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Influence of Pesticide Residues on Honey Bee (Hymenoptera: Apidae) Colony Health in France

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ABSTRACT A 3-yr field survey was carried out in France, from 2002 to 2005, to study honey bee (*Apis mellifera* L.) colony health in relation to pesticide residues found in the colonies. This study was motivated by recent massive losses of honey bee colonies, and our objective was to examine the possible relationship between low levels of pesticide residues in apicultural matrices (honey, pollen collected by honey bees, beeswax) and colony health as measured by colony mortality and adult and brood population abundance. When all apicultural matrices were pooled together, the number of pesticide residue detected per sampling period (four sampling periods per year) and per apiary ranged from 0 to 9, with the most frequent being two (29.6%). No pesticide residues were detected during 12.7% of the sampling periods. Residues of imidacloprid and 6- chloronicotinic acid were the most frequently detected in pollen loads, honey, and honey bee matrices. Several pairs of active ingredients were present concurrently within honey bees and in pollen loads but not in beeswax and honey samples. No statistical relationship was found between colony mortality and pesticide residues. When pesticide residues from all matrices were pooled together, a mixed model analysis did not show a significant relationship between the presence of pesticide residues and the abundance of brood and adults, and no statistical relationship was found between colony mortality and pesticide residues. Thus, although certain pesticide residues were detected in apicultural matrices and occasionally with another pesticide residual, more work is needed to determine the role these residues play in affecting colony health.

KEY WORDS *Apis mellifera*, field survey, pesticides, colony health, France

Honey bees, *Apis mellifera* L., as domesticated pollinators are constantly exposed to pesticides, particularly if their colonies are located in agricultural areas. Exposure to pesticides may result in adverse health impacts such as acute and diffuse mortality or sublethal effects. The effectiveness of a bee colony for pollination or honey gathering depends on the coordination of a suite of worker behaviors in the allocation and collection of nectar and pollen. Exposure to chemicals that compromises the ability of workers to carry out these tasks could impact colony performance (Weick and Thorn 2002).

In 2007, 520 active ingredients from which pesticides could be developed were registered for agricultural use in France. These ingredients were used in ≈3,000 commercial products applied for plant protection. Annually, 80% of commercial pesticide products are used in a limited number of crops (e.g., cereals, corn, canola and vineyards) that correspond to 40% of the total agricultural and area (Ministère de l'Agriculture et de la Pêche 2007). These active ingredients can be found in air, soil, water, or sediments, depending on the conditions of application and the environment.

Our study was initially designed to investigate massive losses of honey bee colonies. From 2002–2005, we conducted a 3-yr field survey in France to study honey bee colony health in relation to bee pathogens and pesticide residues found in the colonies. It was shown that the majority of pollen load samples collected in these colonies between 2002 and 2003 contained detectable concentrations of several pesticides (Chauzat et al. 2006). We previously observed that bee mortality rates were within the expected range (i.e., no acute mortality, unpublished data). In this paper, we sought to examine the possible relationships between low levels of pesticide residues in apicultural matrices, such as honey, pollen collected by honey bees and beeswax, and colony health (adult and brood population levels). To our knowledge, this study is the first attempt to quantify the effects of pesticide residues on honey bee colony health under field conditions.

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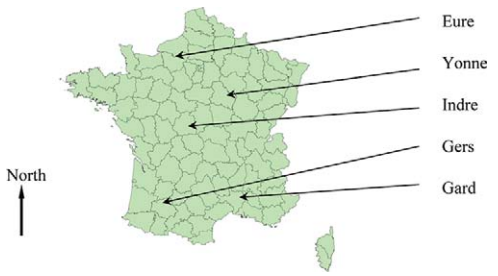

Fig. 1. Location of the surveyed sites in France (online figure in color).

Materials and Methods

General Protocol

The apiaries we studied were distributed among five sites in continental France covering the main zones of French honey production (Fig. 1). The main types of honey were sunflower, canola, chestnut, and local mixed flower honey. Five apiaries in each site were chosen according to the beekeepers' willingness to participate in the experiment. The apiarists were of different status (professionals and hobbyists), possessing 15–1,500 hives. The French beekeeping industry was audited in 2004 (GEM-ONIFLHOR 2005). The estimated total number of hives in continental France was 1.3 million distributed among ≈69,000 beekeepers. Beekeepers possessing at least 150 hives were categorized as professional apiarists. They represented 2.6% of the total number of beekeepers and owned 45% of all French hives. Hobbyist beekeepers possessing <10 hives represented 78.1% of the beekeepers and owned 22% of all French hives. In this study, the apiarists were asked to continue their standard operating procedures with the exception of migratory beekeeping, which was not feasible for the colonies under surveillance. At the beginning of the study, 125 colonies (five honey bee colonies randomly selected in five apiaries from five different locations across France) were monitored for mortality, population level, health status, management system, and pesticide residues over 3 yr (2002–2005). The number of surveyed hives was kept constant by replacing any dead colony by another one randomly selected from the same apiary. Colonies were visited by the same personnel four times per year: at the end of winter (March–April, visit A), before summer (May–June, visit B), during summer (July–August, visit C), and before winter (October–November, visit D). The general activity at each apiary (i.e., mortality in front of hives and flight activity) was recorded before opening the surveyed colonies. The hive entrance was only lightly smoked to ensure minimal disturbance of the clusters. A full clinical examination (i.e., population evaluation and symptoms of diseases) of each surveyed colony was conducted at each visit. Populations were evaluated by the number of interframes occupied by adult bees (including the interframes of the supers if there were any) and by the number of frames occupied by open or capped brood (Faucon et al. 2005). Adult bees were sampled at all visits, honey at visits B, C, and D, and beeswax only at visit D. Adult bees were collected directly from a frame by shaking individuals into a paper envelope. Border frames were chosen in preference to collect the foraging worker class. Pollen loads were sampled from traps and not from bee bread. Pollen traps were fixed on two extra colonies per apiary, and samples were taken at all visits when available. Individual colony samples, taken during the same visit, were pooled per apiary for analyses.

