Mill AC, Rushton SP, Shirley MDF, Smith GC, Mason P, Brown MA, Budge GE.
[Clustering, persistence and control of a pollinator brood disease: epidemiology of American foulbrood](link).
Environmental Microbiology 2014, 16(12), 3753-3763.

Copyright:

DOI link to article:

Date deposited:

22/03/2016

Clustering, persistence and control of a pollinator brood disease: epidemiology of American foulbrood

Aileen C. Mill,[1] Steven P. Rushton,[1]
Mark D. F. Shirley,[1] Graham C. Smith,[2] Phil Mason,[3]
Mike A. Brown[3] and Giles E. Budge[3]*

[1]*School of Biology, Newcastle University, Ridley Building, Newcastle Upon Tyne NE1 4RU, UK.*
[2]*Animal Health Veterinary Laboratories Agency, Sand Hutton, York YO41 1LZ, UK.*
[3]*Food and Environment Research Agency, Sand Hutton, York YO41 1LZ, UK.*

Summary

American foulbrood (AFB), caused by *Paenibacillus larvae*, is the most damaging bacterial brood disease of the honeybee (*Apis mellifera*), causing colony deaths on all continents where honeybees are managed. AFB has been a persistent problem in the UK for over 70 years, with a fluctuating number of cases discovered annually. Once diseased colonies are identified, they are destroyed to reduce pathogen spread. We investigated the pattern of AFB cases recorded over the period 1994 to 2012 using spatial-statistical approaches, with a view to identifying the nature of spread across England and Wales. Our results indicated that AFB exhibits significant spatial aggregation at distances from 10 to 30 km, with aggregations lasting between 1 and 5 years. Kernel smoothing indicated areas of elevated relative risk in different years, and these were further detailed by spatial-scan statistics. We identified disease clusters and successfully estimated their size, location and duration. The majority of clusters did not persist in all years, indicating that management measures may lead to localized extinction of the disease. Whilst less common, persistent clusters likely indicate potential endemic or exotic risk points. The application of robust epidemiological approaches to improve the control of AFB is discussed.

Introduction

Pollinators provide vital pollination services both to agriculture and to wild plant populations. The global value to

agriculture alone was estimated at €153 billion in 2005 (Gallai *et al.*, 2009). Managed pollinators are of particular value because they service the specific needs of crop production, supplement the natural bank of pollinators and yet can be moved to sites as part of husbandry regimes to facilitate pollination. The honeybee (*Apis mellifera*) can be regarded as the most important commercial pollinator in the world, responsible for at least 90% of commercial pollination (McGregor, 1976; Allsopp *et al.*, 2008). However, honeybee populations are threatened globally by a number of endemic and emerging diseases, and it is important to understand the epidemiological processes involved in pollinator disease to minimize the impact of disease on pollination service provision.

American foulbrood (AFB), caused by Gram-positive, spore-forming bacterium *Paenibacillus larvae*, is one of the most deleterious diseases of the honeybee and is a serious problem in global apiculture, causing substantial economic loss to beekeepers all over the world (Genersch, 2010). AFB affects the honeybee larvae and is usually lethal to the colony if left untreated (Hansen and Brodsgaard, 1999). The main mode of AFB transmission is horizontal (Fries and Camazine, 2001) via various bee behaviours, beekeeping practices and infected honey, but it is also known to be transmitted vertically as honeybees swarm (Fries *et al.*, 2006).

Maps of disease incidence over time suggest non-uniform patterns of AFB infection both temporally and spatially (Otten *et al.*, 1998; Wilkins *et al.*, 2007). Investigations in some German states showed that 6% of honey samples from randomly selected apiaries contained spores (4% at a low level and 2% at a high level), whereas 24% of samples from areas with a previous history of AFB contained them (6% at a low level and 18% at a high level), suggesting local dispersal of the pathogen and the possibility of symptomless infection (Otten, 2003). Contaminated honey has been recovered from diseased colonies 2–3 years before clinical symptoms were reported (von der Ohe, 2003), although given that 60% of German beekeepers do not recognize AFB (Otten, 2003), it is not clear whether this represents a failure to report or a long latent phase for the disease. It has been reported that strains of *P. larvae* show different abilities to induce symptoms in laboratory-infected larvae (Genersch *et al.*, 2006)

and that strain prevalence may vary regionally (Otten *et al.*, 1998; Pentikainen *et al.*, 2009). However, a lack of consistent approaches to classifying strains has restricted anything more than local epidemiological studies of *P. larvae* (Genersch, 2010). Experimental work on the epidemiological processes involved in disease transmission at the landscape scale in honeybee disease is practically impossible, yet there exists a need to understand the epidemiology of AFB if we are to develop methodologies for predicting the dynamics of disease and investigate the efficacy of disease control methods (Genersch, 2010).

Burning of diseased colonies and contaminated material is widely considered to be the most appropriate control measure (Genersch, 2010); however, approaches to treatment and control vary across the world. The instigation of an artificial swarm is common, where the frames containing the brood are destroyed and the adult bees are transferred to a new colony (Shimanuki and Knox, 1997b). Oxytetracycline has been used to treat AFB for more than 50 years, and use continues on some continents despite strong evidence that antibiotic resistance has developed in multiple pathogen haplotypes (Evans, 2003). Promoting hygienic behaviour (where adult bees remove dead larvae) through breeding has been shown to reduce disease prevalence in an experimental apiary (Spivak and Reuter, 2001), and candidate genes associated with this behaviour have been identified (Oxley *et al.*, 2010). Indeed, it has also been demonstrated that increased strain virulence at the larval level drives down colony-level virulence of *P. larvae* due to remediation of killed larvae by the hygienic behaviour of nurse bees (Rauch *et al.*, 2009).

The monitoring and control of AFB is under statutory control by a government-funded apiary inspection programme in England and Wales, operated by the National Bee Unit (NBU; http://www.nationalbeeunit.com). The inspection programme uses measures that seek to eradicate infection and any associated AFB spores by burning infected colonies and instigating strict biosecurity measures, such as the sterilization of beekeeping equipment (Wilkins *et al.*, 2007). AFB cases have fallen in recent decades, from over 2000 cases per annum in the 1950s to typically 100–200 cases per annum today (Wilkins *et al.*, 2007). The apiary inspection programme has been in existence in some form since 1942; however, apiary inspection data were not digitized until 1994, when data began being collated in the NBU database, BeeBase. These data now offer an unrivalled opportunity to monitor the seemingly sporadic incidence of AFB from over 400 000 honeybee colony inspections across 19 years.

