Large-scale monitoring of resistance to coumaphos, amitraz and pyrethroids in *Varroa destructor*

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ABSTRACT

Varroa destructor is an ectoparasitic mite causing devastating damages to honey bee colonies around the world. Its impact is considered a major factor contributing to the significant seasonal losses of colonies recorded every year. Beekeepers are usually relying on a reduced set of acaricides to manage the parasite, usually the pyrethroids tau-fluvalinate or flumethrin, the organophosphate coumaphos and the formamidine amitraz. However, the evolution of resistance in the populations is leading to an unsustainable scenario with almost no alternatives to reach an adequate control of the mite.

Here we present the results from the first, large-scale and extensive monitoring of the susceptibility to acaricides in the Comunitat Valenciana, one of the most prominent apicultural regions in Spain. Our ultimate goal was to provide beekeepers with timely information to help them decide what would be the best alternative for a long-term control of the mites in their apiaries. Our data show that there is a significant variation in the expected efficacy of coumaphos and pyrethroids across the region, indicating the presence of a different ratio of resistant individuals to these acaricides in each population. On the other hand, the expected efficacy of amitraz was more consistent, although slightly below the expected efficacy according to the label.

HIGHLIGHTS

1. *Varroa destructor* is causing severe damages to honey bee colonies worldwide.
2. There are very few acaricides available to manage the parasite.
3. The evolution of resistance is limiting our capacity to control the mite.
4. We estimated the expected efficacy of the main acaricides in many Spanish apiaries.
5. The information was shared with beekeepers for them to decide the best treatment to control the mite.

INTRODUCTION

The ectoparasitic mite *Varroa destructor* is considered a major pest of the Western honey bee (*A. mellifera* L.) (Rosenkranz et al. 2010). This mite feeds mostly on the fat body of immature and adult bees and vectors numerous lethal viruses (Boecking and Genersch 2008, Ramsey et al. 2019), compromising the natural honey bee defences. These severe damages make *V. destructor* one of the main factors contributing to the many seasonal losses of honey bee colonies around the world (Genersch et al. 2010, Guzmán-Novoa et al. 2010).

Varroa destructor shifted host from the Eastern honey bee (*Apis cerana* L.) to the Western honey bee in the late 1950's in Asia, but nowadays it is widely distributed throughout the world (Solignac et al. 2005). In Spain, *V. destructor* was first detected in 1985 and currently it can be found all over the country (Llorente 2003, Muñoz et al. 2015).

Varroa destructor reproduces throughout the spring and summer, so the population is larger in autumn. Thus, treatments to control de mite are usually applied in that season to increase the possibility of overwintering success (Underwood and López-Uribe 2020). In this country, as in many others, it is mandatory to apply at least one acaricide treatment per year to manage the parasite (Royal Decree RD608/2006). However, beekeepers usually perform at least another treatment in summer in case they detect mites in their hives. The acaricides authorised to control *V. destructor* in Spain include "hard acaricides" (based on pyrethroids like tau-fluvalinate and flumethrin; the formamidine amitraz, and the organophosphate coumaphos), together with "soft acaricides" (mostly based on formic or oxalic acids, and the essential oil thymol) (FAO, 2000; www.aemps.gob.es/). Integrated Pest Management (IPM) strategies encourage the combined use of both types of acaricides and other beekeeping practices to reach better long-term control of the mite, but beekeepers are relying mainly on hard acaricides because they are faster and usually more effective (Rosenkranz et al. 2010).

The intensive use of pyrethroids to control Varroa for decades resulted in the emergence of resistance to these acaricides in apiaries from several countries (Milani 1995, Elzen et al. 1998,

70 Sammataro et al. 2005, Gracia-Salinas et al. 2006, González-Cabrera et al. 2018). Since the
71 emergence of *V. destructor* resistance to pyrethroids, beekeepers switched to coumaphos as the
72 best alternative to control the parasite, but the intensive treatment regime with this compound
73 resulted in the evolution of resistance in many locations too (Elzen and Westervelt 2002, Maggi
74 et al. 2009, Maggi et al. 2011). In this scenario, the alternatives to control *V. destructor* have been
75 drastically reduced to the use of amitraz and soft acaricide treatments. Currently, the extensive
76 use of amitraz is exerting an intense selection pressure over populations, threatening them with
77 the evolution of resistance to this compound. Indeed, a reduction in the efficacy of amitraz for
78 Varroa control, probably associated with the evolution of resistance, has already been reported
79 elsewhere (Rodríguez-Dehaibes et al. 2005, Maggi et al. 2010, Kamler et al. 2016, Rinkevich
80 2020).

81 The mechanism of resistance to pyrethroids in *V. destructor* is well known. It is associated
82 with mutations at the residue L925 of the major target site for pyrethroids: the Voltage-Gated
83 Sodium Channel (VGSC) (González-Cabrera et al. 2013, Hubert et al. 2014, González-Cabrera
84 et al. 2016, González-Cabrera et al. 2018). To detect these amino acid substitutions, TaqMan®
85 allelic discrimination assays have been developed (González-Cabrera et al. 2013, González-
86 Cabrera et al. 2016, González-Cabrera et al. 2018). This is a high throughput diagnostics
87 technique capable of detecting the mutation in individual mites. On the other hand, the molecular
88 mechanisms causing the resistance to coumaphos and amitraz in *V. destructor* are still unknown,
89 so the reduction in the efficacy to control *V. destructor* in apiaries using treatments based on these
90 two acaricides can only be confirmed by bioassays with a direct exposition of mites to the
91 acaricidal products (Elzen and Westervelt 2002, Maggi et al. 2009, Maggi et al. 2011).

92 The European Union (EU) is the world's second largest honey producer after China. Spain
93 is the country with the most hives in Europe (more than three million hives) and the second largest
94 producer of honey in the EU, with almost 30,000 tons per year (https://ec.europa.eu/info/food-
95 farming-fisheries/animals-and-animal-products/animal-products/honey_en). The Comunitat
96 Valenciana is a Spanish extensive region of 23,255 km² comprising three provinces with an

important professionalized beekeeping sector. It has a census of 358,327 hives in 2,459 beekeeping operations, being the second region with the highest honey production in Spain. Almost all the hives (98 %) are mobile, carrying out migratory beekeeping throughout the year (https://www.mapa.gob.es/es/ganaderia/temas/produccion-y-mercados-ganaderos/indicadoreseconomicossectordelamiel2018comentarios_tcm30-419675.pdf).

