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ARTHROPODS AND MEDICAL ENTOMOLOGY - ORIGINAL PAPER

Assessing the resistance to acaricides in *Varroa destructor* from several Spanish locations

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Abstract

Varroosis is the disease caused by the ectoparasitic mite *Varroa destructor*, one of the most destructive diseases of honeybees. In Spain, there is great concern because there are many therapeutic failures after acaricide treatments intended to control varroosis outbreaks. In some of these cases it is not clear whether such failures are due to the evolution of resistance. Therefore, it is of high interest the development of methodologies to test the level of resistance in mite populations. In this work, a simple bioassay methodology was used to test whether some reports on low efficacy in different regions of Spain were in fact related to reduced *Varroa* sensitivity to the most used acaricides. This bioassay proved to be very effective in evaluating the presence of mites that survive after being exposed to acaricides. In the samples tested, the mortality caused by coumaphos ranged from 2 to 89%; for tau-fluvalinate, it ranged from 5 to 96%. On the other hand, amitraz caused 100% mortality in all cases. These results suggest the presence of *Varroa* resistant to coumaphos and fluvalinate in most of the apiaries sampled, even in those where these active ingredients were not used in the last years. The bioassay technique presented here, either alone or in combination with other molecular tools, could be useful in detecting mite populations with different sensitivity to acaricides, which is of vital interest in selecting the best management and/or acaricide strategy to control the parasite in apiaries.

Keywords *Varroa destructor* · Resistant mites · Bioassay · Acaricides

Introduction

The mite *Varroa destructor* (Anderson and Trueman) is one of the most prevalent bee pathogens worldwide and the causative agent of varroosis, one of the most destructive diseases of honeybees (Rosenkranz et al. 2010). It is an ectoparasite that affects immature as well as adult bees by direct feeding mainly on their fat body (Ramsey et al. 2019) and haemolymph (Annoscia et al. 2019), but also vectoring a set of viruses that cause important physiological alterations to the bees (Shen et al. 2005; Di Prisco et al. 2011; Martin and Brettell 2019). In the absence of an effective management program, the parasitized colony eventually collapse in no more than 2 years (Rosenkranz et al. 2010). There are several alternative treatments to control the mite, but, in some countries, beekeepers usually rely on “hard acaricides” based on pyrethroids (tau-fluvalinate and flumethrin), coumaphos, or amitraz, which have been used extensively for decades. This is because they are very effective removing the mites from the hives, but also because they are easy to apply and show relatively low toxicity to bees (Rosenkranz et al. 2010). As in other arthropods, this intensive and repetitive use of pesticides has led to the evolution of resistance in the populations (Milani 1995; Sammataro et al. 2005; González-Cabrera et al. 2018; Mitton et al. 2018; Rinkevich 2020), making very difficult the control of the parasite. It is known that arthropod species, and mites in particular, have evolved resistance to many pesticides and that this trait may spread swiftly in very few generations

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(Van Leeuwen and Dermauw 2016). Recent studies have shown clear evidence of the mechanism of resistance to synthetic pyrethroids. Indeed, a substitution of an amino acid located in the proposed binding site for these molecules has been associated with the resistance recorded in Europe (González-Cabrera et al. 2013) and the USA (González-Cabrera et al. 2016). This information was further used to develop a high throughput methodology to accurately assess the frequency of resistant mites in the populations. However, so far there is still no information available regarding the mechanism of resistance to other acaricides like coumaphos or amitraz, also heavily used against the mite.

In recent years, the beekeeping sector in Spain has reported a significant increase in the outbreaks of varroosis. In many cases, beekeepers have linked these outbreaks to therapeutic failures of the treatments used for *Varroa* control. However, this correlation has not been confirmed by laboratory assays or by official reports to the Spanish regulator (AEMPS—Spanish acronym for the Agency for Medicines and Sanitary Products). It seems reasonable to associate these reports of therapeutic failures with the presence of mites resistant to the acaricides in the hives. To test this hypothesis, and in absence of high-throughput methodologies to screen populations for the presence of mites resistant to coumaphos and amitraz, we have developed a simple bioassay methodology to analyse, under controlled laboratory conditions, whether the reports on low efficacy are related to reduced *Varroa* sensitivity to these acaricides. This would help to select the most appropriate veterinary medicaments for the adequate control of varroosis in these hives, a treatment that is mandatory in Spain according to the Royal Decree RD608/2006.

