Monitoring honey bee health in five natural protected areas in Italy

Valter Bellucci[1], Stefano Lucci[1], Pietro Bianco[1], Alessandro Ubaldi[2], Antonio Felicioli[3], Claudio Porrini[4], Franco Mutinelli[5], Sabrina Battisti[2], Valentina Spallucci[2], Antonella Cersini[2], Marco Pietropaoli[2] and Giovanni Formato[2*]

[1]*Istituto Superiore per la Protezione e la Ricerca Ambientale, Via Vitaliano Brancati 60, 00144 Rome, Italy.*
[2]*Istituto Zooprofilattico Sperimentale del Lazio e della Toscana 'M. Aleandri', Via Appia Nuova 1411, 00178 Rome, Italy.*
[3]*Dipartimento di Scienze Veterinarie, Università degli Studi di Pisa, Viale delle Piagge 2, 56124 Pisa, Italy.*
[4]*Dipartimento di Scienze e Tecnologie Agro-Alimentari (DISTAL), Alma Mater Studiorum, Università di Bologna, Via Zamboni 33, 40126 Bologna, Italy.*
[5]*Istituto Zooprofilattico Sperimentale delle Venezie, National Reference Laboratory for Beekeeping, Viale Dell'Università 10, 35020 Legnaro (PD), Italy.*

[*]*Corresponding author at: Istituto Zooprofilattico Sperimentale del Lazio e della Toscana 'M. Aleandri', Via Appia Nuova 1411, 00178 Rome, Italy. Tel.: +39 06 79099328, e-mail: giovanni.formato@izslt.it.*

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Summary
The health status of the honey bee populations has attracted a great amount of interest in recent years. We investigated honey bee health in five natural protected areas in Italy from October 2009 to December 2010. Areas were selected to represent a wide range of bio-geographical zones including alpine, continental, and Mediterranean. Within each of these natural protected areas, one apiary of 20 colonies near potential pollution sources (e.g., agricultural areas, industrial areas, or urban settlements) and another apiary of 20 colonies far from possible sources of pollutants have been placed. To monitor honey bee health, colony mortality was related to: honey bee pathologies, environment (Naturality Index, plant protection products and heavy metal exposure), and apiary management. Anthropogenic pollutants and pathogens did not have significant effects on colony mortality while environment and the poor colony management skills of the beekeepers did.

Monitoraggio dello stato di salute delle api in cinque Aree Naturali Protette italiane

Parole chiave
Gestione degli alveari, Inquinamento ambientale, Mortalità delle api, Monitoraggio, Aree Naturali Protette.

Riassunto
Il presente studio riporta i risultati del monitoraggio dello stato di salute delle api effettuato da ottobre 2009 a dicembre 2010 in cinque Aree Naturali Protette italiane, scelte per rappresentare le aree biogeografiche alpina, continentale e mediterranea. All'interno di ciascuna è stato posizionato un apiario di 20 alveari vicino a potenziali fonti di inquinamento (ad esempio aree agricole, aree industriali o insediamenti urbani) e un altro apiario di 20 alveari lontano da possibili fonti di inquinamento. Per monitorare lo stato di salute delle api, è stata messa in relazione la mortalità degli alveari con la presenza di malattie delle api, con l'ambiente (*Naturality Index*, presenza di prodotti fitosanitari ed esposizione a metalli pesanti) e la gestione dell'apiario. Non sono stati osservati effetti significativi degli inquinanti di origine antropica e dei patogeni sulla mortalità degli alveari, mentre la capacità di gestione degli alveari da parte degli apicoltori è risultata strettamente correlata alla mortalità delle colonie.

Introduction

The decline of honey bee (*Apis mellifera* L.) colonies, observed in the last few decades in both Europe and in the United States (Ellis *et al.* 2010, Potts *et al.* 2010), have a multi-factorial origin (Neumann and Carreck 2010) including Plant Protection Products (PPPs), beekeeping practices, pest and pathogens, queen failure, genetic weakness, nutrition, and weather patterns.

Farming techniques and crop protection procedures play a pivotal role in the proper management of honey bee colonies and their possible exposure to PPPs (Alaux *et al.* 2010, Brodschneider and Crailsheim 2010, Johnson *et al.* 2010). Environment pollution, especially pesticides, can negatively affect the health of honey bee colonies. Numerous studies have reported on the negative effects of exposure to PPPs on honey bees (Koch and Wisser 2001, Forster 2009, Anne and Gavin 2010, EFSA 2012, Henry *et al.* 2012, Whitehorn *et al.* 2012). In addition, honey bee pests and pathogens probably play a crucial role in honey bee colony losses, especially losses caused by *Varroa destructor* (*V. destructor*) combined with viruses and *Nosema ceranae* (vanEngelsdorp *et al.* 2009, Neumann and Carreck 2010, Martin *et al.* 2013). The interaction of these pathogens with pesticides also causes losses of hives (Pettis *et al.* 2012, Pettis *et al.* 2013). Aside from PPPs, honey bee pests and pathogens, even beekeeping management contributes to endangering the status of honey bee health (Oldroyd 2007, vanEngelsdorp *et al.* 2008).