Despite the treatments to manage the mite, Varroa parasitism, far from being controlled, it is a persistent problem in all honey-producing countries. It seems plausible that the continuous presence of varroosis in beehives around the world is related to resistance to acaricidal products. In Spain, this correlation has not been confirmed yet since there is no program to track the efficacy of treatments or the evolution of resistance in Spanish apiaries. The lack of knowledge of the incidence and prevalence of varroosis in all the territories of this country led us to plan a systematic study in the Comunitat Valenciana region. The goal of this study was to determine the efficacy of the three groups of hard acaricides in *V. destructor* populations from apiaries located throughout the three provinces of the region. This study was coordinated with the Department of Agriculture, Environment, Climate Change and Rural Development of the regional government of the Comunitat Valenciana (Generalitat Valenciana, www.gva.es) and Sanitary Defence Groups (ADS acronym in Spanish) of the beekeeping sector. Our aim was to provide beekeepers with information about the impact of varroosis in their colonies and to estimate the possible efficacy for each apiary of acaricide treatments based on pyrethroids, coumaphos and amitraz.

117 **MATERIAL AND METHODS**

118 **Mites**

119 *Varroa destructor* females were collected from apiaries located in the three provinces of the
120 Comunitat Valenciana region (Spain): Castellón, Valencia and Alicante. The sample collection
121 was carried out in two consecutive annual beekeeping seasons: first, 90 samples were collected
122 from April to July 2018; and in the second period, 82 samples were collected from November
123 2018 to July 2019. Beekeepers and veterinaries from nine Sanitary Defence Groups (ADS),
124 participated in the collection and shipments of the samples (Table 1). At least two combs with
125 capped brood per apiary were collected, boxed in polystyrene containers and shipped to the
126 laboratory at the University of Valencia by express courier. The samples arrived in less than 24
127 hours after collection to ensure optimal mite conditions before bioassays.

128 **Bioassays with acaricides**

129 Bioassays were conducted as in Higes et al. (2020) using strips of Checkmite+ (coumaphos a.i.,
130 Bayer, Germany), Apitraz (amitraz a.i., Laboratorios Calier, S.A., Spain), Amicel Varroa (amitraz
131 a.i., Maymó S.L., Spain), and Apivar (amitraz a.i., VétoPharma, France). Briefly, parasitized bee
132 pupae were extracted from the brood cells using a pair of soft tweezers (Fig. 1A). The female
133 mites were collected with a soft paint brush and deposited onto a wet filter paper. A piece of
134 approximately 4 cm long of each acaricide strip was placed into a 5.5 cm Petri dish. Amicel
135 Varroa strips were prepared following the manufacturer instructions. Each strip piece maintained
136 its original width (2.5 cm for Checkmite+; 4.0 cm for Apitraz and Apivar; and 4.6 cm for Amicel
137 Varroa). Given the different amount of active ingredient impregnated in the strips of each product,
138 and considering the surface of both sides of the strips, the actual concentration was 13.6 mg/cm^2,
139 2.1 mg/cm^2, 0.8 mg/cm^2 and 3.1 mg/cm^2 for Checkmite+, Apitraz, Amicel and Apivar,
140 respectively. The mites collected (15 mites per replicate, 2-3 replicates for each acaricide product)
141 were laid on top of the strip and their movements were monitored to control that they remain on
142 top of the strip for at least 5 min (Fig. 1B). The dish was sealed with Parafilm® and holed with an

143 entomological needle to allow aeration. Mites onto the acaricide strip were incubated for 1 h at
144 34 ºC, 90 % RH in a wet incubator. After 1 h, the strip was removed and the dish with the mites
145 was incubated for 3 more hours at 34 ºC, 90 % RH. Controls mites were treated the same way but
146 without acaricide strips. After the incubation time was completed, mortality was evaluated by
147 assessing the movement of mites after probing them with a fine paint brush (Fig. 1C). The
148 expected efficacy of each acaricide was estimated using the mortality values obtained in the
149 bioassays.

150 **TaqMan® assays**

151 Genotyping of mites for detecting susceptible and pyrethroid-resistant alleles in *V. destructor*
152 VGSC was carried out using a TaqMan® based allelic discrimination assay as described by
153 González-Cabrera et al. (2013). Genomic DNA was extracted from individual adult mites by an
154 alkaline hydrolysis method and stored at -20 ºC until used. Briefly, reactions mixtures contained
155 1.5 µl of genomic DNA, 7.5 µl of TaqMan® Fast Advanced Master Mix (Thermo Fisher
156 Scientific), 0.9 µM of each primer and 0.2 µM of each fluorescent-labelled probe in a total
157 reaction volume of 15 µl. Assays were run on a StepOne Plus Real-Time PCR system (Thermo
158 Fisher Scientific) using the following temperature cycling conditions: 10 min at 95 °C followed
159 by 40 cycles of 95 °C for 15 s and 60 °C for 45 s. The increase in VIC® and 6FAM™ fluorescence
160 was monitored in real time by acquiring each cycle on the yellow channel (530 nm excitation and
161 555 nm emission) and green channel (470 nm excitation and 510 emission) of the StepOne Plus,
162 respectively. Forty mites were genotyped per apiary. Duplicated control samples corresponding
163 to RR homozygotes, SR heterozygotes, SS homozygotes and negative controls (distilled water)
164 were included in each assay. Since resistance to pyrethroids associated to amino acid substitutions
165 in the VGSC is inherited as a recessive trait (Davies et al. 2007, González-Cabrera et al. 2013),
166 mites carrying the mutant allele in homozygosis (RR) are considered as pyrethroid-resistant,
167 whereas mites carrying the wild-type allele in homozygosis (SS) and the heterozygotes (SR) are
168 considered as pyrethroid-susceptible mites.

169

170 **RESULTS**

171 The samples (capped brood) used in this study were collected from apiaries located across the
172 three provinces of the Comunitat Valenciana region (Spain). In the 2018 season, brood combs
173 were collected from 90 apiaries (Table 2) while in the 2019 season they were collected from 89
174 apiaries (Table 3). As we need at least 120 live mites per sample to carry out the bioassays, not
175 all the samples collected had sufficient mites. Hence, the level of parasitism required for bioassays
176 was found in 58 % and 81 % of the samples in 2018 and 2019, respectively. On the other hand,
177 since it is possible to carry out TaqMan® assays using a smaller number of mites collected either
178 dead or alive, more analyses were carried out with this technique than with bioassays, resulting
179 in 70 % and 94 % of the samples tested in 2018 and 2019 seasons, respectively.