This study set out to use extant data from the apiary inspection programme in England and Wales to examine the pattern and spread of AFB in order to understand whether AFB occurs spatially and temporally at random or whether patterns exist that may infer proximity to potential risk points that may represent disease sources. We use complementary spatial point process models to detect global clustering and characterize local clusters of AFB across England and Wales between 1994 and 2012 with the aim to improve our understanding of the epidemiology of this damaging disease.

Data on disease incidence in apiaries of registered beekeepers were obtained from BeeBase. All colonies testing positive for AFB or another bacterial brood disease, European foulbrood (EFB), and the geographical locations of apiaries sampled in England and Wales were collated for the period 1994–2012. Testing effort (the number of colonies inspected per apiary) and the number of positive cases were also recorded.

Results

Incidence of cases and controls

There were a total of 107 967 apiary inspections between 1994 and 2012; an average of 4.9 colonies were inspected per apiary (range 1–300). There were a total of 1144 cases of AFB recorded over the 19 year period at 819 unique apiary sites. These apiaries had a mean of 9 colonies (range 1–245). Apiaries with fewer colonies (< 10) were less likely to have disease than apiaries with more colonies (10–50 and > 50; AFB, $\chi2 = 151.70$, $df = 2$, $P < 0.001$; EFB, $\chi2 = 1837.012$, $df = 2$, $P < 0.001$). In contrast, there were a total of 7614 cases of EFB recorded over the study period from 3559 unique apiary sites. The overall incidence of AFB and EFB declined over the study period (Fig. 1).

Spatial clustering of AFB cases

Evidence was found that AFB shows a greater degree of spatial aggregation than EFB at distances of 10–20 km using both the 95% confidence envelope and the random labelling envelope methods (Fig. 2). The differences between the *K*-functions are outside the envelope in both cases, indicating significant clustering. At distances of greater than 23 km. the incidence of AFB becomes comparable to that of the control population of EFB cases.

Space–time K-*function.* Given that the bivariate K-function tests indicated significant clustering of AFB up to a distance of 23 km, the occurrence of space–time clustering at this scale was also investigated.

The space–time *K*-function showed significant evidence of clustering of AFB at distances of 10–30 km and over a 3 year period (Fig. 3a). The Monte Carlo significance test statistic (1760×10^9) was significantly greater than the random permutations (Fig. 3b).
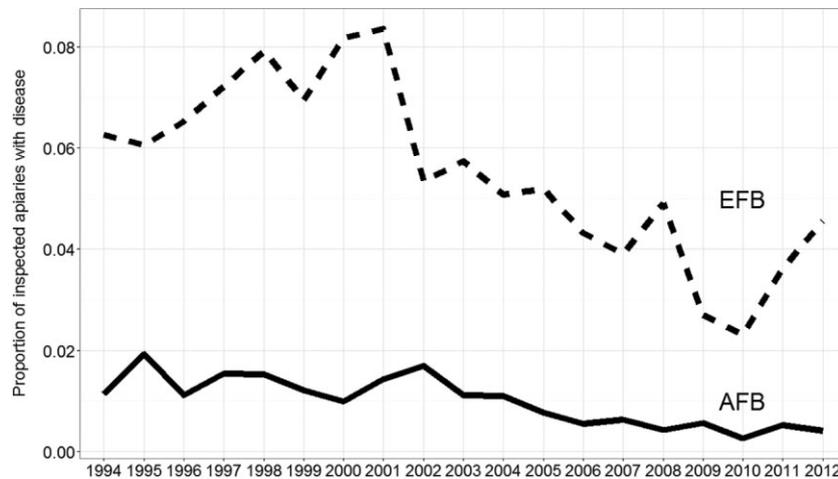
Fig. 1. Annual proportion of inspected apiaries that were found to have American foulbrood (AFB) or European foulbrood (EFB) in England and Wales from 1994 to 2012.

Cuzick–Edwards runs test. In total, 163 AFB clusters were identified with at least two cases, 57 had five or more members, and 19 had 10 or more (Fig. 4, filled circles). Clusters tended to persist over time, with 94 of the 163 clusters having cases in more than one year. The observed clustering pattern represented by T_{run} was highly significant: all 100 Monte Carlo simulations resulted in a T_{run} two orders of magnitude lower than that seen using the true data. The pattern appears to be one of sporadic infection and reinfection rather than a sustained outbreak over multiple years.
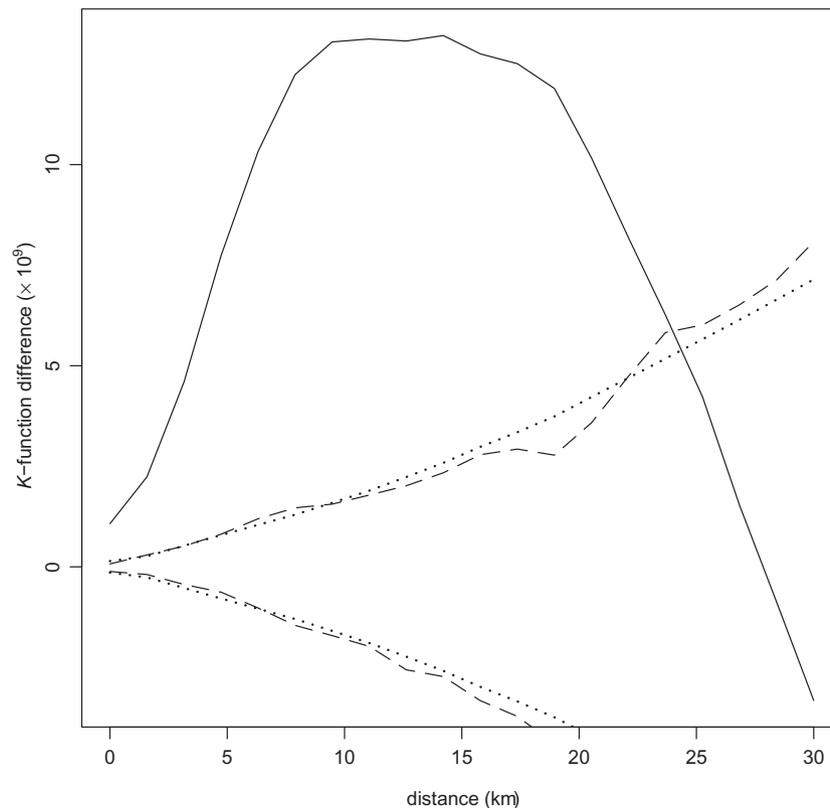

