Title:

American foulbrood in a honeybee colony: spore-symptom relationship and feedbacks between disease and colony development

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Electronic Supplementary Material 1

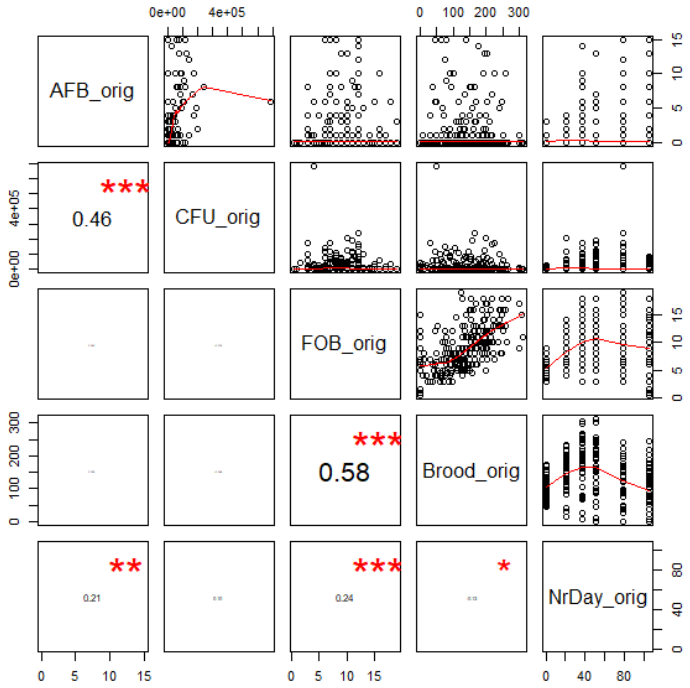


Figure S1: Original data of the five variables plotted against each other on the original scale. Red lines indicate Lowess smoother and numbers show Pearson correlation coefficient and significance (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Table S1: Summary statistic of original data.

	Treatment	n	mean	sd	median	min	max	se
AFB	Control	60	2.4	4.2	0	0	15	0.55
	LAB	60	1.5	3.3	0	0	15	0.42
	LABc	58	1.9	3.4	0	0	13	0.45
	Tylosin	59	0.53	1.2	0	0	6	0.16
CFU	Control	60	21000	40000	917	0	202667	5100
	LAB	60	25000	45000	872	0	191000	5800
	LABc	58	35000	100000	600.5	0	685000	13000
	Tylosin	59	8300	21000	1000	0	139000	2700
Brood	Control	60	140	65	138	0	267	8.3
	LAB	60	130	84	141	0	307	11
	LABc	58	130	75	125.5	0	299	9.8
	Tylosin	59	130	74	128	0	312	9.6
NrDay	Control	60	49	35	44	1	105	4.5
	LAB	60	49	35	44	1	105	4.5
	LABc	58	48	35	37	1	105	4.6
	Tylosin	59	48	35	37	1	105	4.5
FOB	Control	60	9.5	3.3	9	3	18	0.43
	LAB	60	9.7	5	10	1	19	0.64
	LABc	58	8.9	4.1	8	3	19	0.54
	Tylosin	59	8.5	3.6	8	0.5	18	0.46