About 75,000 beekeepers in Italy manage more than 1,317,000 colonies [Commission Implementing Regulation (EU) N. 768/2013]. The responses from 874 surveys conducted in 2007-2009 by the organization named Prevention of honeybee COlony LOSSes, using the COST Action FA0803 framework, showed Winter mortality ranged from 11% (Abruzzo) to 38% (Emilia Romagna), with an average of 23.5% (Mutinelli *et al.* 2010, van der Zee *et al.* 2012). A pan-European epidemiological study on honeybee colony losses called EPILOBEE (Laurent *et al.* 2015), set up from 2012 to 2014 in 17 European Member States, showed a overwintering colony mortality in Italy ranging from 5.5% (winter 2012-2013) and 4.8% (winter 2013-2014).

This study, coordinated by the Italian National Institute for Environmental Protection and Research was promoted in 2009-2010 by the Italian Ministry of Environment, Territory and Sea to evaluate honey bee mortality within five natural protected areas (NPAs) in Italy, in order to verify the effects of chemical pollution, apiary management, and bee pathogens on honey bee's health.

Materials and methods

The monitoring project was conducted from October 2009 to December 2010 using 200 honey bee colonies within five Italian NPAs (Figure 1) as representative of a wide range of the bio-geographical regions in Italy including alpine, continental, and Mediterranean areas. These NPAs were all located near agricultural, industrial, or urban settlements and are listed as follows:

- Parco Nazionale delle Dolomiti Bellunesi (Dolomiti), alpine bioregion, Northeastern Italy;
- Parco dei Gessi bolognesi e dei Calanchi dell'Abbadessa (Calanchi), continental bioregion, Northern Italy;
- Parco di Migliarino San Rossore Massaciuccoli (San Rossore), Mediterranean bioregion, Central Italy;
- Parco dei Monti Simbruini (Simbruini), Mediterranean bioregion influenced by sub-continental conditions, Central Italy;
- Riserva Naturale Statale Litorale Romano (Litorale), Mediterranean bioregion, Central Italy.

For the present study, two apiaries of 20 healthy colonies were established in each NPA (40 colonies/NPA). One apiary was classified as a 'non-exposed' and called 'Apiary A' (combined with site names as Dolomiti A, Calanchi A, San Rossore A, Litorale A, Simbruini A) and located in ecosystems with a modest level of anthropogenic pressure. The other apiary was classified as 'exposed' and called

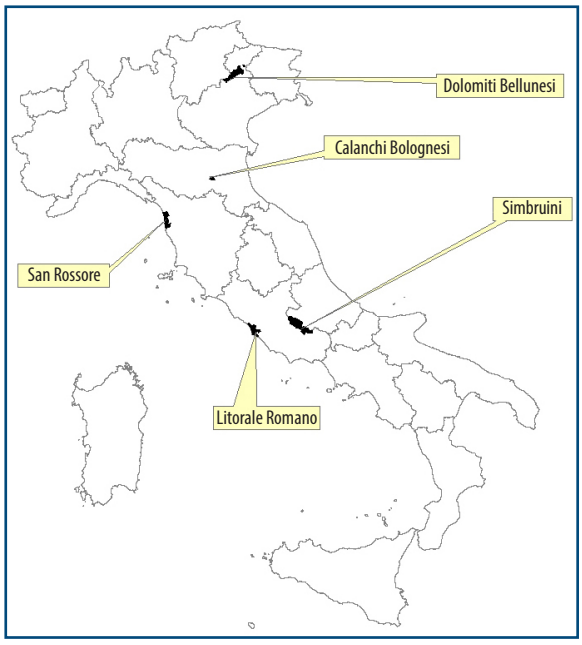

Figure 1. *Locations of the five natural protected areas included in the survey.*

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'Apiary B' (Dolomiti B, Calanchi B, San Rossore B, Litorale B, Simbruini B)and located close to potential anthropogenic pollutant sources with agricultural, industrial, and/or urban contaminants. Apiaries A and B were established in similar climatic conditions.

Data sheets were prepared to standardize data collection related to colony inspections (health status and strength), honey bee mortality, samplings, and the environment. Supplementary inspections and samplings of honey bees and pollen were performed in case of variations in beehive health status (e.g., colony depopulation, disease, death, higher mortality of adult honey bees) detected by the beekeepers.

Laboratory analyses were carried out in an accredited laboratory in conformity with UNI CEI EN ISO/IEC 17025 (International Organization for Standardization and the International Electrotechnical Commission international standards). To assess the health status and the strength of the colonies for each apiary, four *ad hoc* trained inspectors conducted quarterly clinical inspections. Moreover, bee mortality was assessed weekly.

Colony mortality was measured as 'cumulative mortality', 'winter mortality', and 'mortality rate'. A dead colony was assessed if no honey bees of the colony were found alive. 'Cumulative mortality' indicates the ratio of the number of dead colonies in each apiary found throughout the entire observational period (1 year) and the number of colonies (20) monitored in each apiary at the beginning of the project. 'Winter mortality' indicates the ratio of the number of dead colonies in each apiary found throughout the winter season (from 1 October to 1 April) and the number of colonies (20) monitored in each apiary at the beginning of the project (1 October). The 'mortality rate' (colony-month at risk) indicates the mortality rate calculated on a monthly basis during the entire follow-up period (Thrusfield 1995).