Chemical Analyses

Analyses of residues were performed in the GIRPA laboratory (Groupement Interrégional sur les Recherches des Produits Agropharmaceutiques, Angers, France) except for multiresidue analyses (organophosphorus [OP], organochlorine [OC], and pyrethroid [PYR] insecticides) in pollen and insecticides (OP, OC, and PYR) in honey bees that were processed in the AFSSA laboratory. Limits of detection and quantification were calculated following the Guidance document on residue analytical methods (European Commission 2007). Forty-one different molecules were sought through individual (imidacloprid and fipronil) or multiresidue analyses. The metabolites (i.e., 6-chloronicotinic acid for imidacloprid and sulfone and desulfynil for fipronil) were also analyzed. The pesticides were chosen for their high toxicity toward honey bees or because of their frequent use in agriculture. Of the 41 active ingredients analyzed, 30 were common to insecticides and 11 to fungicides (Table 1). The use of these pesticides was legally authorized on crops in 2003 with the exception of lindane, and coumaphos was only used to treat colonies against *Varroa destructor* Anderson and Trueman (Acari: Mesostigmata).

Insecticides and Fungicides in Honey. Insecticidal and fungicidal residues were processed from 5 g of the honey samples that were homogenized with water and cleaned on Chem-Elut cartridges. After complete evaporation of the eluates, the extract was recovered with ethyl acetate for gas chromatography coupled with tandem mass chromatography (GC/MS/MS) analysis (Table 2). The same extract was analyzed by liquid chromatography coupled with tandem mass chromatography (LC/MS/MS) for other pesticide residues (e.g., carbamates and triazoles). In 2003, the limits of detection (LOD) and quantification (LOQ) were 2 and 10 μg/kg, respectively, for all analytes except for tebuconazole, parathion-methyl, hexaconazole, deltamethrin, and azinphos-methyl, for which the LOQ were 20 μg/kg. In 2004 and 2005, the LOD and LOQ were 5 and 10 μg/kg for all analytes except for tebuconazole, parathion-methyl, hexaconazole, deltamethrin, and azinphos-methyl in 2005, for which the LOQ were 20 μg/kg.

Specific analyses were used to detect imidacloprid and 6-chloronicotinic acid. Samples of honey (20 g) were extracted with a mixture of methanol and water containing dilute sulfuric acid. After homogenization and filtration, an aliquot was concentrated to the aque-

Table 1. List of the studied insecticides, acaricides, and fungicides

Insecticides					Fungicides	
Pyrethrinoids	Organochlorines	Organophosphorus	Carbamates	Others	Triazoles	Dicarboximides
Cyfluthrin	Endosulfan	Azinphos-methyl	Aldicarbe	Imidacloprid (*Neonicotinoid*)	Cyproconazole	Procymidone
Cypermethrin	Lindane	Chlorpyrifos	Aldicarbe sulfoxyde	Fipronil (*Phenylpyrazole*)	Epoxyconazole	Vinclozolin
Deltamethrin		Coumaphos	Aldicarbe sulfone		Fluzilazole	
Lambda-cyhalothrin		Dimethoate	Mercaptodimethur		Hexaconazole	
Tau-fluvalinate		Fenitrothion	Mercaptodimethur sulfone		Myclobutanil	
		Fenthion	Mercaptodimethur sulfoxyde		Penconazole	
		Malathion	Carbaryl		Propiconazole	
		Methidathion	Carbofuran		Tebuconazole	
		Mevinphos	Methomyl		Tetraconazole	
		Parathion	Oxamyl			
		Parathion-methyl				

ous residue. This extract was subsequently diluted, washed with *n*-hexane, and cleaned with an Amberlite XAD-4 cartridge. The resulting extract was divided into two equal portions: one for imidacloprid residue determination and the other for total residues (i.e., 6-chloronicotic acid) determination. For imidacloprid residue analysis, the first portion was concentrated, dissolved in water, and cleaned with dichloromethane on a Chem-Elut column. Quantification was by LC/MS/MS method (LOD = 0.3 μg/kg; LOQ = 1 μg/kg). The second portion was concentrated and dissolved in water to oxidize imidacloprid and all its metabolites into 6-chloronicotinic acid. The solution was acidified, and residues of 6-chloronicotic acid were extracted with tert-butyl-methyl ether. The ether phase was dried. Residues were dissolved with an acidified mixture of methanol and water and cleaned with an HLB cartridge. Quantification was by LC/MS/MS (LOD = 0.3 μg/kg; LOQ = 0.6 μg/kg).

