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The health status of Irish honeybee colonies in 2006

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This study assessed the health status of Irish honeybee colonies and provides a snapshot of the incidence of a number of important colony parasites/pathogens including: the mite *Varroa destructor*; three associated viruses (deformed wing virus (DWV), acute bee paralysis virus (ABPV) and Kashmir virus (KBV)); the tracheal mite *Acarapis woodi*; the microsporidian *Nosema* spp., and the insect *Braula coeca*. During June/July 2006, 135 samples of adult bees were collected from productive colonies throughout Ireland and standard techniques were used to determine the presence and absence of the parasites and pathogens. *Varroa destructor* was positively identified in 72.6% of the samples and was widely distributed. Although the samples were analysed for three viruses, DWV, ABPV and KBV, only DWV was detected (frequency = 12.5%). *Acarapis woodi* and *Nosema* spp. occurred in approximately 11% and 22% of the samples, respectively, while *B. coeca*, a wingless dipteran that was once common in Irish honeybee colonies, was very rare (3.7%). Samples where all the pathogens/parasites were jointly absent were statistically under-represented in Leinster and DWV was statistically over-represented in Munster. In Ulster, there was over-representation of the categories where all parasites/pathogens were jointly absent and for *A. woodi*, and under-representation of *V. destructor*.

Keywords: Acarapis woodi; Apis mellifera; *Braula coeca;* honeybee viruses; *Nosema* spp.

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Introduction

The phenomenon of colony losses is well known to beekeepers and, while some winter mortalities are inevitable, there has been a steady increase in losses in Europe from 1985 to 2005 (Potts *et al*. 2010). This period coincides with the arrival of the parasitic mite, *Varroa destructor* Anderson and Trueman, which was first detected in Europe on *Apis mellifera* L. in the late 1970s. Initially, it was controlled successfully using chemical insecticides, but resistance quickly developed (Trouiller 1998). In 2006, high losses were again reported, initially in the US (Cox-Foster *et al.* 2007), but then in Europe and the other continents (Stokstad 2007). This phenomenon was referred to as colony collapse disorder (CCD) (vanEngelsdorp *et al*. 2009) and the causative factors still remain undetermined. Most scientists agree that CCD and the gradual increase in winter mortalities is probably due to a combination of several interacting factors ranging from parasites, pathogens, viruses, single source forage, pesticides and inclement weather (Potts *et al*. 2010). However, *V. destructor* and its associated viruses is emerging as one of the main culprits in winter mortalities in northern climates (vanEngelsdorp *et al*. 2009; Guzmán-Novoa *et al*. 2010) and this agrees with the suggestion that the presence of *V. destructor* in most colonies is an additional pressure on bee health.

Varroa destructor is a relatively recent parasite of the western honeybee *A. mellifera* in terms of co-evolution with its host. In contrast to the original host, the Asian honeybee *A. cerana* Fab., *A. mellifera* is not well adapted to mite infestation, and consequently suffers either severe damage or death of the whole colony in many instances (Potts *et al.* 2010). The intense feeding action of the parasitic mite not only causes physical damage to the adult bee and brood, but it also reduces protein content, body weight and hinders organ development (Bowen-Walker, Martin and Gunn 1999). In addition, the mite is a vector of a wide range of viruses including deformed wing virus (DWV), acute bee paralysis virus (ABPV) and Kashmir virus (KBV) (Tentcheva *et al*. 2004). In many instances these viruses not only weaken the bee's immune system and suppress the expression of immunity-related genes (Yang and Cox-Foster 2007), they also contribute to morphological deformities which reduce longevity, vigour and the homing ability of individuals (Kralj and Fuchs 2006).

Colony survival is further reduced when *V. destructor* co-infects with another parasitic mite, *Acarapis woodi* Rennie (Downey and Winston 2001). This mite is an internal parasite of the adult honeybee which feeds and reproduces in the tracheae inside the thorax of adult honeybees. Infestation reduces longevity of the adult bee (Maki *et al*. 1986), slows colony build-up in spring (Scott-Dupree and Otis 1992) and increases colony mortality (Furgala *et al*. 1989).

