# 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.

44 **INTRODUCTION**

45 The ectoparasitic mite *Varroa destructor* is considered a major pest of the Western honey bee (*A.*
46 *mellifera* L.) (Rosenkranz et al. 2010). This mite feeds mostly on the fat body of immature and
47 adult bees and vectors numerous lethal viruses (Boecking and Genersch 2008, Ramsey et al.
48 2019), compromising the natural honey bee defences. These severe damages make *V. destructor*
49 one of the main factors contributing to the many seasonal losses of honey bee colonies around the
50 world (Genersch et al. 2010, Guzmán-Novoa et al. 2010).

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

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

68 The intensive use of pyrethroids to control Varroa for decades resulted in the emergence
69 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

276 carrying a mutation in the 925 position of the VGSC as described by González-Cabrera et al.
277 (2013). We used the proportion of susceptible and pyrethroid-resistant individuals in each apiary
278 to estimate the efficacy that a pyrethroid treatment would have. The results also showed great
279 diversity in the proportion of resistant mites among apiaries. Remarkably, bees on many
280 operations were parasitized only by pyrethroid-resistant mites, but there were also others with
281 most of the mites labelled as susceptible (Fig. 3 and 4; Tables 2 and 3). The long-track record of
282 treatments only with pyrethroid-based acaricides and their accumulation in beeswax (Calatayud-
283 Vernich et al. 2018) is a likely explanation for the evolution of resistance. Although it has been
284 suggested several times that the mutations associated with the resistance to pyrethroids in Varroa
285 cause a reduced fitness in the mites (Milani and Della Vedova 2002, González-Cabrera et al.
286 2016, González-Cabrera et al. 2018), it seems that this is not enough to remove completely the
287 resistant mites. The Varroa populations may contain a remnant of resistant individuals that would
288 be quickly selected as soon as the treatment with pyrethroids are reinstated. Our data support this
289 hypothesis since the mites collected from apiaries reporting the last treatment with pyrethroids
290 were mainly labelled as resistant (data not shown). This observation is again confirming the idea
291 that, although it is advisable to use pyrethroids to control Varroa in an IPM context when the
292 frequency of resistant individuals is sufficiently low, it is very important to avoid applying
293 continuous treatments with pyrethroid-based acaricides.

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

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 |

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

[2]Number of samples provided for this study.

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

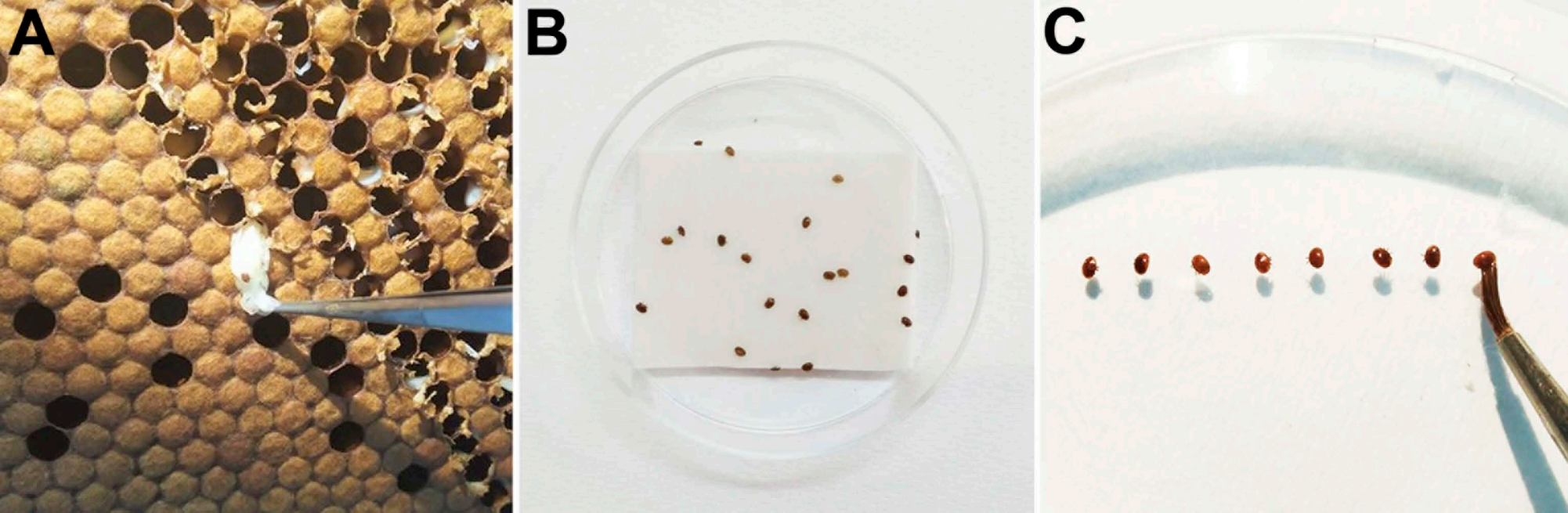
Figure 1. Bioassays with *Varroa destructor*. (A) Parasitized bee nymphs extracted from the brood cells. (B) Female mites laid on the acaricide strip into a Petri dish. (C) Mortality was evaluated by assessing the movement of mites after probing them with a fine paint brush.