Specific analyses were also conducted to detect fipronil and its sulfone and desulfinyl metabolites. Samples of honey (5 g) were extracted with a mixture of water and methanol. After evaporation, the extract was cleaned on an immunologic cartridge (Fiproprep). The eluate was evaporated and dissolved in a mixture of water and methanol for analysis. Fipronil and its two metabolites were quantified by LC/MS/MS. During the study, the LOD decreased from 0.3 to 0.2 μg/kg and the LOQ from 2.0 to 0.5 μg/kg for each compound.

Insecticides and Fungicides in Pollen. Specific analyses were used to search for imidacloprid and 6-chloronicotinic acid. The extraction procedure for pollen samples (20 g) was similar to that used for honey. Quantification was by LC/MS/MS. The LOD for each compound ranged from 0.2 to 0.3 μg/kg during the study. The LOQ for imidacloprid and 6-chloronicotinic acid was 1 and 0.6 μg/kg, respectively.

Fipronil and its two metabolites were also sought through specific analyses. Samples of pollen (1 g) were extracted with acetone. After homogenization and filtration, the extract was allowed to evaporate completely, and the dried residue was dissolved in methanol. This extract was subsequently cleaned on an alumine cartridge, and an immunologic cartridge (Fiproprep) with the same procedure used for honey. Fipronil and its two metabolites were quantified by LC/MS/MS, and the LOD for fipronil and its metabolites was 0.3 μg/kg. During the study, the LOQ decreased from 2.0 to 0.5 μg/kg.

Two multiresidue analyses were conducted: one for carbamates and fungicides and the other for OP, OC, and PYR insecticides. Samples of pollen (2 g) for carbamates and fungicides analysis were extracted with ethyl acetate. After homogenization and filtration, the extract was divided into two equal volumes and evaporated. One portion of the dried residue was dissolved in methanol and water and the other in methanol and water with sodium acetate.

Table 2. List of analytical methods performed for each matrix (honey, pollen loads, honey bees, and beeswax) and pesticide chemical family

Matrix	Analytical method	Family of pesticides
Honey	LC/MS/MS	Neonicotinoids and phenylpyrazoles
		Carbamates, triazoles and iprodione
	GC/MS/MS	Pyrethroids, organochlorines, organophosphorus, procymidone and vinclozolin
Pollen loads	LC/MS/MS	Neonicotinoids and phenylpyrazoles
		Carbamates and triazoles
	GC/ECD et GC/NPD	Pyrethroids, organochlorines and organophosphorus
Honey bees	LC/MS/MS	Neonicotinoids and phenylpyrazoles
		Carbamates and triazoles
	GC/ECD et GC/NPD	Pyrethroids, organochlorines and organophosphorus
Beeswax	GC/MS/MS	Pyrethroids, organochlorines, organophosphorus, procymidone and vinclozolin

Table 3. LOD and LOQ for organophosphorus, organochlorine, and pyrethroid insecticides

Analyte	Honey bees				Pollen loads	
	LOD (μg/kg)	LOD (ng/bee)	LOQ (μg/kg)	LOQ (ng/bee)	LOD (μg/kg)	LOQ (μg/kg)
Azinphos-methyl	57.0	5.7	196.1	19.6	57.0	196.7
Chlorpyrifos	10.0	1.0	34.5	3.5	10.0	34.5
Coumaphos	37.0	3.7	142.8	14.3	37.0	142.6
Cyfluthrin	7.0	0.7	39.5	4.0	7.0	98.7
Cypermethrin	3.8	0.4	32.7	3.3	3.8	93.3
Deltamethrin	0.1	0.01	24.9	2.5	0.1	29.9
Dimethoate	18.0	1.8	59.6	6.0	18.0	59.6
Endosulfan	0.1	0.01	8.0	0.8	0.1	8.0
Fenitrothion	19.0	1.9	66.9	6.7	19.0	66.9
Fenthion	8.0	0.8	30.6	3.1	8.0	30.6
λ-cyhalothrin	0.4	0.04	12.9	1.3	-	-
Lindane	0.1	0.01	1.5	0.2	0.1	4.0
Malathion	9.0	0.9	31.5	3.2	9.0	31.5
Methidathion	13.0	1.3	49.6	5.0	13.0	49.6
Mevinphos	3.8	0.4	18.5	1.9	3.8	27.7
Parathion-ethyl	8.0	0.8	30.4	3.0	8.0	30.4
Parathion-methyl	10.0	1.0	39.5	4.0	10.0	39.5
Tau-fluvalinate	1.1	0.1	11.4	1.1	1.1	76.0