Nosemosis, a disease caused by microsporidian parasites of the genus *Nosema*, also has a negative impact on hive productivity and colony survival over winter (Fries, Ekbohm and Villumstad 1984). Traditionally, the causative pathogen was the microsporidian, *Nosema apis* Zander. However, more recently a related species, *N. ceranae* Fries, originally described in the Asian honeybee (Fries *et al.* 1996) has been identified in *A. mellifera* colonies worldwide (Higes *et al.* 2009). *N. ceranae* causes symptoms similar to *N. apis* (Higes *et al.* 2006); however, some authors suggest that *N. ceranae* is a more virulent pathogen which may be associated with the sudden collapse of colonies (Higes *et al.* 2009). Furthermore, recent studies

suggest that *N. ceranae* infection reduces the longevity of individual bees and affects their feeding behaviour (Nuag and Gibbs 2009).

Many of the above-mentioned causative factors of winter mortalities are known to be present in Irish honeybee colonies (Coffey and Breen 2012). *V. destructor* was first identified in Ireland in 1998 (Kelly *et al*. 2013), but the incidence of viral infection has never been documented. *A. woodi* and *N. apis* are both endemic and the presence of *N. ceranae* was reported for the first time in 2008 (Maloney and Coffey 2008). The main aim of this study was to provide an overview of the health status of Irish honeybee colonies in 2006, with special reference to the occurrence of *V. destructor, A. woodi, Nosema* spp., DWV, ABPV, KBV and *Braula coeca*. We have also attempted an analysis of the co-presence and co-absences of these organisms in Irish honeybee colonies.

Materials and Methods

Sampling

During June–July 2006, samples were collected from 135 different apiaries owned by 78 different beekeepers (Figure 1). Sampling depended on the willingness of beekeepers to participate and, in some instances, the participating beekeepers facilitated sampling from a second apiary provided the two apiaries were at least 10 km apart. Samples were taken from productive colonies which were apparently healthy. Otherwise, sampling was arbitrary as to which colony was chosen. The presence or absence of *V. destructor* was verified by removing and examining 30 purple-eyed drone pupae, while the presence of *B. coeca* was determined by the visual examination of adult bees. A representative sample of approximately 500 adult bees was taken from the outside frames in the brood box in each of the sampled colonies. The sampled bees were immediately placed on ice and when the bees were returned to the laboratory, 50 bees were placed at -80 °C for viral analysis while the remainder were stored at -20 °C.

Acarapis woodi

To assess samples for the presence or absence of *A. woodi*, 30 bees were individually tested by removing the head, forelegs and collar to expose the tracheae. A positive diagnosis was noted if the tracheae had lost the smooth white or fleshy appearance and showed any signs of bronzing, black specks or streaks in either tracheal trunk (OIE 2012).

Nosema spp.

Nosema infection was assessed using the methods described by Cantwell (1970) and no attempt was made to differentiate between the two known species. A composite sample of 30 bees together with 30 ml of distilled water was ground into a suspension using a mortar and pestle. From this suspension, 0.01 ml was transferred using a calibrated loop onto a haemocytometer and covered with a glass slide. The number of spores from five different fields of view was estimated and the number of spores per bee determined according to the equation given in Cantwell (1970). This method does not allow the two *Nosema* spp. to be distinguished. Hence, in this paper, "*Nosema* spp." may refer to infection with *N. apis*, *N. ceranae,* or both.

