Relative contribution of the *Varroa destructor* mite, selected agricultural pesticides and other risk factors to Dutch honey bee losses for winter 2011-2012

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Abstract

This article presents results of an analysis of honey bee losses over the winter of 2011-2012 in the Netherlands, from a sample of 86 colonies located at 43 apiaries. Spatially stratified random sampling was performed. The sample was divided between postal code areas by using the values of postal code random effects calculated from the Dutch National Monitor (for Honey Bee Winter Survival) 2010-2011 data which were collected by beekeeper questionnaires. This resulted in 23 apiaries selected in Dutch postal code areas with higher unexplained honey bee winter mortality, and 22 in postal code areas with lower unexplained winter mortality. Data were collected on colony losses as well as data from laboratory analysis of samples from selected colonies in each of these apiaries. Logistic regression was used to estimate the effects on honey bee winter loss of the *Varroa destructor* mite, and presence of agricultural pesticides, *Nosema* spp. and viruses, allowing for variation in risk between postal code areas of survival over winter 2011-2012. We found that the model which best explained the risk of loss included the variables (1) *Varroa destructor* mite infestation rate in October 2011, (2) the presence of the neonicotinoids acetamiprid or thiacloprid in the first 2 weeks of August 2011 in at least one of the apicultural matrices honey, bees or bee bread (pollen), (3) the presence of *Brassicaceae* pollen in bee bread in early August 2011 and (4) the random effect values at postal code level derived from the analysis of the larger scale data from the National Monitor 2011-2012. No interactions between the

model factors were found. We consider in the discussion that reduced opportunities for foraging in July and August because of bad weather may have added substantially to the effects of acetamiprid and thiacloprid.

Introduction

Honey bee colony losses in the Netherlands have been above 20% since 2006 (van der Zee 2010, van der Zee and Pisa, 2011, 2012, 2013), which is higher than reported from most European countries (van der Zee *et al.*, 2012). Analysis of the annual beekeeper surveys of colony losses in the Netherlands, using an international standardised questionnaire, showed, that beekeepers who treated against the ectoparasite *Varroa destructor* (indicated in this study as varroa mite) – in winter and in summer – always experienced significantly lower risk of winter losses compared with only treating in summer or winter. The mixed model analysis, used in the annual Dutch Winter Survival Monitor reports by van der Zee and Pisa (2011, 2012, 2013), revealed considerable spatial variation in risk of loss between specific administrative (postal code) areas, after allowing for the effects of the significant model factors at beekeeper level. These models incorporated random effects for both beekeeper and postal code area as well as the fixed model factors. Important spatial information about land use or local use of agricultural pesticides was not available in these studies, and as a consequence the variation in the random intercepts at postal code level remained mainly unexplained.

Observational studies at colony level, such as the present one, are necessary to further explore the large variation in risk of colony winter loss between beekeepers and between regions reported in the Dutch annual beekeeper Monitors. One of the main decisions to take is which sampling scheme should be used to reveal information about the crucial period(s) in which colonies are most vulnerable (in the sense that a colony may not be able to recover and will die in winter) to infestation of parasites, effects of pathogens and pesticides. In summer Dutch honey bee colonies produce a population, which must survive, as in most European countries, a winter that is sometimes long. Severe infestation of parasites or bad weather circumstances in summer, resulting in reduced foraging opportunities, are known factors preventing the building up of a winter population with enough lifespan and as a consequence these factors increase the risk of over-winter colony losses (Fluri *et al.*, 1982; van Dooremalen *et al.*, 2012).

Another risk factor for honey bee health, which is explored and discussed in a growing number of publications, is the role of agricultural pesticides in general and neonicotinoids in particular (see Blacquière *et al.*, 2012, for an overview). Pesticide use is high in the Netherlands with its important agricultural sector. In 2008, for example, 5.605 tonnes of active pesticide ingredients were used (CBS, PBL, Wageningen UR (2011)). The few observational surveillance studies that are available (Chauzat *et al.*, 2009; Nguyen *et al.*, 2009; Genersch *et al.*, 2010) report, that apicultural matrices can be severely contaminated with neonicotinoids such as thiacloprid, imidacloprid and acetamiprid. These monitor studies mainly focus on pesticide exposure on specific crops as oilseed rape and maize. Identification of a source of contamination may be important if a control product is only used on a specific crop which might enable taking specific measures to limit risks. The main pesticides investigated in this study are however widely used in agriculture and also in private households. For example the low toxic neonicotinoid thiacloprid, which is considered as safe for honey bees, is widely used for crop and fruit protection, including late blossoming fruits like strawberry, raspberry and species of blueberry. Application of thiacloprid based commercial products is advised during blossoming of fruit in open

culture (Bayer Crop Science, Brochure_Calypso_160805.pdf). We should take into account that these products are present in surface water (Van Dijk *et al.*, 2013) and may contaminate other sources foraged by honeybees.
With these considerations in mind we chose exposure to parasites, pathogens or pesticides in the critical summer period as the basis of our study design, and not a specific source of pesticide contamination. We report whether the presence of agricultural pesticides in three apicultural matrices (honey, beebread/pollen and bees), *Nosema* spp. and a range of honey bee viruses in summer is related to colony loss. We also examine whether or not the degree of varroa mite infestation in October is related to colony loss, as was found in a German study (Genersch *et al.*, 2010). Additionally we report whether the random effects at postal code level, as calculated from the Dutch National Monitor 2012 (van der Zee and Pisa, 2013) of honey bee winter survival 2011-2012, can help to explain the variation in risk between postal code areas in this study. Variation in postal code random intercepts, as calculated from the data of the previous National Monitor 2011 (van der Zee and Pisa, 2012) was used to design a spatially stratified sampling approach for data collection for the present study. To our knowledge this is the first colony level observational study with a study design based on spatial random effects calculated from national monitor survey questionnaire data from beekeepers.

Material and Methods

Data used from the Dutch Monitors of honey bee survival in winter 2010-2011 and 2011-2012
The present study examines factors potentially associated with colony losses for winter 2011-2012. An experimental sampling exercise was conducted to collect colony loss data as well as information on several colony level variables as possible risk factors for loss. Results from the analysis of the colony loss data collected in the Dutch National Monitor 2011 (van der Zee and Pisa, 2012) and Dutch National Monitor 2012 (van der Zee and Pisa, 2013) were used to plan the sampling design and used in the model fitting of the colony level data respectively. The data in these two studies were obtained from the standardised COLOSS questionnaires 2011 and 2012 concerning honey bee colony winter loss and colony management (see van der Zee and Pisa, 2012, 2013). The analysis in these two reports was based on model fitting using a generalised linear mixed model (GLMM; Zuur *et al.*, 2009) with a binomial distribution for the number of colonies lost from the number of wintered colonies (the dependent variable), as also used in van der Zee *et al.*, 2014. These GLMMs incorporated both fixed effects and random intercepts, and allow for correlated observations, i.e. colonies belonging to beekeepers within postal code regions indicated here as PC2 areas. The Netherlands can be grouped into 90 of these PC2 areas, based on the first 2 digits of the number representing the beekeeper location. Beekeeper and PC2 area were both included as random effects in the models, to allow for differences between the effect of beekeeper management and the effect of PC2 area. A series of beekeeper management characteristics such as varroa mite treatment can be specified as fixed effects. The model fitting used the 1 to 50 colony operations, representing most of the beekeepers in the Netherlands.

