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Sudden deaths and colony population decline in Greek honey bee colonies

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ABSTRACT

During June and July of 2009, sudden deaths, tremulous movements and population declines of adult honey bees were reported by the beekeepers in the region of Peloponnesus (Mt. Mainalo), Greece. A preliminary study was carried out to investigate these unexplained phenomena in this region. In total, 37 bee samples, two brood frames containing honey bee brood of various ages, eight sugar samples and four sugar patties were collected from the affected colonies. The samples were tested for a range of pests, pathogens and pesticides. Symptomatic adult honey bees tested positive for *Varroa destructor, Nosema ceranae*, *Chronic bee paralysis virus* (CBPV), *Acute paralysis virus* (ABPV), *Deformed wing virus* (DWV), *Sacbrood virus* (SBV) and *Black queen cell virus* (BQCV), but negative for *Acarapis woodi*. American Foulbrood was absent from the brood samples. Chemical analysis revealed that amitraz, thiametoxan, clothianidin and acetamiprid were all absent from symptomatic adult bees, sugar and sugar patty samples. However, some bee samples, were contaminated with imidacloprid in concentrations between 14 ng/g and 39 ng/g tissue. We present: the infection of Greek honey bees by multiple viruses; the presence of *N. ceranae* in Greek honey bees and the first record of imidacloprid (neonicotonoid) residues in Greek honey bee tissues. The presence of multiple pathogens and pesticides made it difficult to associate a single specific cause to the depopulation phenomena observed in Greece, although we believe that viruses and *N. ceranae* synergistically played the most important role. A follow up in-depth survey across all Greek regions is required to provide context to these preliminary findings.

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1. Introduction

It is widely appreciated that apiculture comprises one of the most important parts of the global agricultural economy. Besides the obvious contribution of hive products (honey, pollen, wax, propolis and royal jelly), honey bees constitute one of the most efficient natural pollinators of a wide range of wild flora (16%) and staple crops in the world (Maheshwari, 2003). Qualitative and quantitative characteristics of agricultural production (fruits, vegetables and seeds) are improved by bee activity and bees contribute greatly to the wider floral biodiversity and improve the balance of the ecosystem (Delaphane and Mayer, 2000; Kevan, 1999).

In Greece there are approximately 1,280,000 beehives, kept by about 25,000 beekeepers and yielding an annual production of approximately 15,000 tons of honey (Bacandritsos et al., 2004). In total, 31.1% of beekeepers in Greece are professionals and the remaining are amateurs or hobbyists.

In recent years many commercial honey beekeepers in America have reported widespread, sudden and unexplained losses of their bee colonies (vanEngelsdorp et al., 2007, 2008). Europe and Middle East had also experienced similar phenomena (Watanabe, 2008). Such losses have occurred throughout the history of apiculture (de Miranda et al., 2010) and information collected through questionnaires administered to Greek beekeepers suggested honey bee losses of 14% were reported during winter 2007/2008 (Hatjina et al., 2010).

Although the exact cause of many large-scale honey bee colony losses remains cryptic, many factors have been implicated such as: honey bee parasites (*Varroa destructor, Acarapis woodi*); pathogens (*Nosema* spp. and bee viruses); contaminated water; use of antibiotics; pesticides poisoning from within-hive and environmental sources; nutritional stress; dietary pyrethrum deficiency and their interactions (Mutinelli and Granato, 2007; Higes et al., 2008; Naug, 2009; vanEngelsdorp et al., 2009; vanEngelsdorp and Meixner, 2010; Sharpe and Heyden, 2009).

Several potential stress factors, such as poor nutrition, drought, and migratory apiculture have all been linked to a weakening of the honey bee immune system, making colonies more susceptible

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to diseases (Mutinelli and Granato, 2007; Naug, 2009; Sharpe and Heyden, 2009). During June and July of 2009, sudden deaths, tremulous movements and population declines of adult honey bees were reported by the beekeepers in the region of Peloponnesus (Mt. Mainalo, altitude: 1200 m), Greece. A preliminary study was instigated to investigate these unexplained phenomena, which had not occurred previously in this region.