Viral analysis

The bees were assessed for the presence of three viruses DWV, ABPV and KBV using separate uniplex reverse transcription polymerase chain reaction

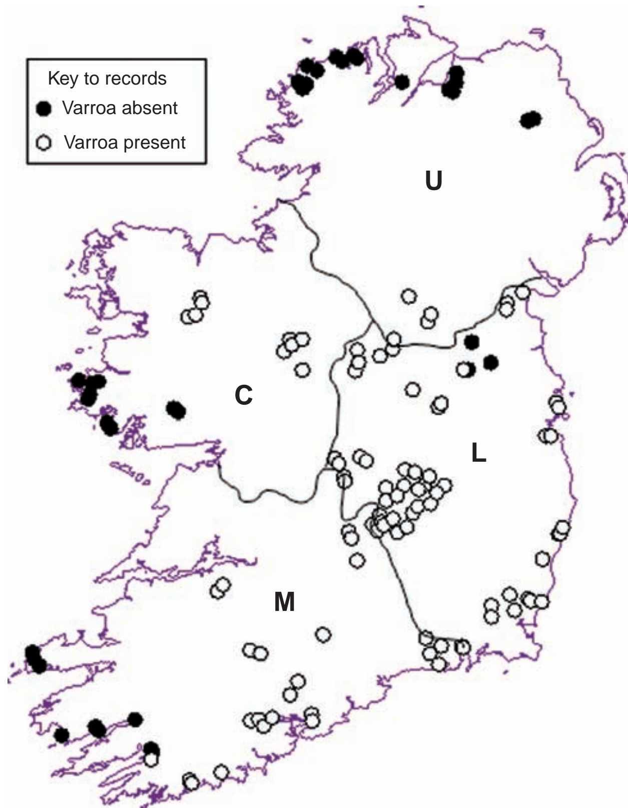

Figure 1. Map of the sampling sites showing where Varroa destructor was present (open symbols) and absent (solid symbols). The four provinces of Ireland (M = Munster; L = Leinster; C = Connacht; U = Ulster) are also indicated.

(RT-PCR). Composite samples of 50 bees were crushed in a mortar in the presence of liquid nitrogen and a subsample homogenised in 500 µl of TRIzol (Sigma-Aldrich, St. Louis, USA) used according to the manufacturer's instructions for RNA extraction. The resultant RNA pellets were re-suspended in diethyl pyrocarbonate-treated (DEPC-water; Sigma-Aldrich) and the Access RT-PCR system (Promega, Madison, Wisconsin) was used to perform RT-PCR. The RT-PCR primers used are given in Table 1. The RNA PCR template samples were amplified using the PTC-100 DNA engine (MJ Research, Boston, USA). The reaction mixture contained: 1 x AMV/Tfl (AMV reverse transcriptase and themostable Tfl DNA polymerase) reaction buffer, 0.2 mM each dNTP (deoxyribonucleotide trophosphate) (Sigma-Aldrich), 1 µM of sense primer, 1 µM of antisense primer, 2 mM $MgSO_4$, 0.1 unit AMV reverse transcriptase (Promega), 0.1 unit Tfl DNA polymerase (Promega) and 500 ng total RNA in a total volume of 25 µl. The profiles of the thermal cycles were as described by Chen *et al.* (2004) and Chen, Pettis and Feldlaufer (2005). Verified negative and positive controls were included in each run of RT-PCR to control for methodological problems, in addition to a positive control for the

Table 1. The RT-PCR primers used for the detection of deformed wing virus (DWV), acute bee paralysis virus (ABPV) and Kashmir virus (KBV).

Virus	Primers	Amplicon size	References
ABPV	F (5′-ttatgtgtccagagactgtatcca-3′) R (5′-gtccctattgctcggtttttcggt-3′)	900	Benjeddou *et al.* (2001)
DWV	F (5′-atcagcgcttagtggaggaa-3′) F (5′-tcgacaattttcggacatca-3′)	702	Chen *et al.* (2004)
KBV	F (5′-gatgaacgtcgacctattga-3′) R (5′-tgtgggttggctatgagtca-3′)	415	Stoltz *et al.* (1995)

F = forward primer and R = reverse primer.

specific virus being tested. Amplification products were analysed together with a 100 bp ladder for determination of the size of the PCR products by electrophoresis through 1% agarose gel containing 0.5 µg/ml ethidium bromide (Sigma-Aldrich) and visualised by UV transillumination (Kodak Imaging Station, Kodak, NewYork, USA). The PCR bands specific for each virus were purified using Wizard PCR Prep DNA purification System (Promega) and sequenced by a commercial sequencing provider to confirm specificity of the RT-PCR assay. The nucleotide sequences of the PCR products were analysed and compared with sequences published for these viruses in Genbank (http://www.ncbi.nlm.nih.gov/nuccore).