180 Results from bioassays conducted with Checkmite+ strips (coumaphos a.i.) showed some
181 variability among samples, with a mean mortality of 50 % (± 21 SD) in 2018 (Fig. 2A), and 54
182 % (± 17 SD) in 2019 (Fig. 2B). Overall, the 75 % percentile value of approximately 66 %
183 mortality obtained from the 122 apiaries tested in both seasons indicated that this product was
184 less effective than expected according to the label.

185 Three commercial acaricides based on amitraz were tested: Apitraz, Amicel Varroa and
186 Apivar. Results from bioassays carried out with Apitraz in 2018 showed a mean mortality of 74
187 % (± 14 SD) (Fig. 2A). In that season, bioassays with Amicel Varroa showed a mean mortality
188 of 79 % (± 12 SD) (Fig. 2A). Statistical analysis showed that these values were not significantly
189 different (t test, P value > 0.05). In 2019, mean mortality with Apitraz and Apivar was 81 % (± 8
190 SD) and 79 % (± 7 SD), respectively (Fig. 2B), again with no significant differences between
191 them (t test, P value > 0.05). Therefore, according to our data, the mortality of the three amitraz-
192 based acaricides tested was found to be similar across the study.

193 The expected efficacy of pyrethroids based acaricides against *V. destructor* was estimated
194 using a TaqMan® genotyping assay. The frequency of pyrethroid-resistant and susceptible mites
195 was determined for each sample after genotyping 40 individual mites for the presence of different

196 alleles of the mutation L925V at the *V. destructor* VGSC. When TaqMan® assays were conducted,
197 a wide range of allele frequency patterns was found in the apiaries evaluated in both, 2018 and
198 2019 seasons. The estimated mean efficacy of pyrethroids was 41 % (± 32 SD) in samples from
199 2018 (Fig. 2A), and 36 % (± 32 SD) in samples from 2019 (Fig. 2B). The Standard Deviation of
200 these means corroborated the high dispersion of pyrethroid expected efficacies throughout the
201 region, ranging from zero (in apiaries with all mites resistant to pyrethroids), to 97 % in some
202 apiaries with almost all mites susceptible to the acaricide.

203 The efficacy of the different acaricides in each apiary was weighed according to its
204 geographic location (Fig. 3 and 4). Our data show that there is no geographic dependent pattern
205 of expected efficacies to the acaricides tested, as it can be noticed looking at the different mortality
206 values estimated in apiaries at nearby locations (Fig. 3 and 4; Tables 2 and 3).

207 Along with the brood combs, information about treatment history was collected from each
208 apiary. These data showed that most of the beekeeping operations used amitraz-based acaricides
209 (88 % of the treatments), while the use of other treatment regimens such as those based on
210 pyrethroids and soft acaricides was much lower, representing 5 % and 7 % of the total treatments,
211 respectively. (Fig. 5).

212

213 **DISCUSSION**

214 The data presented here is the first comprehensive and large-scale monitoring study describing
215 the situation of the resistance to acaricides in populations of *V. destructor*. The analyzed apiaries
216 belong to the Comunitat Valenciana, one of the most prominent apicultural regions in Spain.
217 Moreover, since migratory beekeeping is a common practice among beekeepers, it is possible to
218 hypothesize that the current situation in this region may resembled that of the rest of the country.

219 Varroosis is one of the major threats to the viability of apiculture, not only in Spain, but
220 worldwide. Beekeepers are currently struggling to find good alternatives to reach an adequate
221 management of the mite since there are very few active ingredients and formulations authorized

222 to battle the disease. Therefore, it is crucial that the acaricides already in the market remain
223 effective for as long as possible, although the evolution of resistance is currently jeopardizing this
224 aim. If resistance to an acaricide is detected and the treatments with this compound are not
225 discontinued immediately, resistant individuals will be selected and they will become more
226 frequent in the population, leading to therapeutic failures in more and more places as resistance
227 spreads. For this reason, programs to monitor the efficacy of acaricide products are of critical
228 importance.

229 The huge volume of apiaries in Spain require an adequate management of the active
230 ingredients for *V. destructor* control, so that the most appropriated for each operation is used
231 depending on the susceptibility of the mite population to each acaricide. However, there is no
232 official program in this country recording the efficacy of treatments, nor the monitoring of
233 possible outbreaks of resistance to these treatments. In a recent study, bioassays with Checkmite,
234 Apivar and Apistan were conducted with samples collected in seven Spanish locations (Higes et
235 al. 2020), providing a general idea of the acaricide efficacy in these apiaries. Aiming to perform
236 a more thorough study, we carried out analyses in two consecutive years with a significantly
237 higher number of samples, covering almost completely the area dedicated to beekeeping in this
238 region. To obtain data from a large number of apiaries, we coordinate with the government of the
239 Comunitat Valenciana region (Generalitat Valenciana) and nine sanitary defence groups (ADS)
240 of the beekeeping sector. As a consortium, we started a program to evaluate the efficacy of the
241 authorized active ingredients to control *V. destructor* in apiaries of this Spanish region. The study
242 was carried out during 2018 and 2019 beekeeping seasons testing the three groups of hard
243 acaricides authorized in Spain: coumaphos, amitraz and pyrethroids.

244 Coumaphos has been widely used for many years as an active ingredient in Checkmite+
245 commercial strips. In Spain, a reduction of this product efficacy to control *V. destructor* parasitism
246 was detected a few years ago, with mean efficacies of about 70 to 80 % (Sánchez Escudero and
247 Fernández Tejedor 2016, Calatayud et al. 2018). Actually, the product's distributor in Spain,
248 Bayer Hispania, S.L. (Animal Health), issued a statement informing beekeepers of a possible lack

249 of Varroa sensitivity to coumaphos based on preliminary results obtained in a study with this
250 acaricide in three areas of central and northern Spain (https://www.aeapicultores.org/wp-
251 content/uploads/2017/03/Comunicado_Bayer_Apicultores.pdf). From then on, the use of this
252 acaricide declined dramatically and even the Spanish Association of Veterinary Specialists in
253 Health and Bee Production (AVESPA acronym) demanded the withdrawal of the product from
254 the market and discouraged its use among beekeepers (http://www.colvet.es/node/2663).
255 Tracking the efficacy of Checkmite+ strips with mites from the apiaries of this study confirmed
256 the reduction of its efficacy to control Varroa. According to our data, the mortality observed with
257 this acaricide varied considerably from one apiary to another (Fig. 3 and 4; Tables 2 and 3),
258 although in all cases the expected efficacy would be lower than that indicated by the manufacturer.
259 The significant variation in the mortality registered for coumaphos in the bioassays throughout
260 the study seems to indicate that the reduced efficacy is due to the presence of mites resistant to
261 this acaricide in the hives. Actually, our previous study with mites sampled in different Spanish
262 regions also found that resistance to coumaphos is evolving in this country (Higes et al. 2020).
263 *Varroa destructor* populations resistant to coumaphos were previously reported in America
264 (Elzen and Westervelt 2002, Maggi et al. 2009, Maggi et al. 2011). In particular, the resistance
265 reported in one of these studies does not seem reversible after stopping the treatments with
266 coumaphos (Mitton et al. 2018). Our results support this hypothesis since the apiaries in this study
267 had not been treated with coumaphos for several years and still part of the populations remained
268 insensitive to this compound. The accumulation and persistence of coumaphos residues in
269 beeswax for a long time (Calatayud-Vernich et al. 2018), could favour a constant selection
270 pressure in the colonies, reducing the possibility of reversing the resistance. However, to rule out
271 this hypothesis it may be necessary to identify the molecular mechanism of the resistance, which
272 may also be of help to design molecular tools to identify coumaphos-resistant individuals in the
273 populations.

