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Case Report

A case report of chronic stress in honey bee colonies induced by pathogens and acaricide residues

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Abstract: In this Case Report we analyze the possible causes of the poor health status of a professional *Apis mellifera iberiensis* apiary located in Gajanejos (Guadalajara, Spain). Several factors that potentially favor colony collapse were identified, including *Nosema ceranae* infection, alone or in combination with other factors (eg, BQCV and DWV infection), and the accumulation of acaricides commonly used to control *Varroa destructor* in the beebread (coumaphos and tau-fluvalinate). Based on the levels of residues, the average toxic unit estimated for the apiary, suggests a possible increase in vulnerability to infection by *N. ceranae* due to the presence of high levels of acaricides. These data highlight the importance of evaluating these factors in future monitoring programs, as well as the need to adopt adequate preventive measures as part of national and international welfare programs aimed at guaranteeing the health and fitness of bees.

Keywords: Honey bees, *Apis mellifera*, acaricides, pesticides, Toxic Unit, *Varroa destructor*, *Nosema ceranae*, bee viruses, tau-fluvalinate, coumaphos.

1. Introduction

Bees, including honey bees, bumble bees and solitary bees, are a prominent and economically important group of pollinators worldwide. In fact, 35% of the global food crop production depends on these pollinators [1] and in Europe, the production of 84% of crop species is to some extent dependent on animal pollination [2]. Bees also fulfil an important role in the pollination of wild plants and thus they represent an important element in maintaining the homeostasis of different ecosystems [3,4]. Furthermore, honey bees provide additional economic inputs in temperate areas where honey production is a fundamental source of income to professional beekeepers.

The honey bee colony is a complex system in which thousands of individuals work together to ensure its sustainability. Multiple factors play an important role in colony viability, such as climate, environment, nutrition and pathogens, and consequently, in the colony pollination and production capabilities. Thus, any deterioration of honey bee colonies has a direct negative environmental impact and , in the case of honey bees, economic consequences in countries where there is a large proportion of professional beekeepers as in Mediterranean areas [5].

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Many factors have been related to the decline in honey bee colonies over the past decades (revised in [6]), including changes in land use, pesticide exposure, a reduction in genetic diversity, climate change, etc. [7-9]. However, the impact of pathogens on this decline (mainly parasites and related virus) may be particularly important [10-12], probably in conjunction with the accumulation of pesticide residues in hive matrices. Thus, the combined effect of two or more such stressors could drive mortality of individuals, eventually leading to the colony collapse [13-15].

Accordingly, we present here a screening study of a professional Spanish apiary that reported a problematic health situation. To investigate the factors that had possibly provoked this situation we performed a comprehensive evaluation of multiple drivers including pathogens and pesticides, also analyzing the foraging flora, in an attempt to determine if new factors should be examined in future monitoring programs.

Severe *Nosema ceranae* infection, in conjunction with accumulation of acaricides used to control *Varroa* mite infestations in honey bee hives, represent a real threat for the colonies, indicating that appropriate preventative strategies should be adopted in honey bee health programs.

2. Results

2.1. Veterinary inspection

A professional beekeeper, who managed four hundred honey bee, colonies (*Apis mellifera iberiensis*) at Gajanejos (Guadalajara, Central Spain, lat. 40.8423; long. -2.8933), reported health problems in his colonies. He revised all his colonies in September 2015 while applying a compulsory treatment of *Varroa destructor* with Checkmite® strips (a.m.: coumaphos), according to manufacturer's recommendations. No health problems had previously been noted in the colonies, which appeared to be in satisfactory health at the beginning of autumn 2015, with a normal population of worker honey bees. When the acaricide strips were removed, six weeks after their application (November, 2015), the beekeeper noticed a reduction in the worker honeybee population in some of the colonies and consequently, he began to inspect the apiary more often. The first dead colonies were detected that winter and the losses continued until early spring 2016.

Upon the veterinary inspection in early spring 2016, around 50% of the bee colonies had died. The hives of the dead colonies were stored at the beekeeper's warehouse, in the same conditions that he found then in the field, awaiting cleaning. The beekeeper also conserved the acaricide strips used in the previous autumn in plastic bags (Figure 1).