For the present study we used values of the postal code level random intercepts after including the fixed factors for beekeeper risk of colony loss as derived from the Monitor 2011. We also used the corresponding postal code level random intercepts from the analysis of the Monitor 2012 as a fixed effect (a covariate) in generalized linear regression models (GzLM). See the statistical paragraph below

for further details. In a different context, Drager and Hay, 2012, discuss the benefits of using random intercepts derived from one model as values of a predictor variable in another model. Fig. 1 shows the variation in the postal code random intercepts from the model fitting using the data from the Dutch Monitor 2012.

Statistical analysis

Statistical analysis was performed using the software packages R (R Development Core Team, 2011) and SPPS 20. An ANOVA (F test) was used to test for differences in the mean of variables with a Gaussian or near-Gaussian distribution (i.e. the number of *Varroa* mites per 100 bees), with values of categorical predictor variables. Correlation between the continuous variables operation size and number of V. destructor mites per 100 bees was calculated using the Pearson correlation. Here p-values less than 0.05 are considered significant, and values between 0.05 and 0.1 are considered as a trend.

To determine associations between predictor variables and colony overwinter mortality in the sampled population, a logistic GzLM was used, with the number of colonies dead in spring 2012 as the dependent variable and using a binomial distribution. As the number of colonies studied for each selected beekeeper was the same (see below) it was not thought necessary to allow for possible extra-binomial variation in the data. First, single factor models using the different independent variables as a single predicting factor or covariate in a model were made to identify factors associated with losses. The postcode area random effects from the National Monitor 2012 were used as one of these independent variables. Next the best fitting model was determined by including variables which were significant in the single factor models and which remained significant in the multi-factor model. We investigated the AIC (Akaike's Information Criterion) for the model when each term is dropped, one at a time, and the corresponding likelihood ratio test (LRT) statistic and its p-value. A rise in AIC when the term is dropped, together with a significant LRT p-value, indicates that that term should not be dropped from the model as it does significantly contribute to explaining the risk of colony loss.

Sampling scheme

Beekeeper operations were selected, considering the PC2 random effects values which were calculated from the Dutch Monitor Winter Loss data 2010-2011(van der Zee and Pisa 2011). A randomized sample of 43 operations was taken: 21 operations were situated in PC2 areas with a negative random effect and 22 in PC2 areas with a positive random effect. At each operation 2 colonies were randomly selected and included in the study population (N=86).

Sampling of the colonies and processing of the samples was carried out by the following scheme;

1. **Within the last week of July 2011** a minimum of 35 forager bees were collected between the hours of 11.00 and 14.00 from the closed hive entrance. Samples were conserved in situ in ethanol 70% and sent for analysis at the Warsaw University of Life Sciences, faculty of Veterinary Medicine, Department of Pathology and Veterinary Diagnostics Laboratory of Bee Diseases, Poland for analysis of *Nosema* spp., percentage of infected bees per sample, and band strength of both *Nosema ceranae* and *Nosema Apis*.
2. **Within the first 2 weeks of August 2011** a sample of 50 bees was taken – 25 from frames with brood and 25 from frames without brood – for pesticide residue analysis. The samples were

stored in situ on dry ice (-80° C) and conserved at this temperature for a maximum of 2 days before arriving at the RIKILT laboratory at Wageningen UR, where all samples were conserved at -20° C until analysis.
A minimum of 30 bees was sampled in an identical way for virus analysis. These samples were stored in situ on dry ice (-80° C) and sent on dry ice to the Food and Environment Research Agency in York, England, for analysis.
Further a minimum of 50 cells with bee bread were sampled. Half of the sample was used for pesticide analysis, the other for palynological determination. Mature and half ripe honey was sampled for pesticide residue analysis, stored in situ on dry ice (-80° C) and stored at - 20° C until analysis.

3. **During the last week of July 2011 and last week of October 2011** approximately 200-250 bees per colony were sampled by the beekeeper and sent to the Nederlands Centrum Bijenonderzoek (NCB) for determination of the level of varroa mite infestation.

Chemical Analysis

Chemicals and Reagents
Reference Standards
The majority of the reference standards was purchased from commercial suppliers, and the imidacloprid metabolites were donated by Bayer Crop Science (table 1). Out of stock solutions of individual pesticides a combined stock solution was prepared by mixing different volumes of the individual stock solutions (table 1). Acetonitrile, methanol, (all HPLC grade or better), and HPLC grade water were purchased from Biosolve (Valkenswaard, The Netherlands). Acetic acid, sodium sulphate, and magnesium sulphate were obtained from Merck (Darmstadt, Germany), and formic acid and ammonium formate were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Extraction
Bees.
1.0 (± 0.05) g of frozen sample material was weighed into a 50 mL Greiner tube. Extraction was performed by adding 3.0 mL of water, directly followed by 4.0 mL of extraction liquid (acetic acid/acetonitrile = 1/99 (v/v)). The sample was homogenised for 1 minute with the aid of an ultra-turrax, followed by addition of 1.6 g of $MgSO_4$ and 0.40 g of sodium acetate. After homogenisation (vortex for 30 sec) and centrifugation (5 minutes at 2000 rcf), 250 µL of extract was diluted with 250 mL of water and filtered with the aid of a syringeless filter device (Mini-UniPrep, 0.45 µm, Whatman, Forham Park, NJ), resulting in a matrix equivalent in the extract of 0.125 g/mL.
Honey/Bee bread
The extraction procedure for honey or bee bread was identical to the procedure as described for bees with one exception: the ultra-turrax homogenisation step was replaced by shaking end-over-end for 30 minutes. The samples were homogenized with water first before the extraction liquid was added.

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Analysis
For LC-MS/MS analysis an Shimadzu high performance liquid chromatography (HPLC) system (Shimadzu, 's-Hertogenbosch, the Netherlands) and an Applied Biosystems 5500 triple quadrupole mass spectrometer (Applied Biosystems Bleiswijk, the Netherlands) equipped with an electrospray (ESI) source were used for determination of pesticides. Separation was performed on a 100 mm × 3.0 mm

i.d., 3.0 µm Atlantis T3 C-18 column (Waters, Etten-Leur, the Netherlands) using a flow rate of 0.40 mL/minute. The column temperature was maintained at 35°C. Eluent A was water containing 5 mM ammonium formate and 0.1% (v/v) formic acid. Eluent B was water/methanol 5/95 (v/v) containing 5 mM ammonium formate and 0.1% (v/v) formic acid. The HPLC gradient started with 100% A for 0.5 min, was linearly increased to 100% B over 4.0 minutes, and kept at this percentage for 4.5 minutes. Finally, the gradient was switched to 100% A again over 0.5 minutes and equilibrated for 4.0 minutes before the next injection took place. The injection volume was 10 µL.