Statistical analysis

The geographical distribution of sampling sites was mapped using DMAP for windows (Morton 2001). For the initial analyses, potential links to geography were ignored and patterns in the co-occurrence of parasites and pathogens were tabulated. This showed that a small number of combinations of the parasites/pathogens were sufficient to describe the 135 samples, suggesting that the analysis could be better advanced by combining the parasites/pathogens into new categories and performing further statistical analysis on these new categories. This approach overcomes many of the difficulties arising due to the sparse nature (i.e. many zeros) of the original data. Fisher's Exact Test for testing the null hypothesis of the independence of rows and columns in a contingency table was performed for each species of parasite/pathogen across each province. Parasites or pathogens exhibiting statistically significant regional changes (at $P<0.05$) were noted. The independence of parasite/pathogen patterns across each province was tested using a chi-squared test of independence (at $P<0.05$). Evidence for changes across province was further investigated by a graphical examination of residuals (observed counts against expected counts under independence). The statistics and graphs were produced using the R statistics package (R Core Team 2012).

Results

Varroa destructor was the most frequently occurring parasite (Table 2). In decreasing order of abundances, the other parasites/pathogens were: *Nosema* spp., DWV, *A.*

Table 2. The occurrence of five parasites/pathogens in individual honeybee colonies in 135 different apiaries distributed throughout Ireland

Parasite/pathogen	Total	%
Varroa destructor	98	72.6
Nosema spp.	30	22.2
Deformed wing virus	17	12.6
Acarapis woodi	15	11.1
Braula coeca	7	5.2

woodi and *B. coeca*. No evidence was found for the occurrence of either ABPV or KBV.

The co-occurrence of the five parasites/pathogens of honeybees (Table 3) shows that the top four combinations of co-occurrence accounted for 80% of the samples and that 17.8% of colonies tested were free from all the parasites/pathogens under investigation. *Varroa destructor* occurred most frequently; alone in 37.8% of samples and in 8.1% of the samples as a co-infection with DWV. However of the 17 samples where DWV occurred, two of these were free of *V. destructor*. Co infections of *Nosema* spp. and *V. destructor* were also observed (16.3%), while the incidence of *Nosema* spp. as a sole infection was minimal (<1%). The 15 (11.1%) locations where *A. woodi* was present are not represented in these top four combinations (Table 3), but it was observed as a sole infection or part of a multiple infection. The level of occurrence of *B. coeca* was very low; it co-occurred with *V. destructor* in four samples, and occurred in its absence in three samples. Samples with more than two parasites and pathogens present were also observed (Table 3).

In the samples positively identified for *Nosema* spp., the invasion intensity (spores/bee) ranged from 0.1×10^6 to 24.7×10^6 with a mean (±s.e.) of 0.8 (±0.27)$\times 10^6$. The invasion intensity was categorised into three groups, low ($<1 \times 10^6$ spores/bees), medium ($1–5 \times 10^6$ spores per bee) and high invasion ($>5 \times 10^6$ spores/bee). Low level of invasion was identified in approximately 45% of samples, medium invasion in 38% and high invasion in 16%.

A mosaic plot (Hartigan and Kleiner 1984; Friendly 1994) was used to assess the patterns of co-occurrence of the different parasites/pathogens in the four provinces (Figure 2a). The plot provides a proportional visualisation of a contingency table of expected frequencies. The Pearson's chi-squared test on this contingency table gave $\chi^2 = 62.5$, 15 degrees of freedom, and $P<0.001$. (The chi-squared test on this contingency table is theoretically unreliable as a number of cells have expected values of <5. This theoretical concern can be overcome by excluding the

Table 3. The co-occurrence of five parasites/pathogens of honeybees in Ireland.