2. Materials and methods

2.1. Sampling and recording of symptoms

Colonies displaying atypical behaviour were identified by professional beekeepers resident in the Peloponnesus region (Mt. Mainalo) of Greece (lat: 37°39′45″N – long: 022°05′02″E) between 05/06/09 and 08/07/09. The clinical symptoms of the selected colonies were analytically noted and 37 bee samples collected from five different apiaries suffering similar symptoms. Twenty-two bee samples were collected from the hive entrance, five bee samples from the bees that abandoned the hive and overhanged on tree branches and ten bee samples from the inside of the hive. Also three queen bees were collected from the examined apiaries. In addition, eight crystal sugar (main substance of sugar syrup) samples, four sugar patties (the usual winter food supplement) two representative brood frames were also collected. All the samples were stored at −20 °C prior to commencing investigations.

2.2. Investigations using classical microscopy

Samples positive for *V. destructor* were examined for honey bee tracheal mite (*A. woodi*). Each sample consisted of fifty (50) individual bees. The heads and forelegs were removed, and the thoraces were cut in front of the middle pair of legs and at the base of the forewings. These thin disks were placed into glass vials containing 8% KOH solution and heated in a boiling water bath for approximately 20 min until the muscle tissues were macerated. After heat treatment, the exposed first pair of thoracic trachea was examined under a dissecting microscope (magnification, 20–40×) (OIE, 2008a). Trachea with suspicious colour changes was removed from the thorax and examined at a magnification of 200× under a light microscope to detect infestation with *A. woodi*. The samples were considered positive for *A. woodi* if a single individual was found to be infested, otherwise it was considered negative (Berènyi et al., 2006).

Secondly, all the samples were investigated for the presence of *Nosema* spores. Each sample consisted of thirty (30) individual bees. The abdomens were separated from the thoraces and then crushed and homogenised in 3 ml of water. Three drops of the suspension were placed onto a slide, covered by a slip, and examined under a light microscope, initially at a magnification of 200×, followed by a magnification of 400× (Berènyi et al., 2006; OIE, 2008b).

Thirdly, two representative brood frames containing honey bee brood of various ages were examined for American foulbrood (AFB). Briefly, larval/pupal remains from brood comb were collected with a sterile swab and suspended in 5–10 ml of phosphate buffered saline in a test tube. Culture on MYPGP agar (Mueller–Hinton broth, yeast extract, potassium phosphate, glucose and pyruvate) was performed transferring with a sterile pipette a portion of the sample onto the surface of the solid medium. Inoculated plates were incubated at 34–37 °C for 2–4 days in an atmosphere of 5–10% CO_2 (OIE, 2008c).

2.3. Investigations using molecular methods

Samples of bees that had abandoned hives were collected from five different apiaries, with the most intense field symptoms (high bee mortality, tremulous movements in the front of the hive and depopulation symptoms). These samples were selected to speciate *Nosema* and to determine the presence of five honey bee viruses.

Honey bee exudate, from the microscopic screen for *Nosema* spp. spores, were used for DNA extraction using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions, with a pre-incubation with lysozyme. DNA was analysed according to four different protocols: PCR and sequencing (Higes et al., 2006), two different RFLP–PCR according to Klee et al. (2007) and Giersch et al. (2009) respectively, and specific PCR for *Nosema apis* (Webster et al., 2004). *Nosema ceranae and N. apis* positive controls were included. PCR products were analysed on 7% acrylamide gel and visualized by silver staining. PCR products were subjected to sequencing or digestion with restriction enzymes.

Altogether fifteen bee samples and three queen bees were assessed for the presence of *Chronic bee paralysis virus* (CBPV), *Acute paralysis virus* (ABPV), *Deformed wing virus* (DWV), *Sacbrood virus* (SBV), *Black queen cell virus* (BQCV). Each sample was homogenised by mechanical agitation in a TissueLyser (Qiagen) for 3 min at 30 Hz, in the presence of 7 mm stainless steel beads and lysis buffer (Nucleospin RNA-II kit). After a brief centrifugation, 600 μl of supernatant were used for total RNA isolation by using Nucleospin RNA-II kit (Macherey–Nagel) according to the manufacturer's instructions. Total RNA was re-suspended in diethyl pyrocarbonate-treated water and quantified by spectrophotometry.

Real time RT-PCR protocols were previously described by Chantawannakul et al. (2006) with minor modifications. Real-time PCR primers and a probe for 18S rRNA gene of *Apis mellifera* were used as an internal positive control (IPC) for assessing nucleic acid extraction (Ward et al., 2007). All probes were labelled at the 5′ end with 6-carboxyfluorescin (FAM) reporter dye and at the 3′ with the quencher dye "Black Hole Quencher 1" (BHQ1).