Quantification was by LC/MS/MS. The LOD and LOQ were 5 and 10 μg/kg, respectively, for all analytes, except in 2003, when the LOD and LOQ for hexaconazole and tebuconazole were 10 and 20 μg/kg, respectively. Samples of pollen (10 g) were extracted for the second multiresidue analysis (OP, OC, and PYR) using acetone extraction and liquid partitioning with dichloromethane. One clean-up was performed on a silica gel column for pesticides analysis. The two eluates were concentrated by complete evaporation under reduced pressure in a rotary evaporator using a 40°C water bath. Residues were dissolved in iso-octane for GC analysis. Multiresidue analysis was performed by GC using an electron-capture detector for organochlorine and synthetic pyrethroid pesticides and a nitrogen-phosphorus detector (NPD) for organophosphorus pesticides. The LOD for the different analytes ranged from 0.1 to 57 μg/kg and the LOQ from 4 to 196.7 μg/kg (Table 3).

Insecticides in Beeswax. Beeswax samples (2 g) were extracted with *n*-hexane in an ultrasonic bath heated at 40°C. After freezing in liquid nitrogen and centrifugation, the supernatant fraction was collected and evaporated in a rotary evaporator (40°C) until ≈6 ml remained. Two liquid/liquid separations were performed with a mixture of *n*-hexane and acetonitrile. The acetonitrile phases were pooled together and concentrated on a rotary evaporator. The extract (2 ml) was cleaned on C18 cartridge. After elution with a mixture of acetonitrile and water, 20 ml of acetonitrile were added to the eluate. The solution was dried in a rotary evaporator and dissolved in 1 ml ethyl acetate for GC/MS/MS analysis. The LOD was 5 μg/kg for all molecules, and the LOQ was 20 μg/μg for parathion-methyl and deltamethrin and 10 μg/kg for all the other molecules. During the study, the LOQ of parathion-methyl and deltamethrin decreased from 20 to 10 μg/kg.

Insecticides and Fungicides in Honey Bees. Samples for carbamates and fungicides analysis were extracted with the same procedure as for pollen. The LOD and LOQ were 5 and 10 μg/kg, respectively, for all analytes except hexaconazole and tebuconazole in 2003 for which LOD and LOQ were 10 and 20 μg/kg, respectively. In 2004 and 2005, the LOD and LOQ for hexaconazole decreased to 5 and 10 μg/kg, respectively, whereas those of tebuconazole remained at 10 and 20 μg/kg, respectively. The LOD of carbofuran, mercaptodimethur, and mercaptodimethur sulfoxide decreased from 6.3 to 5 μg/kg during the study. Samples of honey bees were extracted for the second multiresidue analysis (OP, OC, and PYR) using acetone extraction and liquid partitioning with dichloromethane. Clean-up with florisil cartridge was performed for pesticides analysis. The two fractions obtained were concentrated by complete evaporation under reduced pressure in a rotary evaporator using a 40°C water bath. Residues were dissolved in iso-octane for the first eluate and in acetone for the second eluate. The two eluates were analyzed by GC analysis with specific detectors (ECD and NPD). The LOD for the different analytes sought through multiresidue analysis ranged from 0.01 to 5.7 ng/bee. The LOQ ranged from 0.2 to 19.6 ng/bee (Table 3).

Specific analyses to detect imidacloprid and 6-chloronicotinic acid were conducted in 2004 and 2005. The extraction procedure for honey bee samples (20 g) was similar to that used for honey and pollen. Quantification was by LC/MS/MS and the LOD was 0.3 μg/kg for both imidacloprid and 6-chloronicotinic acid, whereas the LOQ was 1 and 0.6 μg/kg, respectively. Specific analyses to detect fipronil and its two metabolites were also conducted in 2004 and 2005. Samples of honey bees (1 g) were extracted with the same procedure as for pollen samples. Fipronil and its two metabolites were quantified by LC/MS/MS. The LOD for both compounds was 0.3 and 0.2 μg/kg in 2004 and 2005, respectively, and the LOQ was 0.5 μg/kg for both years.

Description of the Data Set

Two epidemiological levels were considered: the apiary ($N = 24$) and the colony $N = 120$. One of the original 25 apiaries selected was removed because the apiarist left the program during the study. We measured adult and brood populations including mortality. A case of mortality was recorded when (1) colony mortality was registered by the laboratory team or the beekeeper and (2) a beekeeper refilled a hive with a new population, between sampling periods, after finding the hive empty.

Winter mortality was the percentage of colonies found dead after winter (visit A) compared with the total number of colonies alive before winter (visit D). Seasonal mortality was the percentage of colonies that died during the honey production season (visits B, C, and D) out of the total number of those alive at the beginning of the year plus the number of colonies that had replaced dead ones recorded during visit A.

Only pesticide residues statistically relevant to colony health were given further consideration, and 11 of the 45 initially investigated compounds were retained in the final analysis. New variables were created for fipronil and imidacloprid by pooling parent compounds and metabolites together. A new variable was created for fungicides by pooling results for penconazole, procymidone, propiconazole, tebuconazole, tetraconazole, and vinclozolin residues.