MS/MS Conditions

ESI-MS/MS was performed using scheduled multiple reaction monitoring (sMRM) in positive mode and multiple reaction monitoring (MRM) in negative mode. In sMRM mode the detection window was set to 90 s and the target scan time was set to 0.5 s. In MRM mode acquisition was done with 10 ms dwell time. For both scan types the settling time and pause time was set to 5 ms. The number of data points across the peaks was at least eight. The settings of the ESI-source were as follows: source temperature 300 °C, curtain gas 20 psi, source gas 1 60 psi, source gas 2 60 psi, ion spray voltage -2000 V neg. mode and + 5500 V pos. mode and collision gas (nitrogen) medium. The analyte dependent parameters (declustering potential (DP), collision energy (CE) and cell exit potential (CXP) are listed in table 2.

Verification of Recovery and Matrix Effects

For verification of recovery for the different extraction methods, each matrix was fortified at levels given in table 3. In addition, one non-fortified sample was included in the test set. The extract of the non-fortified sample was also used for preparation of a matrix matched calibration curve. In the LC-MS/MS sequence, for each matrix, the five sample extracts were bracketed by the matrix-matched calibration standard (and a solvent standard at the same concentration). Average recoveries and relative standard deviations (RSDs) were calculated for the fortified samples against matrix-matched standards. Recoveries obtained therefore reflect the recovery from the extraction procedure. (table 3, 4, and 5).

Validation

All three matrixes were fortified in 5-fold at three levels (1x, 2x and 10x spiking level (SL), table 3, 4, and 5). Limits of quantification (LOQ) were set at the lowest spiking level with the exception of imidacloprid urea and thiacloprid in pollen for which higher LOQs of 1.0 and 2.0 µg/kg respectively were established. For certain pesticides it was feasible to determine a limit of determination (LOD) which was lower than the LOQ, and defined as analytical results which complied to ion ratio and retention time parameters (SANCO 2013). Pesticides present at the level of LOD<x<LOQ were reported as “trace”. Matrix matched calibration curves were constructed by diluting the combined stock solution (table 1) 500, 2000, 5000, 20000, and 40000 times with extract of blank honey, bees, and stored pollen. These standards were analysed for verification of linearity of response versus concentration. In the LC-MS/MS sequence, the 2000 times diluted matrix-matched standard was repeatedly analysed every 5-8 injections. Recoveries were calculated based on one-point matrix-matched calibration, using the average of the 2000 times diluted matrix-matched standard preceding and following the sample (bracketing).

Analysis of the presence of pathogens

Varroa mite infestation rate

The proportion of phoretic mites per 100 bees (mite rate) was estimated following OIE protocol (OIE Terrestrial Manual 2008, page 428).

Virus analysis

Samples were tested with an oligonucleotide microarray which enabled the simultaneous detection of nine viruses: chronic bee paralysis virus (CBPV), acute bee paralysis virus (ABPV), Apis iridescent virus (AIV), Kashmir bee virus (KBV), sacbrood virus (SBV), deformed wing virus (DWV), black queen cell virus (BQCV) and Israeli acute paralysis virus (IAPV). Total RNA was extracted using an RNeasy Mini kit (Qiagen, Crawley, UK) following the protocol for animal tissues as supplied. The occurrence of each virus was confirmed using a suite of bee and virus specific TaqMan assays (Chantawannakul *et al.*, 2006; Ward *et al.*, 2008). Labelled cDNA was produced using the CyScribe first-strand cDNA synthesis kit (GE Healthcare, Bucks, UK). The labelled cDNA was then purified using a Qiagen PCR cleanup column (Qiagen, Crawley, UK) and eluted in 120 ll Hyb Buffer #1 (Ambion, Austin, USA). The purified cDNA was heated for 3 minutes at 95° C, applied to the microarray and incubated overnight in a hybridisation chamber at 42° C. Following hybridisation, each slide was washed initially in 2_ SSC (150 mM NaCl, 15 mMsodium citrate) and 0.1% SDS, followed by subsequent washes in 1_ SSC and 0.1_ SSC before being dried and scanned on a GenePix 4000B microarray scanner (Molecular Devices, USA).

Nosema spp. analysis

The abdomens of the collected honey bees were crushed in Eppendorf tubes using liquid nitrogen and sealed pipette tips. 0.5 ml of distilled water was added to each tube and mixed with the powdered abdomens. A pooled sample was made by taking 0.1 ml of the solution from each tube. This pooled sample was centrifuged for 6 minutes at 800g. The pellet was then subjected to DNA extraction using Dneasy Plant Mini Kit (Quiagen) according to the manufacturer's protocol. Extracted DNA samples were stored at -20° C until further processing. Thermal setup and all other PCR conditions were implemented according to Botias *et al.*, 2011. Electrophoresis of the PCR product was performed in 1.5 agarose gel stained with ethidium bromide. Then the product was visualised under UV light. If the result was positive we examined the individual bees from the remaining samples using light microscopy. A quantity of 2 ml of the spore solution was placed on an IFI slide and the presence of spores was checked.

Palynological determinations

Bee bread was collected from approximately 25 honey comb cells to analyse the pollen composition of each sample. The pooled content of these cells was thoroughly mixed and stored in containers with 70% ethanol. Samples for microscopic inspection were prepared from the stored mixed pollen bread by dissolving a small subsample of the mixture in 2 ml water. Two drops of this pollen solution were placed on an observation glass and mounted in glycerine jelly containing basic fuchsin to stain the pollen grains. Identification took place under a light microscope at 400 times magnification with the assistance of a reference pollen collection of approximately 130 species and reference documents (see Kleijn and Raemakers 2008). For each sample we estimated the percentage contribution of each pollen taxon to the total number of pollen grains. Pollen taxa were identified to the lowest possible taxonomic level.

Colony characteristics

During the August 2011 visit the following colony characteristics were assessed: (1) colony size (number of frames with bees), (2) size of the brood nest (number of frames with brood), (3) presence of pollen (categorised as good, reasonable, moderate, bad), (4) age of queen. The two sampled colonies were checked for visible symptoms of bee diseases.

The participating beekeepers were asked in January 2012 and April 2012 about the number of colonies lost between September 1 2011 and December 31 2011 and between January 1 2012 and April 1 2012 respectively. Case definitions as provided in the COLOSS BEEBOOK (van der Zee *et al.*, 2013) were used to determine if a colony was lost.

Results

Lost colonies

Of the 86 colonies in the study sample, 25 (29.1 %) died between September 1 2011 and April 1 2012 Nine colonies (10.5 %) died in the period between September 1 2011 and December 31 2011. Between January 1 2012 and April 1 2012 another 16 (18.6 %) colonies were lost. The colonies lost in March included 1 colony with unsolvable queen problems and 2 reduced to such a small number of bees that these colonies were considered as not able to perform the normal biological activities needed for survival (brood rearing, and resource gathering). No association was found between the observed losses in the study sample and the number of wintered colonies of the participating beekeepers at October 1 2011 ($p=0.681$). A strong relationship was found between increasing risk of colony loss using the data for the two colonies observed for beekeepers in the study sample and increasing loss of those beekeepers during winter 2011-2012 as calculated from the data from the Dutch National Monitor 2012 ($p<0.0001$). No associations were found between colony size or number of frames with brood in August and colony winter loss.