274 On the other hand, the mechanism of *V. destructor* resistance to pyrethroids is well
275 described, so a TaqMan® allelic discrimination assay was used to identify the resistant mites

carrying a mutation in the 925 position of the VGSC as described by González-Cabrera et al. (2013). We used the proportion of susceptible and pyrethroid-resistant individuals in each apiary to estimate the efficacy that a pyrethroid treatment would have. The results also showed great diversity in the proportion of resistant mites among apiaries. Remarkably, bees on many operations were parasitized only by pyrethroid-resistant mites, but there were also others with most of the mites labelled as susceptible (Fig. 3 and 4; Tables 2 and 3). The long-track record of treatments only with pyrethroid-based acaricides and their accumulation in beeswax (Calatayud-Vernich et al. 2018) is a likely explanation for the evolution of resistance. Although it has been suggested several times that the mutations associated with the resistance to pyrethroids in Varroa cause a reduced fitness in the mites (Milani and Della Vedova 2002, González-Cabrera et al. 2016, González-Cabrera et al. 2018), it seems that this is not enough to remove completely the resistant mites. The Varroa populations may contain a remnant of resistant individuals that would be quickly selected as soon as the treatment with pyrethroids are reinstated. Our data support this hypothesis since the mites collected from apiaries reporting the last treatment with pyrethroids were mainly labelled as resistant (data not shown). This observation is again confirming the idea that, although it is advisable to use pyrethroids to control Varroa in an IPM context when the frequency of resistant individuals is sufficiently low, it is very important to avoid applying continuous treatments with pyrethroid-based acaricides.

In the case of both, coumaphos and pyrethroids, no geographical distribution associated with the resistance was observed. On the contrary, the estimated efficacy for these treatments seemed to be randomly distributed. This might be surprising at first sight, but it can be explained by the idiosyncrasy of beekeeping in this region, which beekeepers mainly keeping hives that are seasonally moved across the country. The movement of hives to different areas is most certainly involving a transfer of parasitized bees from the migrant hives to those already in the transient settlement and vice versa. This can justify that each apiary has a specific pattern of acaricide efficacy depending on both, the treatments applied and the places they have traveled.

302 Apitraz, Apivar and Amicel Varroa, commercial products containing amitraz as an active
303 ingredient, are widely used in Spain. The assays conducted showed that the expected efficacy in
304 the field would be very similar for the three of them, and it would also be the highest amongst the
305 different active ingredients tested in this study (Fig. 3 and 4; Tables 2 and 3). The low variation
306 recorded after assaying amitraz in multiple apiaries is an indication of its consistency as acaricide
307 across the country. However, given that our data indicate that the expected efficacy would be
308 below 90 % in most cases, it is clear that the products are performing below the expected efficacy
309 according to the label. These data is in agreement with the actual efficacy of amitraz-based
310 product recorded by beekeepers in the field, with values ranging from 60 to 96 % (Calatayud et
311 al. 2018). Hence, it is possible to anticipate an evolution of resistance to amitraz in the coming
312 seasons unless there is a significant change in the management strategies, currently based on the
313 intensive use of mainly amitraz many times per year. Repeated use of a single acaricide exerts an
314 enormous selection pressure on the mites. If resistance to amitraz evolves, it will be much more
315 difficult to control the parasite, since the other commercial products will not be effective in all
316 apiaries. The recommendations to delay the evolution of resistance encourage the rotation of
317 products with different mode of action (IRAC; https://www.irac-online.org/). Therefore, a more
318 rational use of current acaricides would be desirable, alternating the application of different active
319 ingredients in consecutive seasons. To decide whether a given treatment would be successful in
320 each apiary, it is crucial to monitor the efficacy of the acaricidal compounds in each of them. In
321 this way, the effective products could be rotated, avoiding selection pressures with treatments that
322 can lead to an increase in the frequency of resistant mites.

323 The transfer from Academia to the field is one of the priorities of this work. The final
324 objective was to provide the beekeeping sector with the information obtained in this study. The
325 results from this project were disclosed in informative talks to groups of beekeepers. Moreover,
326 tailored reports with the expected efficacy of acaricide treatments in each apiary were sent to the
327 professionals in charge of the different ADS for them to discuss with the relevant beekeeper the

best management approach for controlling the mites in their apiaries, considering also the previous history of treatments.

Initiatives such as the Honey Bee Health Coalition (https://honeybeehealthcoalition.org/) and the Bee informed partnership (https://beeinformed.org/) in the USA (coalitions of researchers, advisors, and stakeholders from various sides of the honey bee related industry) are good examples of associations that encourage the flow of information among the different actors in the beekeeping world. The rational use of pesticides to manage *V. destructor* is a joint responsibility of public institutions, industry, Academia and the beekeeping sector. Only by acting in a coordinated manner the efficacy of treatments may be prolonged and the evolution of resistances that threaten the viability of apiculture may be delayed.

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439

440 **TABLES**

441

442 Table 1. Sanitary Defence Groups (ADS) that supplied samples of *Varroa destructor*.

Sanitary Defence Groups (ADS)	**Province**	**No. hives[1]**	**No. samples[2]**
ADSAD	Valencia	16901	9
AIXAM	Castellón	16168	9
ALAPI	Alicante	4957	3
APAC	Castellón	46317	25
APIADS	Valencia	93548	50
APICAL	Alicante	44210	27
APIVAL	Valencia	47332	22
CASAPI	Castellón	14904	9
PROAPI	Valencia	43656	18
TOTAL			172

443 [1]Total number of hives belonging to the ADS.

444 [2]Number of samples provided for this study.

