P < 0.001$ ; **Figure 3B**]. An extremely low percentage of infestation on brood was evident in both M and C stocks during spring [0.09% in M and 0% in C;  $\chi^2_{(1)} = 1.05$ ,  $P = 0.31$ ; **Figure 3B**].

Hygienic behavior (HB) differed between stocks and seasons [GLMM results:  $F_{(1,29)} = 5.64$ ,  $P = 0.02$  for stock;  $F_{(1,16)} = 11.21$ ,  $P = 0.004$  for season;  $F_{(1,16)} = 3.79$ ,  $P = 0.07$  for interaction stock  $\times$  season]. Specifically for M stock, HB was similar between spring and autumn, while for C stock, a lower level of HB was observed in autumn than in spring (**Table 1**). M showed higher HB than C only in autumn (**Table 1**).

The percentage of fallen mites on bottom boards differed between stocks and months, with a significant interaction between factors [GLMM results:  $F_{(1,127)} = 23.67$ ,  $P < 0.001$  for stock;  $F_{(5,127)} = 16.16$ ,  $P < 0.001$  for month;  $F_{(5,127)} = 12.55$ ,  $P < 0.001$  for interaction stock  $\times$  month; **Figure 4A**]. Significant variation in this variable was detected across the season for M colonies, with September and February being the months with the highest mite fall percentage (*post hoc* comparisons by Fisher LSD, **Figure 4A**). Conversely, C colonies evidenced a low percentage of fallen mites without significant differences across the season (**Figure 4A**).

The mean percentage of damaged mites over the season was higher in M (25%) than in C (9%) stock [ $F_{(1,97)} = 8.51$ ,  $P < 0.01$ ]. C colonies exhibited a very low and similar number of damaged mites across the season [ $F_{(1,16)} = 0.01$ ,  $P > 0.05$ ; **Figure 4B**]. Conversely, this parameter varied throughout the season for M line [ $F_{(1,71)} = 5.18$ ,  $P < 0.001$ ; **Figure 4B**] with relatively greater