Fig. 2. K-function difference between American foulbrood (AFB) and the background population (European foulbrood) in England and Wales (solid line). Clear evidence of significant AFB clusters, demonstrated by the solid line rising above the 95% confidence envelopes (dashed lines) and relabelling envelopes (dotted lines).
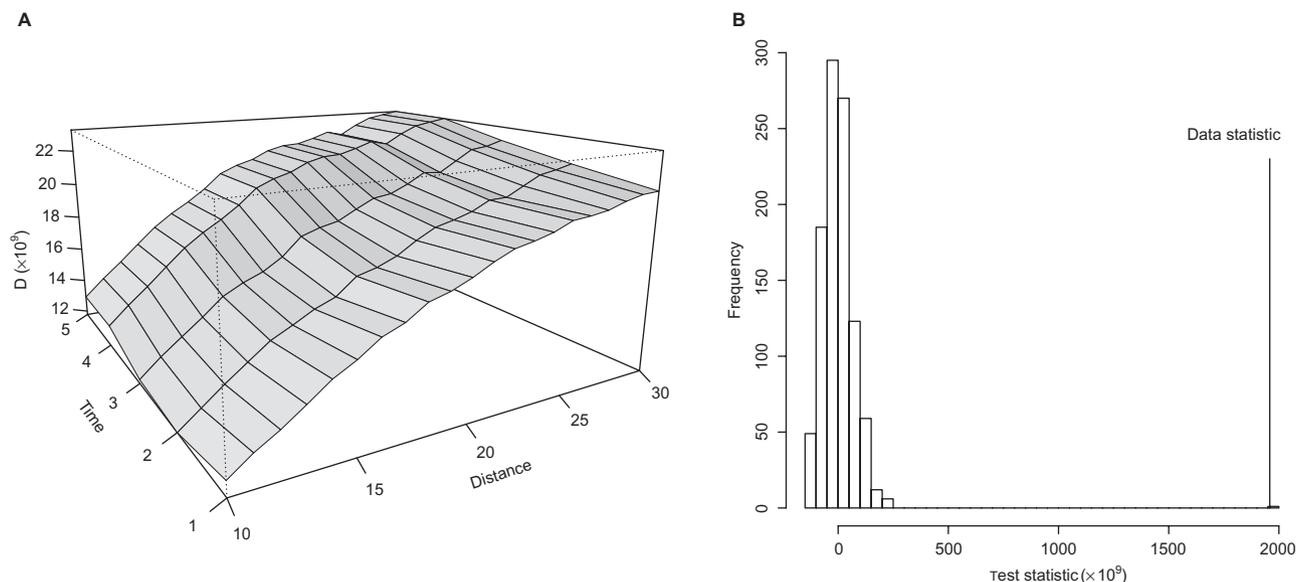
A

B

Fig. 3. Space–time clustering of AFB using *K*-function tests.
A. Three-dimensional plot of the D(s,t) function. High values on the z axis indicate there are more outbreaks within the given spatial and temporal separation than would be expected if there were no clustering.
B. Monte Carlo significance test of a space–time interaction. The bold line indicates the data statistic, which is larger than that of all of the Monte Carlo samples, indicating significant space–time clustering.

Space–time clustering in SATSCAN. The spatial scan identified the most significant cluster and 30 other significant clusters, where likelihood of AFB was greater than background at-risk population. SATSCAN identified 697 records of AFB (61%) that were associated with a significant cluster. These clusters varied in time and space, size and duration (Fig. 4, Table 1).

The most likely cluster (ID = 1) was located in the South East and was small in size (9.7 km radius) and had 86 cases over an 8 year period. The next most likely clusters were larger in area (12–20 km) but varied in duration and number of AFB cases. Cluster ID 1 appeared to be a cluster with recurrence or latent infection at the same apiaries, because the number of infected apiaries was lower than the number of cases observed over the time period. Other clusters (e.g. ID 2–5) appeared not to have high recurrence rates, as the number of apiaries and number of cases were similar.

Spatial scan and runs test clusters. Clusters identified by the space–time scan in SATSCAN correlated well with those identified by the runs test method (Fig. 4). These methodologies differ in their cluster identification methodologies, yet they identified similar clusters. Although there is disagreement in the total number and size of identified clusters, both the methods overlap in identification of cluster occurrence. In some cases, SATSCAN detects a single large cluster in space and time, whereas the runs test identifies multiple smaller clusters that are more geo-

graphically isolated within the SATSCAN cluster. There were only a few runs test clusters with five or more cases that were not identified as significant SATSCAN clusters. The SATSCAN clusters give a likely time domain to the AFB cluster observed, which cannot be determined from the runs test method. However, the space–time cluster method does not report repeat clusters occurring at the same geographic location at a different time period; only the most likely cluster at that location is identified.

Kernel density relative risk. The relative risk maps of the kernel-smoothed density show general areas of elevated risk in each year. The red and orange areas are at increased relative risk of AFB, and the yellow and white areas are at low risk (Fig. 5). Significant clusters of increased risk are indicated by contour *P* values of 0.05 and 0.01. In general, AFB risk across the whole region was lower in 2003 compared with other years. Most years had between three and six discrete clusters of AFB, with locations at increased risk varying between years (Fig. 5). An animation of these colour contour maps is available as (Supporting Information Fig. S1).