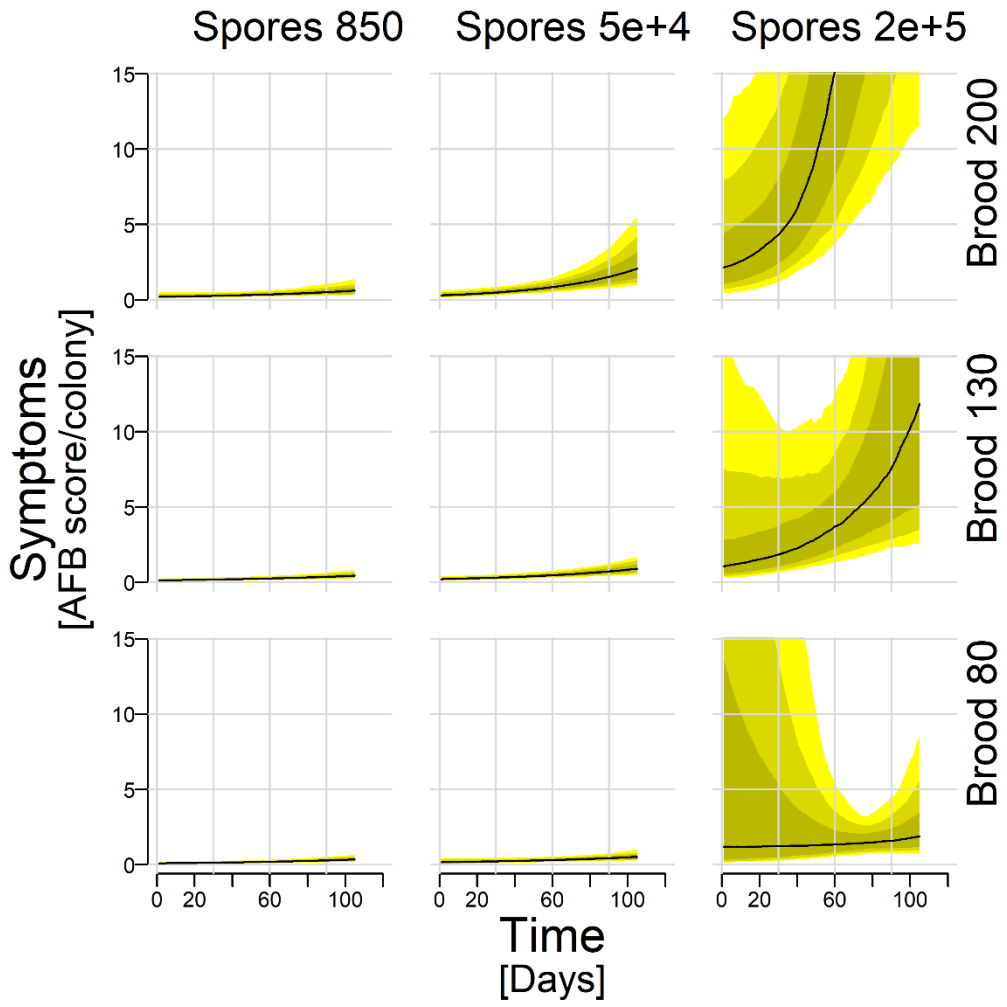


Fig. S2: Clinical symptoms depending on time of the season. Shown are median (with 97, 89, and 67 % credible intervals) posterior distributions along the full range of observation time. Brood sizes are held approximately at their mean (132.6), their 1st quantile (78), and 3rd quantile (191). Spore counts are held approximately at their median (834) and the values 50000 and 200000. Predictions are weighted predictions form four models with different combinations of the three explanatory variables and their interactions.

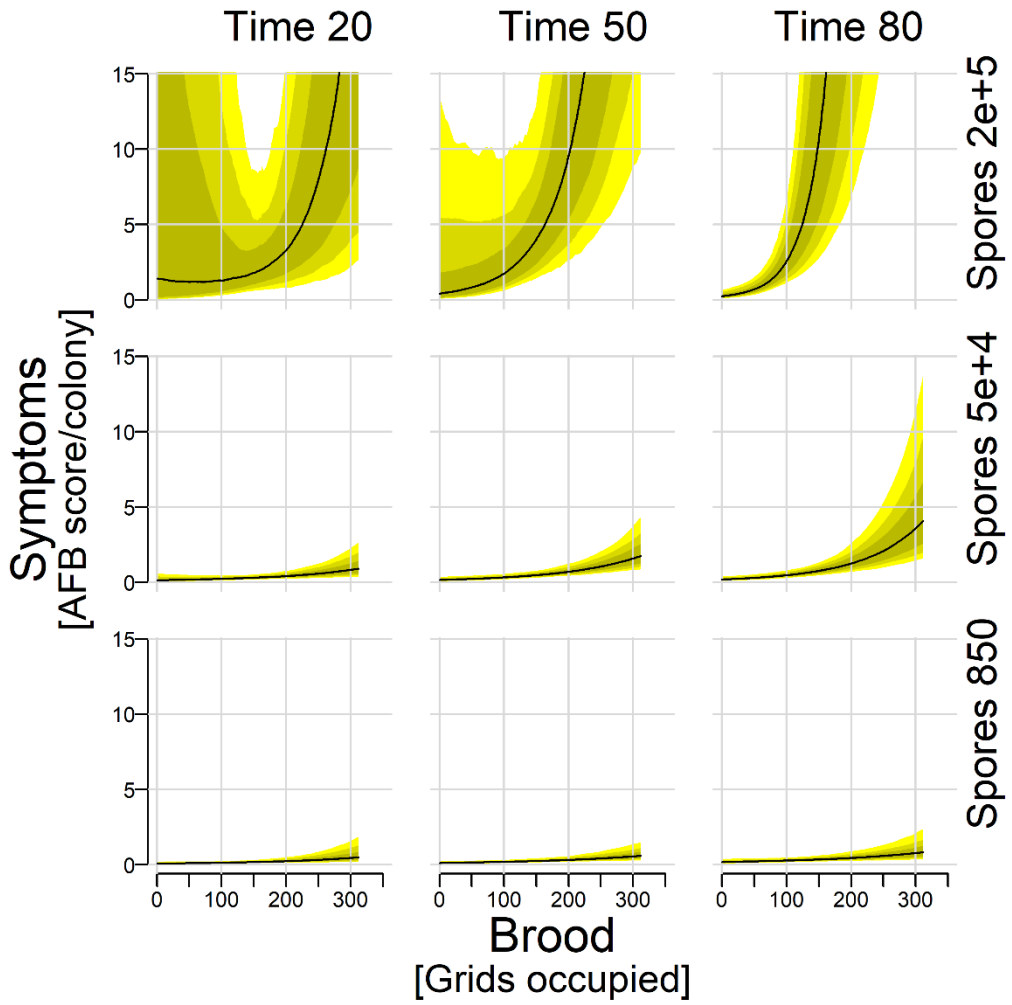


Fig. S3: Clinical symptoms depending on brood size. Shown are median (with 97, 89, and 67 % credible intervals) posterior distributions along the full range of brood size. Days are held approximately at their mean (48.4), their 1st quantile (21), and 3rd quantile (79). Spore counts are held approximately at their median (834) and the values 50000 and 200000. Predictions are weighted predictions form four models with different combinations of the three explanatory variables and their interactions.