445

Table 2. Sample locations and mortality of acaricides recorded in assays from 2018 season.

Apiary	**Location**	**Mortality**						
		Pyrethroids[1]	**Checkmite+**	**SEM[2]**	**Apitraz**	**SEM**	**Amicel**	**SEM**
18_ADSAV6	Monserrat	3	73	ND[3]	77	ND	94	ND
18_AIXAM1	Altura	3	ND		ND		ND	
18_AIXAM2	Castellón	18	69	5	56	5	80	6
18_AIXAM4	Les Alqueries	11	100	ND	93	ND	93	ND
18_ALAPI3	Elche	0	83	5	80	2	90	4
18_APAC01	Betxí	93	ND		ND		ND	
18_APAC02	Onda	0	64	11	72	7	87	4
18_APAC05	Onda	32	46	4	81	4	75	2
18_APAC10	Vila-Real	89	ND		ND		ND	
18_APAC11	San Juan de Moró	50	47	8	58	2	51	3
18_APAC12	Costur	87	41	12	25	10	64	8
18_APAC13	Vall d'Uixò	3	72	4	81	7	82	4
18_APAC14	Sta. Magdalena de Pulpis	33	ND		ND		ND	

18_APAC16	Borriol	72	ND		80	6	85	ND
18_APAC17	Ares del Maestrat	5	56	12	81	6	86	6
18_APIADS0	Caudete	34	34	2	78	4	81	11
18_APIADS01	Llíria	78	38	14	83	11	90	5
18_APIADS02	Bétera	65	5	4	64	9	94	2
18_APIADS03	Llíria	40	ND		ND		ND	
18_APIADS04	Barxeta	77	40	14	94	3	89	6
18_APIADS05	Cheste	75	34	4	54	3	88	3
18_APIADS07	Manises	0	ND		ND		ND	
18_APIADS11	Chiva	51	72	ND	86	ND	97	ND
18_APIADS12	Chiva	76	41	3	80	4	84	2
18_APIADS13	Llíria	59	60	2	72	0	65	7
18_APIADS14	Sagunto	13	71	ND	89	ND	79	ND
18_APIADS15	Monserrat	55	25	6	66	4	68	5
18_APIADS16	Montroi	22	ND		ND		ND	
18_APIADS17	Gestalgar	65	32	2	65	5	63	10

18_APIADS18	Gestalgar	77	19	4	72	5	79	1
18_APIADS19	Yátova	10	ND		ND		ND	
18_APIADS20	Carcaixent	53	49	10	83	7	89	11
18_APIADS21	Guardasuar	0	4	4	59	2	73	6
18_APIADS22	Monserrat	37	37	ND	70	ND	53	ND
18_APICAL01	Beniarrés	65	12	12	80	12	92	9
18_APICAL02	Guardamar del Segura	60	65	9	85	1	81	8
18_APICAL04	Elche	3	68	11	84	4	83	9
18_APICAL05	Guardamar del Segura	62	21	7	89	5	94	3
18_APICAL06	Los Montesinos	68	34	ND	92	ND	81	ND
18_APICAL07	Castalla	26	54	5	76	9	79	6
18_APICAL08	Elche	72	84	7	85	5	82	1
18_APICAL09	Pego	33	55	2	76	6	76	7
18_APICAL10	Pego	8	72	1	74	8	86	ND
18_APICAL11	Beniarrés	53	65	15	92	4	83	2
18_APICAL12	Relleu	10	36	12	55	5	59	10

18_APICAL13	San Vicente del Raspeig	62	ND		91	6	92	4
18_APICAL14	Agres	85	45	11	72	6	84	8
18_APICAL15	Real de Montroy	7	34	6	84	1	82	5
18_APIVAL2	Bufali	15	72	8	85	3	80	6
18_APIVAL3	Polinyà del Xúquer	63	37	10	63	2	58	3
18_APIVAL4	Cheste	0	40	8	73	7	63	9
18_APIVAL5	Fuente la Higuera	87	20	8	60	9	53	5
18_APIVAL6	Anna	3	43	ND	86	ND	97	ND
18_APIVAL7	Mariola	89	44	12	87	1	86	6
18_APIVAL8	Bocairent	87	9	3	54	15	69	16
18_APIVAL9	Ontinyent	82	30	13	68	15	79	16
18_CASAPI1	Torás	4	ND		ND		ND	
18_CASAPI3	Nules	0	27	10	76	ND	76	ND
18_CASAPI5	Artana	5	19	6	52	4	63	5
18_PROAPI2	Tous	2	25	3	40	15	75	16
18_PROAPI3	Ayora	2	39	4	60	12	78	6

18_PROAPI5	Ayora	53	23	ND	90	ND	62	ND
18_PROAPI6	Ayora	68	ND		ND		ND	

[1]Values indicate the percentage of pyrethroid-susceptible mites (RS and SS alleles).

[2]Standard Error of the Mean.

[3]Non determined.

Table 3. Sample locations and mortality of acaricides recorded in assays from 2019 season.

Apiary	**Location**	**Mortality**						
		Pyrethroids[1]	**Checkmite+**	**SEM**[2]	**Apitraz**	**SEM**	**Amicel**	**SEM**
19_ADSAV01	Llíria	8	ND[3]		ND		ND	
19_ADSAV02	ND	40	98	ND	83	ND	75	ND
19_AIXAM02	Castellón	0	71	ND	82	ND	86	ND
19_AIXAM04	Altura	6	ND		ND		ND	
19_ALAPI01	Guardamar del Segura	12	ND		ND		ND	
19_APAC01	Torrechiva	0	ND		ND		ND	
19_APAC02	Vistabella	81	ND		ND		ND	
19_APAC03	Onda	6	54	12	85	4	82	4
19_APAC04	Alquerías del Niño Perdido	10	64	2	72	5	83	7
19_APAC05	Sant Joan de Moró	24	67	6	75	5	76	2
19_APAC06	Borriol	54	73	1	90	3	84	1
19_APAC07	Catí	57	51	2	74	1	74	1
19_APAC08	Xodos	20	48	3	90	0	80	5