Statistical Analysis

We described the frequency distribution of the number of times pesticide residues were detected. We also studied the concurrent presence of pesticide residues in apicultural matrices and the seasonal and geographic influences on concurrent presence. The presence of pesticides in one apicultural matrix (e.g., in bees) was also analyzed to determine their presence in another matrix (e.g., in honey). Significance of associations was assessed using χ^2. We also tested the association between pesticide residues and the occurrence of mortality using χ^2 and used odds ratios as a measure for comparing whether the probability of a certain event was the same for two groups.

We used linear mixed models to analyze the relationship between pesticide residues and the abundance of adult and brood populations (Laird and Ware 1982). An apiary random term was incorporated into the model in accordance with the nested design of the study. Season (i.e., the timing of the sampling period) was included as a fixed effect to account for bee seasonal dynamics. A first order, autoregressive within-the-group correlation structure was assumed. Unequal variances among seasons were also accounted for in the model. Each explanatory variable (i.e., the presence of a pesticide residue) was first added to the model. The variables were checked for interaction with the season. The variables that were significantly linked with model outcomes were introduced into a multivariate model, fitted with backward-selection stepwise regression.

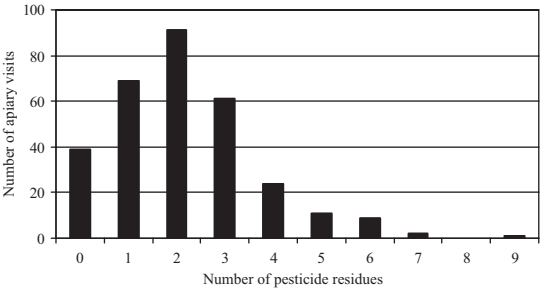

Fig. 2. Frequency distribution of the number of pesticide residues detected within a given apiary. All apicultural matrices were pooled together. Frequencies were calculated from samples collected during the 307 total clinical visits to the apiaries over 3 yr.

Conditional logistic models dependent on the apiary were used to explain mortality associated with the presence of pesticide residues. As with the linear mixed models, an initial screening step was performed using univariate models before developing multivariate models. All statistical tests were performed using the statistical package R, version 2.5.0 (R Development Core Team 2007). Linear mixed models were fitted using the nlme package (Pinheiro and Bates 2000). Conditional logistic regressions were fitted using the survival package.

Results

Pesticide Residues in Apicultural Matrices

An apiary was considered positive if residues of at least one active ingredient were detected in at least one of the matrices. When all the matrices were pooled together, the number of residues across sampling periods and apiaries ranged from zero to nine, whereas the mode was two (29.6%; Fig. 2). No pesticide residues were detected at 12.7% of the sampling periods. Nine different pesticide residues were found at only one sampling period and at only one apiary, although it should be noted that eight residues from this sample were found in beeswax.

The most frequent residue in pollen loads, honey, and honey bee matrices was imidacloprid or 6-chloronicotinic acid (Table 4). Mean concentrations of imidacloprid residue, from those positive samples, were 1.2 μg/kg in honey bees, 0.9 μg/kg in pollen, and 0.7 μg/kg in honey (Table 5). The concentration obtained for imidacloprid and 6-chloronicotinic acid in pollen loads was above the LOD in 40 (75/185) and 33% (61/185) of the samples, respectively. When both were found together, the concentrations were above the LOD in 16% (30/185) of the samples. The maximum residue concentration of imidacloprid and 6-chloronicotinic acid in pollen samples was 5.7 and 9.3 μg/kg, respectively. The second and third highest frequency of occurrence in pollen loads was fungicide and carbaryl residues with 16.0 and 13.5%, respectively (Table 4). Mean concentrations of carbaryl were 142.4, 30.8, and 214.3 μg/kg in pollen loads, honey, and

Table 4. Pesticide residues in samples collected over the 3-yr survey

Matrices	Pollen loads		Honey		Honey bees		Beeswax	
	No. of analyzed samples	Proportion of positive samples (%)	No. of analyzed samples	Proportion of positive samples (%)	No. of analyzed samples	Proportion of positive samples (%)	No. of analyzed samples	Proportion of positive samples (%)
Azinphos methyl	198	0.0	229	4.4	307	0.0	54	5.6
Carbaryl	126	13.5	227	0.9	214	0.5	0	NA
Carbofuran	181	4.4	239	1.3	293	0.7	0	NA
Coumaphos	198	5.1	236	8.5	307	4.6	92	46.7
Deltamethrin	198	0.5	239	0.8	307	5.9	87	1.1
Endosulfan	198	7.6	239	0.0	307	5.5	93	12.9
Fipronils	185	12.4	239	1.7	187	9.1	0	NA
Fungicides	181	16.0	140	0.7	305	9.2	93	1.1
Imidacloprids	185	57.3	239	29.7	187	26.2	0	NA
Lindane	198	1.5	239	0.8	307	2.3	87	2.3
Tau-Fluvalinate	198	3.5	226	0.9	307	4.6	67	52.2

A sample was positive when a residue was detected above the LOD. Fipronils included fipronil, fipronil sulfon, and fipronil desulfinyl. Imidaclprids included imidacloprid and 6-chloronicotinic acid. Fungicides included penconazole, procymidone, propiconazole, tebuconazole, tetraconazole, and vinclozolin.