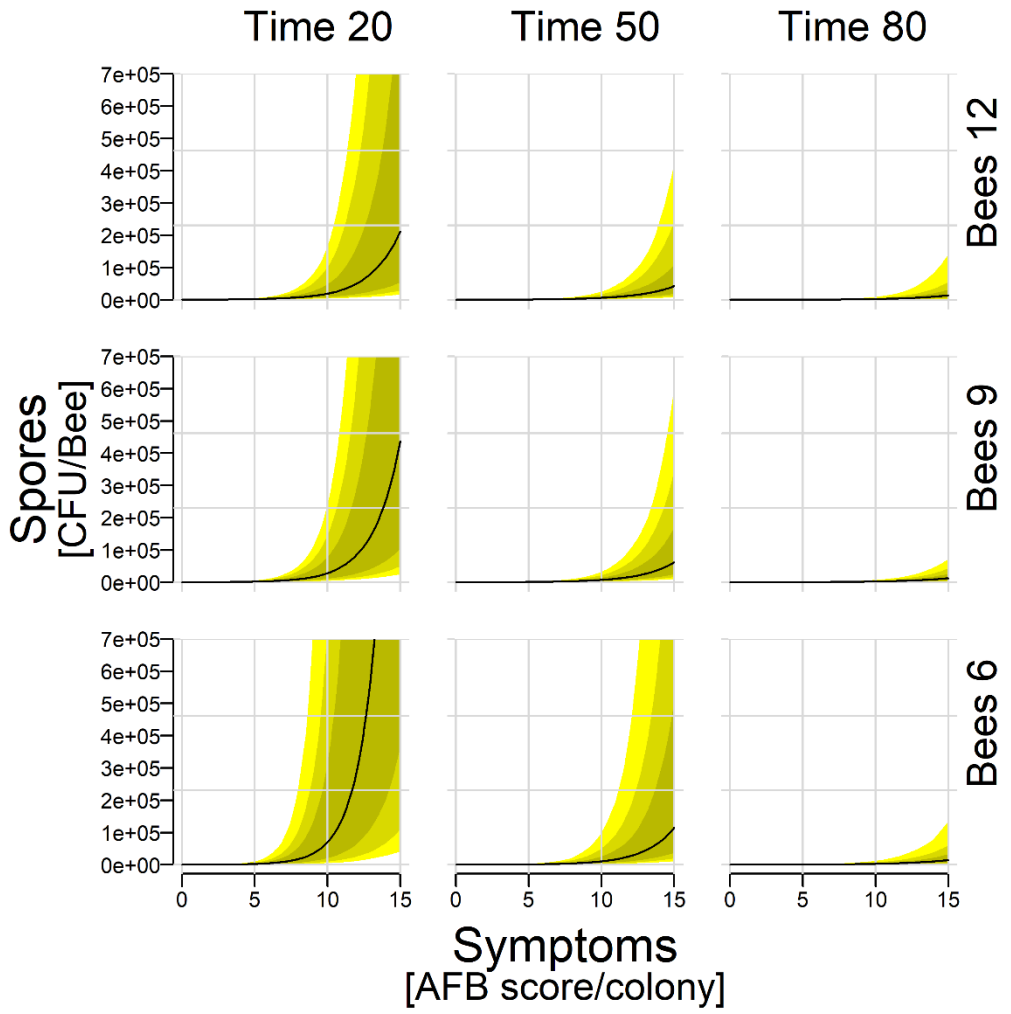


Fig. S4: Spore counts depending on clinical symptoms, time of the season, and number of bees within the colony (FOB = frames of bees). Shown are median (with 97, 89, and 67 % credible intervals) posterior distributions along the full range of observed AFB scores. The remaining continuous predictors are held approximately at their mean (Bees: 9.2; Time: 48.4), their 1st quantile (Bees: 6.0; Time: 21), and their 3rd quantile (Bees: 12.0; Time: 79). Predictions are weighted predictions form four models with different combinations of the three explanatory variables and their interactions.

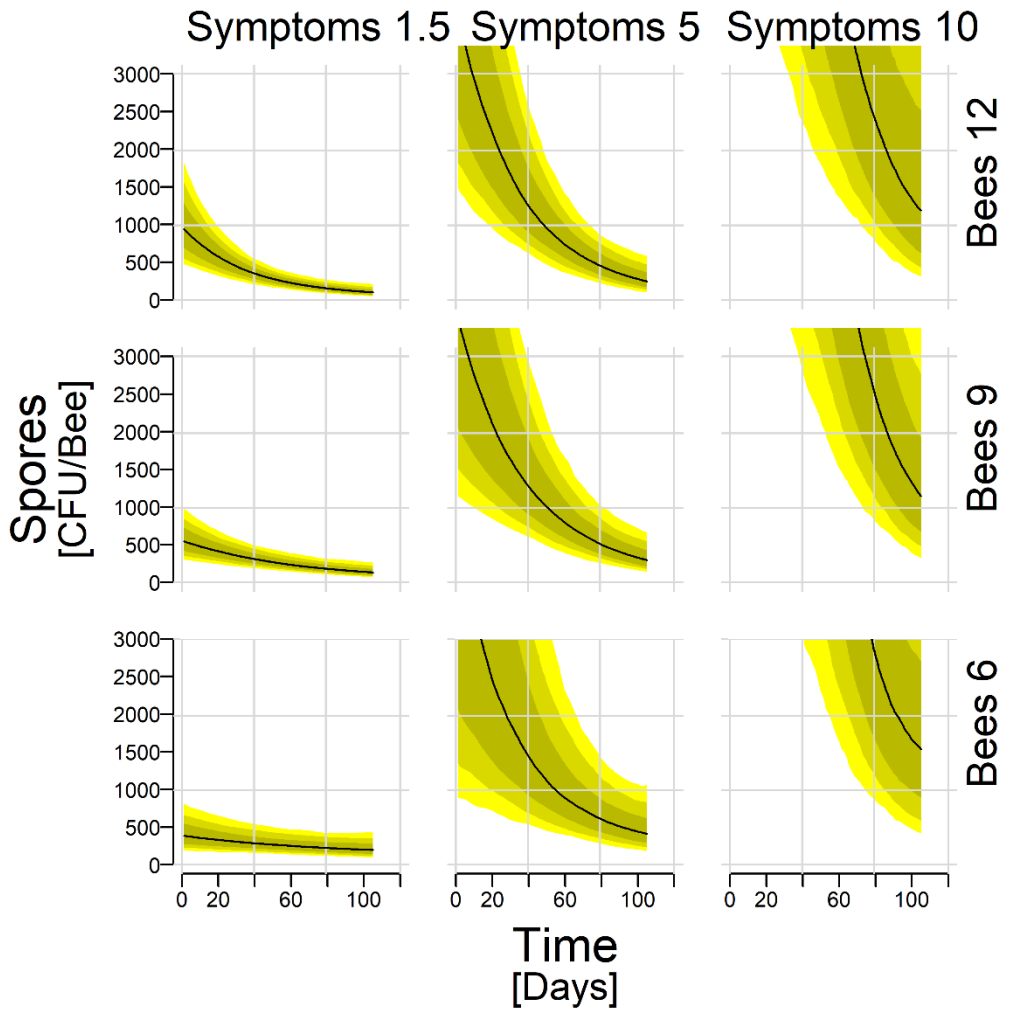


Fig. S5: Spore counts depending on clinical symptoms, time of the season of colony, and number of bees within the colony. Shown are median (with 97, 89, and 67 % credible intervals) posterior distributions along the time of the season. Number of bees are held approximately at their mean (Bees: 9.2), their 1st quantile (Bees: 6.0), and their 3rd quantile (Bees: 12.0). Symptoms are held approximately at their median (1.5) and the values 5 and 10. Predictions are weighted predictions form four models with different combinations of the three explanatory variables and their interactions.

