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Supporting Methods

2.1. Longitudinal monitoring/sampling of commercially managed honey bee colonies

Three Montana-based (Broadwater, Yellowstone, Treasure counties) commercial beekeeping operations that transport their honey bee colonies ~ 1,200 miles to California (Merced and Stanislaus counties) each winter for the almond bloom provided honey bee samples before, during, and after almond pollination (February 2014) (Figure 1). Colony health, using colony population size as a proxy, was assessed by the number of frames covered with honey bees (frame counts) at each sampling event (Delaplane and van der Steen 2013; OSU 2011). Colony strength was defined as follows: weak colonies (< 5 frames covered with bees), average colonies (6-8 frames covered with bees), and strong colonies (> 9 frames covered with bees). Live honey bee samples (~ 100 per sample) were obtained from the top of the frames in the middle of the colony. Samples were composed of female bees of mixed age, including nurse,

24 worker, and forager bees. The samples were collected on ice or dry ice, stored at -20°C,
25 shipped on dry ice, and transferred to -80°C prior to analysis. At the onset of the study
26 in November 2013, each beekeeper identified 15 – 20 colonies of differential health.
27 Specifically, Operation 3 initiated the study with 5 weak, 5 average, and 5 strong
28 colonies and provided samples at three time points; Operation 2 initiated the study with
29 5 weak, 13 average, and 2 strong colonies and provide samples at 4 time points; and
30 Operation 1 initiated the study with 5 weak, 4 average, and 10 strong colonies and
31 provide samples at 4 time points (Supplemental Table S2). A total of 176 honey bee
32 samples with corresponding colony strength observations were obtained and analyzed,
33 4 observations of colony strength lacked corresponding samples, and 8 of the original
34 colonies died during the course of this study (Supplemental Table S2). Operation 3
35 manages ~7,100 colonies, Operation 2 manages ~12,500 colonies, and Operation 1
36 manages ~3,500 colonies. All the colonies in this study were fed protein patties either
37 before (Operation 3 – November and January, Operation 2 – November) or during
38 almond pollination (Operation 1). In addition, Operation 2 treated monitor colonies with
39 an antifungal treatment (Fumagilin-B®) and fed them Pro-Health®. After almond
40 pollination in May 2014, Operation 2 treated for *Varroa destructor* mites (i.e., two strips
41 of Apivar® per colony), fed pollen patties, and treated some colonies with an antibiotic
42 (Terramycin®). Operation 1 treated some colonies with Bee Shield® before almond
43 pollination (December 2013), and fed colonies essential oils in addition to protein patties
44 immediately before the almond bloom. Operation 1 also treated some colonies with the
45 antibiotic Terramycin® immediately after almond pollination, and fed sugar syrup and
46 essential oils and treated for mites prior to the last sample date in May 2014.

47

48 **2.2. Honey bee samples**

49 Five female bees from each sample were used for RNA extraction, cDNA synthesis,
50 pathogen-specific PCR, and qPCR (Runckel, Flenniken et al. 2011). There are varying
51 recommendations of the number of honey bees required to adequately assess the
52 pathogens associated with a single honey bee colony at a particular point in time (Chen
53 et al. 2014; de Miranda et al. 2013; Genersch et al. 2010; Pirk et al. 2013). Successful
54 pathogen detection is dependent upon sensitivity of the assay and signal to noise ratio
55 of each sample (i.e., pathogen RNA to bee RNA ratio). The objective for pathogen
56 screening in our study was to identify the most prevalent pathogens associated with
57 honey bees sampled from individual colonies at each sampling event. Based on
58 empirical data, literature values, and practical sample handling considerations, we
59 assayed five bees per colony per sampling event. The following equation from Pirk et al.
60 2013, $N = \ln(1-D) / \ln(1-P)$ (N=sample size, ln=natural logarithm, D=probability of
61 detection, P=proportion of infected bees) predicts that with a sample size of five bees,
62 pathogenic infections affecting 45% or more of the individuals within a colony would be
63 detected with 95% probability (Pirk et al. 2013); this sample size has proven sufficient
64 for the pathogen-specific PCR detection of highly prevalent pathogens (Daughenbaugh
65 et al. 2015; Runckel, Flenniken et al. 2011).