Material and methods

Mites

Varroa destructor females were collected from nine apiaries located in seven Spanish provinces: La Coruña, Orense, Toledo, Burgos, Guadalajara, Albacete and Badajoz (Fig. 1). The apiaries, except for the two from Guadalajara (experimental apiaries at CIAPA, Marchamalo, Guadalajara), showed high level of *Varroa* infestation after application of acaricidal treatments, that was interpreted as therapeutic failure (Table 1). In the experimental apiaries at CIAPA, the control of varroosis is carried out using an integrated control approach and no therapeutic failures have been detected after treatments with acaricides. At least two combs with capped brood per hive and apiary were collected and shipped to the CIAPA laboratory by express courier. They arrived in less than 24 h after collection.

Once in the laboratory, the combs were kept for 24 h in an incubator at 35 °C (Memmert ® IPP500, 0.1 °C) to mimic the conditions in the hives and to ensure a perfect state of the mites for the bioassay. The combs sampled at CIAPA (Guadalajara) were collected from the hives, transported directly to the laboratory and kept in the same conditions mentioned above.

Bioassays

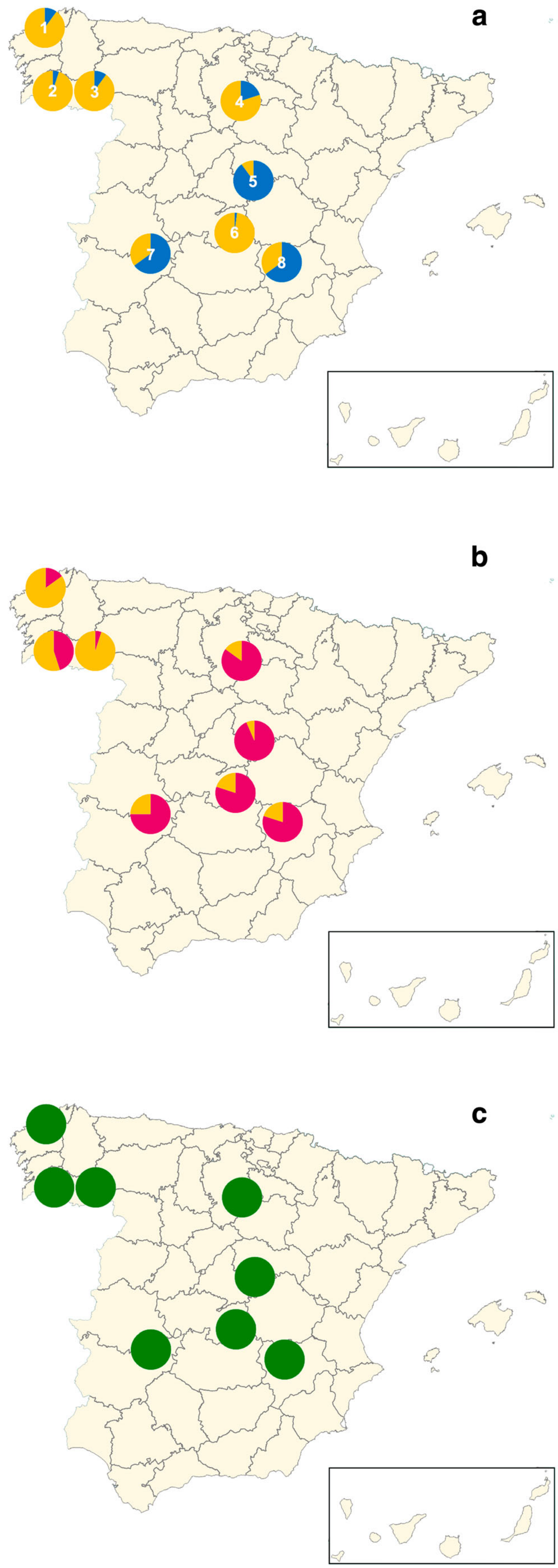
Bioassays were conducted with strips of Checkmite+® (coumaphos a.i., Bayer Hispania, S.L.) Apitraz® (amitraz a.i., Laboratorios Calier, S.A.) and Apistan® (tau-fluvalinate a.i., Vita Europe). Parasitized bee pupae up to 4–6 days old (white to pink eyes) were extracted from the brood cells using a pair of soft tweezers. The pupae were randomly sampled over the entire brood surface until the necessary number of adult mother varroas was obtained. The female mother mites were collected with a soft paint brush and deposited onto a wet filter paper. Only adult *V. destructor* females were used in the tests to limit the interference of age in the bioassay results (Mathieu and Faucon 2000; Kamler et al. 2016).