19_APAC09	Cervera del Maestre	35	44	ND	87	ND	ND	
19_APAC10	Costur	27	40	13	82	ND	79	ND
19_APAC11	Atzaneta del Maestrat	20	63	5	80	0	80	0
19_APAC12	Ares	8	23	4	80	5	76	5
19_APAC13	Torreblanca	0	24	7	74	4	78	0
19_APIADS01	Montroi	10	72	4	65	14	84[a]	2
19_APIADS02	Casinos	80	38	14	83	2	79	10
19_APIADS03	Montserrat	48	23	ND	ND		ND	
19_APIADS04	Higueruelas	0	ND		ND		ND	
19_APIADS05	Picassent	9	ND		ND		ND	
19_APIADS06	Montroi	72	63	2	77	2	75	4
19_APIADS07	Navarrés	20	21	ND	ND		ND	
19_APIADS08	Valencia	84	54	10	90	7	83	0
19_APIADS09	Llíria	90	23	ND	ND		ND	
19_APIADS10	Chiva	54	50	ND	93	ND	82	ND
19_APIADS11	Alzira	57	63	8	89	2	76	8

19_APIADS12	Llíria	32	37	5	80	4	75	3
19_APIADS13	Bunyol	45	62	2	78	4	83	4
19_APIADS14	Torrent	60	82	2	86	1	80	1
19_APIADS15	Chiva	57	53	ND	82	ND	ND	
19_APIADS16	ND	12	ND		ND		ND	
19_APIADS17	Alaquàs	0	69	1	74	1	71	4
19_APIADS19	Villarmarxant	74	73	7	76	1	80	ND
19_APIADS20	Cheste	0	61	6	83	2	83	2
19_APIADS21	Cheste	0	60	ND	87	ND	80	ND
19_APIADS22	Chiva	47	52	3	82	0	83	1
19_APIADS23	Chiva	36	48	ND	83	ND	78	ND
19_APIADS24	Bétera	28	60	5	84	1	84	0
19_APIADS25	Bétera	32	55	4	73	1	75	1
19_APIADS26	Bétera	85	30	1	71	3	67	3
19_APIADS27	Chiva	77	56	7	81	4	78	3
19_APICAL01	Molins	18	67	11	85	4	90a	3

19_APICAL02	Rafol de Almunia	3	66	2	68	4	74	2
19_APICAL03	Gata de Gorjos	78	33	5	66	6	62	13
19_APICAL04	Benitachell	47	44	ND	89	ND	93	ND
19_APICAL05	Vilajoiosa	95	52	ND	90	ND	83	ND
19_APICAL06	San Miguel de Salinas	11	40	3	84	5	87	1
19_APICAL07	Torremanzanas	92	46	7	86	1	80	1
19_APICAL08	Torremanzanas	0	59	6	80	3	77	3
19_APICAL09	Finestrat	0	53	1	82	4	82	1
19_APICAL10	Agres	85	69	1	85	3	69	1
19_APICAL11	Castalla	60	81	1	86	1	83	1
19_APICAL12	Sella	55	53	ND	64	ND	69	ND
19_APIVAL01	Quesa	0	60	4	72	4	77	1
19_APIVAL02	La Font de la Figuera	68	36	4	87	2	87	2
19_APIVAL03	Favara	5	31	ND	93	ND	93	ND
19_APIVAL04	Carcaixent	20	52	5	90	0	86	4
19_APIVAL05	Sumacárcer	0	58	2	86	0	78	2

19_APIVAL06	Bétera	3	29	2	90	2	88	1
19_APIVAL07	Albal	36	57	3	89	1	83	4
19_APIVAL08	Yátova	2	67	4	83	ND	82	ND
19_APIVAL09	Polinyà del Xúquer	47	ND		ND		ND	
19_APIVAL10	Polinyà del Xúquer	40	75	7	76	0	77	1
19_APIVAL11	Bocairent	28	34	3	76	1	77	0
19_APIVAL12	Vall d'Alcalà	5	68	2	76	5	65	6
19_APIVAL13	Villena	97	40	ND	83	ND	81	ND
19_CASAPI01	Mas de Noguera	49	23	4	79	3	69	4
19_CASAPI03	Tales	17	85	4	94	ND	72	ND
19_CASAPI04	Sacañet	20	75	2	72	2	75	2
19_PROAPI01	Ayora	5	67	ND	85	ND	ND	
19_PROAPI02	Énova	0	38	ND	90	ND	83	ND
19_PROAPI03	Orihuela	97	68	1	76	0	72	ND
19_PROAPI04	Requena	89	54	ND	100	ND	72	ND
19_PROAPI05	Moixent	96	40	3	73	3	75	9

19_PROAPI06	Requena	2	85	6	85	2	77	2
19_PROAPI07	Ayora	0	ND		ND		ND	
19_PROAPI08	Ayora	10	83	ND	87	ND	98	ND
19_PROAPI09	Náquera	65	61	1	74	1	89	0
19_PROAPI10	Ayora	72	69	ND	75	ND	ND	
19_PROAPI11	Ayora	50	ND		ND		ND	
19_PROAPI12	Ayora	32	61	10	84	3	79	2

[1]Values indicate the percentage of pyrethroid-susceptible mites (RS and SS alleles).

[2]Standard Error of the Mean.

[3]Non determined.

FIGURE LEGENDS

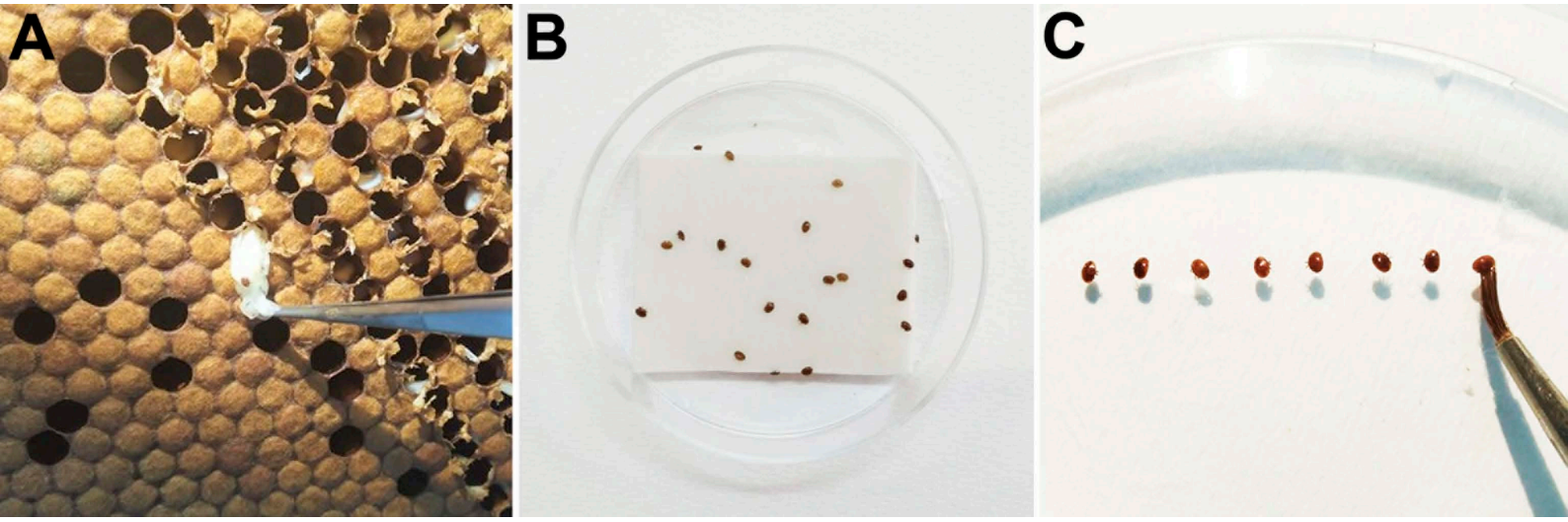
Figure 1. Bioassays with *Varroa destructor*. (A) Parasitized bee nymphs extracted from the brood cells. (B) Female mites laid on the acaricide strip into a Petri dish. (C) Mortality was evaluated by assessing the movement of mites after probing them with a fine paint brush.