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67 **2.3. RNA isolation**

68 Bee samples were homogenized in 800 μL sterile H_2O with sterile beads (3 mm) using a
69 TissueLyzer (Qiagen) at 30 Hz for 2 min. Bee samples were centrifuged for 12 min at

70 12,000*xg* at 4°C to pellet debris, and supernatants were transferred to new 1.5 mL
71 tubes containing an equal volume of Trizol reagent (Life Technologies). RNA was
72 extracted according to the manufacturer's instructions and was suspended in sterile
73 water.
74
75 **2.5. Polymerase Chain Reaction (PCR)**
76 PCR was performed according to standard methods (Runckel, Flenniken et al. 2011, de
77 Miranda et al. 2013; Govan et al. 2000; Lanzi et al. 2006; Maori et al. 2007). In brief, 1
78 μl cDNA template was combined with 10 pmol of each forward and reverse primer, and
79 amplified with ChoiceTaq polymerase (Denville) according to the manufacturer's
80 instructions using the following cycling conditions: 95°C for 5 min; 35 cycles of 95°C for
81 30 s, 57°C for 30 s, and 72°C for 30s, followed by final elongation at 72°C for 4 min. The
82 PCR products were visualized by gel electrophoresis/fluorescence imaging. To
83 minimize the number of negative PCR-tests, we pooled samples (<10) for initial PCR
84 analysis and then assessed individual samples for each pathogen detected in pooled
85 analysis. Positive and negative control reactions were included for each set of reactions.
86
87 **2.6. Quantitative PCR (qPCR)**
88 Quantitative PCR was used to analyze the relative abundance of the most prevalent
89 pathogens in select samples to investigate the relationship between pathogen
90 abundance and honey bee colony health. Five hundred ng of RNA from each of these
91 samples was reverse transcribed with M-MLV as described above. All qPCR reactions
92 were performed in triplicate with a CFX Connect Real Time instrument (BioRad) and the

93 following reaction conditions: 2 µL of cDNA template in 20 µL reactions containing 1X
94 ChoiceTaq Mastermix (Denville), 0.4 µM each forward and reverse primer, 1X SYBR
95 Green (Life Technologies), and 3 mM $MgCl_2$. The qPCR thermo-profile consisted of a
96 single pre-incubation 95°C (1 min), 40 cycles of 95°C (10 s), 58°C (20 s), and 72°C (15
97 s). Plasmid standards, containing from 10^9 to 10^3 copies per reaction, were used as
98 qPCR templates to assess primer efficiency and quantify the relative abundance of
99 each pathogen. The linear standard equations generated by plotting the crossing point
100 (Cp) versus the $\log_{10}$ of the initial plasmid copy number for each primer set were as
101 follows: LSV2: $y = -3.8147x + 44.805$, $R^2 = 0.980$; BQCV: $y = -3.7336x+ 42.849$, $R^2 =$
102 0.996; LSV1: $y = -3.1994x + 38.71$, $R^2 = 0.982$, and SBV: $y = -3.3768x + 39.484$, $R^2 =$
103 0.996. In addition, qPCR of a host encoded gene, *Apis m*. Rpl8, was performed using 2
104 µL cDNA template on each qPCR plate to ensure consistency and cDNA quality. qPCR
105 products were analyzed by melting point analysis and 2% agarose gel electrophoresis.