Honey and pollen

In total 68 of 86 colonies received supplemental sugar solutions before or during the visit in the first 2 weeks of August. In 5 colonies honey and pollen were absent. Pollen presence was estimated as good in 5, reasonable in 14, moderate in 27, nearly absent in 35, and absent in 5 colonies. No significant associations were found between differences in degree of pollen presence and risk of colony loss.

Varroa mite related effects

No associations were found between risk of winter loss and the varroa mite infestation in the last week of July. The varroa mite infestation rate in the last week of October could be calculated for 81 of the 86 colonies in the study population. For the other 5 colonies, data was missing for 3 colonies which died before the sampling in the last week of October took place, and for another 2 because one beekeeper could not be contacted in this period. A strong association ($p<0.001$) was found between increasing mite load in October and higher risk of colony loss, which confirms the outcome of other studies (Genersch *et al.*, 2010; Liebig, 2001).

Associations between pesticide presence and losses

In 15 colonies, trace amounts of neonicotinoids were present at above the limits of detection (LOD) but below the limits of quantification (LOQ). These observations were considered as positive samples and were allocated a value of 0.05 if a value was required for the analysis.

Imidacloprid (including its metabolite imidacloprid desnitro), acetamiprid and thiacloprid were found in bee bread, honey or bees of 37 colonies in the study sample (n=86). No other neonicotinoids were detected.
The chemical synergist piperonyl-butoxide was found in the bee bread of 6 colonies. Fipronil, fipronil-sulphide, fluvalinate-tau and propiconazole were only detected in low numbers. A remarkably high value (371 µg/kg) for propiconazole contamination was found in one of the samples (see table 6 for more detailed descriptive information).
The statistical analysis was limited to the group of neonicotinoids rather than all the pesticides, because only this group was present in a sufficient number of samples (table 6).
The odds of colony loss in the group of colonies in which the neonicotinoids acetamiprid, imidacloprid (or imidacloprid desnitro) or thiacloprid were present in one or more matrices (bee bread, honey and bees), were significantly higher (p=0.014) compared with colonies without the presence of these neonicotinoids. We also tested the effect on the risk of loss of the presence of any of these neonicotinoids in colonies for each matrix separately. Presence of neonicotinoids in bee bread was not associated (p=0.209) with higher odds of loss compared with colonies without neonicotinoids, however a strong association was found between higher odds of loss and presence of neonicotinoids in honey (p=0.002). Presence of neonicotinoids in bees was also associated with higher odds of loss (p=0.014) compared to the colonies without neonicotinoids.
We also considered the presence in at least one of the matrices of the substances thiacloprid, acetamiprid and imidacloprid singly and examined the effect on the risk of loss compared to colonies in which the neonicotinoid was absent.
Colonies in which thiacloprid was present in at least one matrix had significantly higher odds of loss (p=0.009) than colonies for which it was absent. A trend (p=0.065) was found for higher odds of loss if acetamiprid was present in at least one of the matrices. Such an effect was not found for imidacloprid (or imidacloprid desnitro) (p=0.410). Considering thiacloprid further, the risk of early losses (before December 31) was also significantly higher (p=0.0461) if thiacloprid was present in colonies compared with colonies where it was absent. Presence of thiacloprid in honey was also associated (p=0.007) with higher odds of loss compared with colonies without this neonicotinoid. Such effects were also found for presence of thiacloprid in bees (p=0.003) and bee bread (p=0.039).
The honey, pollen and bee matrices, contaminated with the cyano-substituted neonicotinoids thiacloprid and acetamiprid, were pooled to one new factor 'acetamiprid or thiacloprid present (yes/no)', to optimise the statistical validity of the estimated model effects. If thiacloprid or acetamiprid was present odds of colony loss were significantly higher (p= 0.0025) compared with all colonies in which thiacloprid or acetamiprid were not detected in any of the three matrices.

Losses related to specific plant families and genera.

The bee bread samples showed that the colonies collected pollen from a variety of plant families. *Brassicaceae, Fabaceae, Liliaceae, Ericacaea, Lamiaceae, Asteraceae, Apiaceae* and *Rosaceae* were present. The most prevalent pollen families, or genera if these could be identified, were used for further statistical analysis. We used presence or absence of '*Brassicaceae*' as a model factor, although we observed *Brassica napus* and *Sinapis genera*, but it was difficult to differentiate between the two genera in bee bread. At family level only *Brassicaceae* in bee bread was associated with higher odds of colony loss (p=0.0112).

At genus level no associations were found between presence of *Zea maize* in bee bread and risk of loss (p=0.9904). An effect close to a trend (p=0.112) was found for lower odds of loss if *Calluna vulgaris* was present in bee bread.

Virus and Nosema spp. related losses

Only the viruses BQCV, SBV, DWV, ABPV were found in the bees, which were sampled in July. No associations were found between the presence of each of these viruses in the colonies and risk of winter loss, nor for interactions between these viruses and other factors. No significant associations were found relating the presence of *Nosema* spp. in the colonies in July to the risk of colony loss.

Postal code area related losses

There was a strong relationship (p=0.002) between the PC2 random intercept values (from the National Dutch Monitor 2012) attributed to the colonies in those PC2 areas and higher odds of loss for increasing (from negative to positive) random effect values. Only 1 of the 25 lost colonies was located in an area with a negative postal code random effect (suggesting lower risk of loss). A significant association was also found between the varroa mite ratio in October and the attributed postal code random effects (p= 0.0129). A positive association was found between the presence of thiacloprid or acetamiprid in one of the matrices and a positive value of the attributed postal code random effect (p= 0.0203). *Zea maize* in bee bread was significantly associated (p=0.015) with higher presence in areas with negative random effects.

Most parsimonious model

Colonies (n=79) were included in the final modelling if data was available on, at least, varroa mite infestation and neonicotinoids. A full model was constructed including the significant variables (1) varroa mite rate, (2) contamination with the cyano-substituted neonicotinoids acetamiprid or thiacloprid (yes/no) in honey, pollen or bee matrices, (3) *Brassicaceae* present in bee bread (yes/no), and (4) the attributed PC2 random effects (from the National Monitor 2012).
These 4 factors were all significantly associated with winter loss (see table 7 for detailed information). The AIC decreased from 95.459 in the null model to 72.334 in the most parsimonious model, residual deviance decreased from 93.459 to 62.334 (table 8). Fig.2 shows the odds ratios for the 4 terms in the model. No interaction effects between the 4 model factors/covariates were found.
Table 9 shows the AIC for the model when each term is dropped, one at a time, and the corresponding likelihood ratio test (LRT) statistic and corresponding p-value. A rise in AIC with a significant LRT p-value indicates that that term should not be dropped. The most significant term is the varroa mite rate in October, followed by contamination with the neonicotinoids acetamiprid or thiacloprid (yes/no) in honey, pollen or bee matrices, then *Brassicaceae* found (yes/no) in bee bread, with areal random effect 2012 being the least significant of the predictor variables. The estimated effects are about 5 times as large for the last 3 of these compared to the effect of an increase of size one in the varroa mite load, however they cannot be estimated as precisely from the available data, so the standard errors of the effects are larger than for varroa mite effect.