Under-basket cages were used as a supplementary tool to monitor bee mortality within each colony (Human *et al.* 2013). These cages were placed in front of each colony for a weekly count of the number of dead adult honey bees in each colony. Whenever the number of dead honey bees exceeded the threshold of 200 honey bees/week in the same colony (Porrini *et al.* 2003), an additional inspection of the colony was combined with samplings for pathogens and pollutants (PPPs and heavy metals) to find the cause of the increased mortality. To compare data related to cumulative mortality, survival curves in exposed and non-exposed colonies were drawn using the non-parametric method of Kaplan-Meier (Kaplan and Meier 1958). This model represents the survival function as the probability that a colony will survive over a given period. The present study used 1 week as a unit of time; this corresponds to the weekly beekeeper check of hive status. The differences between the survival curves of A and B colonies of the five apiaries were evaluated through a log-rank test (Thrusfield 1995). The association of both the cumulative and winter mortality indices with honey bee diseases and the weekly count of the under-basket mortality (> 200 bees/colony) was measured by the Spearman's rank correlation coefficient (r_s); $p < 0.05$ was selected as the level of significance. STATA 12.0 software was used for statistical analysis.

The honey bee diseases investigated were: Varroasis, seven main honey bee viruses (Acute Bee Paralysis Virus, ABPV; Black Queen Cell Virus, BQCV; Chronic Bee Paralysis Virus, CPBV; Deformed Wings

Table I. *Pathogens, contaminants, methods and matrices used to monitor the honey bee health of the apiaries located in the natural protected areas.*

Pathogen/ contaminant	Diagnostic methods	Matrix
Varroasis (*Varroa destructor*)	Visual identification of the parasite	Adult honey bees with symptoms of disease; honey bee brood of hives with symptoms of disease
Main honey bee viruses: DWV, ABPV, CPBV, BQCV, SBV, KBV, IAPV	Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR) (Singh *et al.* 2010)	Adult honey bees (10 adult honey bees)
American Foulbrood - AFB (*Paenibacillus larvae*)	Cultural method (OIE 2008)	Honey bee brood (3-5 affected larvae)
European Foulbrood - EFB (*Melissococcus plutonius*)	Cultural method (OIE 2008)	Honey bee brood with symptoms of the disease
Nosemosis (*N. apis* and *N. ceranae*)	Polymerase Chain Reaction (PCR) and microscopic examination (OIE 2008)	Adult honey bees (10 adult honey bees and 30 honey bees, respectively)
Ascosphaera apis	Cultural method	Honey bee brood (affected larvae)
Main Plant Protection Products (PPPs): Organochlorine, Organophosphorous, Pyrethroids, Neonicotinoids and Carbamates	High resolution gas chromatography separation analyses method for neonicotinoids	Adult honey bees (200 bees/hive)
	High resolution gas chromatography	Honey (500 g/apiary)
	High resolution gas chromatography (for neonicotinoids only)	Pollen (10 cc)
Heavy metals: Pb, Cd, Cr and Cu	Atomic absorption spectrophotometry	Honey (500 g/apiary)
Palynological analysis	Optical microscopy	Pollen (10 g/colony)

Virus, DWV; Israeli Acute Paralysis Virus, IAPV; Kashmir Bee Virus, KBV; Sac Brood Virus, SBV), American Foulbrood (*Paenibacillus larvae*), *Ascosphaera apis*, European Foulbrood (*Melissococcus plutonius*), and nosemosis (*Nosema apis* and *Nosema ceranae*). Table I provides the laboratory methods adopted for the above-mentioned analyses. To compare the frequency of each infectious and parasitic disease within exposed and non-exposed apiaries, a series of 2 × 2 tables for each disease and NPAs was developed and the Fisher's exact test was conducted. When appropriate, risk measure was expressed as a risk ratio (RR). When the colonies experienced depopulation, death, relatively high mortality of adult bees or honey bee pathologies, extra-inspections and extra-samplings were conducted to assess chemical and/or biological causes of the related problems.

To investigate the relationships between honey bee mortality and environment, data were collected related to a Naturality Index, including land use, wild vegetation, and crops. Farming techniques were also recorded. Around each apiary, a 1.5-km radius buffer area (honey bee flight area) was evaluated; maps of land use and vegetation coverage were produced using a scale of 1:10,000. Land use and vegetation polygons were delineated using both photo interpretation and field surveys. The identified polygons were referred to the European University Information Systems, Co-ordination of Information on the Environment Biotopes, and Natura 2000 (Council Directive 92/43/EEC) categories according to the European Environment Agency (EUNIS 2007). The current agricultural use (presence of vegetable crops, vineyards, and corn) was extrapolated in detail from these buffer maps. The number of vegetation categories and the surface area for each of them were calculated with the Naturality Index (expressed as natural + natural like/urban + agricultural surfaces), Shannon Diversity Index, and the Simpson Dominance Index. Naturality Index was associated with cumulative mortality through Pearson chi square test with Yates correction, furthermore a linear regression was used to analyze correlation between cumulative and agricultural land coverage.