One-step real time RT-PCR was performed using the QuantiFast probe RT-PCR Kit (Qiagen), according to the manufacturer's instructions for individual component concentrations, in a LightCycler 2.0 instrument (Roche Applied Science). The samples were prepared in triplicates and for each reaction 2 μl of total RNA (corresponding to 100 ng of total RNA) was added to 18 μl of master mix in a glass capillary. Negative and positive controls were included in each run of one-step real time RT-PCR reaction. The thermal cycling profile consisted of a RT step which was performed at 50 °C for 10 min, of a HotStartTaq plus activation step at 95 °C for 5 min, followed by 40 cycles consisting of denaturation at 95 °C for 10 s and annealing/extension at 60 °C for 30 s. All acquired fluorescence data were analysed using LightCycler software (version 3.5) and quantification point (Cp), was determined automatically. Positive samples were defined as $Cp \leqslant 35$, low positive as $35 > Cp < 40$ and negative as $Cp = 40$.

2.4. Chemical analysis using LC–MS

The five bee samples, eight sugar samples and four sugar patties were examined for the presence of systemic insecticide residues (hard substances such as amitraz, imidacloprid, thiametoxan, clothianidin and acetamiprid). In order to detect these substances, 5 g of sample (sugar or bees) were homogenised with whole sachet of EXtrelut (20 g) and transferred in a reservoir. The column was eluted with six portions of dichloromethane (25 ml) to obtain 150 ml.

For sugar patty samples the extraction was performed twice with 30 ml of dichloromethane and the solvent was mixed with EXtrelut powder and was transferred in a reservoir. The elution

was completed with the remaining 90 ml of dichlorometane to obtain 150 ml of total volume. The eluted was collected in a 250 ml round bottom flask and evaporated under vacuum at 40 °C.

For honey bee samples, extracts were re-dissolved in 10 ml cyclohexane–dichlorometane (50:50 v/v) and 1 ml was injected in a Gel Permeation Chromatography (GPC) system for further purification. The eluted from GPC was collected in test-tube and evaporated under nitrogen stream at 40 °C. The dried material was re-dissolved in 1 ml of mobile phase and was filtered in vial. Chemical analysis was performed by LC–MS analysis (SHIMADZU LCMS – 2010 EV, SIM ESI+) with an Ascentis Express C18. 150 × 3.0 mm × 2.7 μm, mobile phase composed of 0.5% formic acid and methanol gradient, flow of 0.3 ml/min and injection volume of 10 μl. Quantification was achieved using external standard solutions.

3. Results

3.1. Sampling and recording of symptoms

The following clinical symptoms were recorded in all five apiaries: a large number of dead bees in front of the hive entrance; a large number of trembling bees clustered outside the hive entrances; the appearance of black adults bees after the onset of trembling; a large number of bees absconding the hives and aggregating in nearby trees; apiary losses varying between 50% and 70% while some colonies containing non-laying queens and some were queenless. Clearly, less intense symptoms (trembling bees, dead bees at the bottom of the hive and the feeder) were observed inside the hive (Table 1). The onset of symptoms coincided with the bees beginning to forage on fir honeydew, and symptoms continued to intensify during this period. According to the history, death of adult bees had been recorded during the period of the pine honeydew collection albeit to a lesser degree. The pine honeydew collection period had preceded the transfer to fir forest.

3.2. Investigations using classical microscopy

All the adult bee samples were found to be negative to tracheal acariosis (0/37). All samples of adult bees that were collected from surrounding trees or from outside the hive entrance were positive to *Nosema* spp. using microscopy (27/37), whereas all samples of adult bees collected from inside the hive were negative for *Nosema* spp. (10/37). Both brood comb samples proved negative for AFB.

3.3. Investigations using molecular methods

Sample from all five apiaries selected for *Nosema* speciation tested positive only for *N. ceranae* using RFLP-PCR protocols. Interrogation of a 252 bp PCR product using the *blastn* search algorithm suggested highest nucleotide sequence homology with nucleotide sequence from *N. ceranae*. Neither *N. apis* alone nor *N. apis/N. ceranae* co-infection were detected in any of the five apiaries tested and no PCR products were obtained using specific primers for *N. apis*.

All fifteen bee samples tested positive for multiple viruses using one-step real-time RT-PCR. BQCV was found to be the most prevalent, present in all 15 samples of bees. SBV and DWV exhibited the same prevalence of 87%. CBPV was detected in 73% of samples while ABPV in 67%. All the samples collected from outside the hive tested positive for CBPV and DWV and most tested positive for ABPV and SBV. In samples collected from the inside of the hive, SBV was detected in 90%, while DWV, CBPV and ABPV exhibited a prevalence of 80%, 60% and 60% respectively (Table 2). All three queen bees tested positive for DWV only (Table 3).