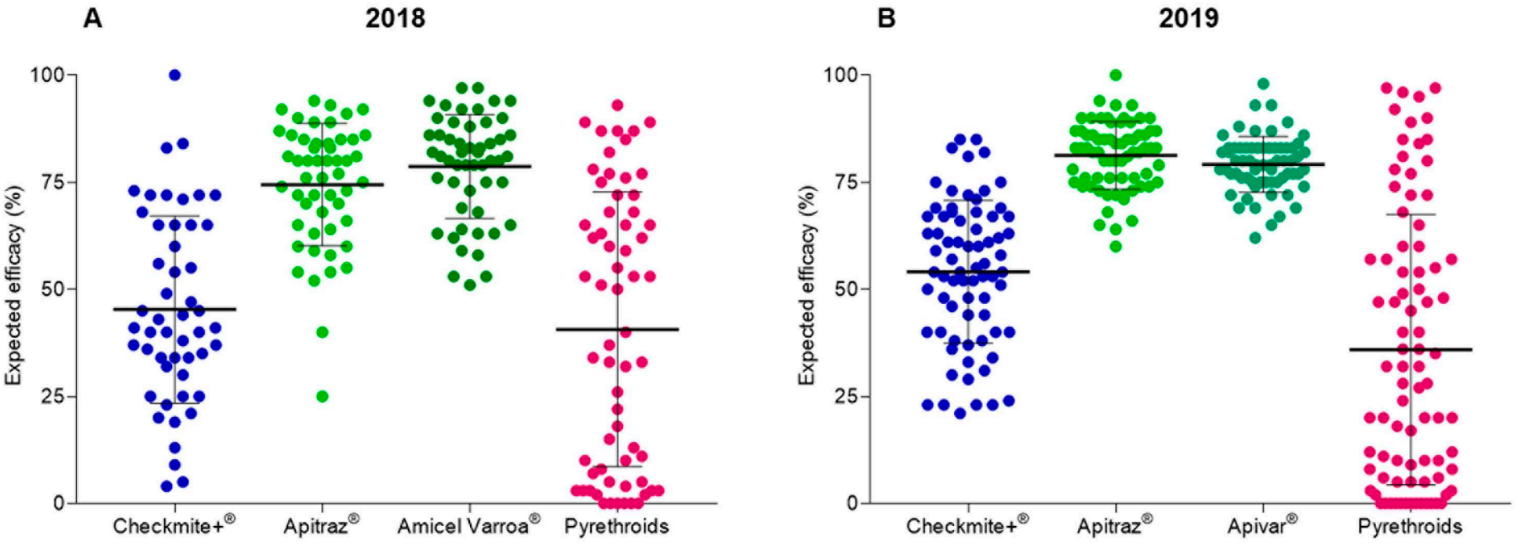
Figure 2. Expected efficacy of commercial acaricides against *Varroa destructor* in 2018 season (A) and 2019 season (B). For Checkmite+, Apitraz, Amicel and Apivar, the expected efficacy corresponds to mortality recorded in the bioassays. The expected efficacy of pyrethroids-based acaricides was estimated using the frequency of pyrethroid-resistant and susceptible mites after genotyping individual mites for the presence of different alleles of the mutation L925V at the *V. destructor* VGSC.

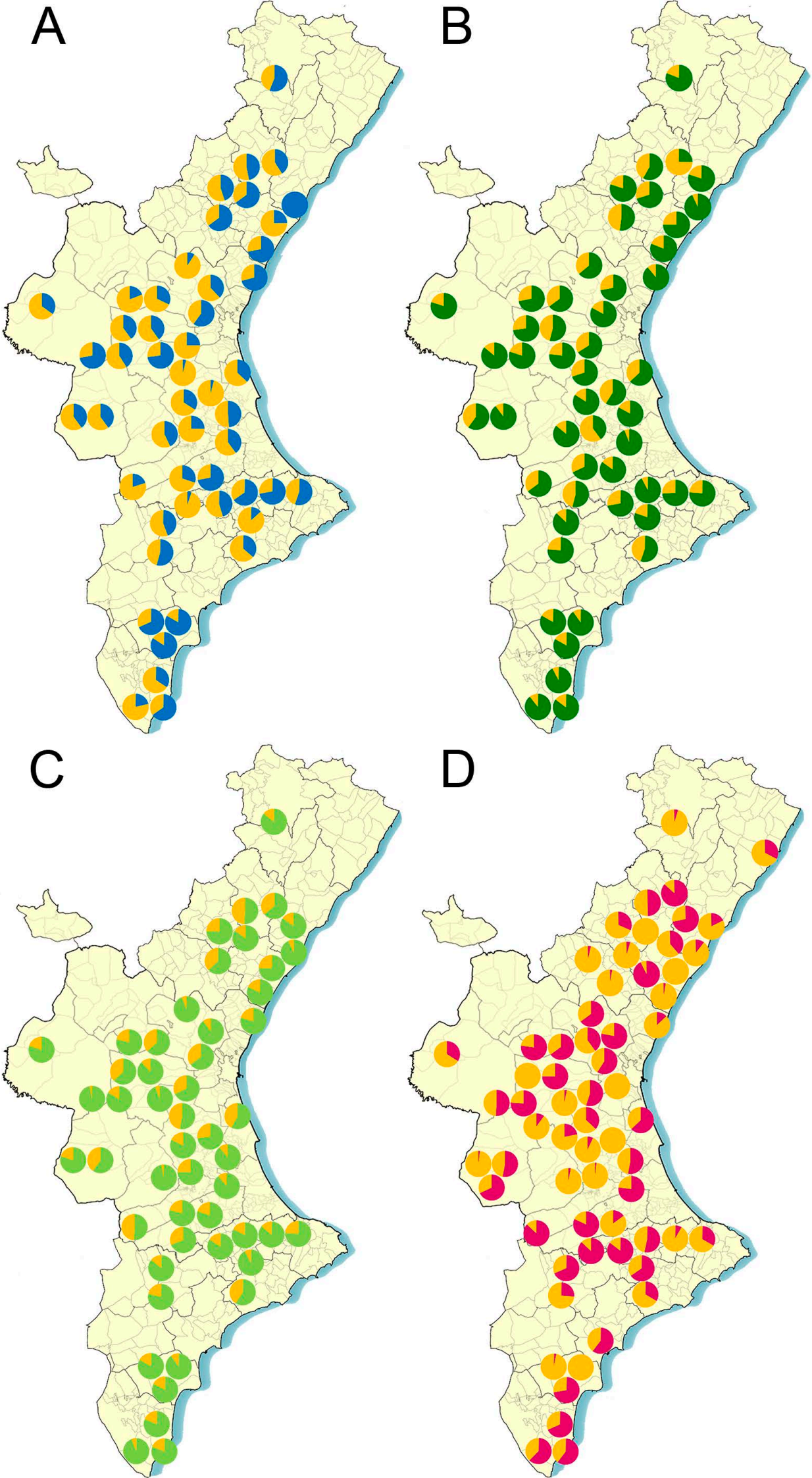
Figure 3. Sampling locations and expected efficacy (expressed as percentage) of commercial acaricides against *Varroa destructor* in 2018 season. A) Coumaphos (efficacy in blue); B) Apitraz (efficacy in dark green); C) Amicel Varroa (efficacy in light green); D) Pyrethroids (efficacy in pink).