Honey samples were collected monthly from each colony to analyze any heavy metal residues of Pb, Cd, Cr, and Cu with atomic absorption spectrophotometry (Table I). To test for differences in the average concentration of heavy metals in honey taken from the A and B apiaries, the Student's t-test was performed for each NPA and each individual metal. To investigate the residues of PPPs, adult bees and honey were sampled monthly from each apiary. Moreover, the same types of samples were collected from each colony in all cases of abnormally high colony mortality to monitor the exposure to PPPs and heavy metals. Table I provides the methods adopted for analyzing PPPs. Because neonicotinoid compounds are thermally unstable, high-resolution gas chromatography separation analysis was preferred instead of high-resolution liquid chromatography. Palynological analyses were used to identify pollen grains using optical microscopy (Model B-500tph, Optika Srl, Ponteranica, BG, Italy) on the beebread samples from dead or depopulated colonies, so as to relate the poisonings of honey bees by PPPs to the treated plants.

Table II. *Scoring criteria used to evaluate the management commitment and the success in adopting good beekeeping practices.* Scores range from 1 (weak) to 5 (excellent).

		Commitment		
		Excellent	**Sufficient**	**Weak**
Management	**Excellent**	5	4	2
	Sufficient	4	3	2
	Weak	2	2	1

To evaluate the apiary management, a score from 1 (weak) to 5 (excellent) was given to the beekeeper skills, considering both their commitment to beekeeping and their ability to adopt good beekeeping management practices. The grades were assigned according to an evaluation grid designed and approved by the working group (Table II). In four NPAs, the two apiaries were managed by different beekeepers according to their customary practices. For the San Rossore NPA only, the same beekeeper managed both apiaries. All the beekeepers had to follow the same measurement protocols. The relationship between the skill of beekeepers (beekeepers score) and the mortality indices (cumulative mortality, winter mortality) was measured by the Spearman's rank correlation coefficient. Then, linear regression was used to measure their reciprocal influence.

Results

A total of 826 clinical inspections of beehives and 733 laboratory analyses were conducted to check the health status of honey bees and their exposure to pollutants. Table III shows detailed data about the analytical activities conducted for the project. Table IV reports the results of hive mortality for the ten apiaries as cumulative mortality, mortality rate, and winter mortality.

The colonies of the B apiaries of Litorale and San Rossore Parks showed higher mortality compared with the corresponding A apiaries.

The log-rank test applied on Kaplan-Meier curves representing aggregated data of colony mortality rates according to apiary exposure to pollution

Table III. *Activities performed to verify the health status of honey bees and their exposure to pollutants.*

Activities	Numbers
Clinical inspections of the hives	826
Samples for honey bee viroses	117
Samples for nosemosis	108
Samples for American Foul Brood (AFB)	24
Samples for European Foul Brood (EFB)	1
Samples for *Ascosphaera apis*	3
Samples for neonicotinoids	109
Samples for other PPPs	123
Samples for heavy metals	96
Palynological analyses	27

(Figure 2) showed that the mortality rate detected in the exposed (B) apiaries was significant higher compared to the mortality rate detected in the non-exposed (A) apiaries ($p = 0.0002$). Colony survival on the 30th week (210 days) was 87% for non-exposed and 75% for exposed apiaries. Table V reports the excesses of adult honey bee weekly mortality (> 200 dead honey bee) found in the under-basket cages, for each individual hive. This parameter was not observed to be related to colony mortality (both cumulative and winter mortality) in our study.

The vegetation surveys conducted in the honey bee flight area provided a critical contribution to the study. Table VI shows the synthetic characteristics of land use, vegetation coverage, naturalness, and the diversity of the above-mentioned buffer zones. In all five NPAs, linear regression showed only a weak correlation ($r_2 = 0.0373$) between increased mortality of the colonies with an increase in agricultural land coverage (cultivation of carrots, forage crops, mixed crops, horticultural crops, alfalfa, corn, melons, olive plantations, potatoes, rape, savoy cabbage, sorghum, vineyards, and watermelon; Figure 3) index of the possible use of PPP.

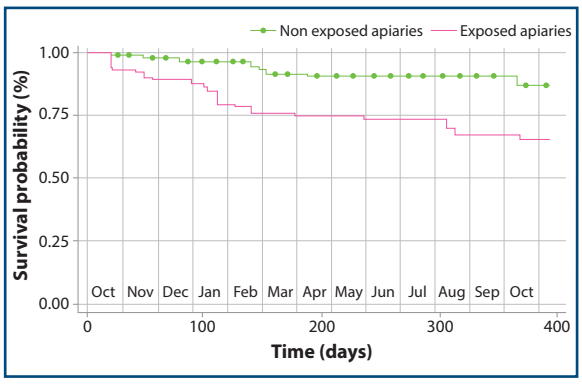

Figure 2. *Kaplan-Meier survival estimate curves for apiaries that were exposed and not exposed to high levels of pollutants.*

Table IV. *Mortality rates (cumulative and winter mortalities) observed in non exposed and exposed apiaries located in natural protected areas.*

Apiary	Dolomiti		Calanchi		Litorale		San Rossore		Simbruini	
	A	B	A	B	A	B	A	B	A	B
Cumulative mortality	15%	15%	0	0	20%	70%	25%	70%	5%	45%
Winter mortality	5%	15%	0	0	15%	45%	25%	70%	5%	45%

A = non exposed apiaries; B = exposed apiaries.