Discussion

Analysis of annual Dutch questionnaire data (van der Zee and Pisa 2011, 2012, 2013; van der Zee, 2013), showed that varroa mite treatment was the strongest factor in explaining honey bee colony

winter losses. The outcome of the present observational study of potential risk factors at colony level confirms the important contribution of this ecto-parasite to honey bee winter losses. A high varroa mite load in October, indicating failing varroa control, was the strongest factor in the best fitting model. This is in line with the findings in many studies (such as the German longitudinal surveillance study by Genersch *et al.*, 2010 and the pan-European study by van der Zee *et al.*, 2014).
The direct motivation for this observational pilot study was the need expressed in the Dutch Parliament for a first exploration of the presence of agricultural pesticides in honey bee matrices and possible effects on winter loss. Genersch *et al.*, 2010, found no association between contamination with agricultural pesticides in oilseed rape pollen and winter loss. We also found no significant evidence of such an association, although in our study the presence of *Brassicaceae* in bee bread was a significant model factor associated with higher losses. We also found no significant association with higher losses, when we investigated interaction between *Brassicaceae* in bee bread and the presence of thiacloprid or acetamiprid in honey, pollen or bees. This was expected, since thiacloprid and acetamiprid were not accepted pesticides for oilseed rape in the Netherlands at the time. The effect of interactions of *Brassicaceae* with other agricultural pesticides on risk of colony winter loss could not be estimated, because in this study only neonicotinoids were detected in sufficient quantities to allow for a statistical analysis.

Presence of thiacloprid or acetamiprid present (yes/no) in apicultural matrices in the beginning of August was another strong model factor in the best explaining model and was associated with higher odds of winter loss. To our knowledge such a result has not been found in other observational studies. This outcome was not expected, since cyano-substituted neonicotinoids are considered to have a low toxicity. A possible explanation could be as suggested by Laurino *et al.* (2011), who found in an experimental study that '*Acetamiprid and Thiacloprid, as also evidenced in other acute toxicity trials (Iwasa et al., 2004; Maccagnani et al., 2008), were apparently not dangerous to the honey bees unless they were starved. This result suggests that there is a repellent effect of both a.i. as also reported for Imidacloprid (Ramirez-Romero et al., 2005) and a food preference test would prove such an effect. If so, and disregarding sub-lethal effects, some hazards can arise when colonies are severely short of stores or after prolonged seclusion'*. Conditions of food deprivation were observed in the colonies in the present study. The Royal Netherlands Meteorological Institute reported that July 2011 was ranked 6th in the wettest July-months since the year 1901. The temperature was 2 degrees below the long-term average, the amount of sunshine was 158 hours compared to 212 hours normally, and a total monthly precipitation of 179 mm in 96 hours compared to the long term average of 81 mm and 40 hours. August 2011 showed a similar pattern, with 153 hours of sunshine where 195 hours is the long term average, and 110 mm precipitation, while 78 mm is the long term average. These weather conditions resulted in bad foraging opportunities for honey bee colonies during the high summer period. September, October and November were warmer and drier than normal. These bad foraging conditions during the main honey flows in early summer were reflected in the low general honey and pollen reserves in the colonies observed in the first two weeks of August 2011, even though most of the beekeepers had started supplementary feeding of their colonies. As a consequence, starvation may have played a role. Also, exposure to contaminated honey and pollen may have been at a relatively high level because of reduced opportunities for colonies to detoxify the limited stored food supplies by the uptake of noncontaminated foods and so decrease the concentration of acetamiprid or thiacloprid

by diluting these ingredients (Wallner, 1999).
Some colonies were in a position to compensate for these bad weather circumstances. The analysis of the national Dutch Monitor 2012 (van der Zee and Pisa, 2013) showed that colonies with access to *Calluna vulgaris* honey flow in August 2011 and September 2011 had lower odds of winter loss compared with colonies without this access ($p<0.0001$). Relatively warm weather continued in October and November, which provided these colonies with opportunities to produce a winter population in late summer. An indication of such an effect of lower odds on loss, close to a trend ($p=0.112$), was found in the study sample for the limited number of colonies (12 colonies wintered, 1 lost) with *Calluna vulgaris* pollen in beebread.
The PC2 area random effects/intercepts, which were calculated from the national Monitor Dutch monitor 2012 and were attributed to the colonies in the present study as a fixed model covariate, was the least significant factor in the most explaining model. This may indicate that the spatial pattern of losses in the present study is similar to the regional variation in colony losses observed in the National Monitor 2012. Spatial variation in the study sample may be partly due to differences in mite load between PC2 areas, since higher varroa mite rates in October were correlated ($p=0.0154$) with higher positive values of the attributed PC2 random effect. Comparable correlations were found for presence of acetamiprid or thiacloprid in colonies. Colonies with acetamiprid or thiacloprid were more often present in PC2 regions with higher positive values of the attributed PC2 random effect. We conclude that the regional variation in the present study could to some extent be explained by variation in mite loads and acetamiprid and thiacloprid presence in colonies.
Using postal code area effects in this way was the best available option at the time this study started. Adding information on land use to the modelling is crucial for further exploration of local effects. Using database information on land use and use of pesticide on agricultural crops may also be helpful in the modelling of data from the annual National Monitor (data collection using questionnaires). This approach would also allow for a better understanding of increases and decreases of other pollinators.

This study illustrates the need for honey bee colonies to have a period offering good opportunities to produce a strong winter population. In this study both a high infestation of the varroa mite and the presence of the agricultural pesticides thiacloprid and acetamiprid in colonies were associated with higher winter losses of colonies. Such colonies were significantly more often present in postal code areas which were identified in the national Dutch Monitor as areas with higher odds of loss. *Brassicaceae* in bee bread was also associated with higher odds of loss, although not associated with any of the agricultural pesticides investigated in this study.
Bad weather may have played an important role in the observed losses, because reduced foraging conditions in July and August may have had impacts on the production of a healthy winter population, not only by deprivation of the necessary food supply, but also because starvation may have increased the toxic effects of thiacloprid and acetamiprid. These effects may be playing a role in areas with reduced food sources in summer under less extreme weather conditions also. A longitudinal study with more colonies is necessary to further clarify the findings in this pilot study.