A piece of approximately 4 cm long of each acaricide strip was placed into a 5.5-cm Petri dish. Each strip piece maintained its original width (2.5 cm for Checkmite+®, 4 cm for Apitraz® and 3.0 cm for Apistan®). Given the different amount of active ingredient impregnated in the strips of each product, the actual concentration was 13.6 mg/cm^2, 2.2 mg/cm^2 and 1.9 mg/cm^2 for Checkmite+®, Apitraz® and Apistan®, respectively. The mites collected (15 mites per replicate, 3 replicates for each acaricide substance) were laid on top of the strip and their movements were monitored to control that they remain on top of the strip for at least 5 min. The dish was sealed with Parafilm® and holed with an entomological needle to allow aeration. The Petri dishes were incubated for 1 h at 34 °C, 90% RH in an incubator (Memmert ® HCP240. Precision: 0.1 °C and 0.5% RH). After 1 h, the strip was removed and the dish with the mites was incubated for 3 more hours at 34 °C, 90% RH. The controls were mites treated the same way but without acaricide strips. After the incubation time was completed, mortality was evaluated by assessing the movement of mites by probing with a fine paint brush.

Results and discussion

The devastating effect of *Varroa* parasitism on honeybee health is threatening the sustainability of beekeeping worldwide. A small set of synthetic acaricides based on organophosphates, pyrethroids or formamidines have been widely and intensively used against mites in the last decades resulting in the evolution of resistance to all of them in the populations (Rosenkranz et al. 2010), although amitraz (formamidine) remains largely effective (Evans and Cook 2018).

In our experiment, *Varroa* mites sampled from different Spanish locations displayed high variability in the level of



◀ **Fig. 1** Sampling locations and results of the bioassays with each acaricide, expressed as percentage. **a** Results obtained with coumaphos (yellow: survivors, blue: dead). **b** Results obtained with tau-fluvalinate (yellow: survivors, pink: dead). **c** Results obtained with amitraz (green: dead). Locations: 1, Culleredo (La Coruña); 2, Castrelo do Miño (Orense); 3, Bande (Orense); 4, Salas de los Infantes (Burgos); 5, Marchamalo (Guadalajara); 6, Madridejos (Toledo); 7, Herrera del Duque (Badajoz); 8, Barrax (Albacete)

mortality after exposition to the acaricides commonly used to treat the colonies (Table 1). For coumaphos, *Varroa* survival ranged from 98 (Madridejos) to 11% (Marchamalo) (Fig. 1a) and for tau-fluvalinate from 95 (Bande) to 4% (Marchamalo) (Fig. 1b), and finally, for amitraz, survival was 0% in all cases (Fig. 1c). The control mites showed a very high survival, between 100 and 98% (Table 1), higher than that reported before for other bioassay methodologies (Kamler et al. 2016; Stara et al. 2019). The low mortality in the controls evidences the reliability of our methodology, allowing the correct assessment of the acaricide effect on the mortality of *Varroa* as previously reported by Milani and Della Vedova (2002). Therefore, mites surviving after the exposure to acaricides could be considered as potentially resistant. In this sense, the results obtained suggest the presence of *Varroa* resistant to coumaphos and fluvalinate in many of the apiaries sampled for this study.

Coumaphos is an organophosphate-based acaricide used globally for decades (Ritter 1985; Kamrin 1997). In beekeeping, the use of coumaphos for the control of *Varroa* began towards the 1980s of the last century, being one of the alternatives to the use of pyrethroids, especially after the detection of resistance to fluvalinate in many locations (Milani 1995; Elzen et al. 2000). However, the intensive treatment regime also resulted in *Varroa* resistance in both laboratory assays and field trials (Spreafico et al. 2001; Kanga et al. 2010). In our experiment, mites coming from colonies previously treated with coumaphos (Table 1) showed a high survival rate to this acaricide (80 to 98%). These results are in agreement with the therapeutic failures reported by beekeepers in those apiaries and could be explained by the selection of resistant mites that survived the selection pressure of the acaricide. On the other hand, the presence of mites that survived in the bioassay but coming from hives that were not treated with coumaphos last year is a relevant fact (11 to 51% of surviving mites). Especially interesting are the results obtained with *Varroa* sampled in the experimental apiary at CIAPA (mite survival around 11 to 15%) where the colonies had not been treated with coumaphos for at least 5 years, which suggests the presence of a significant rate of resistant mites despite the time elapsed without coumaphos treatments. These results are in agreement with those reported by Maggi et al. (2010) and Mitton et al. (2018) in South America, suggesting that coumaphos resistance is maintained over time, probably associated