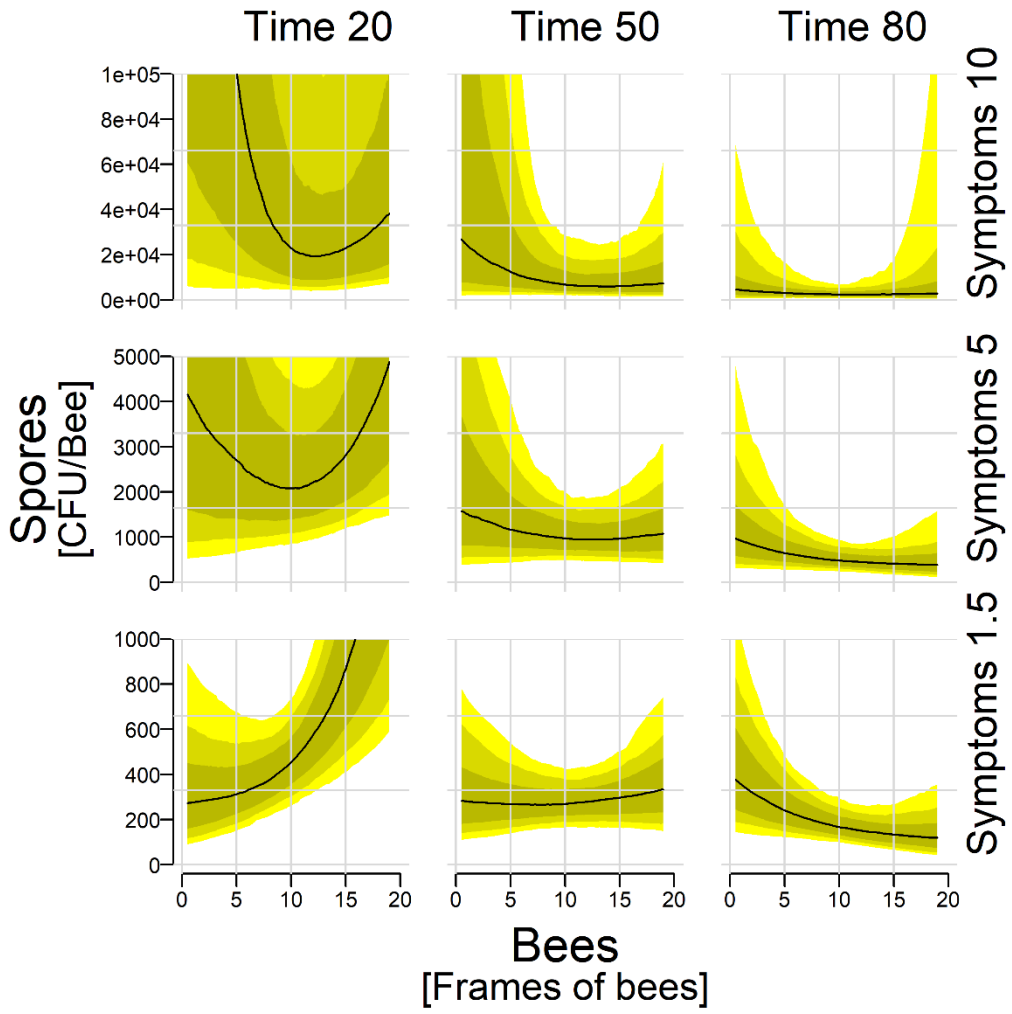


Fig. S6: Spore counts depending on clinical symptoms, time of the season, and number of bees within the colony. Shown are median (with 97, 89, and 67 % credible intervals) posterior distributions along the full range of observed number of bees. Time is held approximately at its mean (48.4), their 1st quantile (21), and 3rd quantile (79). Symptoms are held approximately at their median (1.5) and the values 5 and 10. Predictions are weighted predictions form four models with different combinations of the three explanatory variables and their interactions. Mind that ranges of y axis are chosen in order to see the relationship.