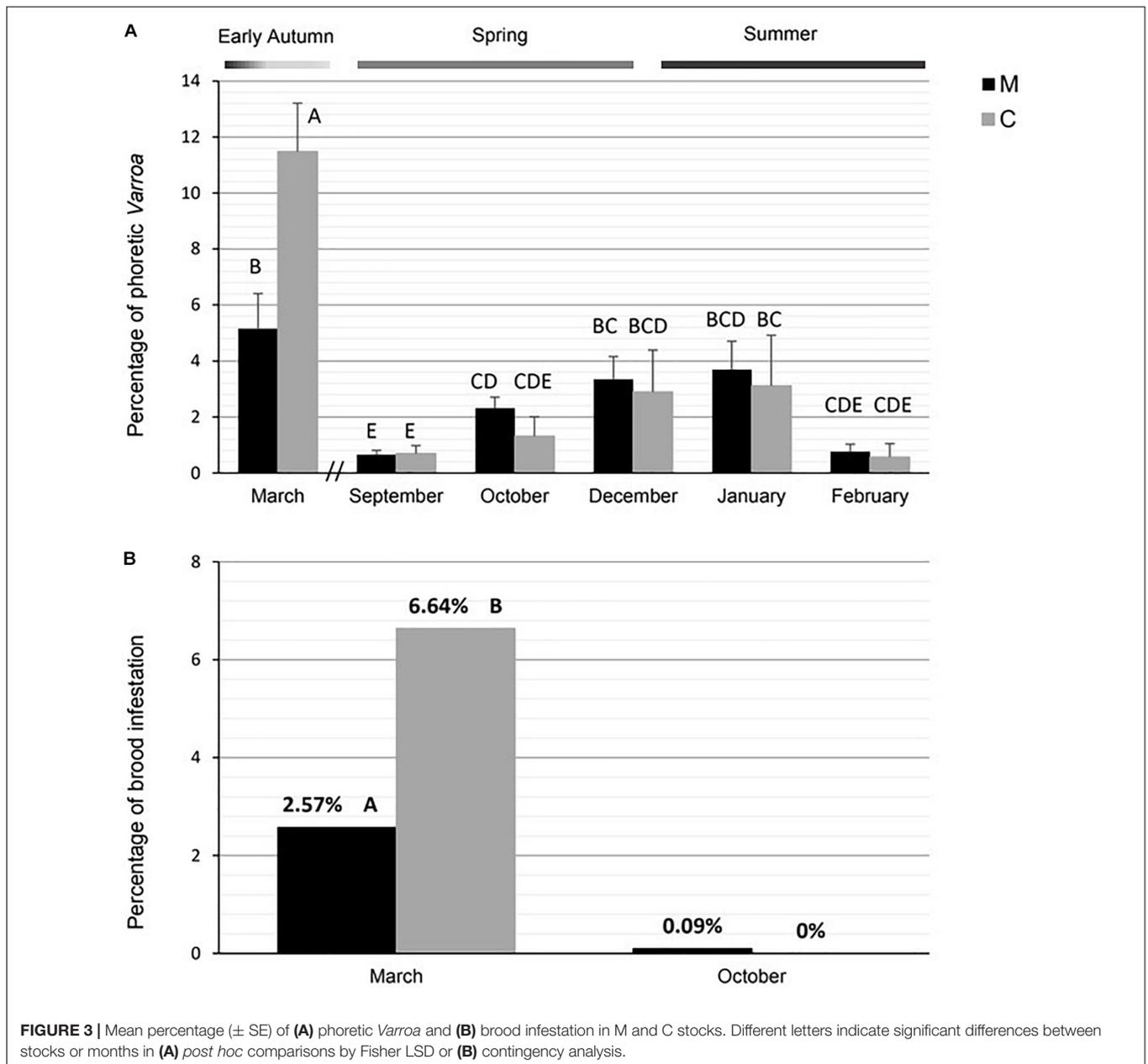


**FIGURE 2 |** Mean number ( $\pm$  SE) of frames fully occupied by (A) adult bees and (B) brood in M and C stocks. Different letters indicate significant differences in *post hoc* comparisons by Fisher LSD ( $\alpha = 0.05$ ).

damage during spring (September and October) and end of summer (February) (Figure 4B).

An association between the percentage of fallen mites and that of damaged mites was evident for M (Spearman's rank correlation:  $r = 0.45$ ,  $P < 0.001$ ) but not for C colonies. No association was found between the percentage of phoretic *Varroa* and grooming parameters (fallen and damaged mites) in M stock. Conversely, a positive correlation was detected between the percentage of phoretic *Varroa* and that of fallen mites in C stock (Spearman's rank correlation:  $r = 0.4$ ,  $P < 0.05$ ).

Different categories of damage to the mite were recorded in this study (Figures 5A–F). These categories were present in mites from colonies of both stocks, but at different relative frequencies depending on the colony origin (Table 2). Damaged leg (total or partial loss of one or more legs) was the predominant type of physical injury to the mite recorded in both M and C lines in similar percentages (Table 2), but with different intensity. In fact, significant differences were detected in the proportion of mites that presented more than 2 damaged legs in M (63.3%) than in C (10.5%) stock [ $\chi^2(1) = 20.98$ ,



**FIGURE 3 |** Mean percentage ( $\pm$  SE) of (A) phoretic *Varroa* and (B) brood infestation in M and C stocks. Different letters indicate significant differences between stocks or months in (A) *post hoc* comparisons by Fisher LSD or (B) contingency analysis.

**TABLE 1 |** Percentage of hygienic behavior ( $\pm$ SE) measured in early autumn (March) and spring (October) for M and C stocks.

	C	M
Autumn	68.7 (3.9) B	82.4 (3.0) A
Spring	89.8 (5.7) A	88.8 (3.4) A

Different letters indicate significant differences between stocks or months by Fisher LSD ( $\alpha = 0.05$ ).