Table 1 Results obtained in the bioassay and description of the treatments applied in each of the apiaries. Last *Varroa* treatment applied (active product) in each apiary and surviving *Varroa* mites after conducting the bioassay

Locality (Province)	Last treatment (a.i)	Surviving *Varroa* (%) (mean ± SD)			
		Coumaphos	tau-Fluvalinate	Amitraz	Control
Culleredo (La Coruña)	Checkmite+® (coumaphos)	91 ± 3	84 ± 4	0	100
Castrelo do Miño (Orense)		95 ± 4	55 ± 4	0	98 ± 4
Bande (Orense)		91 ± 3	95 ± 4	0	100
Madridejos (Toledo)		98 ± 4	20 ± 7	0	100
Salas de los Infantes (Burgos)		80 ± 0	15 ± 4	0	98 ± 4
Marchamalo (Guadalajara)	Apitraz® (amitraz)	11 ± 3	11 ± 3	0	100
Marchamalo (Guadalajara)		15 ± 4	4 ± 4	0	100
Barrax (Albacete)		35 ± 8	20 ± 7	0	100
Herrera del Duque (Badajoz)		51 ± 4	24 ± 3	0	100

with a remnant selection pressure due to the presence of residues in the hive matrices, undeclared treatments, etc. A more in-depth research is needed to investigate the dynamics of coumaphos resistance in *V. destructor* and the possibility of resistance reversion.

Pyrethroids acaricide/insecticides are nerve poisons that were introduced around the 1970s for pest control (Elliott et al. 1978). Historically, tau-fluvalinate has been the acaricide of choice in apiculture for *Varroa* control due to their efficacy and low toxicity to bees. By the end of the 1980s most of the worldwide control of *Varroa* relied on this active substance (Trouiller 1998). This intensive treatment regime led to the evolution of resistance, first detected in Italy in the 1990s (Milani 1995), but spread to many locations afterwards. In our study, none of the beekeepers declared to have used tau-fluvalinate (or other pyrethroid) as an acaricidal treatment in the last year. However, our data showed that a significant number of mites survived the treatment with this acaricide (Table 1; Fig. 1b). The most extreme cases were detected in samples from La Coruña and Orense, where 55–95% of the mites exposed to tau-fluvalinate survived after the bioassay. In the rest of the apiaries studied, survival ranged from 4 (Guadalajara) to 24% (Badajoz) (Table 1; Fig. 1b). Again, the result obtained in our experimental hives at CIAPA, which have not received acaricidal treatment with fluvalinate in the last 4 years, is relevant. The mites from this apiary were the most sensitive to tau-fluvalinate. In this sense, previous data suggested a decrease in *Varroa* resistance to pyrethroids over a 3-year period (Milani and Della Vedova 2002). Similar results were obtained in a *Varroa* population from Florida, USA, where a significant increase in susceptibility was observed short after the treatment was discontinued (Elzen and Westervelt 2004). Milani and Della Vedova (2002) hypothesized that resistance reversion could happen due to reproductive fitness cost associated with pyrethroid resistance. In this line, González-Cabrera et al. (2018) also reported a rapid decrease in the frequency of pyrethroid-resistant *Varroa* once the treatment was discontinued and also suggested a lower fitness of resistant mites, which is a common trait in species sharing the same mechanism of resistance than that described in *Varroa* resistant to pyrethroids (Anstead et al. 2007). Hence, it is likely that the high frequency of *Varroa* surviving after exposure to tau-fluvalinate in our bioassays was caused by a strong selection pressure maintained over time in the hives of origin. Indeed, the selection pressure that would allow the continuous presence of resistant mites in the hives could be due to the direct and repeated application of acaricide treatments (like in the hives of the present study treated with coumaphos) but also, as we suggested previously, it could be due to the constant presence of acaricide residues inside the hives (Onstad 2013).