Discussion

Reports of the local reoccurrence of AFB have taken place over several centuries (Rocca, 1790; Pentikainen *et al.*, 2009); however, the absence of comprehensive, spatially explicit disease distribution data has prevented
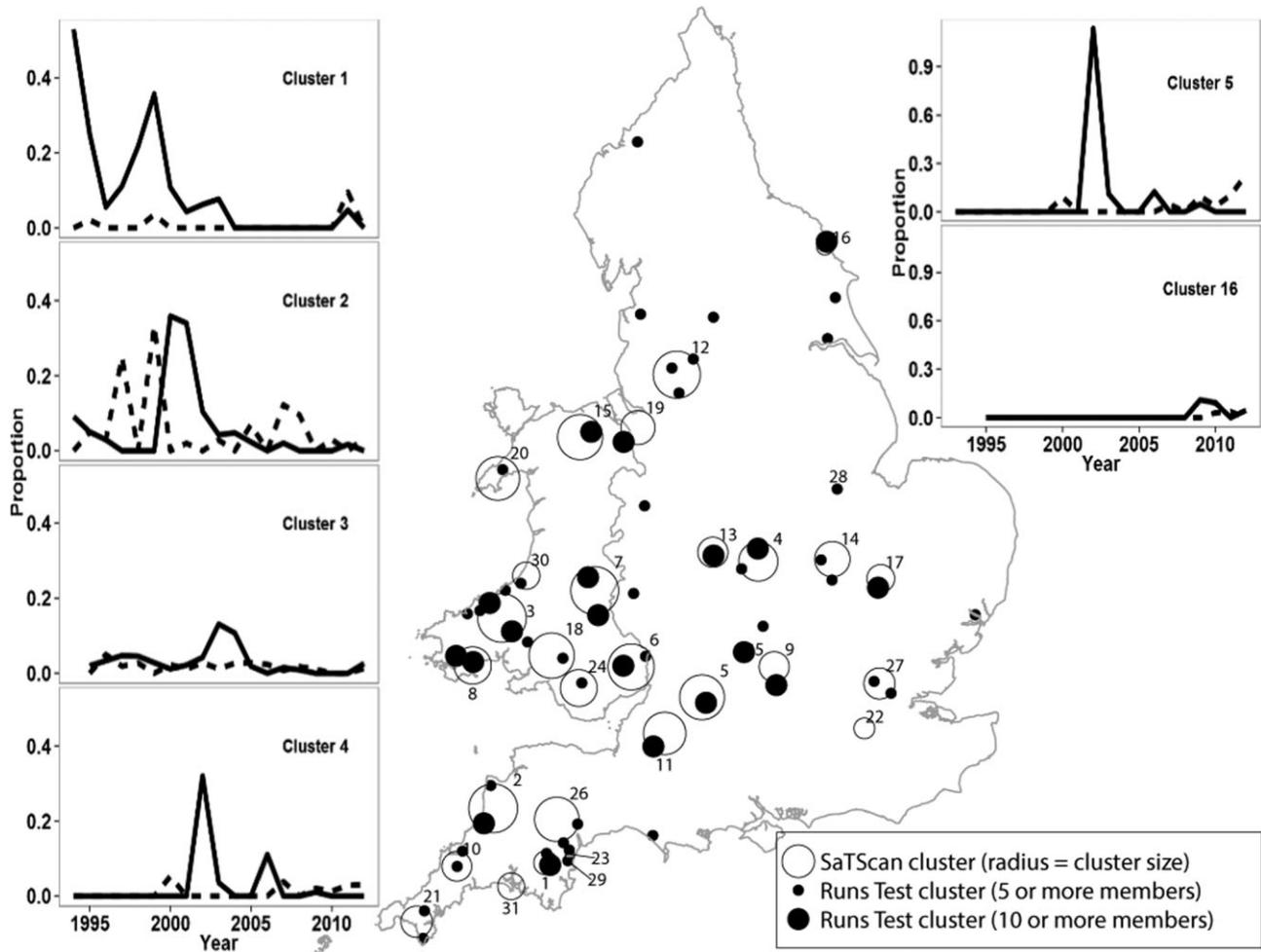
Fig. 4. Correlation between runs test clusters (filled circles; large, ≥ 10 cases and small, ≥ 5 cases) and significant SATSCAN clusters (open circles of approximate radius). Numbers refer to cluster rank 1 being the most likely cluster. Clusters presented are for all year combinations; a particular cluster may not have been in that location for all years (see Table 1 for full description of each cluster). Inset graphs show disease intensity of American (solid line) and European (dashed line) foulbrood in numbered clusters over time.

full understanding of the epidemic status of this disease. For the first time we demonstrate that AFB shows significant spatial and temporal clustering related to epidemiological processes across England and Wales. Furthermore, we quantify cluster sizes, measure frequency of occurrence and even observe possible localized extinction to provide new and important information about the epidemiology and control of this damaging pollinator disease.

The methodologies used in the current study have advanced the field of honeybee pathology beyond repeated incidental observations of apparent clustering to epidemiologically significant inference. The most informative works currently in the literature (Gensersch & Otten, 2003; Peters *et al*. 2006; Pentikainen *et al*., 2009) all infer that strains of *P. larvae* may differ between different geographical locations; they do not, however, confirm disease clustering in any formal epidemiological sense as they are

not based on rigorous sampling of the population at risk. Understanding clustering is essential to assess the extent to which disease risk is dependent on apiary density, husbandry practices or environmental factors.

Clusters occurred at a peak of 10–20 km, providing strong evidence of local between-apiary spread facilitated by honeybee or beekeeping behaviours. Honeybee colonies can produce daughter colonies by splitting to produce a swarm. Swarms tend to disperse locally, often within a few hundred metres of the parent colony (Seeley and Morse, 1977), and have the potential to explain some local spread. However, such vertical transmission events rarely result in clinically diseased daughter colonies (Fries *et al*., 2006), and because swarming is initiated as a response to overcrowding, heavily diseased colonies are less likely to swarm, suggesting this route of local AFB spread is unlikely to be of major importance (Fries and Camazine, 2001). The honeybee behaviours of drifting

Table 1. Space–time clusters of American foulbrood in England and Wales from 1994 to 2012 identified by SaTScan.

Cluster ID	Radius (km)	Start year	End year	Number of apiaries in cluster	Test statistic	P value of cluster	Observed number of cases	Expected number of cases	Ratio of observed to expected cases	Relative risk ratio
1	9.7	1994	2002	31	168.65	< 0.001	86	11.5	7.48	8.01
2	19.9	2000	2004	59	103.73	< 0.001	56	7.71	7.27	7.59
3	19.8	1997	2004	55	80.18	< 0.001	55	9.01	6.1	6.36
4	16.0	2002	2006	39	63.46	< 0.001	31	4.05	7.65	7.84
5	18.3	1997	2005	21	47.01	< 0.001	23	3	7.65	7.79
6	18.8	1996	2004	34	45.01	< 0.001	24	3.27	7.35	7.48
7	19.5	2000	2006	39	44.13	< 0.001	29	4.57	6.34	6.48
8	15.2	2005	2012	16	40.99	< 0.001	22	3	7.32	7.45
9	12.3	1994	2002	33	36.98	< 0.001	20	2.74	7.29	7.4
10	12.1	2003	2011	19	34.71	< 0.001	17	2.22	7.65	7.76
11	17.3	1994	1996	37	29.00	< 0.001	16	2.22	7.2	7.29
12	19.2	1997	2002	26	28.73	< 0.001	19	3	6.32	6.41
13	12.3	1995	2000	10	26.52	< 0.001	13	1.7	7.65	7.73
14	14.4	2006	2011	10	26.52	< 0.001	13	1.7	7.65	7.73
15	18.4	1994	1999	20	26.52	< 0.001	13	1.7	7.65	7.73
16	6.6	2009	2012	11	24.73	< 0.001	15	2.22	6.75	6.83
17	11.3	2001	2009	14	24.48	< 0.001	12	1.57	7.65	7.73
18	18.5	2003	2009	17	21.09	< 0.001	12	1.7	7.07	7.13
19	13.9	1997	2002	13	20.39	< 0.001	10	1.31	7.65	7.71
20	17.8	2002	2005	6	18.35	< 0.001	9	1.18	7.65	7.71
21	12.8	2006	2012	27	17.82	0.001	15	2.87	5.22	5.28
22	8.5	1994	1995	28	17.39	0.002	12	1.96	6.12	6.18
23	3.8	2007	2012	11	16.31	0.004	8	1.05	7.65	7.7
24	15.0	1994	1995	24	16.31	0.004	8	1.05	7.65	7.7
25	4.8	1994	1995	12	15.26	0.010	10	1.57	6.38	6.43
26	18.1	1997	1999	71	15.26	0.010	10	1.57	6.38	6.43
27	12.7	1997	1998	14	15.24	0.011	9	1.31	6.89	6.94
28	2.0	2003	2005	2	14.27	0.034	7	0.91	7.65	7.7
29	0.7	1995	1996	4	14.27	0.034	7	0.91	7.65	7.7
30	11.1	2001	2009	5	14.27	0.034	7	0.91	7.65	7.7
31	10.9	2001	2008	10	14.27	0.034	7	0.91	7.65	7.7

The cluster ID cross-references with locations in Fig. 4.