NA, not applicable.

honey bees, respectively (Table 5). Tau-fluvalinate and coumaphos were the two most frequently found residues in beeswax samples (Table 4), with mean concentrations of 220.0 and 647.5 μg/kg, respectively. Pesticide residues were rarely found in honey samples with the exception of coumaphos and the imidacloprid group, which were found in 8.5 and 29.7%, respectively, of honey samples (Table 4).

Fipronil, fipronil sulfone, and fipronil desulfinyl residues were all detected as single substances in samples. Residues of fipronil were higher than the LOD in 11 of the samples, fipronil sulfone in 12 of the samples, and fipronil desulfinyl in 6 of the pollen load samples. The mean concentrations of fipronil residues was 0.5 in honey bees and 1.2 μg/kg in pollen loads. Fipronil was not detected in honey (Table 5). The maximum values of fipronil sulfone and fipronil desulfinyl residues detected were 3.6 and 1.5 μg/kg, respectively.

Table 5. Mean concentrations of pesticide residues (μg/kg) in positive samples collected from 2002 to 2005 in 120 French hives maintained under field conditions

Matrices	Pollen	Honey	Honey bees	Beeswax
6-chloronicotinic acid	1.2	1.2	1.0	NA
Azinphos-methyl	<LOD	21.8	<LOD	228.2
Carbaryl	142.4	30.8	214.3	NA
Carbofuran	32.7	16.1	13.0	NA
Coumaphos	423.5	38.0	1545.6	647.5
Deltamethrin	39.0	2.6	16.9	14.7
Endosulfan	45.8	<LOD	8.3	51.0
Fipronil	1.2	<LOD	0.5	NA
Fipronil desulfinyl	1.0	<LOD	1.2	NA
Fipronil sulfone	1.7	<LOD	0.4	NA
Imidacloprid	0.9	0.7	1.2	NA
Lindane	7.0	8.5	10.5	18.8
Penconazole	17.6	<LOD	7.5	NA
Procymidone	NA	<LOD	NA	27.7
Propiconazole	<LOD	<LOD	<LOD	NA
Tau-Fluvalinate	334.1	44.7	65.5	220.0
Tebuconazole	16.5	<LOD	18.2	NA
Tetraconazole	<LOD	<LOD	17.3	NA
Vinclozoline	NA	109.4	NA	21.5

NA, not applicable.

Concurrent Detection of Residues in Apicultural Matrices

Several pairs of active ingredients were significantly and concurrently detected in honey bees (nine pairs of ingredients) and pollen loads (three pairs of ingredients) but not in beeswax and honey samples (Table 6). Residues of imidacloprid and its metabolite were involved in five of these significant pairings: three with fungicides (epoxyconazole and tebuconazole) and one with each of the following compounds: tau-fluvalinate, deltamethrin, and fipronils (active substance and metabolites).

The profiles of the matrices for the detected residues of a given pesticide were also calculated (Table 7). Residues of fipronils in pollen were statistically linked to their presence in honey bee ($\chi^2 = 5.6$; df = 1; $P = 0.02$). When residues of fipronils were detected in pollen loads, there were seven times as likely to be also detected in honey bees (Table 7). There was a trend for honey bees and pollen loads, and honey and pollen, to also be related to the presence of imidacloprids (active substance and/or 6-chloronicotinic acid)

Table 6. Statistical testing of the concurrent detection of residues in pollen loads and honey bees

Matrices	Active substances		n	P value of the χ^2 test
Honey bee	Epoxyconazole	Imidacloprid	186	0.007
	Epoxyconazole	Tebuconazole	303	0.000
	Tebuconazole	Fluzilazole	277	0.006
	Imidacloprid	Tebuconazole	187	0.039
	Imidacloprid	Tau-fluvalinate	186	0.017
	Lindane	Tau-fluvalinate	307	0.000
	Myclobutanil	Fluvalinate	302	0.001
	Deltamethrin	6-chloronicotinic acid	186	0.047
	Deltamethrin	Coumaphos	307	0.000
Pollen	Carbaryl	Tebuconazole	126	0.002
	Coumaphos	Endosulfan	198	0.006
	Fipronils	Imidacloprid	185	0.034

Fipronils included fipronil, fipronil sulfone, and fipronil desulfinyl.

Table 7. Statistical testing of the concurrent detection of residues in pollen loads and honey bees and in pollen loads and honey

Active substances	Matrices		*n*	*P* value	OR	CI_{95}
Imidacloprids	Pollen loads	Honey bees	103	0.06	2.26	0.97–5.37
Imidacloprids	Pollen loads	Honey	127	0.11	1.86	0.86–4.06
Fipronils	Pollen loads	Honey bees	103	0.02	7.00	1.12–47.30

Fipronils included fipronil, fipronil sulfone, and fipronil desulfinyl. Imidacloprids included imidacloprid and 6-chloronicotinic acid.
OR, odds ratio; CI, confidence interval.

residues. In this case, *P* values were, respectively, 0.06 and 0.11.