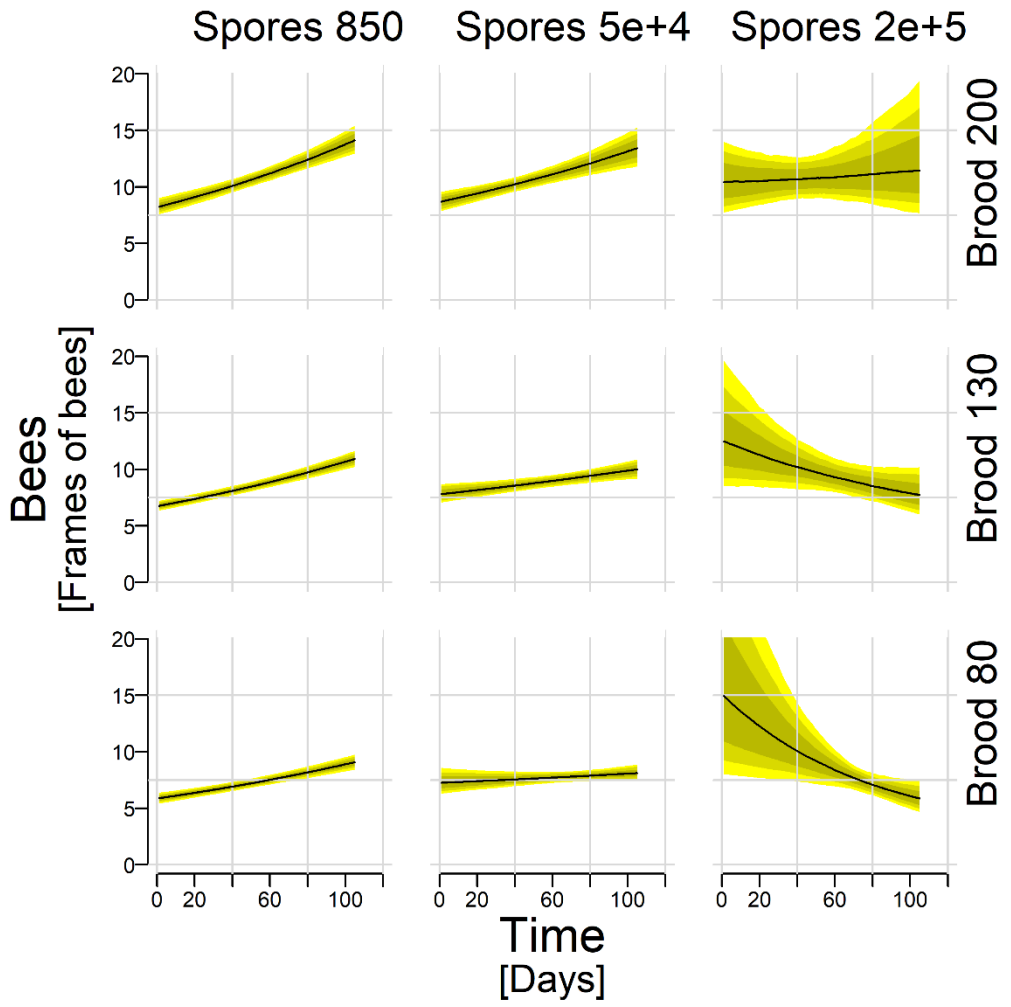
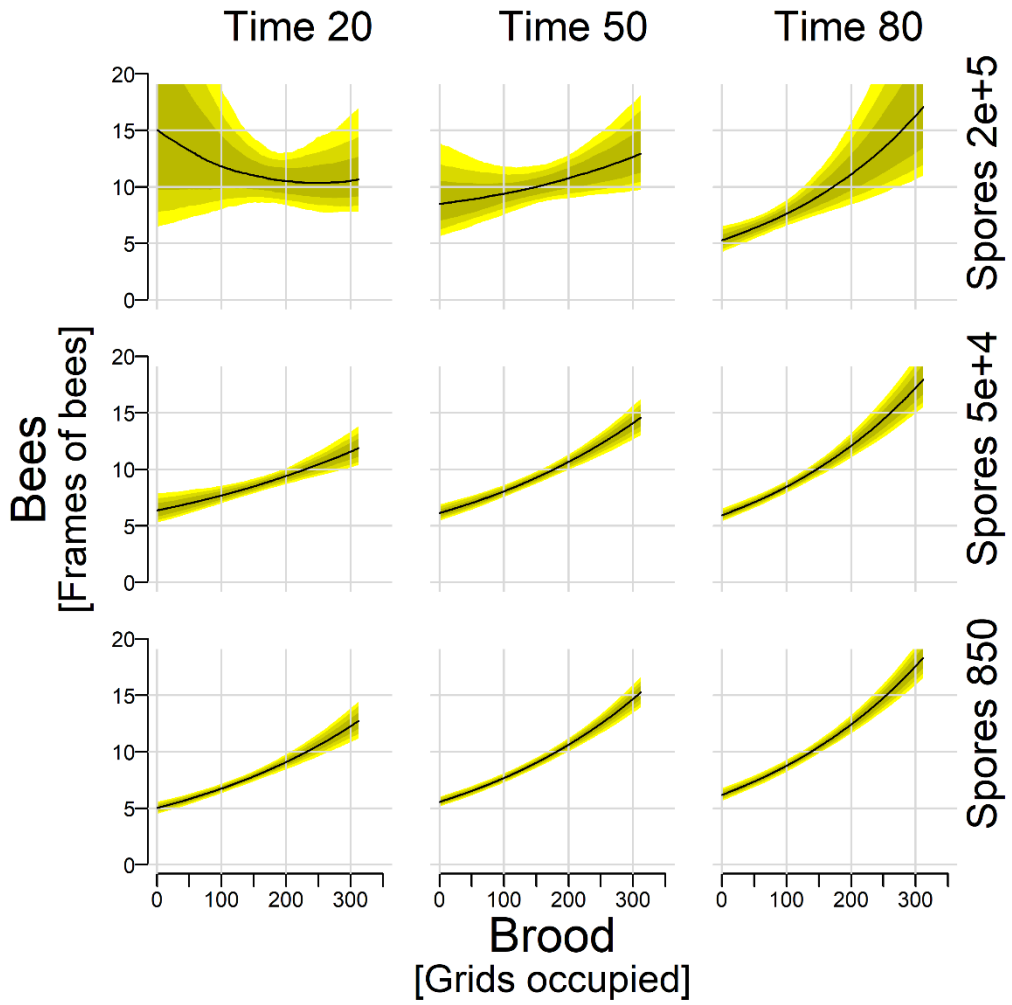


Fig. S7: Colony size depending on spore count, time of the season of colony, and brood size. Shown are median (with 97, 89, and 67 % credible intervals) posterior distributions along the time of the season. Brood sizes are held approximately at their mean (132.6), their 1st quantile (78), and 3rd quantile (191). Spore counts are held approximately at their median (834) and the values 50000 and 200000. Predictions are weighted predictions form four models with different combinations of the three explanatory variables and their interactions.