Varroa destructor	*Nosema* spp.	Deformed wing virus	*Acarapis woodi*	*Braula coeca*	Frequency	%
+	—	—	—	—	51	37.8
—	—	—	—	—	24	17.8
+	+	—	—	—	22	16.3
+	—	+	—	—	11	8.1
—	—	—	+	—	6	4.4
+	—	—	+	—	5	3.7
+	+	+	—	—	3	2.2
+	—	—	—	+	2	1.5
—	—	—	—	+	2	1.5
+	+	—	+	—	2	1.5
—	—	+	—	—	2	1.5
—	—	—	+	+	1	0.7
+	—	+	—	+	1	0.7
+	+	—	—	+	1	0.7
—	+	—	+	—	1	0.7
—	+	—	—	—	1	0.7

'+' = present; '—' = absent.

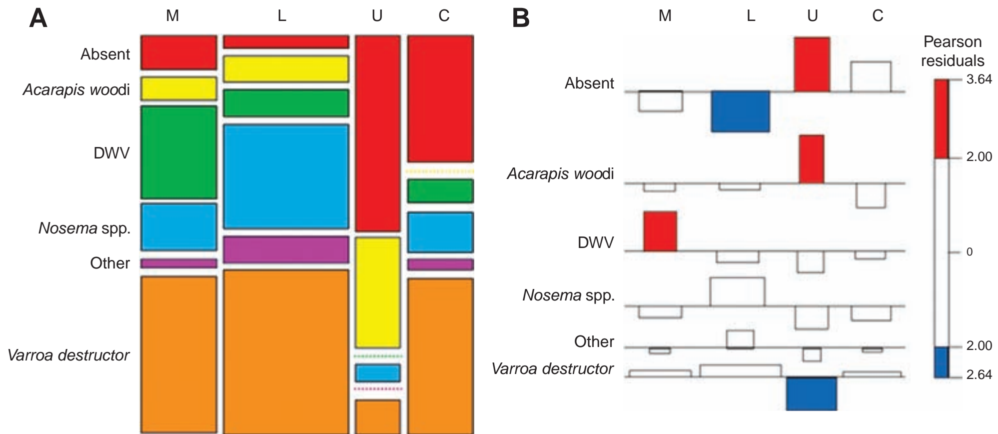

Figure 2. (a) Mosaic plot showing the sole occurrence or co-occurrence of five parasites/pathogens of honeybees in Ireland during 2006, broken down according to province (M = Munster; L = Leinster; C = Connacht; U = Ulster). The column widths are proportional to the number of sites surveyed in each province, and the column heights to the frequency of occurrence of the different parasites and pathogens in each province. The category "Absent" (red) refers to the sites where all of the parasites/pathogens were jointly absent. The category "Varroa destructor" (orange) refers to the sites where the parasitic mite V. destructor was the only parasite/pathogen present. The categories "Acarapis woodi" (yellow), "deformed wing virus (DWV)" (green), and "Nosema spp."(blue) refer to sites where these parasites/pathogens occurred solely or co-occurred with V. destructor. The category "Other" (purple) refers to the small number of sites with other combinations of parasites/pathogens. (b) Plot of Pearson residuals from the Chi-squared test of independence between parasites/pathogens of honeybees and province surveyed. The positive (red) and negative (blue) values of the residual indicates significant over- and under-representation in each province.

category "Other", but this did not change the outcome. Hence, we retained all categories for the purpose of the graphical displays.) When the residuals of the contingency table analysis were examined (Figure 2b), colonies with DWV were statistically over-represented in Munster and colonies where all pathogens or parasites were jointly absent ("Absent") were under-represented in Leinster. The pattern of co-occurrences was quite different in Ulster where there was over-representation of the "Absent" and *A. woodi* categories, and under-representation of *V. destructor*. *Nosema* spp. was over-represented in Leinster and under-represented in the other three provinces, but this pattern in co-occurrences was not significant. *Braula coeca* was not represented in this figure because of its small number of occurrences.