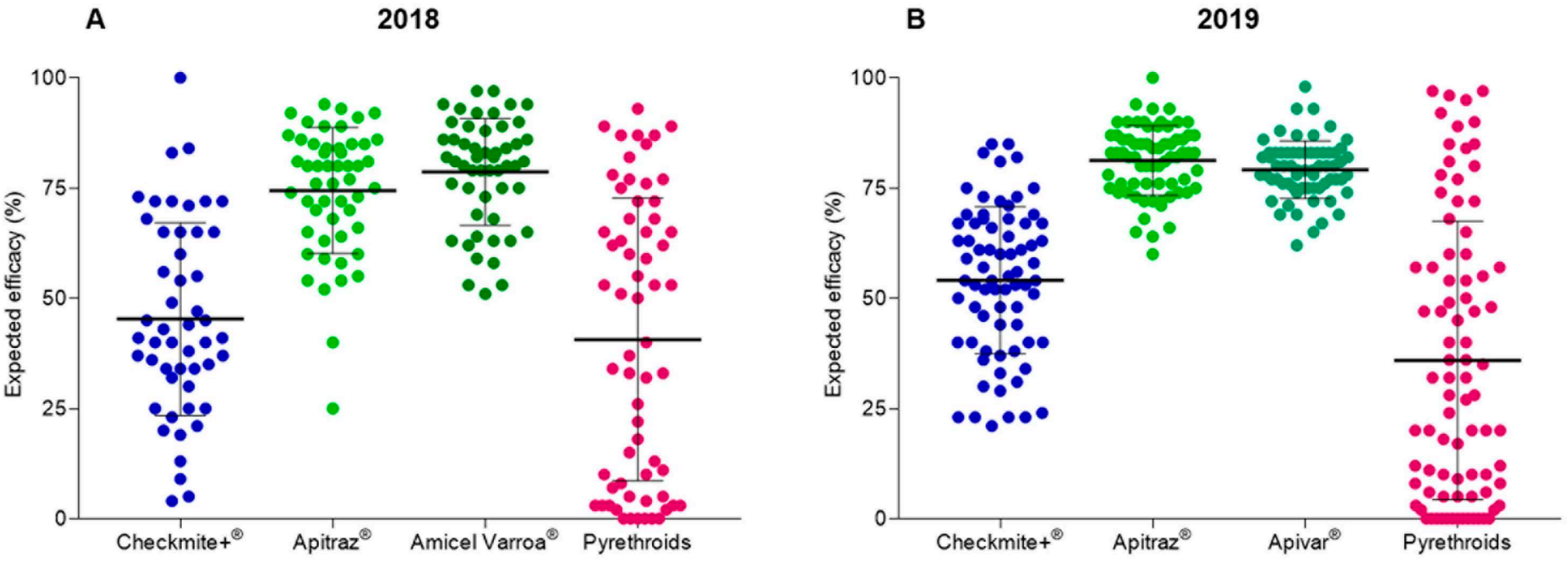
Figure 2. Expected efficacy of commercial acaricides against *Varroa destructor* in 2018 season (A) and 2019 season (B). For Checkmite+, Apitraz, Amicel and Apivar, the expected efficacy corresponds to mortality recorded in the bioassays. The expected efficacy of pyrethroids-based acaricides was estimated 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.