The comparison of the cumulative mortality between colonies placed in buffers at different Naturality Index values showed higher values for cumulative mortality in areas with a lower Naturality index (Figure 4). This difference resulted significant ($p = 0.047$)

Table VII shows the amounts of several heavy metals found in the honey samples; the statistical analysis showed no significant differences between exposed (area B) and non-exposed (A) apiaries. In addition, no residues of PPPs were found in the monthly honey samples with the exception of one case where the active principle imidacloprid was detected in a dead honey bee sample, at a concentration of 0.0096 mg/kg [equivalent to about ¼ of the Lethal Dose 50% (LD_{50}) for honey bees], in March 2010, in the non-exposed Apiary A of Calanchi.

Varroosis was detected at different levels in all the apiaries (Table VIII). The Spearman's rank correlation coefficient used to measure the relationship between heavy *Varroa* infestation and the mortality

Table V. *Weekly mortality threshold excess found in the under basket cages of the apiaries located in selected natural protected areas (NPA).*

NPA	Excesses of weekly mortality threshold (> 200 honey bees/ hive/week)	Month
Simbruini A	2	May (1st week)
Simbruini B	0	-
Litorale A	9	May (1st and 2nd week)
Litorale B	10	November (2nd week) December (4th week) August (1st week)
Dolomiti A	0	-
Dolomiti B	2	May (4th week) June (4th week)
Calanchi A	0	-
Calanchi B	1	October (1st week)
San Rossore A	1	May (3rd week)
San Rossore B	1	October (3rd week)

Table VI. *Characteristics of the honey bee flight areas used for statistical analyses.*

	Dolomiti		Calanchi		Litorale		San Rossore		Simbruini	
	A	B	A	B	A	B	A	B	A	B
N. Polygons	491	1,806	610	789	273	333	120	298	748	1,381
Average size of polygons (ha)	1.44	0.39	1.16	0.90	2.61	2.12	5.94	2.37	0.94	0.53
N. CORINE/EUNIS Categories	30	30	40	39	32	39	29	34	33	42
N. Natural categories	16	7	18	16	15	20	21	14	17	16
Shannon Diversity Index*	2.73	2.1	1.80	2.23	2.64	2.24	2.39	2.23	2.30	1.80
Simpson Dominance Index**	0.03	0.12	0.04	0.06	0.06	0.15	0.05	0.01	0.06	0.08
Rate naturalness (Natural+ semi-natural/Agricultural+Urban)	6.32	0.74	1.38	0.29	0.53	0.98	8.68	0.34	6.79	1.04
% Forests	73.93	17.54	20.90	13.89	6.24	18.24	37.51	10.27	56.27	45.16
% Meadows and pastures	8.73	21.41	19.92	7.12	36.53	26.23	4.75	11.91	25.19	4.18
% Built	5.08	23.86	2.53	13.52	6.83	11.56	1.19	9.67	5.79	19.08
% Agricultural	7.99	29.09	27.04	58.86	73.35	33.94	3.68	55.38	6.20	28.41
% Vegetable crops	1.87	5.94	2.52	18.22	0.60	7.03	0	0.05	1.87	5.94
% Vineyards		0.06	3.30	0.40						0.45
% Corn	1.8				2.12					

A = non-exposed apiaries; B = exposed apiaries; *Calculated on the percentage of coverage; **Calculated on the number of polygons.

indices (cumulative mortality, winter mortality) showed no correlation.

American Foulbrood (AFB) was detected in two colonies of the non-exposed Dolomiti Apiary A, in three colonies of the Litorale Apiary A, in four colonies of the Litorale Apiary B, and in nine colonies of the Simbruini Apiary B (Table VIII). The prevalence of AFB ranged from 0 to 45% between the apiaries, with an average of 5% in the A apiaries and of 13% in B apiaries. The Spearman's rank correlation coefficient calculated between the frequency of AFB and both cumulative and winter mortality showed no correlation between them. However, no case of European Foulbrood (EFB) was detected during the study.

Seven main honey bee viruses (ABPV, BQCV, CBPV, DWV, IAPV, KBV, and SBV) were investigated in 65 samples of honey bees (Table VIII). The frequency of the seven viruses did not significantly differ between A and B apiaries, with the exception of the following five cases: ABPV in Calanchi, CBPV in Dolomiti and Simbruini, KBV in Litorale, and SBV in Calanchi. However, the high variability of the RR did not allow any statement on a presumed major risk of viral diseases in the B apiary areas compared with the A ones. The Spearman's rank correlation coefficients (r_s) used to measure correlation among the prevalence of the seven main honey bee viruses listed above and the cumulative and winter mortality indices showed that the variables are unrelated for most of the cases with the exception of ABPV and KBV. Acute Bee Paralysis Virus was positively and significantly related with both cumulative and winter mortality (r_s = 0.6862 and 0.6790, p = 0.028 and 0.031, respectively). Kashmir