Only a few dead bees were found in the brood chamber frames of these hives, with no anatomical deformities and no *Varroa* mites detected at the bottom of the hives or in workers bees and sealed brood (Figure 1b). Moreover, there were no clinical signs of chalkbrood, American or European foulbrood.

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Figure 1. Pictures obtained during the inspection of colonies. (**a**) Hives stored in the beekeeper´s warehouse; (**b**) Brood combs from the dead colonies with few honey bees and sealed brood in the frames; (**c**) Checkmite® strips kept by the beekeeper after their removal; (**d**) Apiary with many empty spots and a few surviving colonies; (**e**) Weak surviving colony with a small honey bee population; (**f**) Detail of some Checkmite® strips with low interaction with honey bees.

In the surviving colonies (Figure 1d) there were no more than two combs from the brood chamber that were covered with adult honey bees, much less than the five to seven combs that would be expected to be covered in this geographical area at that time (Figure 1e). The acaricide strips used in autumn treatment were essentially untouched by the honey bees (Figure 1f) and there were no clinical signals of varroosis or other diseases in the brood, nor in adult worker honey bees. *Varroa* mites were not identified in brood cells or at the bottom of the hives.

The presence of accessible pollen and honey reserves in both the dead and surviving colonies, ruled out death by starvation.

2.2. Pathogen screening.

Five collapsed colonies had sufficient dead honey bees to carry out pathogen screening and in only one of these was there a 5% of prevalence of *V. destructor* and the mite was not detected in any of the other hives. Of the surviving colonies sampled, only two were positive in *Varroa* with infection rate of 25% and 1%.

All samples were positive for *N. ceranae*, with severe percentages of parasitization that were statistically higher in dead colonies (p-value = 0.001312).

The presence of Deformed Wing Virus (DWV) was confirmed in surviving colonies infected by *Varroa* and in six other samples. Moreover, Black Queen Cell Virus (BQCV) was present in six samples, one of which was positive for *V. destructor* (Table 1). Finally, *N. apis, Acarapis. woodi*, Trypanosomatids, Neogregarines Lake Sinai Virus complex complex(LSV) and Acute Bee Paralysis Virus-Kashmir Bee Virus Israeli Acute Paralysis Virus complex (AKI) were not detected in any sample.

Table 1. Results of pathogen and pesticide residue screening and the palynological analysis of beebread samples from dead (D) and weak yet surviving (S) colonies. **Pathogen screening:** V. destructor parasitization (% VD), prevalence of N. ceranae (% NC) and N. apis (% NA) and detection(+ or -) of A. woodi (AW), Trypanosomatids (Tryp), Neogregarines (Neog), Lake Sinai Virus complex complex(LSV), Acute Bee Paralysis Virus-Kashmir Bee Virus Israeli Acute Paralysis Virus complex (AKI), Deformed Wings Virus (DWV) and Black Queen Cell Virus (BQCV). **Residues quantified in beebread (ppb):** tau-fluvalinate (FVT) and coumaphos (CMF). Natural logarithm of the toxic unit of the mixture in each colony (Ln(TUm)).**Palynological analysis:** percentage of wild foraging plants (%WP). Grey cells in the table indicate parameters not analyzed

Status of the colonies	%VD	%NC (*)	DWV	BQCV	FVT (*)	CMF (*)	LN(TUm) (*)	%WP
Dead	5	70			7	435	-6.38	96.3
	0	80			7	415	-6.43	45.1
	0	75			<LOQ	202	-7.154	89.3
	0	92			13	350	-6.59	65.1
	0	89			9	323	-6.68	79.4
Surviving	25	20	+	+	7	283	-6.81	90.5
	1	30	+	-	9	545	-6.16	30.7
	0	25	+	-	10	2230	-4.75	15.2
	0	60	+	+	15	465	-6.31	30.5
	0	36	+	+	20	1165	-5.42	33.7
	0	20	+	-	16	305	-6.73	77.7
	0	30	-	+	18	775	-5.80	92.1
	0	45	-	+	19	850	-5.71	55.6
	0	35	+	-	13	936	-5.62	85.4
	0	35	+	+	13	845	-5.72	93.7

(*) statistically significant differences between dead and surviving colonies at α=0.05
LOQ: level of quantification.