Figure 4. Sampling locations and expected efficacy (expressed as percentage) of commercial acaricides against *Varroa destructor* in 2019 season. A) Coumaphos (efficacy in blue); B) Apitraz (efficacy in dark green); C) Apivar (efficacy in light green); D) Pyrethroids (efficacy in pink).

Figure 5. Acaricide treatments (%) in the apiaries that provided samples for this study in 2018 and 2019 seasons.


A
B
C


A
2018
Expected efficacy (%)
100
75
50
25
0
Checkmite+®
Apitraz®
Amicel Varroa®
Pyrethroids
B
2019
Expected efficacy (%)
100
75
50
25
0
Checkmite+®
Apitraz®
Apivar®
Pyrethroids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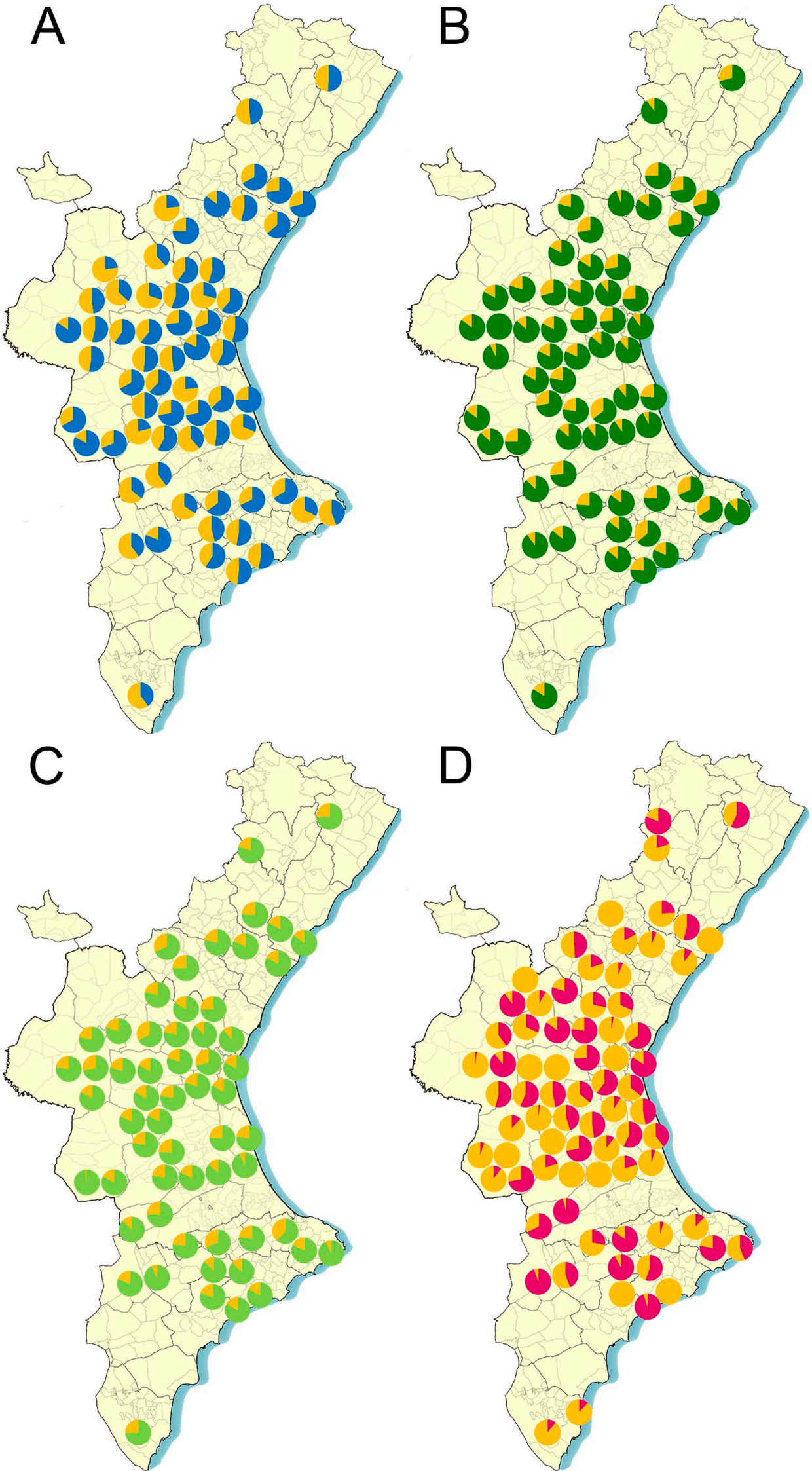
A
B
C
D


Amitraz
Amitraz + soft acaricides
Soft acaricides
Pyrethroids