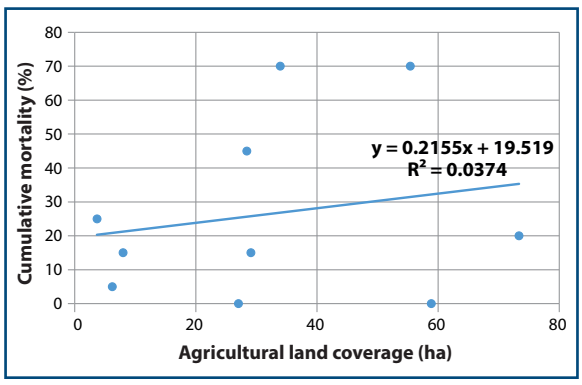

Figure 3. *Relationship between the cumulative beehive mortality and the agricultural land coverage.*

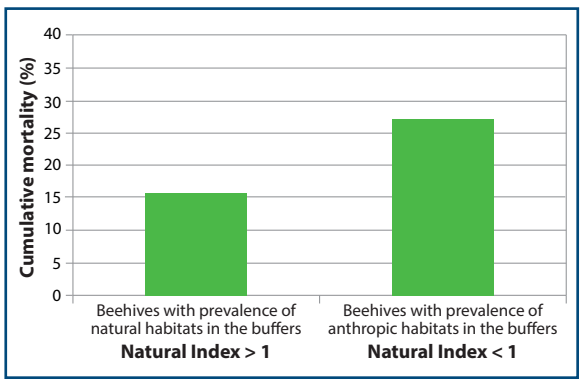

Figure 4. *Cumulative mortality in colonies placed in buffers at different Naturality Index values.*

Bee Virus was positively and significantly related with cumulative mortality ($r_s = 0.6351$, $p = 0.048$).

The diagnosis of Nosemosis was performed on 64 honey bee samples. While *N. ceranae* was present in the samples from both A (average of 78.1%) and B (average of 65.6%) apiaries of the five NPAs, *N. apis* was never found (Table VIII). Statistical analysis showed that the frequency of *N. ceranae* in the A and B apiaries was not significantly different. The Spearman's rank correlation coefficient used to measure correlation between the prevalence of *N. ceranae* and the cumulative and winter mortality indices showed no significant correlation between them.

Table IX provides the results of the beekeeping skill assessment. The worst score for beekeepers' management skill was given to Litorale B and Simbruini B (score 2) and the best was given to apiary A as well as apiary B within both the Dolomiti and Calanchi sites (score 5). The relationship between the beekeeper score and the winter and cumulative mortality indices, showed that the beekeepers' skill was significantly related to both indices. The Spearman's rank correlation coefficients showed a negative correlation between the beekeeper score and those two parameters, with values of - 0.7730 and - 0.7722, respectively ($p = 0.009$ in both cases). Two linear regressions showed that a one-point increase in the beekeeper score corresponded to a 14% and 16% decrease in winter (Figure 5) and cumulative mortality (Figure 6), respectively.

Discussion

The weekly counts of dead adult honey bees in the under-basket cages in our study were not related to

Table VII. *Average and standard deviation of heavy metal concentration detected (mg/kg) in each apiary of selected natural protected areas.*

Heavy metal	Dolomiti		Calanchi		Litorale		San Rossore		Simbruini	
	A	B	A	B	A	B	A	B	A	B
Pb	0.042±0.021	0.045±0.032	0.042±0.027	0.045±00.041	0.033±0.015	0.035±0.020	0.038±0.028	0.053±0.019	0.033±0.036	0.053±0.042
Cu	0.369±0.402	0.551±0.436	0.178±0.035	0.165±0.075	0.567±0.461	0.304±0.288	0.204±0.082	0.150±0.058	0.183±0.075	0.299±0.218
Cd	0.005±0	0.005±0	0.005±0	0.005±0	0.005±0	0.005±0	0.005±0	0.005±0	0.005±0	0.005±0
Cr	0.038±0.029	0.047±0.020	0.101±0.082	0.104±0.096	0.091±0.080	0.054±0.030	0.066±0.052	0.076±0.095	0.063±0.030	0.084±0.055

A = non-exposed apiaries; B = exposed apiaries.

Table VIII. *Pathogens detected in apiaries located in selected natural protected areas for the non-exposed and the exposed apiaries.*