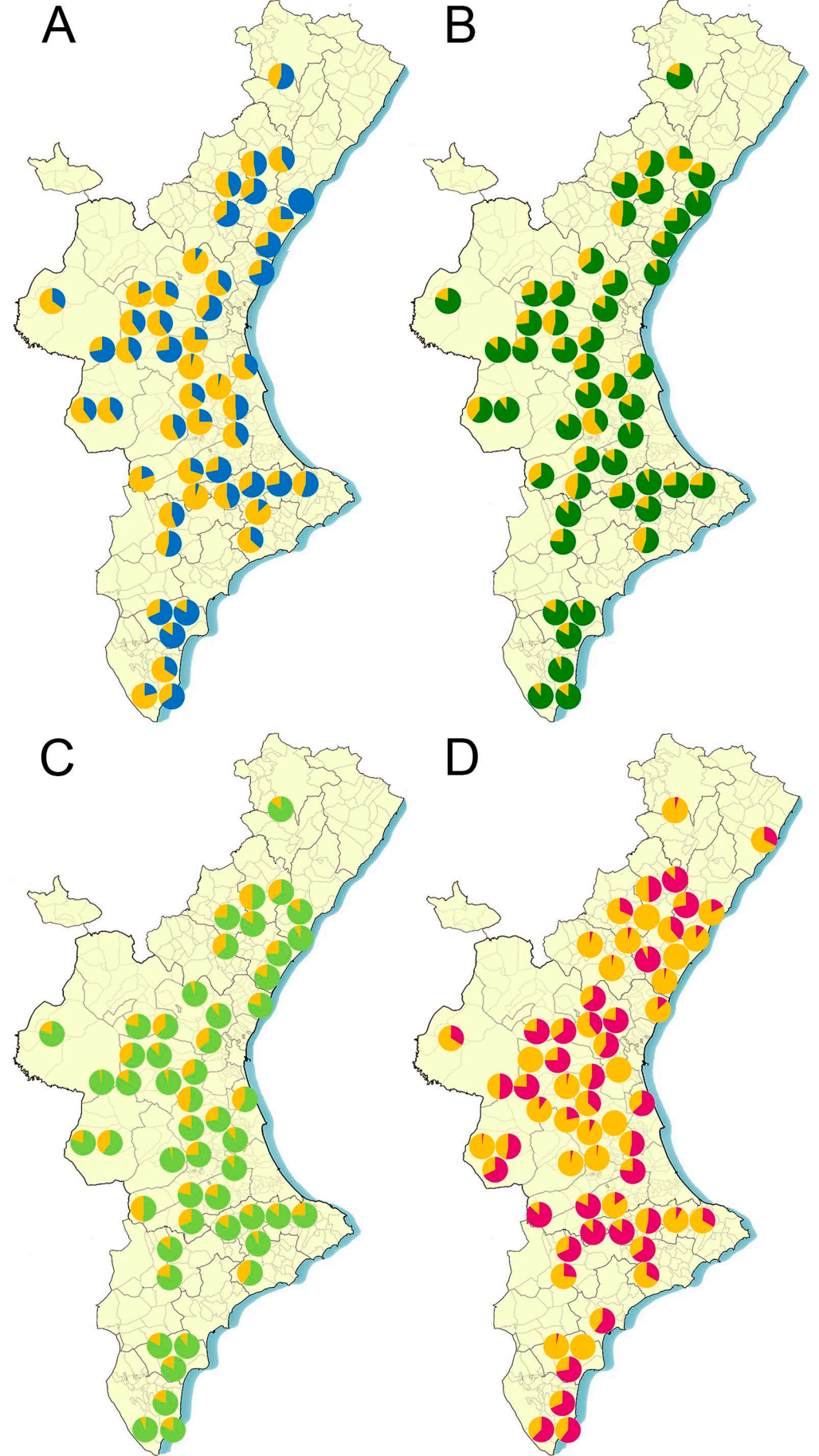
Figure 3. Sampling locations and expected efficacy (expressed as percentage) of commercial acaricides against *Varroa destructor* in 2018 season. A) Coumaphos (efficacy in blue); B) Apitraz (efficacy in dark green); C) Amicel Varroa (efficacy in light green); D) Pyrethroids (efficacy in pink).