2.1.1. *Varroa mite* resistance to acaricides

Only one of the fifteen colonies sampled, complied with the criteria to conduct the resistance test [16] , and this colony did not show any evidence of *Varroa* mites resistant to acaricides (Table 2).

Table 2. Results of the screening to identify *Varroa mite*s from S1 colony resistant to acaricides

Incubation time	Mites	Control	Checkmite® (coumaphos)	Apistan® (tau-fluvalinate)	Apitraz® (amitraz)
6 h	Dead	1	10	5	8
	Alive	7	0	0	0
24 h	Dead	2	11	7	8
	Alive	9	0	1	1

2.2. Stored pollen analysis

Of the 67 substances analyzed in beebread samples, only tau-fluvalinate and coumaphos were detected (Table 1) and there were statistically higher concentrations of tau fluvalinate and coumaphos in the beebread samples from the surviving colonies (W = 43.5, p-value= 0.01312; W = 42.0; p-value = 0.02165, respectively; Table 1). The mean TUm value of the whole apiary was 0,00262± 0.00199. TUm values were < 1 in both the dead and surviving colonies (Table S1), indicating that the residue levels did not in principle reach the threshold of acute toxicity. Tau-fluvalinate represented less than 1% of TUm of the colonies (Table S1). All the compounds identified in a given mixture contribute to the TUm in

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accordance with their potency and the levels of their residues. Thus, as the level of residues were significantly higher in the surviving colonies, the TUm was also significantly higher in these hives.

No significant differences were found between the dead and surviving colonies regarding the presence of wild flora in the beebread samples (W = 18.0, p-value = 0.21299). In four of the five samples from the dead colonies, wild plants were majority present in the beebread. The most frequent taxa identified of wild plants were: *Araliaceae*, *Labiatae*, *Asteraceae*, *Chenopodiaceae* and *Diplotaxis* taxa. The major cultivated taxa detected was sunflower (*Helianthus annus*). In the surviving colonies, the predominant pollen was from wild plants in six out of ten samples, belonging to eight taxa: *Araliaceae*, *Labiatae*, *Caryophyllaceae*, *Cichorioideae*, *Convolvulaceae*, *Asteraceae*, *Chenopodiaceae* and *Diplotaxis* taxa. In the remaining four samples the predominant taxa identified in the beebread were *H. annus*, *Prunus* spp. and *Brassicaceae*. In these, residues of neonicotinoids are not detected in the corresponding residue analyses, therefore the use these insecticides is ruled out, in the surroundings of the apiaries under study

3. Discussion

Here we have investigated a specific case study of the weakening and death of honey bee colonies in the field, a situation that has occurred quite frequently in Spain over recent years. The first suspected cause of colony death in this apiary was the action of the *Varroa* mite due to a failure in the acaricidal treatment, often proposed to cause these effects [17]. However, the visual examination and pathogen screening suggested a different origin of collapse and in fact, *V. destructor* was only detected in 20% of the colonies sampled (3 out of 15) there were no clinical signs consistent with generalized varroosis in the apiary or in the hives in which the honey bee colonies died. Moreover, two of the positive colonies had a parasitic mite load of only 1 and 5%, not apparently representing an immediate risk to the bee health [17]. Only one of the surviving honey bee colonies sampled (10%) had a higher parasite load (25%) and was thus, at risk of suffering the negative effects of this mite [17]. After decades of miticide use against varroosis, there is a general concern about the selection of *Varroa* mites tolerant of acaracides [18]. However, the preliminary results of the acaricide resistance test and the absence of clinical signs of varroosis upon inspection, suggest that resistant *Varroa* mites do not affect treatment efficacy in this apiary. Hence, it was concluded that the symptoms observed were not due to clinical varroosis after therapeutic failure. Overall, these results indicate the *Varroa* mite might have exerted a degree of pressure on some individual colonies, but it is insufficient to provoke the collapse/weakening evident across the entire apiary.