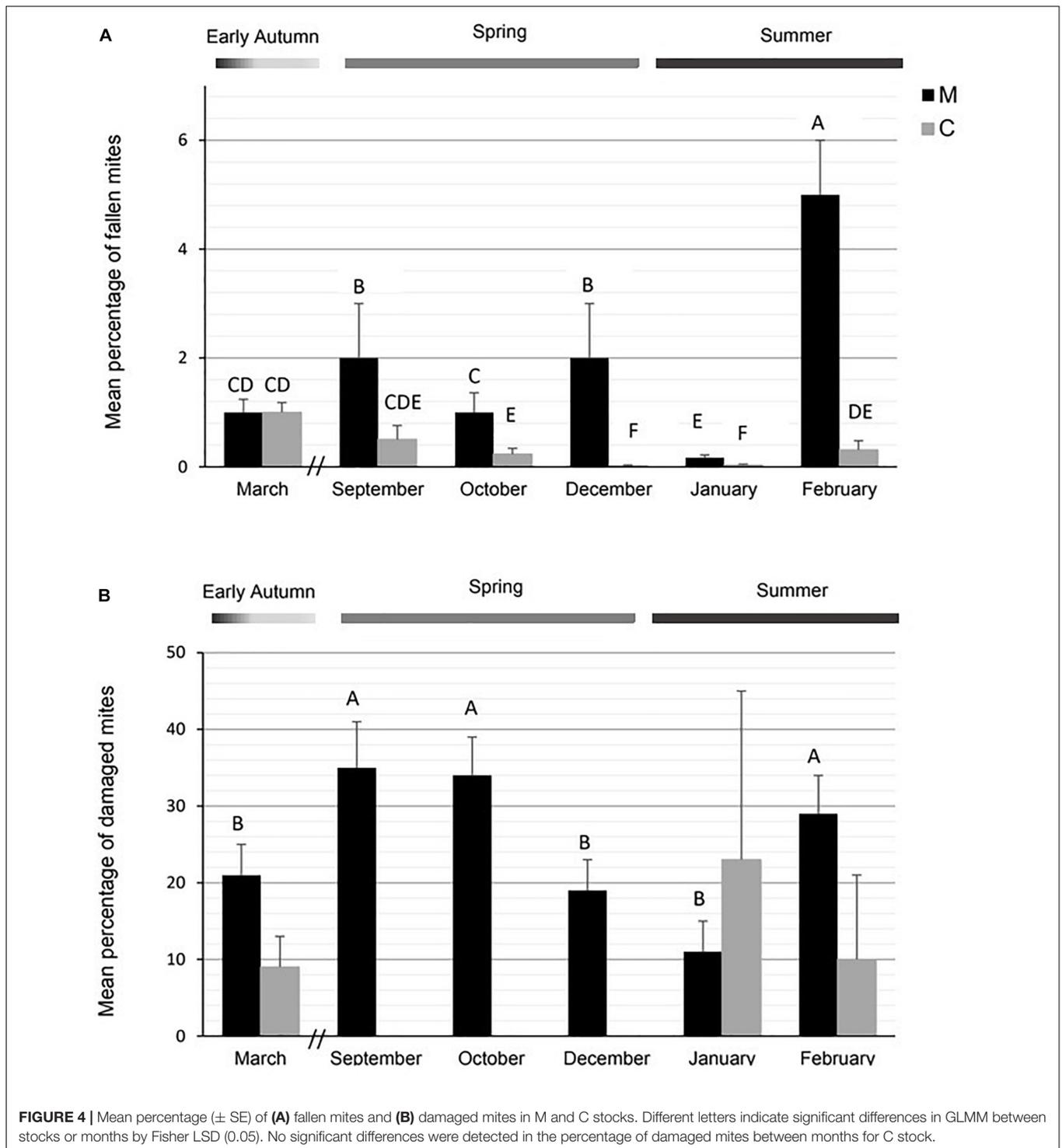
$P < 0.001$ ; **Supplementary Table S1**]. Moreover, 52.7% of the damaged mites from M colonies presented 4 or more damaged legs (**Supplementary Table S1**). Combined injury in body and legs (damaged legs + damaged gnathosoma or dorsal shield) was detected in 20.6 and 5% of the injured mites from M

and C colonies, respectively. This difference was marginally significant (**Table 2**).