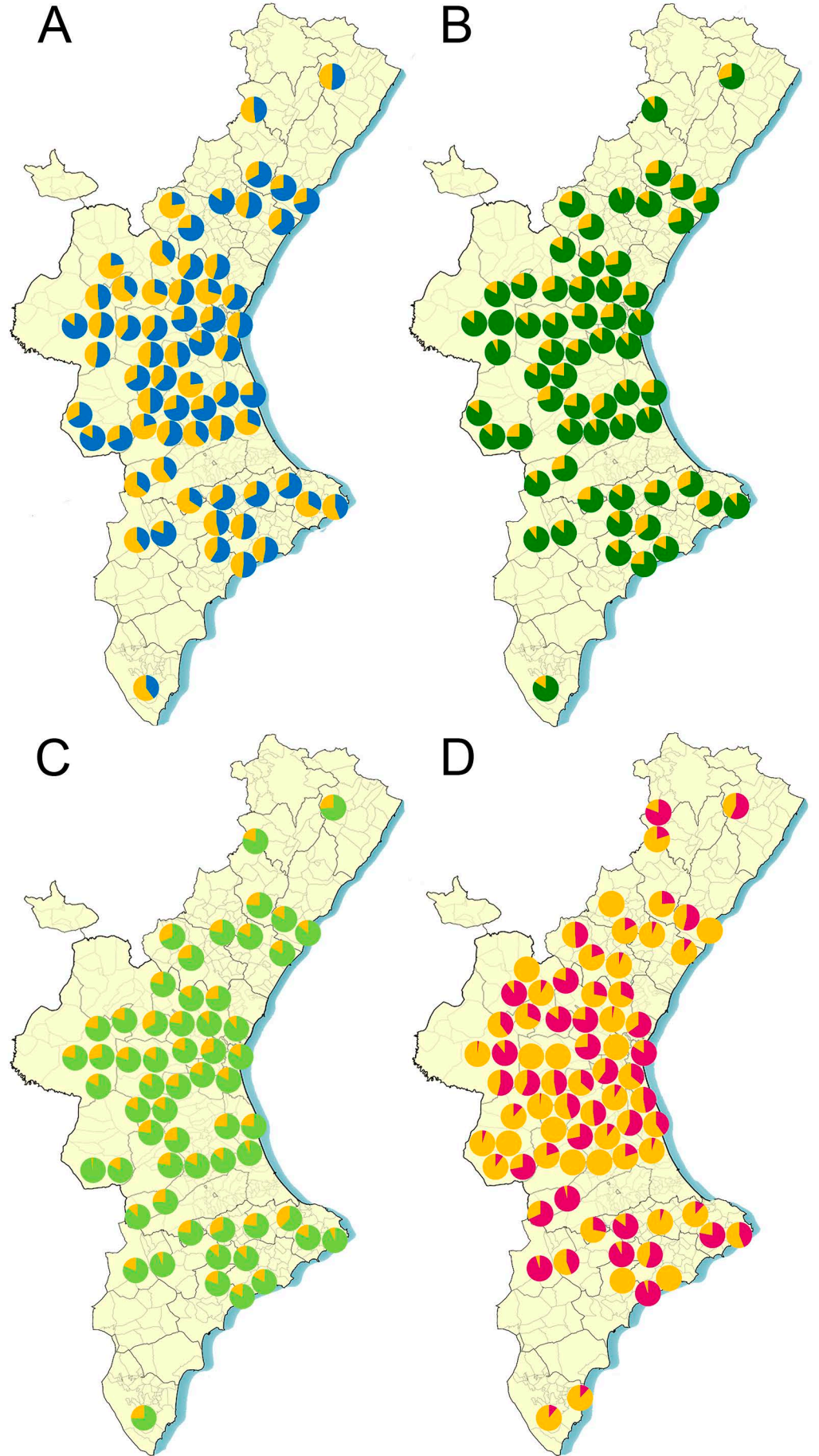
Figure 4. Sampling locations and expected efficacy (expressed as percentage) of commercial acaricides against *Varroa destructor* in 2019 season. A) Coumaphos (efficacy in blue); B) Apitraz (efficacy in dark green); C) Apivar (efficacy in light green); D) Pyrethroids (efficacy in pink).

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


A
B
C


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


A
B
C
D


A
B
C
D

Amitraz
Amitraz + soft acaricides
Soft acaricides
Pyrethroids