Discussion

Varroa destructor and its associated viruses have been indentified as the main culprit for the death and reduction of honeybee populations in northern climates (Guzman-Novoa *et al*. 2010). First reported in Ireland in 1998, *V. destructor* had established itself in many parts of the country by 2006 (approximately

73% of samples), although as indicated in the present study geographical differences were still apparent. In Ulster, the significantly lower co-presence of *V. destructor* and the significant over-representation of disease-free colonies are consistent with the suggestion that the presence of *V. destructor* in a colony weakens the bee immune system by suppressing the expression of immune related genes and increasing DWV concentrations, both of which have negative impact on worker survivorship and colony fitness (Yang and Cox-Foster 2007).

A total of 23 honeybee viruses have been identified worldwide, but DWV, ABPV and KBV and others have been specifically associated with varying degrees of *V. destructor* infestation (Tentcheva *et al*. 2004). However, infection with multiple viruses is generally accepted as a normal occurrence in honeybee colonies (Carreck, Ball and Martin 2010). In the present study, although the samples were analysed for DWV, ABPV and KBV, only DWV was identified and it occurred in relatively low proportions (approximately 12.6%) when compared with studies from other European countries. For example in France (Tentcheva *et al.* 2004), Germany (Genersch *et al.* 2010) and Austria (Berényi *et al*. 2006), the prevalence of DWV was found to be greater than 90%, while in Denmark (Nielsen *et al*. 2008), the reported frequency of occurrence was approximately 55%. In these studies, there was a general consensus that a strong correlation existed between mite infestation levels and colony collapse (Nielsen *et al*. 2008) and more recently Landaverde *et al*. (2012) found a high incidence of DWV following a sharp decline in bee population, suggesting that DWV is acting as an opportunist rather than a cause of decline. The health status of the colony has also been indentified as an influential factor and a study by Berényi *et al.* (2006) showed that the virus load in bees from a healthy colony can be ten to 126 times lower than in bees from a diseased colony. In the present study, the low incidence of viral infection may be attributed in part to all the samples being taken from productive colonies and, at the time of sampling, *V. destructor* was still being successfully controlled with the chemical acaricide, flumethrin (Bayvarol®). Furthermore, samples for this study were collected in June/July and according to Tentcheva *et al*. (2004) and Santillán-Galicia *et al*. (2010) the prevalence and infectivity of DWV is highest in early autumn.

The symptoms of DWV are directly related to DWV concentration, multiple mite infestation, infestation from mites from previously symptomatic bees or the ability of the DWV to replicate in the mite (Yue *et al.* 2007). Without pupal infestation, bees are generally asymptomatic and remain so irrespective of the concentration of the virus (Yue *et al.* 2007). However, in a recent study, Forsgren, Fries and de Miranda (2012) identified deformed bees in a confirmed *V. destructor*-free colony. In the present study, although the bees analysed from the *V. destructor*-free colonies showed no clinical symptoms, two samples tested positive for DWV. Since the distance of these colonies from *V. destructor*-infested colonies was not noted at the time of sampling, it is likely that the DWV may have been transmitted vertically through the drones (Yue *et al*. 2007) and queens (Fievet *et al*. 2006) and/or horizontally though robbing and drifting and larval food (Chen, Evans and Feldlaufer 2006).

Although widespread in some European countries the reported incidence of ABPV is quite variable and it was not detected in the present survey. In France (Tentcheva *et al.* 2004), Hungary (Bakonyi *et al*. 2002), Austria (Berényi *et al.* 2006) and Denmark

(Nielsen *et al.* 2008) the prevalence was 58% , 67%, 69% and 11% respectively, while in Belgium the reported incidence rate was lower at 8% (Nguyen *et al.* 2011). As with DWV, ABPV is positively correlated with the *V. destructor* population (Genersch *et al.* 2010), but annual differences in the prevalence of ABPV (5.8%–11.7%) have been documented in Germany from 2005 to 2007 (Genersch *et al*. 2010). The absence of KBV was not surprising as it is rare throughout Europe (Allen and Ball 1996). It was identified in the UK for the first time in 2006 after an extensive study (458 samples) found it in only three colonies at two locations (Ward *et al.* 2007).