(where adult bees move between colonies) and robbing (where adult bees steal honey from weaker colonies) are more likely to be important for local, horizontal transmission of AFB.

Drifting is a common occurrence in apiaries; its intensity is influenced by colony arrangement, hive colour and environmental factors (Pfeiffer and Crailsheim, 1998). Hornitzky (1998) reported no apparent risk of disease spread by drifting bees, whereas Goodwin and colleagues (1994) reported an AFB transmission rate to 8% of neighbouring healthy colonies when the quantity of drifting was low. Robbing is considered to be of major importance for local AFB transmission (Shimanuki and Knox, 1997a) and occurs when foragers from a strong colony remove honey from a weaker colony. The likelihood of a colony being robbed increases as it is weakened by disease, and the resilient spores of *P. larvae* are frequently found to be viable in the honey from diseased colonies (de Graaf *et al.*, 2001). The quantity of viable *P. larvae* spores is directly related to the disease intensity within a beekeeping operation (Pernal and Melathopoulos, 2006). Furthermore, when AFB-killed colonies were allowed to be robbed, disease was readily transmitted to the robbing

colonies less than 1 km away, and spores were detected in colonies at greater distances (Lindstroem, 2008). The honeybee behaviour of robbing can therefore be seen as an important contributory factor in the localized spread of AFB.

The observed pattern of disease must be linked to bee management by humans, which operate across different spatial scales reflecting apiary ownership. Cluster size and the association of AFB with larger apiaries could reflect the activity of local beekeeping networks. Exchanging material between colonies and managing numerous colonies in a confined area are known to facilitate disease spread (Genersch, 2010). Reoccurrence of AFB within the same beekeeping operation and long-distance transmission of AFB through apicultural practices have both been demonstrated by typing *P. larvae* isolates (Pentikainen *et al.*, 2009). The apiculture sector in the UK is dominated by amateur beekeepers who manage an average of only five colonies, often on a single apiary site close to their home (Wilkins *et al.*, 2007). Single-beekeeper apiaries must have a lower chance of becoming diseased than multi-beekeeper apiaries. Larger apiaries frequently belong to one of the few professional beekeepers or to
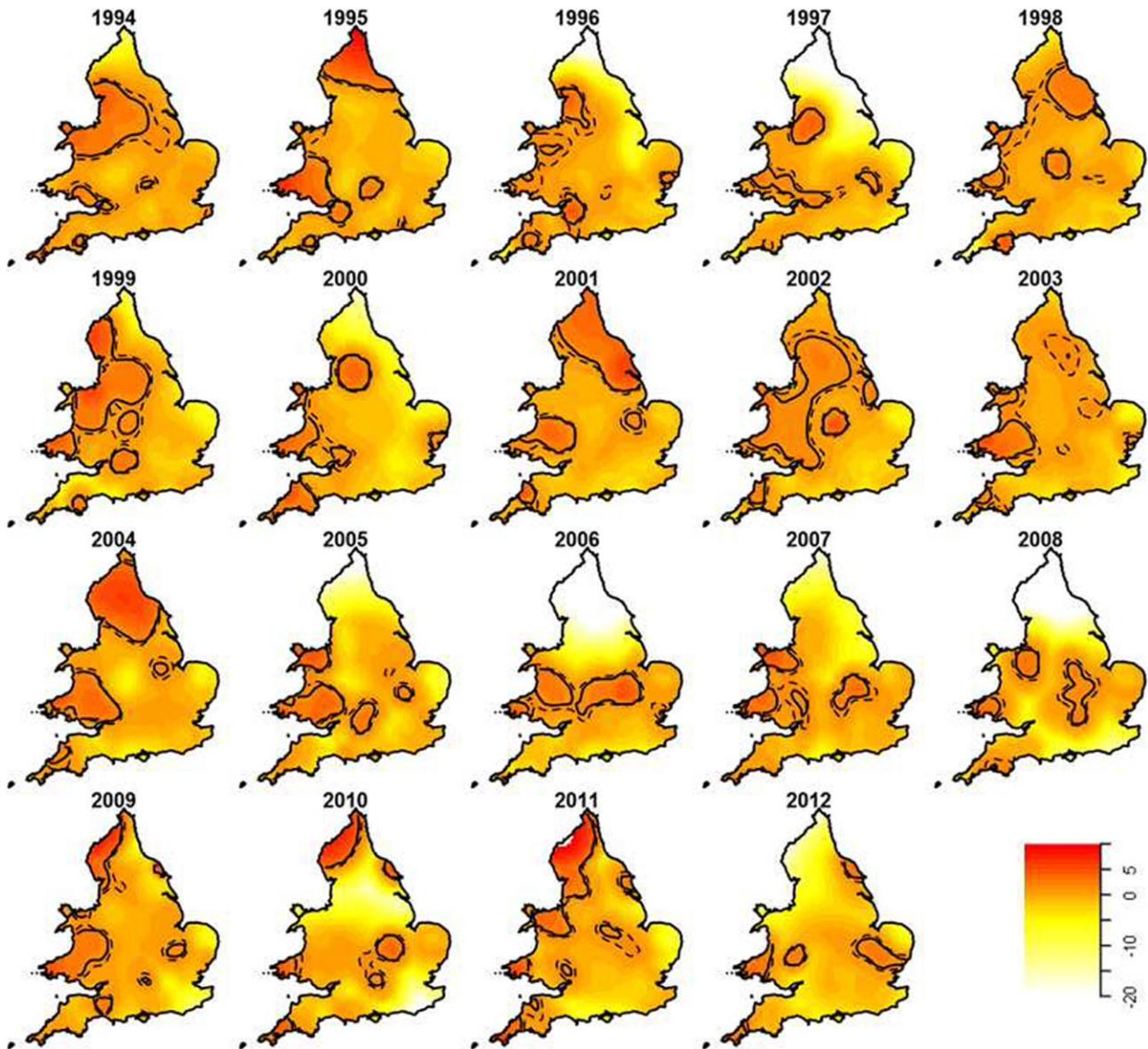
Fig. 5. Kernel density relative risk by year (1994–2012); increased-risk areas are shown in red, whereas low-risk areas are white/yellow. Tolerance *P* values of 0.05 (dashed line) and 0.01 (solid line) are shown as contours.

enthusiastic amateurs who collaborate to form larger shared apiary sites. Larger apiaries in the UK are likely exposed to increased disease risk by either sharing ownership (large amateur sites) or having multiple apiaries (locations) within the same operation (professional apiaries). The important observation that larger apiary sites are more likely to suffer from AFB will inform training and education efforts, which should concentrate on the owners of such sites to reduce future disease risk.