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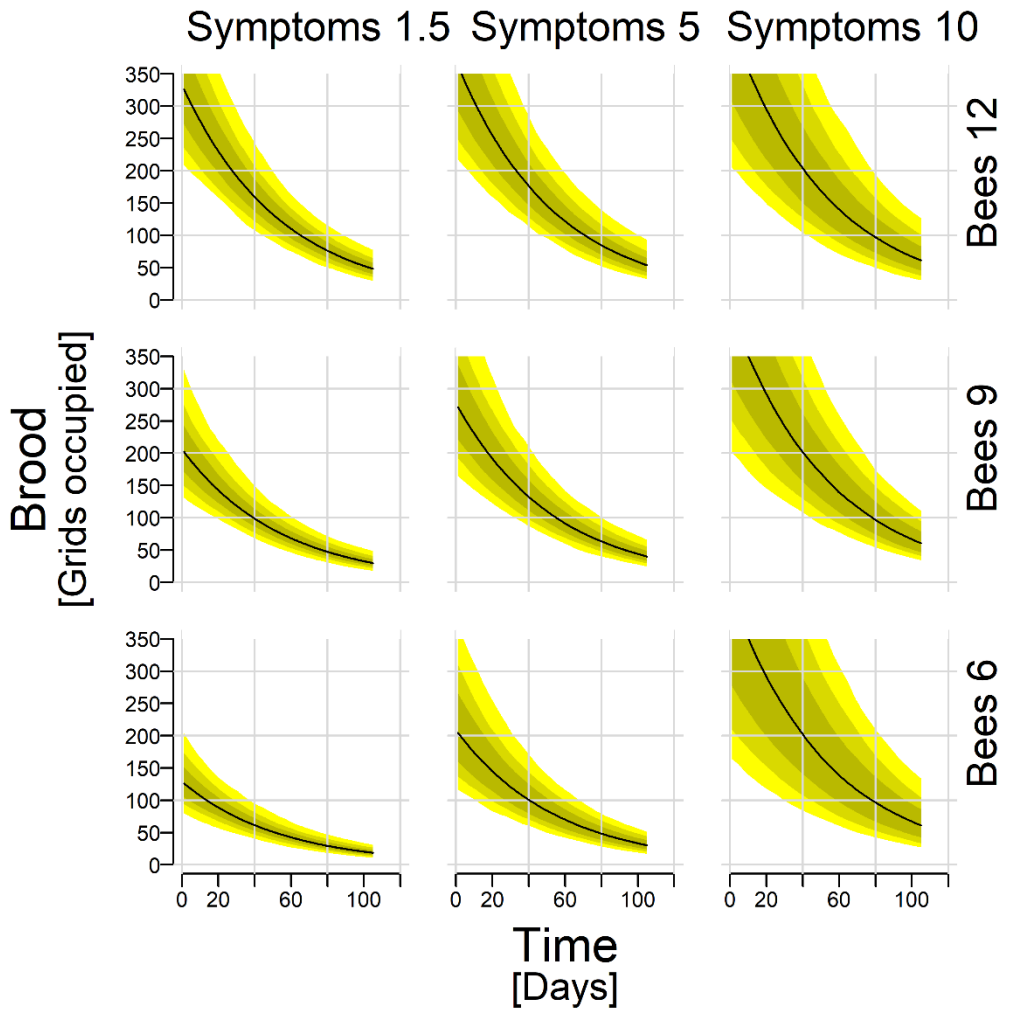
69 Fig. S8: Colony size depending on spore count, time of the season of colony, and brood size.
70 Shown are median (with 97, 89, and 67 % credible intervals) posterior distributions along the
71 full range of observed brood size. Time is held approximately at its mean (48.4), their 1st
72 quantile (21), and 3rd quantile (79). Spore counts are held approximately at their median (834)
73 and the values 50000 and 200000. Predictions are weighted predictions form four models
74 with different combinations of the three explanatory variables and their interactions.



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76 Fig. S9: Brood size depending on spore count, time of the season of colony, and colony size.
77 Shown are median (with 97, 89, and 67 % credible intervals) posterior distributions along the
78 full range of observed colony size. Number of bees are held approximately at their mean
79 (Bees: 9.2), their 1st quantile (Bees: 6.0), and their 3rd quantile (Bees: 12.0). Symptoms are
80 held approximately at their median (1.5) and the values 5 and 10. Predictions are weighted
81 predictions form four models with different combinations of the three explanatory variables
82 and their interactions.