	Dolomiti		Calanchi		Litorale		San Rossore		Simbruini		Mean	
	A	B	A	B	A	B	A	B	A	B	A	B
Varroa observations on adult honey bees	33%	33%	8%	13%	10%	5%	62%	47%	25%	20%	27.6%	23.6%
American Foul Brood	2/20 (10%)	0/20 (0%)	0/20 (0%)	0/20 (0%)	3/20 (15%)	4/20 (20%)	0/20 (0%)	0/20 (0%)	0/20 (0%)	9/20 (45%)	5/100 (5%)	13/100 (13%)
Acute Bee Paralysis Virus	0/7 (0%)	2/7 (28.6%)	4/7 (57.1%)	0/7 (0%)	7/7 (100%)	7/7 (100%)	3/5 (60%)	4/5 (80%)	2/6 (33.3%)	3/7 (42.8%)	16/32 (50%)	16/33 (48.5%)
Black Queen Cell Virus	0/7 (0%)	0/7 (0%)	1/7 (14.3%)	0/7 (0%)	5/7 (71.4%)	5/7 (71,4%)	3/5 (60%)	0/5 (0%)	0/6 (0%)	0/7 (0%)	9/32 (28.1%)	5/33 (15.2%)
Chronic Bee Paralysis Virus	6/7 (85.7%)	0/7 (0%)	5/7 (71.4%)	4/7 (57.1%)	7/7 (100%)	7/7 (100%)	2/5 (40%)	0/5 (0%)	1/6 (16.7%)	6/7 (85.7%)	21/32 (65.6%)	17/33 (51.5%)
Deformed Wings Virus	3/7 (42.8%)	0/7 (0%)	4/7 (57.1%)	5/7 (71.4%)	7/7 (100%)	6/7 (85.7%)	4/5 (80%)	4/5 (80%)	6/6 (100%)	6/7 (85.7%)	24/32 (75%)	21/33 (63.6%)
Israeli Acute Paralysis Virus	0/7 (0%)	0/7 (0%)	0/7 (0%)	0/7 (0%)	0/7 (0%)	0/7 (0%)	0/5 (0%)	0/5 (0%)	0/6 (0%)	0/7 (0%)	0/32 (0%)	0/33 (0%)
Kashmir Bee Virus	0/7 (0%)	0/7 (0%)	0/7 (0%)	0/7 (0%)	2*/7 (28.6%)	0/7 (0%)	0/5 (0%)	4*/5 (80%)	0/6 (0%)	2*/7 (28.6%)	2/32 (6.3%)	6/33 (18.2%)
Sac Brood Virus	5/7 (71.4%)	5/7 (71.4%)	6/7 (85.7%)	1/7 (14.3)	6/7 (85.7%)	6/7 (85.7%)	4/5 (80%)	2/5 (40%)	1/6 (16.7%)	1/7 (14.3%)	22/32 (68.7%)	15/33 (45.5%)
Nosema ceranae	7/7 (100%)	7/7 (100%)	6/7 (85.7%)	3/7 (42.9%)	7/7 (100%)	5/7 (71.4%)	1/5 (20%)	2/5 (40%)	4/6 (66.7%)	4/6 (66.7%)	25/32 (78.1%)	21/32 (65.6%)
Nosema apis	0/7 (0%)	0/7 (0%)	0/7 (0%)	0/7 (0%)	0/7 (0%)	0/7 (0%)	0/5 (0%)	0/5 (0%)	0/6 (0%)	0/6 (0%)	0/32 (0%)	0/32 (0%)

A = non-exposed apiaries; B = exposed apiaries.

Table IX. *Beekeepers' honey bee management skill score in the investigated apiaries.* Scores range from 1 (weak) to 5 (excellent).

NPA	Apiary	Beekeepers score
Dolomiti	A	5
	B	5
Calanchi	A	5
	B	5
San Rossore	A	3
	B	3
Litorale	A	4
	B	2
Simbruini	A	3
	B	2

A = non-exposed apiaries; B = exposed apiaries.

the colony mortality observed in the five NPAs; this was probably due to an absence of strong acute toxic effects caused by PPPs during the year of monitoring activity.

The Kaplan-Meier survival estimates (Figure 2) expressed mortality events more evident in exposed apiaries respect to non-exposed apiaries during October/November, January/February, August. In these months, hive mortalities are usually related to *V. destructor*. The Spearman's rank correlation coefficient used to measure the relationship between severe *V. destructor* infestations and the mortality indices showed no correlation between them. However, in acquiring this information, we should also consider that a more accurate method may be used to assess the level of varroa infestation; this would have to be applied to achieve a robust conclusion, avoiding the different interpretations of the four inspectors that evaluated the hives in the five NPAs. Indeed, the on-field evaluation methods to detect the level of varroa infestation in adult honey bees, such as the use of icing sugar (Lee *et al.* 2010) or detergent solutions (Rinderer *et al.* 2004), were still not in use at the time of our protocol definition.

While EFB was not found in the present study, AFB was found with a higher prevalence in apiary B (13%) respect to apiary A (5%) (Table VIII). The Spearman's rank correlation coefficient did not highlight any correlation between the mean AFB prevalence and the mortality indices. At Simbruini B apiary, AFB caused the highest mortality with nine colonies affected (45%); this was caused by the poor awareness of the beekeeper.

With regard to the honey bee viruses, ABPV was positively related to both winter and cumulative mortality, while KBV was positively related only to cumulative mortality. This could be explained by the prevalence of KBV, which appears to be higher in summer than in winter (Formato *et al.* 2012, Cersini

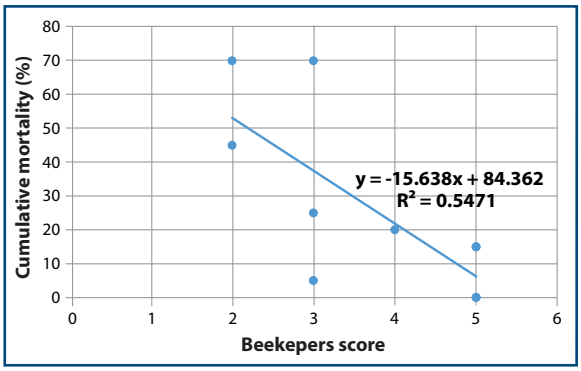

Figure 5. *Relationship between beekeeper management skill score and cumulative mortality (%).*