By contrast, severe *N. ceranae* infection was detected in all cases, especially in dead colonies, with clinical signs and symptoms in all cases (dead and surviving) in line with the infections observed previously in colonies that collapse in winter due to such infestation [19]. As expected for nosemosis C, a lower infestation was seen in early spring, as described in the phases three and four of the disease [19] and consistent with its evolution in different seasons [19-21]. The fact that the acaricide strips did not change of color or underwent propolisation, probably reflects a change in honey bee behavior due to *N. ceranae* infection, which could produce a serious risk that the acaricide treatment would lose efficacy if the *V. destructor* mites were abundant [21,22]. In fact, *N. ceranae* alters various physiological processes in individual honey bees, involving immunomodulation [23-25] and energetic stress ([26,27], and inducing early foraging activities ([28,29]. These alterations have a direct impact on the colony ([19,30,31], especially in geographical areas with warmer climates where there is a large concentration of professional beekeeping [32-34], in contrast to colder climates ([35-38] reviewed in [21]).

Although there was a scattered presence of BQCV and DWV in the samples, their detection may be a consequence of a side effect of the presence of *Varroa* mites [17,39-41] and *Nosema* spp.[39,40,42,43].

Neonicotinoids and other agrochemicals were not detected in the beebread, consistent with the fact that honey bees mainly visited wild flora. Nevertheless, the high concentrations of tau-fluvalinate and coumaphos detected in beebread samples were assumed to have a beekeeping origin as these chemicals are registered in Spain to control *Varroa* mite. Moreover, tau fluvalinate and coumaphos have an octanol: water partitioning coefficient (logKow) > 3 [44,45], indicating a high lipophilicity and potential to accumulate in wax [46,47] and other hive matrices [48],where they may remain relatively stable for long periods of time [49-51]. Indeed, both of these acaricides are estimated to need 5 years to completely disappear from bee matrices [52]. Moreover, their concentration in wax may increase due to the wax recycling processes [49,50], explaining why their residues are frequently found in wax and beebread worldwide [47,53-55]. If the acaricide residue levels in beebread reaches toxic levels, the health of the honey bee colony might be compromised. Thus, a synergetic toxic effect between these acaricides cannot be ruled out. Indeed, acute contact toxicity of coumaphos increases up to 3-4 fold when 4 days old honey bees were pretreated with tau-fluvalinate at a dose of 1 or 3 ug/bee, and the contact toxicity of tau-fluvalinate increases up to 32 fold when the individuals were pretreated with coumaphos at a dose of 10 ug/bee [56]. In addition, high concentrations of acaricides may have made the honey bee colonies more sensitive to *N. ceranae* infection [57]. In this sense, following the Toxic Unit (TU) approach and based on acute toxicity LC50, it has been proposed that a Ln (TU)= -6.706 may represent as a preliminary break point regarding the increment of *N. ceranae* when assessed in the presence of a mixture of xenobiotics [13]. The mean Ln(TUm) in the present apiary was -5.95, suggesting it may have been more vulnerable to *N. ceranae* infection [57]. However, Ln(TUm) values were higher in surviving colonies, which also had lower *N. ceranae* infection. This may lead to the erroneous conclusion that a high miticide concentration contributes to colony survival. However, this was not the case because their viability was compromised in early spring due to the small adult honey bee population. Thus, while more than 50% of the dead honey bees are expected to be infected in colonies that collapsed due to nosemosis C in the cold months, this percentage is lower when colonies collapse later in the year, probably due to an increment in the proportion of uninfected newborn honey bees [19].

4. Material and Methods

4.1. Sampling

Dead colonies with sufficient honey bees in the frames were sampled, taking 100-150 individuals per hive to study the pathogens.

Most of the surviving colonies (approximately 200) did not have a large enough adult population to ensure their future survival and thus, given that it is necessary to take at least 300 worker honey bees to reach a diagnosis of varroosis, samples were only taken from a representative part of the apiary with the largest number of individuals. Therefore, and with the authorization of the beekeeper, 10 surviving colonies with a sufficiently large population of honey bees were selected randomly for sampling.