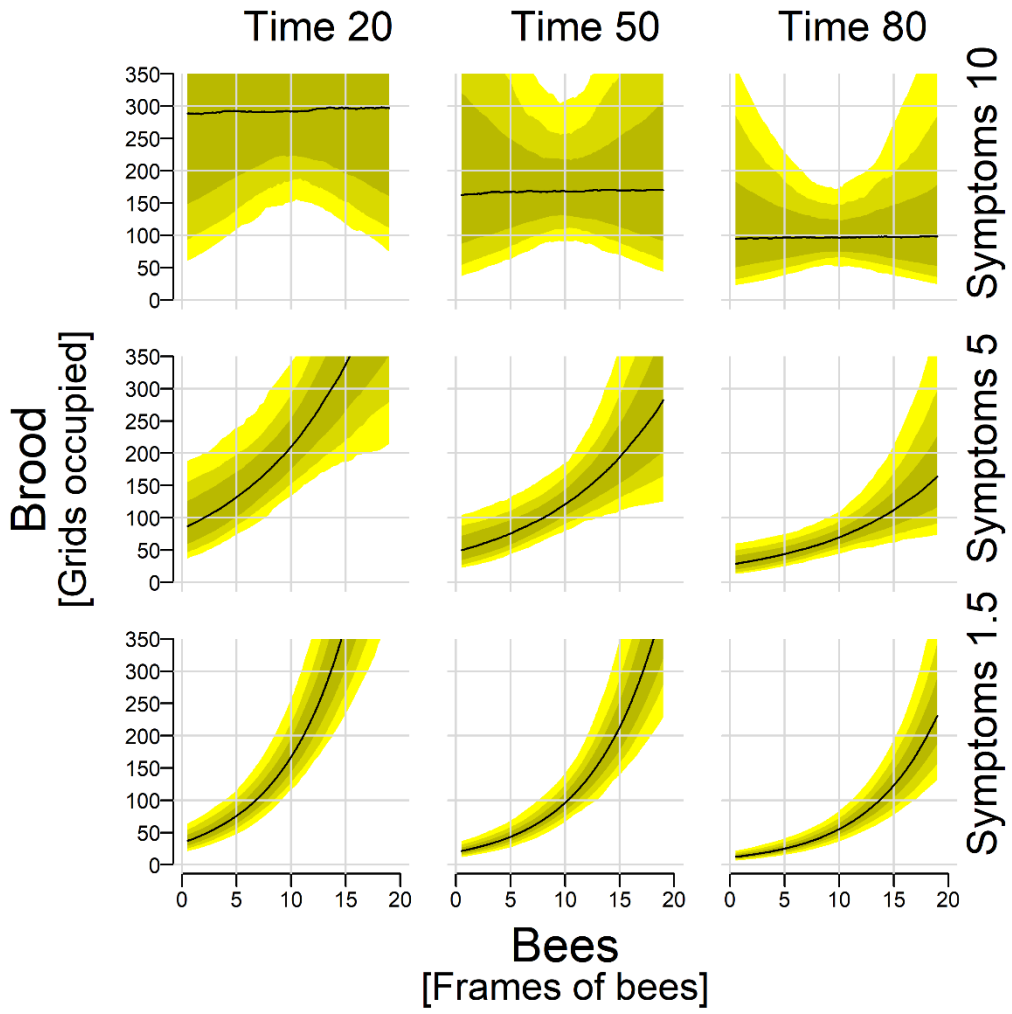

Fig. S10: Brood size depending on spore count, time of the season of colony, and colony size. Shown are median (with 97, 89, and 67 % credible intervals) posterior distributions along the time of the season. Time is held approximately at its mean (48.4), their 1st quantile (21), and 3rd quantile (79). Symptoms are held approximately at their median (1.5) and the values 5 and 10. Predictions are weighted predictions form four models with different combinations of the three explanatory variables and their interactions.

Electronic Supplementary Material 2

Colony treatment

The colonies were assessed and adult bees sampled on April 23rd, and then 21, 37, 51, 79, and 105 days after the first assessment. On each sampling occasion approximately 200 adult bees were collected from the brood chamber per colony and the samples were stored at -20°C until spore estimation in the lab.

The experiment was originally designed to test the effect of the antibiotic tylosin and honey specific lactic acid bacteria on AFB [1]. All colonies received the same amount of food in the previous autumn and were fed three times with a 1:1 sucrose:water solution meaning the starting conditions of the colonies were comparable. The colonies were divided into four groups of ten colonies each (tylosin treatment, hbsLAB treatment, hbsLAB placebo and untreated control) using a stratified random design. On April 23 the colonies were inspected for the presence of AFB and on May 1st all colonies were inoculated with a suspension of *P. larvae* spores to boost the onset of AFB. This was done by spraying two combs of unsealed brood with 5 ml of a sucrose solution containing the spores (approximately 200 million *P. larvae* spores per colony). The hbs-LAB treatment/placebo was administered twice, on April 23-25 and May 7-9, while the Tylosin treatment was administered on May 13th. The effects of the various treatments on AFB and colony development have been presented in a separate publication [1]. Although some treatments affected some of the predictors (e.g.: the treatment tylosin lowered the clinical symptoms over all sampling occasions [1]) we decided to include all colonies rather than excluding a fourth of all data for several reasons. First we modelled the variability originating from the treatments (see below) and, secondly, were interested in modulations of the relationship between two predictors by a third predictor. Excluding one treatment would mean excluding the data from all other predictors that are unaffected by the treatment meaning the interactive

effects would have been less precise. Lastly, if symptoms are slightly higher while spore counts are not affected would mean we slightly overestimated the spore count at symptom levels of zero and the minimum detection. For example the current model resulted in 228 spores at the minimal AFB score of 1, which was already considerable lower than previous studies and a lower count of e.g. 100 spores is irrelevant given the magnitudes of spore counts.

Spore counting

Samples of 100 adult worker bees were crushed in 20 mL of sterile 0.9 % NaCl in a filter grinding bag (Neoreba®). The fluid produced was centrifuged for 10 min at 27,000g and the resulting pellet was re-suspended in 2 mL sterile NaCl, heat shocked at 85 °C for 10 min and spread out over 3 MYPGP-agar plates (10 µL each). The numbers of *P. larvae* colonies were counted on each plate after an incubation period of 7 days at 36 °C in 5 % CO_2 and a mean value for the 3 plates calculated. The numbers of *P. larvae* bacteria colony forming units (CFU) were counted and the data presented as CFU per bee.

Motivation of using Bayesian approach

A Bayesian approach was used for the statistical modelling and analyses, since this examines the validity (probability) of a hypothesis (*i.e.* "spore levels can predict symptoms") given the available data, as opposed to the frequentist approach (and P-value use), which examines the validity of the data for rejecting a null hypothesis [2–4]. Generally speaking, the Bayesian approach produces more reliable and accurate predictions and the results are posterior probability distributions from which probabilistic statements about the size and direction of an effect can be made.

Model building and validation

We used a large data set recording the development of AFB disease in experimentally infected colonies across a single bee season, as well as the effects of the disease on colony

development, in order to evaluate the ability of certain variables to predict the outcome of the other variables.