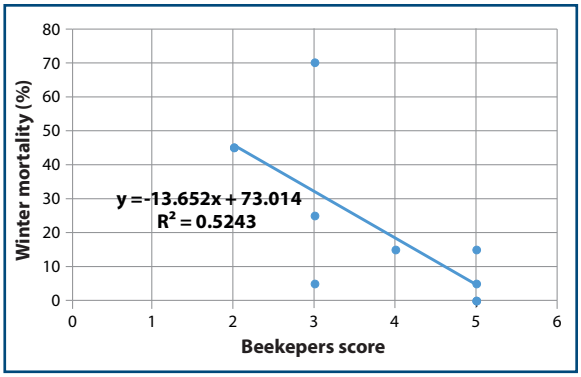

Figure 6. *Relationship between beekeeper management skill score and winter mortality (%).*

et al. 2013). A more accurate study is needed to substantiate the relationship between ABPV and KBV and colony mortality in combination with the varroa infestation level. The prevalence of *N. ceranae* did not significantly differ in A and B apiaries.

No PPP residues were found in this 1-year study, excluding one case in which, during spring 2010, a low level of imidacloprid (0.0096 mg/kg) was found in a dead honey bee sample of apiary B at Calanchi. The active amount of imidacloprid found corresponded to about ¼ of the LD_{50} for honey bees. However, in that apiary, bee mortality did not exceed the mortality threshold in the under-basket cages, and no abnormal mortality was found. It should be stressed that, in Italy, a rule[1] (OJ of Italian Republic n. 221 of 20 September 2008) established an immediate precautionary suspension of the use of PPPs for seed dressing (but not other formulations, e.g., spray applications) when those PPPs contain any of several active substances such as clothianidin,

[1] Decreto dirigenziale. 2008. Sospensione cautelativa dell'autorizzazione di impiego per la concia di sementi, dei prodotti fitosanitari contenenti le sostanze attive clothianidin, thiamethoxam, imidacloprid e fipronil, ai sensi dell'articolo 13, comma 1, del decreto del Presidente della Repubblica 23 aprile 2001, n. 290. *OJ*, **221** of the 20.09.2008.

fipronil, imidacloprid, and thiamethoxam. As a consequence, since 2009 during corn sowing season, no neonicotinoid-dressed seeds were allowed in Italy and only two honey bee mortality outbreaks related to neonicotinoid-dressed seeds were recorded in that year in Italy. For this reason, the ban was then extended until June 2013, and no further cases of this type of mortality have been reported. It could have somehow reduced the possibility of detecting neonicotinoid residues in the investigated matrices, despite the permitted use of neonicotinoids on fruit trees, vineyards, and other shrubs.

In NPAs, the vegetation coverage resulted related with the health status of colonies, even if it is not clear the influence of intensive agricultural techniques. However, it has to be considered that, within the NPAs, large areas of industrial or agricultural lands were not usually present. The failure to find a direct link between colony mortality and the crop coverage may be caused by the heterogeneity of the farming procedures used in the study area (e.g., the diversity of the treatments and cropping systems employed). Despite the presence of large areas of farmland, vegetable crops, and vineyards, the low colony mortality recorded in both types of apiaries of Calanchi NPA, should be attributed to the spreading of sustainable and organic agricultural production promoted by the park authorities (Naylor and Ehrlich 1997), as well as to the apiary management skills of highly professional beekeepers..

The colonies of the apiaries B in Litorale and San Rossore Parks, showed significantly higher mortality compared to the associated apiaries A. In both of these NPAs, some factors could have influenced this mortality trend, such as the presence of intensive horticultural crops (Litorale), an autumn weed control treatment with herbicides (San Rossore), and the presence of an airport (Litorale).

The heavy metal concentrations detected in the honey samples did not statistically differ between the A and B apiaries. In fact, heavy metals do not decompose and are easily transported at considerable distances by air currents, being spread out in an area independently from their natural, rural, urban, or industrial characteristics (Devillers *et al.* 2002).

Finally, the managerial skills of beekeepers were significantly related both to the winter and cumulative mortality, confirming that beekeeping management, like honey bee pathogens and environmental pollution, can contribute to threaten the health of honey bee colonies (Oldroyd 2007; vanEngelsdorp *et al.* 2008).

Conclusions

Our monitoring activity did not reveal a significant effect on colony mortality caused by either anthropogenic pollutants or honey bee pathogens that were observed in all the monitored apiaries in different proportions, even if there was a significant increase in risk in exposed areas (B apiaries) compared to unexposed ones (A apiaries). The results demonstrate that the application of the under-basket cage criterion used to evaluate colony mortality is not effective in apiaries that are not involved in acute toxic effects (e.g., acute toxic PPP effects). The relation between honey bee mortality and Naturality index is interestingly observed even in the context in which the present study was conducted, i.e. Natural Protected Areas. In the areas most heavily affected by the colony mortality (Litorale, San Rossore, and Simbruini), the poor colony management skills of the beekeepers played the most important role in colony losses.

This study confirms previous studies showing that colony collapse is the consequence of the interaction of multiple factors, both natural and anthropogenic, that affect honey bee health.

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