Effect of Pesticide Residues on Bee Populations

There was no significant effect of individual pesticide residues on the abundance of adults or broods whether different apicultural matrices were considered separately or whether data were pooled across all matrices. Also, none of the mixed model analyses showed any significant relationship between the presence of pesticide residues and population abundance.

Winter colony mortality was 7.3% in 2003, 9.2% in 2004, and 5.8% in 2005. Seasonal mortality was 6.7% in 2003, 10.8% in 2004, and 7.6% in 2005. After the first univariate screening step, only endosulfan and coumaphos residues were found to have a potential link to mortality and thus each was selected for further analysis. The residues of both pesticides were found mostly in samples collected during the autumn (e.g., 53 of 78 observation for coumaphos), and colony losses were also observed mostly after the autumn sampling period (27 of 56 observations). Fifteen of the 20 observations of colony mortality specifically associated with the presence of coumaphos residues were obtained after the last visit of the year. Seven cases of winter colony mortality, of a total of 10 cases, were recorded at the same time as when endosulfan residue was detected in at least one apicultural matrix. When including a binary variable into the models to account for the seasonal (i.e., winter) effect on mortality, results indicated a significant seasonal effect but no significant effect of coumaphos and endosulfan. Mortality was three times more likely to occur in winter than in any other season (Table 8). Thus, in this study, no statistical relationships were found among honey bee populations, colony mortality, and the presence of pesticide residues.

Table 8. Conditional logistic regression models of mortality caused by the presence of coumaphos and endosulfan and when including the main and interacting effect of winter mortality

Variables	OR	*P* value	CI_{95}
Coumaphos			
Winter (yes/no)	3.1	0.08	0.9–10.5
Presence of coumaphos (yes/no)	2.0	0.36	0.45–8.9
Interaction winter × coumaphos	0.8	0.86	0.1–6.4
Endosulfan			
Winter (yes/no)	3.1	0.02	1.2–8.4
Presence of endosulfan (yes/no)	1.9	0.42	0.4–9.3
Interaction winter × endosulfan	1.3	0.84	0.1–11.5

Likelihood-ratio test: $P < 0.05$ for both models.
OR, odds ratio.

Discussion

This 3-yr field survey to study colony health under natural conditions involved 120 colonies randomly selected in 24 apiaries. We conducted four full clinical visits each year to evaluate colony health, mortality, and the presence of pesticide residues. Although we detected pesticide residues, and often in the presence of another residue, their presence was not statistically associated with bee population decline or colony mortality.

Imidacloprid and 6-chloronicotinic acid residues were the most frequently detected molecules in honey bees, honey, and pollen loads, although at low mean concentrations (Table 5). Imidacloprid has a half-life of 4.5 h in a honey bee, resulting in two main metabolites: 6-chloronicotinic acid and the urea derivative (Suchail et al. 2004b). These substances might be considered as late metabolites, appearing mainly in the midgut and rectum (Suchail et al. 2004a). Metabolic pathways for other neonicotinoid insecticides, such as acetamiprid, also metabolize into 6-chloronicotinic acid (Brunet et al. 2005). This suggests that 6-chloronicotinic acid residues might not only be derived from exposure to imidacloprid but also from exposure to other neonicotinoid insecticides such as acetamiprid. The toxicity of imidacloprid and its metabolites to honey bees has been previously discussed and debated (Faucon et al. 2005, Maxim and van der Sluijs 2007).

Synergism between neonicotinoid insecticides and pathogens or other active substances has been studied (Iwasa et al. 2004). The 14α-demethylase inhibitor-(DMI-) fungicides, which include epoxyconazole and propiconazole, are an important group of fungicides widely used in crop protection. In laboratory tests, the toxicity of acetamiprid or thiacloprid to bees generally increased when applied after a pretreatment with DMI-fungicide (synergistic ratio of 559 when propiconazole was added). In contrast, the synergistic ratio for imidacloprid was much lower (1.52) under the same conditions. However, toxicity of some of these associations was much lower when tested under semi-field conditions (Iwasa et al. 2004). Mortality of the German cockroach, *Blattela germanica* L. (Blattodea: Blattellidae), when fed on imidacloprid bait increased significantly when previously inoculated with suspensions of spores of the entomopathogenic fungus *Metar-*

hizium anisopliae (Kaakeh et al. 1997). Thus, our results regarding mortality should be interpreted conservatively given that (1) various residues are present at the same time within hives and (2) there was a concomitant presence of pathogens within honey bees. Data on the potential for synergistic mechanisms, similar to those described in other insects, within the hives and how they could affect honey bee populations are lacking.