Crucially, 87.5% of disease clusters failed to persist (Table 1). This likely reflects the prolonged effort of a risk-based inspection programme. The programme design explicitly accounts for colony ownership and proximity to previous known cases. Whilst it is known that colonies can maintain low levels of spores for several years without developing clinical disease symptoms (Hansen and Rasmussen, 1986; Fries *et al.*, 2006) and viable spores can persist for long periods (Hasemann, 1961), some disease clusters were last observed 17 years ago, providing good evidence that the activities of the disease management programme have likely eradicated AFB from these areas. The ability to eradicate disease locally suggests that a sustained surveillance effort focused on previous disease can overcome the mobility of this host and pathogen combination.

The extent to which the pathogen might go undetected both within an apiary and in those adjacent remains unknown. Current inspection policy is to maintain vigilance in an area affected by disease for 3 years after the last remaining case was reported. The space–time clustering of AFB using *K*-function tests (Fig. 3) suggests that 3 years is appropriate. Some disease persisted for 8 years; this highlights two points of consideration for control programmes: Firstly, no monitoring strategy is guaranteed to be 100% successful, and there will be some failure to detect disease (Table 1); secondly, the implementation of control is based on the observation of clinical symptoms, but colonies can maintain low levels of *P. larvae* spores for several years and remain asymptomatic (Hansen and Rasmussen, 1986; Fries *et al.*, 2006). It is therefore likely that successful control will only result from sustained surveillance efforts that extend well beyond the longest latent period.

Whilst local spread of AFB may explain the spatial aspects of disease clustering, it does not explain cluster location or timing, which appears to be sporadic. The global trade in honeybee products can facilitate long-distance movement of *P. larvae*-infected material (Genersch, 2010). The UK is not self-sufficient in honey or apiary products such as beeswax, and over the last 30 years there has been an expansion in importations of hive products and honey. UK beekeepers also import a vast number of queens and caged bees from Europe and third countries. Whilst this material is screened for clinical symptoms at dispatch, no diagnostic procedures are used to detect the hardy infectious endospore of *P. larvae*. Movements of infected honeybee stocks within the UK might also account for such disease movements. Whilst migratory beekeeping, where hives are moved from one location to another, is not common for the predominantly amateur UK beekeeping community, sales of stocks occur readily. Given the low level of disease, such movements of infected material would likely be rare. It is therefore possible that the haphazard importation of apiary products and within-territory movement of infected material may cause the foci of AFB clusters, leading to the observed sporadic pattern of outbreaks. An internationally accepted typing scheme to characterize *P. larvae* would help to determine whether the disease clusters within England and Wales are related to each other or more related to international pathogen populations. Such a method should be developed as a priority in order to help scientists understand the importance of importation in AFB aetiology.

Persistent clusters occurring in the same location represent a particularly interesting observation with clear management implications. BeeBase is a voluntary register of beekeepers and as such does not contain a complete list of apiary locations. Persistent clusters could represent repeated escapes from non-registered beekeepers into the registered population. Alternatively, business premises that receive a constant supply of imported hive products (in particular honey) such as restaurants or honey importers could also explain the persistent clusters. A comprehensive screen of import risk pathways could be conducted to determine whether the presence of such industries tallies with those regions exhibiting persistent AFB clusters.

Our results clearly indicate the occurrence of local disease clusters of AFB in England and Wales over the last 19 years and provide important new insight into AFB epidemiology. Future work will concentrate on understanding the risk associated with disease cluster location and the development of typing methods for *P. larvae* to help identify the extent of local epidemics.

Experimental procedures

Data

Sampling. The NBU operates a combined prioritized risk-based inspection protocol for the control of AFB and EFB. Each apiary within BeeBase is allocated a different class of risk based on proximity to previously known cases of disease or known exotic pest risk points (e.g. shipping ports or airports). Apiaries are designated as high-risk if they are within 3 km of a focal case of disease and/or within 10 km of a known exotic risk point, medium-risk if they are between 3 and 10 km from a focal case of disease, and low-risk if they are further than 10 km from any known disease or exotic risk point.

Disease risk classifications are retained for 3 years after the initial report of disease, and the discovery of new disease cases or exotic pest risk points leads to a modification of the risk designation. The discovery of disease raises the risk to high for all other apiaries possessed by the same owner. Given the large number of apiaries in England and Wales (approximately 30 000), 40% of known high- and medium-risk apiaries were inspected.

Clinical disease was confirmed after visual identification combined with a positive lateral flow test and/or microscopic confirmation using published protocols (Wilkins *et al.*, 2007; Tomkies *et al.*, 2009).

Case–control data. All of the clustering and cluster detection methods that we explore require knowledge of a background population or control group. Complete census data are not available for apiaries in England and Wales, so the true background population is not known. Commonly in epidemiological studies of this nature, a group of controls is selected from the population at risk by random allocation of a similar disease (Diggle *et al.*, 2007). BeeBase data show that apiary distribution is not uniform in space. Apiaries that tested positive for EFB were selected as controls because, although the disease aetiology differs, EFB is subject to the same non-random stratified inspection regime as AFB. EFB is endemic in the UK and, over the same time frame, was recorded at 13.5% ($n = 3500$) of known apiary locations in England and

Wales, giving a good approximation of the background apiary population distribution.

All colony disease data were transposed into binomial apiary level data [i.e. 1–9 positive colonies present (1) or no positive colonies (0)]. The data were summarized to the locations of positive AFB tests (cases) and the locations of positive EFB tests (controls) over the 19 year period.