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Table 1. Reference material pesticides

Pesticide	Origin [1]	Purity (%)	Concentration stock solution (ug/ml)	Solvent stock solution [2]	Concentration mix stock solution (µg/ml)
6-Chloronicotinic acid	S	99.0	2000	MeOH	20
Acetamiprid	S	99.9	2000	MeOH	1.0
Clothianidin	S	99.9	2000	MeOH	4.0
Coumaphos	E	96.0	2000	ACN	4.0
DMA	S	99.0	2000	MeOH	50
DMF	S	97.0	2000	MeOH	10
DMPF	S	99.7	500	MeOH	10
Fipronil	E	98.0	2000	MeOH	1.0
Fipronil-sulfone	E	99.5	2000	ACN	1.0
Fipronil-carboxamide	E	96.5	100	ACN	1.0
Fipronil-desulfinyl	E	96.5	100	ACN	1.0
Fipronil-sulfide	E	99.5	1000	MeOH	1.0
Fluvalinate-tau	E	91.0	2000	ACN	20
Imidacloprid	E	99.0	2000	MeOH	1.0
Imidacloprid olefin	B	97.9	500	MeOH	10
Imidacloprid urea	B	99.4	500	MeOH	1.0
Imidacloprid, 5-hydroxy	B	96.7	500	MeOH	10
Imidacloprid, desnitro	B	97.9	500	MeOH	1.0
Imidacloprid, desnitro olefin	B	99.9	500	MeOH	1.0
Piperonyl butoxide	E	92.5	2000	ACN	1.0
Propiconazole	E	97.0	2000	MeOH	10
Thiacloprid	E	99.5	1000	MeOH	2.0
Thiamethoxam	E	99.5	2000	MeOH	4.0
Triflumizole	S	99.8	2000	MeOH	2.0

(1) S = Sigma-aldrich, Zwijndrecht, the Netherlands
E = Dr. Ehrenstorfer, Augsburg, Germany
B = Bayer Crop Science, Mijdrecht, the Netherlands

(2) ACN = acetonitrile; MeOH = methanol

Table 2. List of analytes with MS/MS parameters

Pesticide	m/z Precursor ion	DP (V)	m/z Product ions	CE (V)	CXP (V)
6-Chloronicotinic acid	155.9	-50	111.9	-14	-13
Acetamiprid	223	51	126.0/73.0	29/79	10/12
Clothianidin	249.9	46	169.1/132.0	17/23	12/10
Coumaphos	363	85	227.0/307.0	35/23	15/15
DMA	122	91	107.1/77.1	23/37	8/10
DMF	150	41	107.1/77.0	27/53	8/12
DMPF	163	76	122.1/107.2	23/33	8/8
Fipronil	434.9	-50	329.7/249.9	-18/-36	-21/-17
Fipronil-sulfone	450.9	-30	281.9/414.7	-40/-22	-19/-29
Fipronil-carboxamide	452.9	-80	348.0/303.8	-22/-34	-23/-21
Fipronil-desulfinyl	387	-50	350.8/281.8	-20/-44	-25/-17
Fipronil-sulfide	418.9	-40	261.9/382.8	-40/-16	-17/-11
Fluvalinate-tau	503	30	181.0/208.1	35/17	12/14
Imidacloprid	256.1	41	209.0/175.1	21/25	14/12
Imidacloprid olefin	253.9	56	171.2/204.9	25/21	12/14
Imidacloprid urea	212.2	41	128.0/99.1	25/25	10/8
Imidacloprid, 5-hydroxy	271.9	51	146.1/225.1	39/23	10/16
Imidacloprid, desnitro	210.9	76	90.1/72.9	47/77	8/6
Imidacloprid, desnitro olefin	208.9	66	126.0/73.0	29/67	10/8
Piperonyl butoxide	356.2	31	177.1/119.1	19/47	25/25
Propiconazole	342	106	159.0/69.1	37/23	10/6
Thiacloprid	253	90	126.0/186.0	29/19	15/15
Thiamethoxam	292	41	210.9/180.9	17/31	14/12
Triflumizole	346	26	278.0/73.1	15/21	18/10

Table 3. Validation for Honey

Pesticide	SL (µg/kg)	Recovery's								
		1x SL			2x SL			10x SL		
		n	Avg. (%)	RSD (%)	n	Avg. (%)	RSD (%)	n	Avg. (%)	RSD (%)
6- Chloronicotinic acid	10	10	26	15	8	23	21	10	22	19
Acetamiprid	0.50(1)	10	94	4.5	8	94	4.6	10	90	5.7
Clothianidin	2.0	10	92	9.3	8	93	5.7	10	92	5.3
Coumaphos	2.0(2)	8	92	6.7	6	90	6.8	8	88	7.6
DMA	25	10	82	12	8	85	7.6	10	88	14
DMF	5.0	10	88	5.0	8	89	5.5	10	87	3.9
DMPF	5.0(3)	10	89	4.0	8	89	5.9	10	65	20
Fipronil	0.50	10	108	15	8	99	12	10	103	7.2
Fipronil carboxamide	0.50	10	94	8.3	8	92	7.4	10	96	3.0
Fipronil-desulfinyl	0.50	10	104	12	8	95	6.9	10	98	4.3
Fipronil-sulfide	0.50	10	106	15	8	95	9.2	10	101	5.0
Fipronil-sulfone	0.50	10	99	12	8	92	7.9	10	96	3.5
Fluvalinate-tau	10(4)	10	78	6.8	8	79	5.9	10	82	10
Imidacloprid	0.50(5)	10	90	14	8	91	7.1	10	91	3.2
Imidacloprid olefin	5.0	10	96	10	8	94	13	10	94	5.4
Imidacloprid urea	0.50	10	93	5.7	8	92	3.7	10	91	3.9
Imidacloprid, 5-hydroxy	5.0	10	94	2.8	8	92	6.1	10	91	5.0
Imidacloprid, desnitro	0.50	10	70	4.2	8	67	4.6	10	95	6.8
Imidacloprid, desnitro olefin	0.50	10	74	5.2	8	73	5.4	10	91	3.8
Piperonyl-butoxide	0.50	8	85	3.3	6	82	3.3	8	84	2.9
Propiconazole	5.0(6)	10	96	3.5	8	93	5.0	10	93	4.4
Thiacloprid	1.0(7)	10	93	2.5	8	93	6.1	10	91	5.4
Thiamethoxam	2.0	10	95	5.4	8	94	5.0	10	92	4.8
Triflumizole	1.0	10	93	4.1	8	92	5.9	10	91	5.1

(1) LOD = 0.20 µg/kg
(2) LOD = 1.5 µg/kg
(3) LOD = 2.5 µg/kg
(4) LOD = 4.0 µg/kg
(5) LOD = 0.30 µg/kg
(6) LOD = 1.0 µg/kg
(7) LOD = 0.25 µg/kg