Table 1
Clinical symptoms, losses and forage areas of each apiary in relation to parasitological and chemical results (nd: not detected).

Apiary	Symptoms	Losses % (of the total population)	Forage areas	Imidacloprid (ng/g)	*N. ceranae*
1	Dead bees in front of the hive entrance, less at the bottom and the feeder Trembling bees clustered outside the hive entrances Bees absconding the hives and aggregating in nearby trees Black adults bees (<2%)	70	Wild flowers away from cultivations, fir forest	nd	Positive
2	Dead bees in front of the hive entrance, less at the bottom and the feeder Trembling bees clustered outside the hive entrances Bees absconding the hives and aggregating in nearby trees Black adults bees (<2%)	60	Wild flowers away from cultivations, fir forest	nd	Positive
3	Dead bees in front of the hive entrance, less at the bottom and the feeder Trembling bees clustered outside the hive entrances Colonies containing non-laying queens (~10% of the total colonies) Queenless colonies (−15% of the total colonies)	50	Olive, citrus, fruit cultivations and fir forest	39	Positive
4	Dead bees in front of the hive entrance, less at the bottom and the feeder Trembling bees clustered outside the hive entrances Colonies containing non-laying queens (−10% of the total colonies) Queenless colonies (−10% of the total colonies)	50	Olive, citrus, fruit cultivations, pine and fir forest	28	Positive
5	Dead bees in front of the hive entrance, less at the bottom and the feeder Trembling bees clustered outside the hive entrances Bees absconding the hives and aggregating in nearby trees Colonies containing non-laying queens (~10% of the total colonies)	60	Olive, citrus, fruit cultivations pine and fir forest	14	Positive

Table 2
Results of one-step real time RT-PCR for viruses detection and RFLP–PCR for *Nosema* speciation. Acquired fluorescence data were analysed using LightCycler software and the crossing point (Cp), was determined automatically. Positive samples were defined as Cp < 35, low positive as 35 > Cp < 40 and negative as Cp = 40. Samples from 1 to 5 were collected from outside of the hive, while samples from 6 to 15 were collected from inside the hive. IPC*: internal positive control 18S rRNA gene of *Apis mellifera*.

Sample	CBPV	Cp value	ABPV	Cp value	DWV	Cp value	BQCV	Cp value	SBV	Cp value	IPC* Cq value	*N. ceranae*
1	Positive	12.62 13.29 13.14	Low positive	>35 >35 >35	Low positive	>35 >35 >35	Positive	26.77 26.23 25.79	Positive	27.2 27.11 27.12	10	Positive
2	Positive	12.11 12.65 12.31	Positive	30.65 31.09 31.12	Low positive	>35 >35 >35	Positive	25.54 25.23 25.13	Positive	34.81 34.91 34.88	11	Positive
3	Positive	17.07 16.16 16.05	Negative	40 40 40	Positive	31.96 32.68 32.17	Positive	28.79 28.84 29	Negative	40 40 40	11.5	Positive
4	Positive	10.61 12.12 12.52	Positive	33.41 33.46 32.74	Low positive	>35 >35 >35	Positive	24.81 25.12 25.02	Low positive	>35 >35 >35	11	Positive
5	Positive	26.51 27.03 26.86	Low positive	>35 >35 >35	Low positive	>35 >35 >35	Positive	27.28 27.45 27.47	Low positive	>35 >35 >35	11	Positive
6	Negative	40 40 40	Negative	40 40 40	Positive	31.74 31.69 31.69	Positive	17.31 17.79 17.89	Positive	26.31 25.75 26.88	8.85	Negative
7	Positive	26.42 25.68 26.01	Positive	26.23 25.66 25.27	Positive	34.28 32.74 32.38	Positive	26.93 26.23 26.17	Positive	26.52 26.49 25.19	10.1	Negative
8	Negative	40 40 40	Negative	40 40 40	Positive	30.5 30.48 31.09	Positive	23.89 23.62 23.94	Positive	28.62 28.83 29.51	8.68	Negative
9	Positive	13.09 12.44 12.3	Positive	30.28 31 31.16	Negative	40 40 40	Positive	23.97 24.28 23.66	Positive	32.36 32 32.1	8.75	Negative
10	Positive	29.22 28.77 29.55	Negative	40 40 40	Positive	31.28 32.71 32.2	Positive	26.93 26.9 26.62	Positive	31.96 32.1 30.97	8.71	Negative
11	Positive	29.01 29.36 28.94	Low positive	>35 >35 34.5	Positive	30.92 30.42 30.08	Positive	26.38 26.13 25.79	Positive	28.48 28.08 28.35	9.71	Negative
12	Negative	40 40 40	Positive	28.39 28.75 28.29	Positive	29.29 29.6 29.18	Positive	26.29 26.21 25.94	Positive	31.87 31.73 32	10.1	Negative
13	Negative	40 40 40	Negative	40 40 40	Negative	40 40 40	Positive	28.21 28.31 28.2	Negative	40 40 40	9.04	Negative
14	Positive	19.09 18.62 18.86	Positive	30.5 29.9 27.9	Positive	22.68 22.89 22.59	Positive	24.93 24.85 24.8	Positive	28.66 29.49 29.04	9.61	Negative
15	Low positive	>35 >35 >35	Positive	29.53 29.22 29.26	Positive	21.97 21.89 22.14	Positive	24.08 24.02 23.9	Positive	28.51 28.33 27.92	9.95	Negative