Fipronil is a "new generation insecticide" in that its mode of action differs from that of the organophosphorus and carbamates (both cholinesterase inhibitors) and some pyrethroids (sodium channels activators) (Gunasekara et al. 2007). Fipronil interferes with the function of γ-aminobutyric acid (GABA)-gated channels. This phenylpyrazole insecticide is used to control many soil and foliar insects on a variety of crops and is also formulated as flea and tick sprays for pests (Le Faouder et al. 2007). Fipronil is highly toxic to nontarget organisms, such as aquatic species, terrestrial game birds, and honey bees (Le Faouder et al. 2007). In sunflower, the transfer of fipronil by xylem from seeds to plants has been shown, although at very low levels, not surprising considering its low systemic property (octanol/water partition constant $K_{ow} = 3.5$; Aajoud et al. 2006, Le Faouder et al. 2007). In our study, honey, pollen loads, and honey bees could be considered as nontarget components and were not often contaminated by fipronil, or one of its metabolites, and when they were, concentrations were low (Table 5).

The acaricide coumaphos is used to treat varroa mites and was frequently found in apicultural matrices at high concentrations (Table 5), although its residues were not detected in honey at two of our five locations (Eure and Yonne sites). The commercial honey bee veterinarian medication containing coumaphos (Perizin) was not available in France when samples were collected; however, some apiculturists prepared home-made coumaphos formulations using the dog drug Asuntol. We observed that the doses of these coumaphos preparations applied in hives were often higher than the recommended dose. Coumaphos residues were mostly found in samples collected during the autumn visit, which was consistent with treatments against varroa mites. The concentration of fat-soluble stable ingredients increase in beeswax and migrate from the combs into stored honey. The higher the concentration in the wax, the higher the risk of residues in honey (Wallner 1992). Similar results were found in past studies (Wallner 1999, Martel et al. 2007).

When in contact with active ingredients, bees react by significantly increasing their uptake of noncontaminated foods that in turn decreases the concentration of the active ingredient in the honey sac, thus diluting the toxin (Wallner 1999). Within the hive, bees regurgitate a portion of the contaminated food into the cells, potentially contaminating the remaining cell contents and the cell walls (Wallner 1999). This transfer from outside the hive to inside the colony, through individual insects, lowers the pesticide concentration, which is why bees are considered to act as natural filters (Bogdanov 2006). This is consistent with our study, in which there was a low frequency of positive honey samples and a low mean concentrations relative to pollen loads or honey bees.

Honey bee sensitivity to pesticides is also dependent on bee health quality. Although past studies are limited, it has been shown that infection by *Nosema* spp. (Microsporida: Nosematidae) is more severe in honey bees as they age (Wahl and Ulm 1983). It was also shown that the presence of *Nosema* spp. spores in honey bees lowered the DL_{50} for DDT (Ladas 1972).

Honey bee exposure to some pesticides may have an adverse effect on colony health. These chemicals may affect the synthesis, transport, action, or elimination of natural molecules, such as hormones or enzymes, that are responsible for maintaining development, immune mechanisms, and behavior. Although these patterns are less known in insects than in mammals, several studies have shown their importance specifically in honey bee biology (Yang and Cox-Foster 2005, Evans et al. 2006, Sadd and Schmid-Hempel 2006). However, additional research is needed to better understand how pesticide residues, especially when present at low concentrations, influence colony health. In this study, no statistical relationship was found between pesticide residues and honey bee populations in the field. Although laboratory tests have shown adverse effects on the biology when examining individual honey bees, the honey bee colony has not been collectively studied under field conditions in such a detailed way (Pham-Delègue et al. 2002, Weick and Thorn 2002, Decourtye et al. 2005). This research could provide useful information regarding the impact of pesticide residues at the colony level.

One impact of large-scale agriculture with extended expanses of a single cultivated crop species to honey bees is the availability of pollen, which is the only source of proteins and lipids in the bee diet and thus crucial for their survival and development. Agricultural trends toward larger monoculture farming systems can place pollinating honey bees in situations where they have a restricted choice of dietary pollen. In these highly managed systems, factors such as feeding preference and nutrition can deeply affect the bee behavior and health (Schmidt et al. 1995). Three crops grown on an increasingly larger scale in Europe are rape, sunflower, and corn. In France, from 2002 to 2005, the area dedicated to corn, sunflower, and canola production represented ≈1.83–1.66, 0.60–0.64, and 1.04–1.23 million ha, respectively (French Ministry of Agriculture 2008). The nutritional value of the collected pollen has been previously studied in laboratory tests, and it was concluded that bees should not experience nutritional or longevity problems when limited to rape as the only food source (Schmidt et al. 1995). Sunflower pollen was readily consumed but seemed to be nutritionally poor and consequently reduced survival (Schmidt et al. 1995). Because bees are

polylectic, modifications to current agricultural practices to provide greater pollen diversity have been suggested, such as the cultivation of small areas of other crops near monocultures or to permit weedy areas to grow along the edges of the fields (Schmidt et al. 1995).

In our study, we found that pesticide residues were present at low levels in all apicultural matrices (Table 4). The concentrations were comparable with those found in other studies from Europe and the United States (Waller et al. 1984, Wallner 1999). We found no association between the presence of pesticide residues and with variation in populations of adult or broods or with colony mortality. However, additional studies are needed to better understand possible synergistic mechanisms between small amounts of pesticide residues within the hive and other potential sources of mortality, such as pathogens, to better quantify their synergistic effect to honey bee colony health.

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