The data involved 237 observations (40 colonies * 6 sampling occasions minus 3 missing observations due to colony death). Thirteen of these observations were free of *P. larvae* spores. However, since these observations were randomly distributed in time, between colonies and between treatment groups, all colonies were considered to be infected for the statistical analyses.

When used as response the variables were modelled with a Poisson likelihood (log link function). Although Symptoms represents a mixture of ordinal (0-3) and continuous (sum of 0-3 per frame for each colony) scales, the latter was used here since it more accurately represents the total AFB burden in the colony and is therefore the logical counterpart to the colony-level spore count. Hence, all predictor variables were used as continuous variables and were scaled and centred [5].

The models were constructed in two steps. First, two similar models with different random structures were compared, where the first model included just colony ID and every data point, while the second model also included the treatment groups, as Gaussian random effects. Each observation was assigned its own likelihood which removed the possibility of model over-dispersion [6] while the inclusion of colony ID accounted for the repeated measure structure across time. The difference between the two models' predictions identifies the extent to which the different treatment groups affected our conclusions. These comparisons showed that only in the models for Symptoms was it important to specifically account for the different treatment groups. This is logical, since one of the treatments, tylosin, affects AFB symptoms, but not spore levels [1]. For the other models, colony ID and the individual observations sufficed as random effects. In step two, we compared eight models for each of the four response variables. Each model included the three main effects (Time, a

predictor of colony strength and the most relevant predictor for AFB prevalence) and all combinations of their interactions. For example the model on Brood used Symptoms as most relevant disease predictor, since clinical symptoms only occur in the brood. The four most important models were then used in the analysis by weighting the predictions in order to include modulations of one predictor by the other two predictors. The weighted predictions of these four models were subsequently back-transformed to the original scale. To understand the effect of each predictor we calculated the posterior of the response variable along the full observed range of one explanatory variable while keeping the remaining two explanatory variables constant, conventionally at their mean/median value. For a better understanding of the complex interaction between the three continuous predictors two additional values were selected for each of the 4 models to investigate interactive effects. The selection of all tree values was straight-forward for Brood, Bees, and Time (mean, 1^{st} and 3^{rd} quantile), but less so for the highly skewed distributions of Spores and Symptoms. The mean (1.5), 5, and 10 AFB scores were used for Symptoms, while the median (~850), 50000, and 200000 spores per bee were used for Spores. Nevertheless, each variable was also used on the x axis but only the most relevant is shown (see Fig. S2 to S10 for the remaining combinations).

The models were validated running 3 chains (no major differences were found between these), using the Gelman and Rubin diagnostic ([7]; $\hat{R}$ was always between 1 and 1.02), inspecting the effective number of independent samples from the posterior, and performing posterior predictive checks. In both steps described above we used the Akaike weight based on the Widely Applicable Information Criterion (WAIC) of each model [8] to identify those models that are important. In all 36 models (eight regular models for all combinations of the three predictors, plus one model to determine the random structure, for each of the four response variables) the chains were stable, the posteriors were uncorrelated, and each model was able to predict the data.

Electronic Supplementary Material 3

Dilution of sampling bees

We found that the spore counts within a colony decreased over time while the number of bees increased. Yet, we attribute this to the sampling protocol of taking 100 bees regardless of how many bees are in the colony due to the following. Although spore loads in individual bees increase with the proportion of infected bees in a sample, spores are very unevenly distributed among workers [9]. Therefore the chance of sampling infected workers decrease with increasing number of bees in a colony and the 100 bee sample becomes diluted. This becomes very obvious if we look at the spore counts in relation to the number of bees (Fig. S6). In line with the SIR model, an increase of spore levels with increasing number of bees was observed earlier in the season. However, later in the season the spore counts decreased with increasing number of bees (Fig. S6). This is most evident if the symptoms are low, which further strengthens our interpretations as hygienic behaviour as cause of the decline would show the opposite (stronger decline in diseased colonies). Hence, the decrease in spores is probably entirely due to a dilution effect, especially since the behaviour that lowers the transmission rate (brood removal) is not directly relevant for the adult bees.

Another practical question that may arise is how diluted bee samples from larger colonies are, and should the number of bees in a sample be adjusted to colony size. Certainly in extreme cases (Table S1: Extreme dilution: comparing the Spores estimates of the minimum and maximum Bees at low Symptoms late in the season) this is relevant as there is a 99.5% chance that small colonies will have larger spore counts. If the number of susceptible hosts and this dilution effect are working in opposite directions in determining the Spores-Bees relationship we can calculate where both cancel out each other (slope of zero; posteriors of small Bees are not different to large Bees). This point also lays at values that are likely to be observed in colonies (Table S1: Likely dilution 1 and 2). Therefore, if the aim is to detect

P. larvae it does seem sufficient to sample a composite sample of 100 bees as the dilution effect will not majorly increase the false-negative results [9]. However, if the aim is to precisely measure the spore levels in a colony this dilution effects calls for a colony size adjusted number of bees in a sample by taking more bees in larger colonies.

Response	Parameter	Posterior distribution	P[effect>0]
Spores	*Extreme dilution*	*625.84 ± 555.11 (-65.47, 1835.53)*	*99.5*
	Likely dilution 1	*16.33 ± 73.38 (-116.44, 224.07)*	*53.3*
	Likely dilution 2	*210.02 ± 660.78 (-905.44, 1640.00)*	*55.9*

Table S1: Posterior distributions for the main parameters on original scale. Show are mean ± standard deviation (with 97% credibility intervals) of the main effects and the effect probability. We predicted three scenarios (*Extreme dilution*: Symptoms = 1, Time = 105, Bees = 0.5 versus 19; *Likely dilution 1*: Symptoms = 2, Day = 50, Bees = 5 versus 10; *Likely dilution 2*: Symptoms = 4, Time = 40, Bees = 4 versus 13; see text for further explanations).

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