3.4. Chemical analysis using LC–MS

Chemical analysis revealed that three out of five bee samples (3/5) contained imidacloprid in concentrations between 14 ng/g and 39 ng/g tissue with a mean value of 27 ng/g tissue. Amitraz, thiametoxan, clothianidin and acetamiprid were not detected in any samples of adult bees. Neither sugar nor sugar patty samples contained residues of any target chemicals (Table 1).

4. Discussion

This study reports honey bee colony losses associated with specific symptomatology that occurred in the summer in Greece. Whilst substantial losses have been attributed to a heavy infestation by *V. destructor* and its close associate DWV (de Miranda et al., 2010), mite infestation was low in adult honey bee samples collected from Greek colonies suffering summer losses.

The current study reports the presence of five bee viruses (ABPV, CBPV, SBV, BQCV, DWV) in adult bee populations in Greece. Although several bee viruses had been detected in extracts of infected brood by electron microscopy in Greece (Allen and Ball, 1996), this study presents the first detection of viruses in Greek adult bee populations with the use of molecular techniques. Whilst it is impossible to interpret these results in the absence of a large-scale virus survey to include healthy and diseased apiaries across Greece, the high proportion (73%) of CBPV observed in the current study appears unusual when compared to reports from other countries where 5–28% is more typical (Nielsen et al., 2008; Tentcheva et al., 2004). It is also noteworthy that high percentages (60–100%) of samples collected from inside and outside the affected colonies tested positive for viruses. Samples positive for *N. ceranae* and

Table 3
Results of one-step real time RT-PCR for virus detection in three queens from the examined apiaries. Acquired fluorescence data were analysed using LightCycler software and the crossing point (Cp), was determined automatically. Positive samples were defined as Cp < 35, low positive as 35 > Cp < 40 and negative as Cp = 40. IPC*: internal positive control 18S rRNA gene of *Apis mellifera*.

Sample	CBPV	Cp value	ABPV	Cp value	DWV	Cp value	BQCV	Cp value	SBV	Cp value	IPC* Cp value
Queen 1	Negative	40	Negative	40	Positive	18.35	Negative	40	Positive	34.78	9.71
		40		40		18.29		40		34.31	
		40		40		18.06		40		34.78	
Queen 2	Negative	40	Negative	40	Positive	15.6	Negative	40	Negative	40	8.64
		40		40		15.6		40		40	
		40		40		18.73		40		40	
Queen 3	Negative	40	Negative	40	Positive	21.31	Positive	28.72	Negative	40	8.18
		40		40		21.31		28.69		40	
		40		40		21.64		28.17		40	

CBPV that were collected from the outside of the hive had more intense symptoms and average Cp value = 16.07 ± 5.8, presumably, indicating high level of CBPV infestation, compared with the *Nosema* negative samples from the inside of the hive where average Cp value = 30.6 ± 9.8 indicates low level of CBPV infestation (Table 2). Since only three queen bees were examined for viruses and all of them were positive for DWV, no correlation with clinical signs of the colony, namely queenless colony and non-laying queens, can be hypothesized. The symptoms seen in 2009 were similar but more severe than those observed in previous years in Greece and many resemble CBPV infection. Although virus infection has been linked to *Nosema* spp. or/and mite infestation (Chen and Siede, 2007; Higes et al., 2008), in our cases multiviruses infections were detected in samples *Nosema* negative, with low mite (*V. destructor*) infestation.