Amitraz was the acaricide that showed the best results killing *V. destructor*. Amitraz is a formamidine acaricide that was first synthesized in 1969 and has since been used to control ticks on cattle (Hollingworth 1976). It is also used to treat honeybee mites as an alternative to pyrethroids. The evolution of resistance to amitraz is a serious problem, although it remains in use for the control of ticks, mites and fleas (Kita et al. 2017). The resistance of *Varroa* to amitraz has been also described, although fewer cases were reported compared with other acaricides (Elzen et al. 1999; Maggi et al. 2010; Kamler et al. 2016; Rinkevich 2020). Indeed, *Varroa* control failures due to amitraz resistance continue to be rare despite the first reports of amitraz resistance nearly 20 years ago (Elzen et al. 1999), suggesting that the selection pressure should be lower than that exerted by the other acaricides (probably associated with the metabolic fate of amitraz, see below). In our study, all mites exposed to amitraz in the bioassay died after treatment, regardless the treatment regime of the colony (Table 1). This suggests the absence of resistant mites in the hives studied, even in our experimental apiary at CIAPA, where amitraz has been used as an acaricide treatment for 4 consecutive years once a year. In the apiaries of Badajoz and Albacete, beekeepers reported a possible therapeutic failure of amitraz treatment due to the high number of *Varroa* observed after treatment. However, our data indicates that the acaricide was toxic for these mites. Similar discrepancy of results has been described before (Subirana 1999) and could be explained by other factors as either errors in the application of treatments, reinfestations or the presence of other pathogens that may be altering the behaviour of the honeybee colony and the treatment efficacy (Botías et al. 2012).

Pesticides may accumulate in the different hive matrices according to their physico-chemical properties, often finding higher levels of varrooacides residues in beeswax and beebread than in honey (Johnson et al. 2010). In Spain, lipophilic compounds like coumaphos and tau-fluvalinate are frequently detected in beeswax and beebread at high concentrations (Bernal et al. 2010; Calatayud-Vernich et al. 2018; Alonso-Prados et al. 2020). In this sense, Medici et al. (2015) demonstrated a positive correlation between coumaphos residues in beeswax and the level of *Varroa* resistance to this acaricide. Thus, the results reported in our study could be understood under the light of this scenario with a significant presence of mites resistant to coumaphos and tau-fluvalinate in colonies with no obvious treatment with these acaricides. In addition, Fulton et al. (2019) suggested a transfer of tau-fluvalinate from beeswax into larvae and adult bees as an important route of exposure and bioaccumulation of this acaricide. This way, *Varroa* would get in contact with the acaricide while feeding on the bees. On the other hand, amitraz itself does not accumulate in the hives because it is quickly degraded to N^2-(2,4-dimethylphenyl)-N^1-methyformamidine (DPMF) and N-(2,4-dimethyl-phenyl)-formamide (DMF) that can be detected as residues (Mullin et al. 2010; Lozano et al. 2019; Murcia Morales et al. 2020). Although both amitraz and DPMF are agonists of the octopamine receptor, the metabolite DPMF is more potent than amitraz (Kita et al. 2017). In contrast to the lipophilic compounds mentioned above, DPMF is a polar compound and does not tend to accumulate in wax (Lozano et al. 2019). This would suggest that the selection pressure they would exert on *Varroa* populations would not be so strong than that exerted by lipophilic compounds.

Overall, our data is a showcase of the situation in several Spanish locations regarding the efficacy of acaricides and management strategies for controlling *V. destructor*. In this scenario it is very important to screen populations before treatment to determine the frequency of resistant mites to select the best management strategy. The bioassay technique presented here as well as the TaqMan® (González-Cabrera et al. 2013) or PCR-RFLP (Millán-Leiva et al. 2018) assays described before for detecting mites resistant to pyrethroids are straightforward methodologies that will provide this information to the beekeeping community so they can take an informed and scientific-based decision on the most convenient way to manage the parasite.

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Data availability Not applicable.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval In Europe, the EU Directive 2010/63/EU on the protection of animals used for scientific purposes describes the ethical framework for the use of animals in scientific experiments. The scope of this directive also includes specific invertebrate species, but no insects. Thus, according to European legislation no specific permits were required for the described studies.

Consent to participate Not applicable.

Consent for publication All authors consent to the publication of this work.

Code availability Not applicable.

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