## DISCUSSION

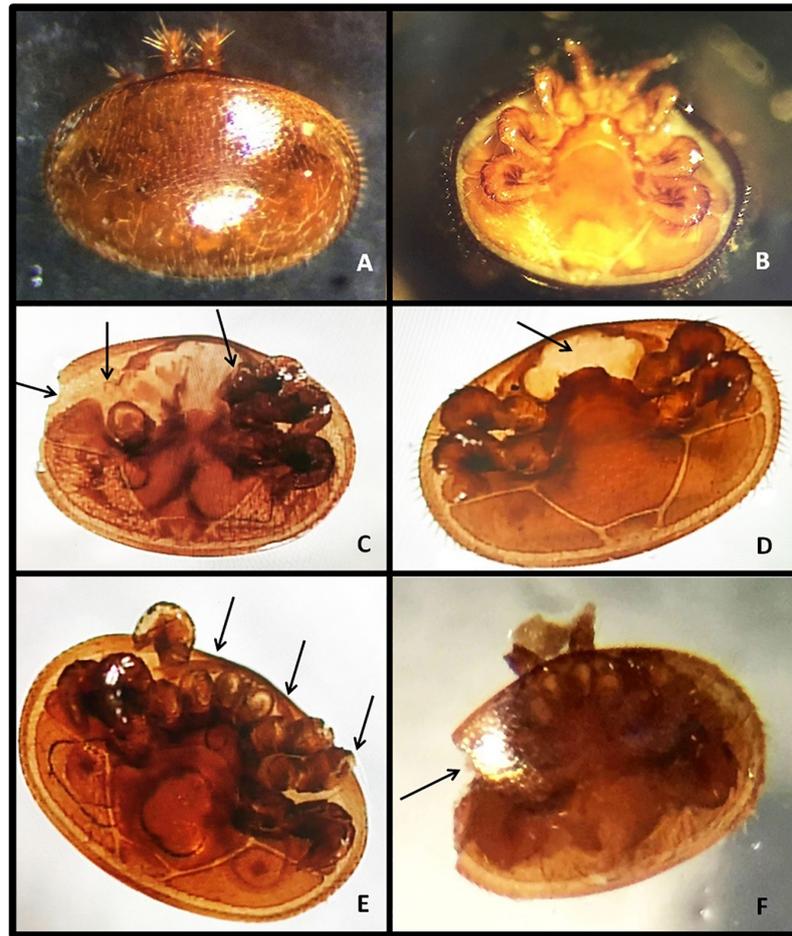
Here, we present a field survey of a naturally mite-surviving honey bee stock from north-east Argentina and explore the contribution of grooming behavior and colony dynamic to *Varroa*-resistance.

Our findings revealed that the *Varroa*-surviving honey bee stock (M) expressed a higher grooming behavior than that of the susceptible local control stock (C). This was evident in the higher mite damage recorded on the screened



bottom boards of M compared to C colonies. The mean percentage of mite damage observed in our M population during spring (34.5%) appeared to be intermediate between those recorded in *A. m. ligustica* (26.4%) by Fries et al. (1996) and in Africanized *A. mellifera* (38.5%) by Moretto et al. (1991). We also detected phenotypic variation among

stocks for the percentage of mite infestation in adults (phoretic *Varroa*) and in brood, particularly in early autumn. At this time of the season, C stock evidenced 2.2 and 2.6-fold more mites in adults and brood, respectively, compared to M stock. The difference in *Varroa* infestation between stocks in early autumn appeared to largely impact on the



**FIGURE 5 |** Photographs showing the different damage patterns in *V. destructor* mites. Arrows indicate the injuries on mite's body, legs, and chelicerae. **(A,B)** Dorsal and ventral views of non-damaged mites, **(C)** damaged dorsal shield + damaged gnathosoma, and missing legs + chelicerae, **(D,E)** missing legs + chelicerae, **(F)** damaged dorsal shield + damaged gnathosoma. Classification of damage to the mites was previously reported by Corrêa-Marques et al. (2000). All the pictures were taken with an Olympus BX40 Microscope at 40x magnification.

**TABLE 2 |** Mean percentages for the different categories of damage to *V. destructor* recorded in the colony debris of M and C stocks.

Category of damage	C (%)	M (%)	P
Damaged legs (DL)	70	66.6	0.123
Damaged dorsal shield (DDS)	15	2.3	<b>0.001</b>
Damaged gnathosoma (DG)	10	8.5	0.172
Damaged body (DB)	0	2.0	0.369
Multiple damage (MD)	5	20.6	0.062

DL includes total or partial loss of one or more legs. DDS includes partial loss of dorsal shields and/or the presence of fissures; DG includes loss of chelicerae and/or pedipalps; DB includes DG + DDS; MD includes mites with DL + DG + DDS. P-values (after Bonferroni correction for multiple comparisons) from  $\chi^2$ -tests are shown. Significant comparisons ( $P < 0.05$ ) are in bold.

observed overwintering survival and the colony strength at the beginning of the season.

The high percentage of damaged mites observed during the active season and the more intense injuries inflicted on the mites by M bees, as discussed in more detail below, suggest

that grooming behavior could increase mite mortality and hence modulate its population growth in the colonies. Our results are in line with a growing body of evidence (Morse et al., 1991; Moosbeckhofer, 1992; Ruttner and Hänel, 1992; Boecking and Ritter, 1993; Moretto et al., 1993; Bienefeld et al., 1999; Arechavaleta-Velasco and Guzmán-Novoa, 2001; Guzman-Novoa et al., 2012; Invernizzi et al., 2015; Nganso et al., 2017; Russo et al., 2018) suggesting that grooming behavior may be an important mechanism conferring resistance to honey bee colonies toward *V. destructor*, even in honey bee populations of European origin. Moreover, this trait may evolve by natural selection (as shown by the present results) and can be further developed or improved in ongoing selected stocks (e.g., Hunt et al., 2016).