Analysis of spatial clustering

K-*function.* First, a bivariate *K*-function test (Ripley, 1977) was used to estimate the extent to which cases of AFB were clustered in space. We then went on to investigate spatio-temporal clustering, using the spatial range identified from the first analyses as a guide. The *K*-function, $K(s)$, is defined as the expected number of events within a given distance, *s*, of an arbitrary event (Diggle and Chetwynd, 1991). The bivariate *K*-function method estimates the spatial dependence of the two diseases within a region, identifying distances within which spatial dependence is evident. The significance of clustering was tested relative to expected distribution of points if there was no spatial dependence. Simulation envelopes, used to assess the peaks of significance, were calculated in two ways: firstly as 95% confidence intervals (the standard error of the differences between the $K(s)$ cases and $K(s)$ controls) and secondly by a random permutation of cases and controls.

To consider clustering in both time and space, the *K*-function was extended to $K(s,t)$, which is defined as the expected number of events within distance *s* and time *t* of an arbitrary event. The $K(s,t)$ can be calculated for any range of time and space over the upper and lower limits of the data set; however, this can be computationally demanding for large data sets collected at fine scales.

Analysis of the data for clusters occurring over larger distances may be influenced by edge effects, where the spatial distance approaches the magnitude of the geographic range of the data. An upper limit of 33% of the ranges of *s* and *t* was selected to ensure that the edge correction factors did not become unbounded. The lower limit of the spatial range was set to 10 km, as clusters detected between 0 and 10 km may be an effect of the testing regime.

Analyses were undertaken over a spatial range of 0–30 km in 1 km increments and over a time range of 1–19 years in 1 year increments. Significance testing of clustering was carried out using 999 randomizations in each test, giving significance values to three significant figures. To take account of edge effects, we defined all apiaries within a convex polygon enclosing all points. We used the *splancs* package (Rowlingson and Diggle, 1993) in the public-domain statistical package R (R Development Core Team, 2012) to analyse the spatial aspects of disease clustering within the time and spatial domains of the dataset.

Cuzick-Edwards. The Cuzick–Edwards analysis of nearest neighbours (Cuzick and Edwards, 1990) was used to investigate the extent to which cases of AFB were clustered spatially, relative to the known disposition of apiaries reported as having EFB. The Cuzick–Edwards runs test takes the ordered sequence of proximity to each case or control in turn and records the number of consecutive cases from the index

location, with the expectation that a cluster will have more consecutive cases than controls. The test statistic T_{run} is the sum of each run of cases over all cases or controls.

Significance of T_{run} was determined using a Monte Carlo approach. Cases and controls were randomly reassigned 100 times, and the test statistic was recalculated each time. The number of occasions where the calculated statistic for the real cases was higher than the randomly permuted statistic was recorded.

The sequences of consecutive cases from other cases were used to build a network of cases based on 'hot spots' identified using the Cuzick–Edwards methodology. First, each sequence was curtailed by restricting the maximum distance between case-pairs to an arbitrary distance of 20 km. All remaining case-pairs formed the links (or 'edges') of a network, while the cases themselves formed the nodes (or 'vertices'). Clusters were identified from the resultant network by identifying maximal connected components of the network (Newman, 2003), i.e. components of the network from which it was possible to reach some vertices but not others. These fragments of the network were clusters of the presence of AFB in the landscape, though independent of time. All network operations were performed in R using the 'igraph' package (Csárdi and Nepusz, 2006).

Detection of local clusters.

Spatial scan statistic. We used Kulldorff's spatial scan statistic, implemented in the SATSCAN software program (Version 9.1.1; www.satscan.org) to test for local clusters in time and space (Kulldorff, 1997). For case–control data SATSCAN uses a Bernoulli model to calculate local rates within circular scans of various sizes, ranging from the smallest interpoint distance to a user-defined maximum. This method is extended to include a temporal element by adding a height to the circular base to correspond to time (Kulldorff, 1997). This cylindrical window is moved though time and space to identify potential clusters. Each potential cluster is tested with a likelihood ratio test, assuming the alternative hypothesis that there is elevated risk within the window as compared to outside. The likelihood function is maximized across all window locations and sizes, and the one with the maximum likelihood constitutes the most likely cluster, i.e. the least likely cluster to occur by chance. *P* Values are generated using Monte Carlo hypothesis testing. Subsequent likely clusters are identified according to their likelihood ratio statistic. No spatially overlapping clusters were reported as these would be numerous and very similar. To avoid detection of artificially large clusters the scan size was limited to a maximum of 20 km and up to 33% of the temporal extent of the data frame in the first instance.

Kernel density relative risk. The spatial variation of disease risk was estimated using kernel-smoothed relative risk functions for case–control data (Kelsall and Diggle, 1995). Kernel smoothing is used to estimate the density of case and control data and then the log relative risk for a given location on a grid. When all points on a grid are considered this gives a surface relative risk. The kernel function requires a bandwidth to determine the kernel size and resulting smoothness. We used a Gaussian bivariate kernel in the 'sparr' package in R (Davies *et al.*, 2011). Bandwidth was determined from the

pooled data set, and smoothing was carried out over a 100×100 grid over the study area. Pointwise *P* values are estimated by asymptotic theory (Hazelton and Davies, 2009) and are displayed on risk maps as tolerance contours.

Acknowledgements

References

Allsopp, M.H., de Lange, W.J., and Veldtman, R. (2008) Valuing insect pollination services with cost of replacement. *PLoS ONE* **3:** 9.

Csárdi, G., and Nepusz, T. (2006) The *igraph* software package for complex network research. *Intern J Complex Syst* 1695.

Cuzick, J., and Edwards, R. (1990) Spatial clustering for inhomogeneous populations. *J R Stat Soc Ser B Methodol* **52:** 73–104.

Davies, T.M., Hazelton, M.L., and Marshall, J.C. (2011) Sparr: analyzing spatial relative risk using fixed and adaptive kernel density estimation in R. *J Stat Softw* **39:** 1–14.

Diggle, P.J., and Chetwynd, A.G. (1991) 2nd-Order analysis of spatial clustering for inhomogeneous populations. *Biometrics* **47:** 1155–1163.

Diggle, P.J., Gomez-Rubio, V., Brown, P.E., Chetwynd, A.G., and Gooding, S. (2007) Second-order analysis of inhomogeneous spatial point processes using case-control data. *Biometrics* **63:** 550–557.

Evans, J.D. (2003) Diverse origins of tetracycline resistance in the honey bee bacterial pathogen *Paenibacillus larvae*. *J Invertebr Pathol* **83:** 46–50.

Fries, I., and Camazine, S. (2001) Implications of horizontal and vertical pathogen transmission for honey bee epidemiology. *Apidologie* **32:** 199–214.

Fries, I., Lindstrom, A., and Korpela, S. (2006) Vertical transmission of American foulbrood (*Paenibacillus larvae*) in honey bees (*Apis mellifera*). *Vet Microbiol* **114:** 269–274.