Table 4. Validation for Bees

Pesticide	SL (µg/kg)	Recoveries								
		1x SL			2x SL			10x SL		
		n	Avg. (%)	RSD (%)	n	Avg. (%)	RSD (%)	n	Avg. (%)	RSD (%)
6- Chloronicotinic acid	10	9	23	24	9	24	28	9	28	10
Acetamiprid	0.50(1)	13	87	8.5	13	82	8.5	11	81	7.8
Clothianidin	2.0	13	90	23	13	83	23	11	71	24
Coumaphos	2.0(2)	13	82	3.9	13	79	11	11	81	8.9
DMA	25	13	70	24	13	74	7.4	11	82	13
DMF	5.0	13	85	9.1	13	86	8.1	11	88	7.3
DMPF	5.0	13	85	4.2	13	85	6.7	11	59	21
Fipronil	0.50	9	86	10	9	90	8.1	9	87	6.9
Fipronil carboxamide	0.50	13	83	10	13	88	11	11	86	10
Fipronil-desulfinyl	0.50	9	85	9.3	9	87	10	9	93	9.0
Fipronil-sulfide	0.50	9	83	6.8	9	90	4.7	9	90	7.8
Fipronil-sulfone	0.50	9	85	8.6	9	90	5.1	9	86	3.4
Fluvalinate-tau	10	13	69	22	13	76	18	11	84	8.3
Imidacloprid	0.50(3)	13	100	15	13	94	11	11	85	21
Imidacloprid olefin	5.0	13	103	14	13	102	18	11	100	19
Imidacloprid urea	0.50	13	87	7.9	13	85	7.2	11	87	9.7
Imidacloprid, 5-hydroxy	5.0	13	111	19	13	109	31	11	105	25
Imidacloprid, desnitro	0.50(4)	13	68	3.8	13	73	13	11	100	16
Imidacloprid, desnitro olefin	0.50	13	69	4.4	13	75	12	11	95	19
Piperonyl-butoxide	0.50	8	79	8.7	8	79	17	6	86	6.1
Propiconazole	5.0(5)	13	80	7.5	13	80	9.8	11	81	11
Thiacloprid	1.0(6)	13	83	21	13	103	19	11	121	37
Thiamethoxam	2.0	13	94	13	13	92	15	11	86	14
Triflumizole	1.0	13	83	5.7	13	79	10	11	79	8.1

(1) LOD = 0.20 µg/kg

(2) LOD = 0.40 µg/kg

(3) LOD = 0.30 µg/kg

(4) LOD = 0.20 µg/kg

(5) LOD = 2.0 µg/kg

(6) LOD = 0.20 µg/kg

Table 5. Validation for Stored Pollen

Pesticide	SL (μg/kg)	Recoveries								
		1x SL			2x SL			10x SL		
		n	Avg. (%)	RSD (%)	n	Avg. (%)	RSD (%)	n	Avg. (%)	RSD (%)
6- Chloronicotinic acid	10	6	21	8.5	6	21	14	6	21	7.1
Acetamiprid	0.50(1)	6	95	16	6	82	5.8	6	82	4.7
Clothianidin	2.0	6	85	13	6	85	10	6	80	11
Coumaphos	2.0(2)	6	85	3.0	6	80	6.8	6	84	2.4
DMA	25	6	61	9.8	6	62	6.8	6	68	4.3
DMF	5.0(3)	6	105	21	6	86	12	6	90	7.6
DMPF	5.0	6	74	4.2	6	74	7.8	6	45	10
Fipronil	0.50	6	78	9.7	6	80	18	6	81	9.0
Fipronil carboxamide	0.50	6	90	7.2	6	83	15	6	91	9.1
Fipronil-desulfinyl	0.50	6	70	20	6	76	5.7	6	84	4.6
Fipronil-sulfide	0.50	6	85	4.5	6	81	9.4	6	86	3.9
Fipronil-sulfone	0.50	6	77	10	6	76	8.7	6	87	4.5
Fluvalinate-tau	10	6	73	21	6	61	25	6	70	10
Imidacloprid	0.50(4)	2	128	28	6	129	30	6	91	17
Imidacloprid olefin	5.0	6	85	12	6	80	9.0	6	81	4.3
Imidacloprid urea	0.50(5)	6	61	40	6	87	9.4	6	82	16
Imidacloprid, 5-hydroxy	5.0	6	79	15	6	78	13	6	78	6.9
Imidacloprid, desnitro	0.50(6)	6	50	10	6	51	6.0	6	64	2.6
Imidacloprid, desnitro olefin	0.50	6	44	19	6	40	6.6	6	53	1.3
Piperonyl-butoxide	0.50	6	100	21	6	78	14	6	77	5.8
Propiconazole	5.0(7)	6	81	5.5	6	78	8.4	6	85	3.5
Thiacloprid	1.0(8)	6	55	52	6	69	29	6	73	3.0
Thiamethoxam	2.0	6	81	6.8	6	86	9.1	6	86	3.7
Triflumizole	1.0	6	82	3.6	6	78	5.8	6	83	2.9

(1) LOD = 0.25 μg/kg

(2) LOD = 1.0 μg/kg

(3) LOD = 4.0 μg/kg

(4) LOD = 0.75 μg/kg; LOQ = 1.0 μg/kg

(5) LOQ = 1.0 μg/kg

(6) LOD = 0.20 μg/kg

(7) LOD = 2.5 μg/kg

(8) LOD = 0.80 μg/kg; LOQ = 2.0 μg/kg

Table 6. Number of analysed samples per matrix for each component

Component	Honeybees			Stored pollen			Honey		
	Number analysed samples	No. pos. [1]	Range[2]	Number analysed samples	No. pos. [1]	Range[2]	Number analysed samples	No. pos. [1]	Range[2]
6_chloronicotinic_acid	42	0/0	na	42	0/0	na	42	0/0	na
Acetamiprid	84	1/1	0.52	79	2/3	0.65-5.20	80	1/6	0.8-3.7
Clothianidin	43	0/0	na	42	0/0	na	42	0/0	na
Coumaphos	84	2/2	5.0-12.0	79	9/5	4.1-26.0	80	0/2	2.3-2.7
DMA [3]	43	0/0	na	42	0/0	na	42	0/0	na
DMF [4]	84	0/0	na	79	0/1	6.2	80	0/2	17.8-22.0
DMPF [5]	84	0/0	na	79	0/2	7.6-9.8	80	1/1	6.4
Fipronil	84	0/0	na	79	0/2	2.1-2.9	80	0/0	na
Fipronil_carboxamide	43	0/0	na	42	0/0	na	42	0/0	na
Fipronil-desulfinyl	43	0/0	na	42	0/1	0.9	42	0/0	na
Fipronil-sulfide	43	0/0	na	42	0/0	na	42	0/0	na
Fipronil-sulfone	43	0/0	na	42	0/0	na	80	0/0	na
Fluvalinate-tau	84	0/0	na	79	0/2	13-23	80	0/0	na
Imidacloprid	84	2/0	na	79	3/1	1.1	80	2/2	1.0
Imidacloprid_5-OH	43	0/0	na	42	0/0	na	42	0/0	na
Imidacloprid_desnitro	84	0/1	0.65	79	5/0	na	80	0/0	na
Imidacloprid_desnitro_olefin	43	0/0	na	42	0/0	na	42	0/0	na
Imidacloprid_olefin	43	0/0	na	42	0/0	na	42	0/0	na
Imidacloprid_urea	43	0/0	na	42	0/0	na	42	0/0	na
Piperonyl-butoxide	84	0/0	na	79	0/6	0.5-2.3	80	0/0	na
Propiconazole	84	2/0	na	78	0/2	8.3-371	80	0/0	na
Thiacloprid	84	5/5	1.1-3.8	79	0/19	1.0-66.0	80	8/13	1.4-15.0
Thiamethoxam	43	0/0	na	42	0/0	na	42	0/0	na
Triflumizole	43	0/0	na	42	0/0	na	42	0/0	na

[1] Number of positive samples: >LOD< LOQ (trace amount) / >LOQ, [2] range of positive samples >LOQ in µg./kg., [3] amitraz metabolite: dimethylaniline, [4] amitraz metabolite: dimethylphenylformamide, [5] amitraz metabolite: dimethylphenyl-N-methylformamide, na: not available

Table 7. Risk factors for colony loss: results from the best explaining model for the 79 colonies.