The percentage of damaged mites showed seasonal variation, as previously suggested for grooming behavior (Büchler, 1994; Moosbeckhofer, 1997). Specifically, M colonies exhibited a high percentage of damaged mites during spring, where the phoretic infestation is low, in agreement with previous observations by

Mondragón et al. (2005). In this sense, M (in contrast to C) colonies may strategically respond to mite phoretic infestation below a load threshold, slowing the population growth of the mite and ensuring fewer loads to deal with overwintering. Even though Kruitwagen et al. (2017) suggested that grooming would not be mite-density dependent and speculated that it would only be beneficial at high levels of mite infestation, this pattern was specifically observed in control colonies (like our C colonies) and at a small mite infestation range. On the one hand, our results can be used to recommend specific times of the season to measure and select the grooming behavior performance at the colony level. On the other hand, our results are in line with observations on colonies bred for hygienic behavior, which are more efficient at removing *Varroa*-infested brood only under low mite parasitism (Spivak and Reuter, 1998, 2001; Ibrahim and Spivak, 2006). This hypothesis must be further evaluated in controlled assays that test the response of groomer colonies against different parasite loads.

As the proportion of damaged mites can be a time-consuming measurement in field surveys (Rosenkranz et al., 1997; Bienefeld et al., 1999; Aumeier, 2001), it has been suggested that mite fall could be a simpler alternative to select the grooming behavior of a colony (e.g., Kruitwagen et al., 2017). The present results evidenced a positive correlation between the percentage of fallen and damaged mites in M stock, but not in C stock. Therefore, the validity of using only the percentage of mite fall as a measure to select colonies for increasing grooming abilities must be further evaluated on different stocks, specifically if the selection is initiated on a honey bee population or is used to increase this trait in already groomer stock (as in the present case). For unselected stocks, as our C colonies, the mite fall may reflect mainly the passive fall of the mite [consistent with its use as an estimator of mite infestation at colony level (Branco et al., 2006; Guzman-Novoa et al., 2012; Hunt et al., 2016)], and it may not strictly represent a measure of grooming activity by the adult bees.

To characterize the differences in grooming behavior intensity between the stocks, we analyzed the patterns of damage in mites using the known classification performed by Corrêa-Marques et al. (2000). In agreement with previous studies (Ruttner and Hänel, 1992; Lodesani et al., 1996; Rosenkranz et al., 1997; Corrêa-Marques et al., 2000; Stanimirovic et al., 2003), we found that leg damage was the most frequent damage in mites from colonies of both origins. While the percentage of this kind of damage did not differ between M and C colonies, the number of damaged legs was higher in mites of M colonies than C colonies. In fact, more than 50% of the mites from M colonies evidenced damage in 4 or more legs. This, together with an apparent higher frequency of multiple injuries (legs and gnathosoma or dorsal shield damage) to the mites from M stock, would reflect that more intense grooming, possibly collective behavior (allogrooming), was displayed by adult bees from this origin. Accordingly, Nganso et al. (2017) detected the same kind of combined injuries to mites from both African and European honey bee colonies, but at higher frequency in the former. Overall, the high rate of mite mutilations observed in our M stock reflects how robust is the mite damage as indirect measurement of grooming behavior at the colony level. Even this measurement is tedious and time-consuming in field surveys, it is the only reliable

phenotypic trait to breed for increased grooming behavior so far known. Alternatively, the mite population growth, estimated by determining the difference between two measurements of mite fall assessments over time, could represent a simpler and less time-consuming method to predict *Varroa*-resistance in honey bee populations (Emsen et al., 2012) since it may estimate several mechanisms of mite resistance simultaneously (e.g., grooming behavior, VSH, etc.).