Gallai, N., Salles, J.M., Settele, J., and Vaissiere, B.E. (2009) Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecol Econ* **68:** 810–821.

Genersch, E. (2010) American foulbrood in honeybees and its causative agent, *Paenibacillus larvae*. *J Invertebr Pathol* **103:** S10–S19.

Genersch, E., and Otten, C. (2003) The use of repetitive element PCR fingerprinting (rep-PCR) for genetic subtyping of German field isolates of *Paenibacillus larvae* subsp larvae. *Apidologie* **34:** 195–206.

Genersch, E., Forsgren, E., Pentikainen, J., Ashiralieva, A., Rauch, S., Kilwinski, J., and Fries, I. (2006) Reclassifica-tion of *Paenibacillus larvae* subsp. *pulvifaciens* and *Paenibacillus larvae* subsp. *larvae* as *Paenibacillus larvae* without subspecies differentiation. *Int J Syst Evol Microbiol* **56:** 501–511.

Goodwin, R.M., Perry, J.H., and Tenhouten, A. (1994) The effect of drifting honey-bees on the spread of American foulbrood infections. *J Apic Res* **33:** 209–212.

de Graaf, D.C., Vandekerchove, D., Dobbelaere, W., Peeters, J.E., and Jacobs, F.J. (2001) Influence of the proximity of American foulbrood cases and apicultural management on the prevalence of *Paenibacillus larvae* spores in Belgian honey. *Apidologie* **32:** 587–599.

Hansen, H., and Brodsgaard, C.J. (1999) American foulbrood: a review of its biology, diagnosis and control. *Bee World* **80:** 5–23.

Hansen, H., and Rasmussen, B. (1986) The investigation of honey from bee colonies for *Bacillus larvae*. *Dan J Plant Soil Sci* **90:** 81–86.

Hasemann, L. (1961) How long can spores of American foulbrood live? *Am Bee J* **101:** 298–299.

Hazelton, M.L., and Davies, T.M. (2009) Inference based on kernel estimates of the relative risk function in geographical epidemiology. *Biom J* **51:** 98–109.

Hornitzky, M.A.Z. (1998) The spread of *Paenibacillus larvae* subsp *larvae* infections in an apiary. *J Apic Res* **37:** 261–265.

Kelsall, J.E., and Diggle, P.J. (1995) Kernel estimation of relative risk. *Bernoulli* **1:** 3–16.

Kulldorff, M. (1997) A spatial scan statistic. *Commun Stat Theory Methods* **26:** 1481–1496.

Lindstroem, A. (2008) Distribution of *Paenibacillus larvae* spores among adult honey bees (*Apis mellifera*) and the relationship with clinical symptoms of American foulbrood. *Microb Ecol* **56:** 253–259.

McGregor, S. (1976) *Insect Pollination of Cultivated crop Plants*. Washington, DC, USA: USDA.

Newman, M.E.J. (2003) The structure and function of complex networks. *Siam Rev* **45:** 167–256.

von der Ohe, W. (2003) Control of American Foulbrood by using alternatively eradication method and artificial swarms. *Apiacta* **38:** 137–139.

Otten, C. (2003) A general overview on AFB and EFB pathogen, way of infection, multiplication, clinical symptoms and outbreak. *Apiacta* **38:** 106–113.

Otten, C., Reiche, R., Martin, K., and Otto, A. (1998) Comparative studies on *Paenibacillus larvae* subsp. *larvae* of different origin. *Apidologie* **29:** 419.

Oxley, P.R., Spivak, M., and Oldroyd, B.P. (2010) Six quantitative trait loci influence task thresholds for hygienic behaviour in honeybees (*Apis mellifera*). *Mol Ecol* **19:** 1452–1461.

Pentikainen, J., Kalliainen, E., and Pelkonen, S. (2009) Molecular epidemiology of *Paenibacillus larvae* infection in Finland. *Apidologie* **40:** 73–81.

Pernal, S.F., and Melathopoulos, A.P. (2006) Monitoring for American foulbrood spores from honey and bee samples in Canada. *Apiacta* **41:** 99–109.

Peters, M., Kilwinski, J., Beringhoff, A., Reckling, D., and Genersch, E. (2006) American foulbrood of the honey bee: occurrence and distribution of different genotypes of *Paenibacillus larvae* in the administrative district of

Arnsberg (North Rhine-Westphalia). *J Vet Med B Infect Dis Vet Public Health* **53:** 100–104.

Pfeiffer, K.J., and Crailsheim, K. (1998) Drifting of honeybees. *Insectes Sociaux* **45:** 151–167.

R Development Core Team (2012) *A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing. ISBN 3-900051-07-0, [WWW document]. URL http://www.R-project.org/.

Rauch, S., Ashiralieva, A., Hedtke, K., and Genersch, E. (2009) Negative correlation between individual-insect-level virulence and colony-level virulence of *Paenibacillus larvae*, the etiological agent of American foulbrood of honeybees. *Appl Environ Microbiol* **75:** 3344–3347.

Ripley, B.D. (1977) Modelling spatial patterns. *J R Stat Soc Series B-Methodological* **39:** 193–195.

Rocca, D. (1790) *Traité complet sur les abeilles [A Complete Summary of Bees]*. Paris, France: Chez Bleuet.

Rowlingson, B.S., and Diggle, P.J. (1993) Splancs – spatial point pattern-analysis code in s-plus. *Comput Geosci* **19:** 627–655.

Seeley, T.D., and Morse, R.A. (1977) Dispersal behavior of honey bee swarms. *Psyche (Stuttg)* **84:** 199–209.

Shimanuki, H., and Knox, D.A. (1997a) Bee health and international trade. *Rev Sci Tech* **16:** 172–176.

Shimanuki, H., and Knox, D.A. (1997b) Summary of control methods. In *Honey Bee Pests, Predators and Diseases*, Morse, R.A., and Flottum, K. (eds). Medina, USA: A.I. Root company, pp. 493–514.

Spivak, M., and Reuter, G.S. (2001) Resistance to American foulbrood disease by honey bee colonies *Apis mellifera* bred for hygienic behavior. *Apidologie* **32:** 555–565.

Tomkies, V., Flint, J., Johnson, G., Waite, R., Wilkins, S., Danks, C., *et al.* (2009) Development and validation of a novel field test kit for European foulbrood. *Apidologie* **40:** 63–72.

Wilkins, S., Brown, M.A., and Cuthbertson, A.G. (2007) The incidence of honey bee pests and diseases in England and Wales. *Pest Manag Sci* **63:** 1062–1068.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. An animation of the Kernel density relative risk by year (1994–2012), increased risk areas are shown in red low risk areas are white/yellow. Tolerance p values of 0.05 (dashed line) and 0.01 (solid line) are shown as contours.