The incidence of *Nosema* spp. in Irish honeybee colonies (22.2%) is lower than that reported in most other studies. In Britain and Finland, Varis, Ball and Allen (1992) documented an incident rate of *N. apis* of 33% and 35%, respectively, while in more recent studies *Nosema* spp. prevalence in Australia (Giersch *et al.* 2009) Turkey (Aydin *et al.* 2005) and France (Chauzat *et al.* 2007) was estimated at 60% to 65%. In these studies, the frequency of occurrence of *N. apis* and *N. ceranae* were separately reported and in the French study (Chauzat *et al.* 2007) 65% of the samples were positive for *N. ceranae*, 1.6% for *N. apis* and 6.6% had both species present. This possible replacement of *N. apis* by *N. ceranae* is further reflected in the recent analysis of historic samples by Paxton *et al.* (2007). Infection by *N. apis* is traditionally considered as a low prevalence disease, declining during the summer months and resurging in autumn (Doull and Eckert 1962). In contrast, *N. ceranae* demonstrates an even seasonal virulence and is consequently considered a major threat to honeybee colonies (Higes *et al.* 2008). It is this epidemiological difference between these two species, especially in relation to temperature tolerance, which may be related to the higher prevalence of *N. ceranae* worldwide (Higes *et al.* 2010). However, in Canada (Williams *et al.* 2008) and Sweden (Fries and Forsgren 2008) and possibly Ireland and Britain (Fries 2010), pure infections of *N. apis* may still exist. If we assume that the predominant species in this study was *N. apis*, the intensity of invasion (spores/bee) presented in this study compares well with the data reported by Pickard and El-Shemy (1989) for Britain. They observed that during June/July, the invasion intensity of *N. apis*, ranged from 0.06×10^6 to 2.94×10^6. In the present study, the mean (±s.e.) invasion intensity recorded for samples collected in late June/July was 0.8×10^6 spores/bee, with more that 80% of the samples analysed having low-medium invasion intensity. However, since *N. ceranae* has been positively identified in Irish colonies (Maloney and Coffey 2008) and the frequency of occurrence of the two species was not differentiated in this study, direct comparisons such as this should be treated with some caution.

The incidence of *A. woodi* (11.1%) in this study, although higher than that reported for Greece (Bacandritsos and Saitanis 2004) or Turkey (Çakmak *et al.* 2003) would suggest that it is not a threat to honeybee colonies. However, Downey and Winston (2001) showed that the co-infection of these parasitic mites in a colony caused a greater mortality risk than either of the mites acting alone. In the present study, the significant co-presence of *A. woodi* in colonies where the presence of *V. destructor* is significantly under-represented can be attributed to the non-use of annual varroacides in these colonies. In Spain, Orantes Bermejo *et al*. (1997) reported a reduction in prevalence of *A. woodi* from 18.4% to 11.4% after repeated annual treatments of varroacides.

However, the negative impact of chemical acaricides on *A. woodi* is inconclusive (Scott-Dupree and Otis 1992), although thymol-based products are emerging as the most effective treatment for this species (Rice *et al.* 2002).

The bee louse, *Braula coeca* was once a cosmopolitan inhabitant of honeybee colonies (Smith and Caron 1985), but the extensive use of acaricides for the control of *V. destructor* may threaten its survival at least in some localities. Although Kulincevic *et al.* (1991) reported that fluvalinate was more toxic to *B. coeca* that amitraz, in Ireland, flumethrin, a close relative to fluvalinate has been used against *V. destructor* since its arrival in 1998 and its negative impact on *B. coeca* over a relatively short period (8 years) is already reflected in its low frequency of occurrence reported in this study.

In conclusion, many of the parasites and pathogens identified in this study and the observed complex interaction with each other are now known to be key factors in the increased number of colony mortalities being reported world wide. In Ireland during 2009 to 2011, winter mortalities were approximately 20% (van der Zee *et al.* 2012), consequently, an updated study is warranted to ascertain the status of parasites and pathogens in Irish honeybee colonies today, 15 years after the arrival of *Varroa destructor.*

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