Despite the European mitochondrial lineage of our stocks, as the analyzed region represents a hybrid zone where Africanized and European honey bee populations coexist (Agra et al., 2018), our stock may be a local ecotype that carries genes from both origins. In fact, we observed differences in the temperament of the stocks during field monitoring, with more excitable behavior in M than in C workers. Consistently, previous studies revealed that subspecies of *A. mellifera* described as more excitable or even aggressive differed from other subspecies in their grooming behavior in laboratory assays (Aumeier, 2001; Wilde et al., 2003; Bık and Wilde, 2015). Further laboratory assays on this stock will allow us to investigate the apparently greater intensity of the grooming reactions of M worker bees against *V. destructor* and to test the association between the proportion of damaged mites in field monitoring and the proportion of mites dislodged by the bees in lab grooming assays (as previously detected by Andino and Hunt, 2011; Guzman-Novoa et al., 2012; Invernizzi et al., 2015). Moreover, these experiments will enable us to elucidate the weight of individual (autogrooming) and social (allogrooming) responses in the behavioral resistance against *V. destructor* in this stock and the best parameters to quantify each response.

It is important to note that grooming behavior may not be the only sanitary trait involved in regulating *Varroa* parasitism in M colonies. In fact, this stock expressed a higher hygienic behavior toward dead brood than did the control stock during early autumn, when the percentage of brood infestation is high. This result suggests that the bees of the surviving stock display higher hygiene and can behaviorally respond to the infestation status of the colony. However, since the method used here to test hygienic behavior may overestimate the expression of this behavior (Espinosa-Montaño et al., 2008), these results have to be taken with caution and confirmed in future research using more reliable methods for testing this complex behavior. Additionally, although hygienic behavior against dead brood does not necessarily imply greater resistance to *Varroa* (e.g., Danka et al., 2013), it would be linked to other associated behaviors such as *Varroa* Sensitive Hygiene (VSH; Spivak, 1996; Visintini, 2018), which were not measured in this work. In this sense, the analysis of other host traits that can jointly determine the surviving phenotype of our M stock (as previously evidenced in other naturally surviving stocks: Fries et al., 2006; Harris et al., 2010; Locke and Fries, 2011; Panziera et al., 2017; Oddie et al., 2018) is needed.

In addition, analyzing the performance of these colonies under different environments may help to clarify the influence of genotype x environment interactions (Büchler et al., 2014; Meixner et al., 2014) on grooming. It must be noted that this behavior can be influenced by environmental factors (Stanimirovic et al., 2003; Currie and Tahmasbi, 2008) and that *Varroa* damage thresholds can change under different

environmental conditions (Meixner et al., 2014; Giacobino et al., 2017). In this sense, the development of regional breeding programs for mite-resistant honey bees that take advantage of the locally-adapted stocks deserves consideration, especially in Argentina where contrasting eco-regions coexist.

Given the complexity of measuring the mite damage at the colony level (the best way to phenotype grooming behavior according to our results) and the efforts involved in selecting it at a large scale, the genetic characterization of M stock would facilitate the identification of candidate genes associated with this trait. In turn, this identification would help to further develop marker-assisted selection tools for facilitating breeding efforts (Grozinger and Robinson, 2015; Guarna et al., 2017). Recent findings demonstrated a significant correlation between the expression of the gene *neurexin* and direct observations of grooming behavior (Hamiduzzaman et al., 2017). Furthermore, Morfin et al. (2019) found a correlation between this gene and mite mutilation, which reinforces the validity of analyzing mite damage as an indirect measurement of grooming behavior until the development of robust markers for marker-assisted selection. Finally, efforts are being made to characterize the productivity of the selected stock under standard beekeeping management. This information will greatly contribute to incorporating this genetic material into the breeding program conducted by INTA and to making it available for commercialization in the region.

## CONCLUSION

Our data show that increased grooming behavior seems to be an important factor in reducing autumn *Varroa* infestation and enhancing overwintering survival of honey bee colonies of European origin, and suggest that mite damage would be the best proxy to evaluate and select this trait in the field. The characterization of this Argentinian stock, together with previously reported cases, clearly shows that honey bee populations can develop (different) traits and specific colony dynamics to overcome *V. destructor* infestations by means of natural selection. Taking advantage of these cases would be useful for a practical application in the apiculture and conservation of locally adapted honey bee populations.

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## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

## AUTHOR CONTRIBUTIONS

AS, JM, HP, SL, GR, and MP conceived and designed the research. RR, LL, HF, HP, JM, and IM conducted the experiments. ML contributed to statistical analyses. RR, SL, and AS analyzed the data and wrote the manuscript. All authors read and reviewed drafts of the manuscript and approved its final version.

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## SUPPLEMENTARY MATERIAL

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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