Lost colonies and risk factors: most parsimonious model for 79 colonies						
Predicting variables	No. of positive cases	Odds Ratio (95% confidence interval)	Slope parameter	Std. Error	Z-test statistic	p value
Varroa mite rate Oct. 2011		1.17 (1.04-1.31)	0.155	0.060	2.585	0.01
Presence of acetamiprid or thiacloprid	30	5.24 (1.37-20.07)	1.887	0.686	2.415	0.018
Brassicaceae in bee bread	20	5.81 (1.34-25.13)	1.760	0.747	2.355	0.021
PC-2 random effects		6.60 (1.08-40.41)	1.656	0.925	2.040	0.042

Table 8. Summary results of model fitting: AIC = Akaike's Information Criterion, DF = Degrees of Freedom, Residual Deviance = -2 logLik; a low AIC, and low Residual Deviance indicate a better model.

Model	AIC	DF	Residual Deviance
Null (intercept only) model	95.459	78	93.459
Model with only acetamiprid/thiacloprid as a factor	91.792	77	87.792
Model with only *Brassicaceae* in bee bread as a factor	91.324	77	87.324
Model with only PC2 2012 random effects as a covariate	85.632	77	81.632
Model with only varroa mite rate (October) as a covariate	83.262	77	79.262
Full model with all 4 sign. factors	72.334	74	62.334

Table 9. Analysis of the best explaining model: DF = Degrees of Freedom for the term dropped, AIC = Akaike's Information Criterion, LRT=Likelihood Ratio Test statistic for the change in model fit and the p-value of the test.

Term dropped	Df	AIC	LRT	p-value
None		72.334		
Varroa mite rate	1	78.226	7.8918	0.004966**
Presence of thiacloprid or acetamiprid	1	76.906	6.5719	0.010360*
Brassicaceae in bee bread	1	76.362	6.0280	0.014081*
PC2 random effect from Monitor 2012	1	75.287	4.9531	0.026044*
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1				

Figure 1: Choropleth map showing the spatial variation in the postal code level random intercepts in a binomial GLMM allowing for significant fixed factors for beekeeper risk of colony loss in the Netherlands, and with a beekeeper random effect, for the 1 to 50 colony operations used in the model fitting. The data are derived from the Dutch Monitor for Honey Bee Winter Loss 2012. The legend in the figure shows the key to the colour coding of the random effects. Darker green indicates areas of lower risk and darker red areas of higher risk of loss. The value of the postal code random effect was used as a predictor in the final model of the data in the present study.

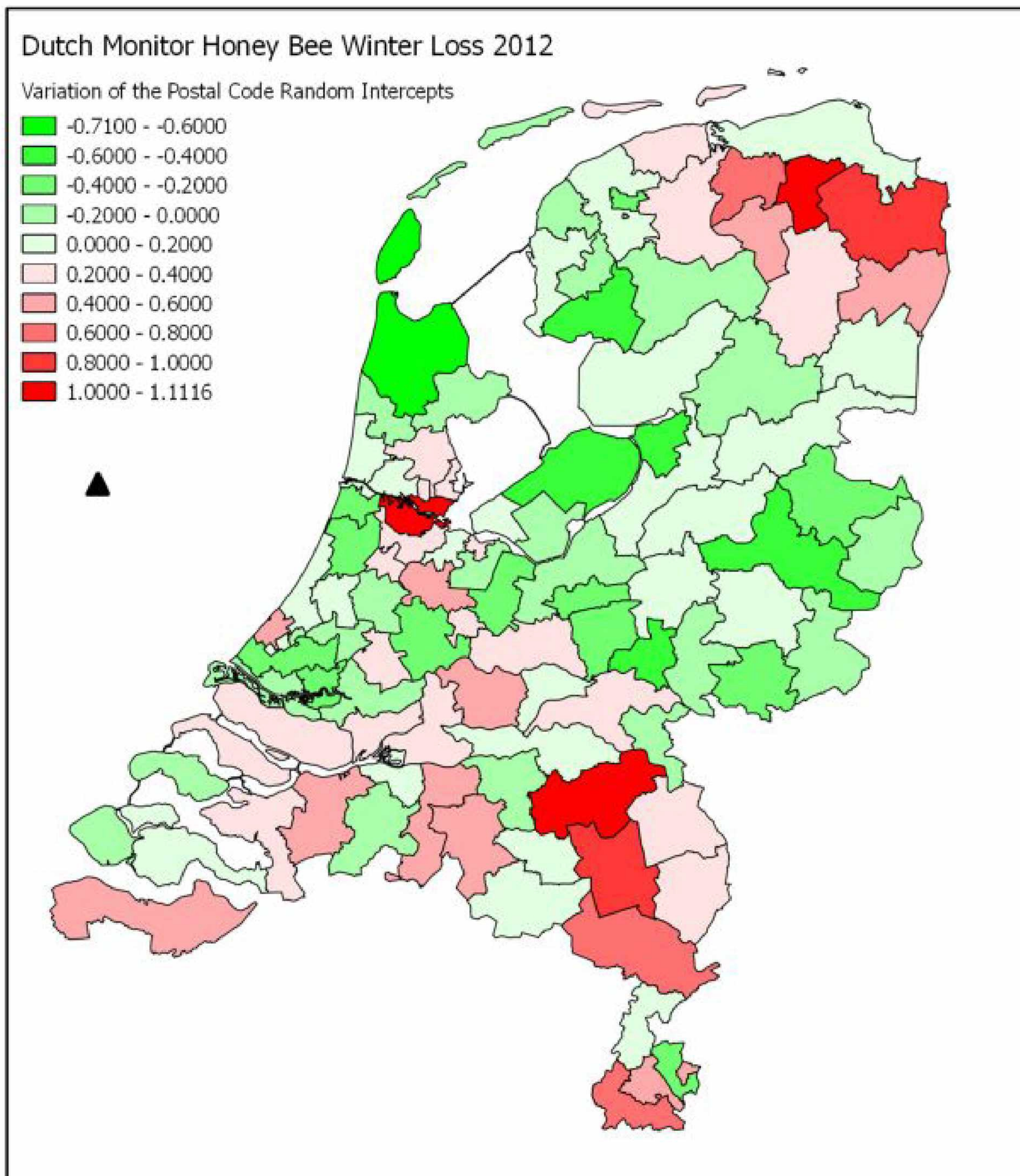

Figure 2: Odds Ratios from the best explaining model for the fixed model variables, with 95% confidence intervals. Fixed model terms are acetamiprid or thiacloprid present in summer 2011, brassicaceae present in bee bread in summer 2011, attributed postal code random effects 2011-2012 and percentage varroa mite load in October 2011. Baseline categories used were: no acetamiprid or thiacloprid present in honey, bee bread or bees and no *Brassicaceae* in bee bread.