106 **2.7.1. Statistical analysis of PCR**

107 For this study, we use “pathogen prevalence” to refer to the total number of pathogens
108 detected by PCR out of a target list of 16. Though our interest is in the relationship
109 between strength rating and pathogen prevalence, graphical analyses indicated that
110 there were likely relationships between pathogen prevalence and sampling time as well
111 as between strength and sampling time. Thus, we used a Poisson log-linear regression
112 model and accounted for an interaction between sample date (time period), beekeeping
113 operation, colony strength, and pathogen prevalence. Colonies were evaluated and
114 sampled multiple times (i.e., either 3 or 4 times), but since subsequent measurements
115 on a single colony were both temporally and geographically distant and graphical

116 analyses did not suggest a relationship between pathogen prevalence and subsequent
117 measures on a given colony, this parameter was not included in our model.
118 Observations with average strength rating were not included in the analysis to simplify
119 the inferences between strong (S) and weak (W). The natural logarithm (ln) of the
120 pathogen prevalence data was used in comparisons between each beekeeping
121 operation and time period combination; for the model, we used beekeeping Operation 1,
122 before almond pollination (time period 1), and weak colonies as the base level.
123 In all, our model can be expressed

$$y_i \sim \text{Poisson}(\mu_i)$$

$$\log(\mu_i) = \beta_0 + \beta_1 \times \text{Operation2}_i +$$

$$\beta_2 \times \text{Operation3}_i + \beta_3 \times (S{:}\,period1)_i +$$

$$\beta_4 \times (\text{W: period2})_i + \beta_5 \times (\text{S: period2})_i +$$

$$\beta_6 \times (\text{W: period3})_i + \beta_7 \times (\text{S: period3})_i +$$

$$\beta_8 \times (\text{W: period4})_i + \beta_9 \times (\text{S: period4})_i$$

124 • y_i = the total abundance/prevalence for the ith observation $i = 1,2,\ldots,180$.

125 • $\text{Operation2}_i = 1$ if observation i came from beekeeping Operation 2 and 0
126 otherwise.

127 • $\text{Operation3}_i = 1$ if observation i came from beekeeping Operation 3 and 0
128 otherwise.

129 • $\text{period2}_i = 1$ if observation i was taken during and 0 otherwise.

130 • $\text{period3}_i = 1$ if observation i was taken after pollination and 0 otherwise.

131 • $\text{period4}_i = 1$ if observation i was taken in the second after pollination sampling time
132 and 0 otherwise.

133 • $\text{A}_i = 1$ if observation i was Average (colony strength) and 0 otherwise.

134 • $\text{S}_i = 1$ if observation i was Strong (colony strength) and 0 otherwise.

135 In the equation above, $\gamma_{j(i)}$ is the random effect for colony. We assume
136 $\gamma_{j(i)} \sim N(0, \sigma^2_{colony})$, $\epsilon_i \sim N(0, \sigma^2_y)$ and $\gamma_{j(i)}$ and ϵ_i are independent for all $j = 1,2,\ldots,60$,
137 $i = 1,2,3,\ldots,180$. S and W are indicators for strong and weak colony ratings. Here, we
138 defined weak colonies from beekeeping Operation 1 during time period1 (before almond
139 pollination) as the base-level for comparisons. μ_i is the expected pathogen prevalence
140 given the covariates. Since we observed an interaction between time period and
141 strength rating, our question of interest must be evaluated in each of the time periods.
142 Thus our final inferences are based on our estimates of β_3, the difference between β_4
143 and β_5, β_6 and β_7, and β_8 and β_9; the values for parameters in the equation that are not
144 required to address specific questions become 0.

145 **<u>References associated with longitudinal monitoring of honey bee colonies.</u>**

146 Longitudinal monitoring of colony health and pathogen prevalence and abundance is
147 critical to determining the role of pathogens in colony losses (Berényi et al. 2006; Chen
148 et al. 2014; Clermont et al. 2014; de Miranda et al. 2013; Delaplane and van der Steen
149 2013; Ellis et al. 2010; Gajger et al. 2014; Genersch et al. 2010; Gisder et al. 2010;
150 McMenamin and Genersch 2015; Nielsen et al. 2008; Ravoet et al. 2013; Runckel,

151 Flenniken et al. 2011; Spleen et al. 2013; Steinhauer et al. 2014; Tentcheva et al. 2004;
152 van der Zee et al. 2012; van Engelsdorp et al. 2008; vanEngelsdorp et al. 2012;
153 vanEngelsdorp et al. 2009; vanEngelsdorp et al. 2013).
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