In addition, samples of 4 or 5 pieces of honey bee comb (10 x 15 cm each) that contained stored pollen (bee bread) were also taken from different areas of the brood chamber from each colony surveyed to carry out chemical and palynological analysis.

4.2. Pathogen screening

The detection of acari *V. destructor* loads was established as described previously [31,58]. The presence of *Nosema* spp., Trypanosomatids, Neogregarines and *Acarapis woodi* was evaluated in a subsample from each colony. The presence of different viruses was only analyzed in the surviving colonies samples because the viral RNA integrity could not be assured in the dead colonies.

To obtain nucleic acids, sub-samples of 60 bees were macerated in AL buffer (50%, Qiagen) as described previously [11,59]. The samples were then centrifuged (1811 x g)

extracting DNA from the pellet [60], and RNA from the supernatant. The corresponding cDNAs were generated from the total RNA recovered [59].

PCR or RT-PCR was carried out to detect *Nosema apis* and *N. ceranae* ([60], Trypanosomatids and Neogregarines [61], LSV complex [62], AKI complex [63], DWV and BQCV [64]. In addition, the proportion of *Nosema* spp. infection was determined by PCR on 25 individual worker honey bees from each colony sampled [60].

4.2.1. *Varroa* mite resistance test to acaricides

When, possible, mite resistance to acaricides was determined using the respective marketed products Checkmite® (a.m.: coumaphos), Apistan® (a.m.: tau-fluvalinate) and Apitraz® (a.m.: amitraz) according to the protocol described previously [16] with the following modifications:

1. Inclusion of an additional batch for a 24 h incubation period,
2. Feeding the honey bees with syrup during the incubation periods,

and

3. Freezing the honey bees at the end of the incubation period to collect the remaining *Varroa* mites (-80 °C, 15 minutes).

The honey bees were kept at 35 °C during the test and after incubation periods, a control test without treatment was used to determine how the basal conditions affected *Varroa* mite mortality.

4.3. Stored pollen analysis

Beebread was extracted aseptically from the combs, removing the wax and preparing a composite sample for each colony by mixing the corresponding subsamples. Finally, each pollen sample was divided into two 100 g aliquots, for chemical and palynological analysis, and stored at -80 °C.

A multiresidue chemical analysis of 60 substances was carried out following the method described elsewhere [53], assessing acaricides (AC), fungicides (FU), herbicides (HB), and insecticides (IN) In addition, 7 neonicotinoid INs (acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid and thiamethoxam), were measured as described previously [65].

Based on the results of the multi residue analysis, and the toxicity data reported previously ([13],supplementary information Table A.2.) the toxic unit of the mixtures (TUm) was calculated following the approach given elsewhere [13] to assess the risk of the chemical mixture found in each hive sampled. Subsequently, the natural logarithm (Ln(TUm)) was estimated for comparison purposes [13].

Finally, the type of foraging flora was confirmed by analyzing beebread samples as previously described [10,19,31] and estimating the proportion of pollen from wild (WP) and cultivated (CP) plants.

4.4. Statistical analysis

One-tailed Mann Whitney test (α= 0.05) was used to analyze possible differences between the dead and surviving colonies in terms of the different experimentally parameters measured (pathogens, % wild pollen in beebread and chemical residues and TUm). The analysis was carried out with Statgraphics Centurion 18 ©.

5. Conclusions

The veterinary inspection and analytical evidence presented here indicate that nosemosis C infection was the underlying cause of the colony weakness and collapse at the professional apiary studied, probably accelerated by the presence of high levels of miticides. In conjunction with the unchecked concentrations of acaricide that accumulated in honey bee hives, *N. ceranae* infection represents a real danger for honey bee colony sur-

vival. Therefore, in addition to the correct use of veterinary products to control *V. destructor*, appropriate wax renewal of the combs should be introduced to develop specific preventive strategies aimed controlling possible infections from prevalent pathogens.

Supplementary Materials: Table S1: Details of calculation of TUm values.

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