All 27 adult bee samples collected from outside of the hives contained *Nosema* spp. spores, whereas no samples from within hives were *Nosema* positive by microscopy. This finding might be attributed to the fact that house bees are younger than field bees and consequently not yet infected by the parasite. When the *Nosema* in the five of the positive samples was speciated, were all found to contain *N. ceranae,* a finding consistent with reports referring to Greek areas (Hatjina et al., 2010).

Chemical analysis of bee tissues revealed the presence of imidacloprid (neonicotinoid) in 60% of the samples analysed and in an average concentration of 27 ng/g tissue. This represents the first report of imidacloprid contamination in honey bee samples from Greece. Furthermore, this contamination was unlikely to have originated from food supplements applied by the beekeepers, because both sugar syrup and sugar patties tested negative for imidacloprid residues. Imidacloprid acts on the nicotinic acetylcholine receptor of many invertebrates (Tomizawa and Casida, 2005) and has a low mammalian toxicity. Coupled with high effectiveness and high mobility in plant and mammalian tissue, imidacloprid is registered in numerous countries for a wide range of uses including: soil, seed, and foliar insecticide to control sucking insects such as leaf and plant hoppers, aphids, thrips, termites and coleopteran pests on crops including rice, cotton, cereals, maize, sugar beet, potatoes, vegetables, citrus, and stone fruit (Liu and Casida, 1993; Mullins, 1993; Yamamoto et al., 1998). The widespread use imidacloprid as a systemic insecticide, and its possible translocation to pollen and nectar, has raised concerns for the possible detrimental impact on beneficial insects (James and Price, 2002; Oldroyd, 2007). There is a considerable debate about the chances of this happening to the degree that bees are threatened. Chauzat et al. (2006) reported residues of imidacloprid in nectar and pollen at levels that are potentially dangerous to bees, while Schmuck et al. (2001) detected no residues. It has been assumed that the homing ability and behaviour of bee foragers may be severely affected by residual imidacloprid (Bonmatin et al., 2005; Yang et al., 2008), although Nguyen et al. (2009) suggest that imidacloprid seed-treated maize has no negative impact on honey bees. It is difficult to identify the source of the imidacloprid residues detected in the current study. What is known, is that the contaminated bees were from apiaries located in Argolida and Messinia (Peloponnesus), prior to transportation to fir forests at Mt. Mainalo. Bees foraging in these regions were likely exposed to imidacloprid and other insecticides which are widely applied to olive, citrus and fruit cultivations in Greece.

A key commonality between all the apiaries suffering symptoms is that all belonged to migratory beekeepers. The overall procedure of transportation from the one foraging area to another, along with the corresponding environmental changes encountered by the bees, are undoubtedly stress factors for bees. The constant relocation typified by migratory beekeeping is stressful for the bees, probably depresses the immune system and advances contagious diseases (Cooper, 2007). Long distance mass migration is one of the suspected risk factors for a disorder that has led to large-scale colony losses in the USA (Oldroyd, 2007). Migration often serves to pollinate or derive a particular honey crop at a particular time of year, and so can result in a sudden increase in local colony density. This increases the risk of disease spread between colonies, and is typical of the fir honeydew foraging period in Greece (Mt. Mainalo). The interchanges of the environmental conditions as well as the honeydew type could also be two stress factors. The colonies included in the current study were subjected to different weather conditions in a very short time. Just after the bee-colonies transportation to Mt. Mainalo area (altitude: 1200 m) the weather conditions altered from low temperature and heavy rain to high temperature without rain. Furthermore, foraging in fir forests seems to be quite fatiguing for bees workers due to the low water content (13–14%) of this honeydew type and might be a stressful condition for them.

In conclusion the current study, whilst focused on a single outbreak of bee mortality, has identified five possible factors that may have contributed to the summer losses experienced in 2009: (i) multiple virus infection by five different viruses along with infection by *N. ceranae* (ii) imidacloprid residues in bee tissues, stress induced by (iii) transportation, (iv) temperature and humidity fluctuations and (v) the collection of the fir honeydew (low water content).

Our data demonstrates the need to complete an in-depth study across all Greek regions, taking into account the five putative risk factors identified in this pilot study. Such additional context is essential to provide a more complete picture of colony losses in Greece, and would provide the evidence to help ensure the future welfare of over a million Greek honey bee colonies.

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