

Changes in transcript abundance relating to colony collapse disorder in honey bees (*Apis mellifera*)

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Colony collapse disorder (CCD) is a mysterious disappearance of honey bees that has beset beekeepers in the United States since late 2006. Pathogens and other environmental stresses, including pesticides, have been linked to CCD, but a causal relationship has not yet been demonstrated. Because the gut acts as a primary interface between the honey bee and its environment as a site of entry for pathogens and toxins, we used whole-genome microarrays to compare gene expression between guts of bees from CCD colonies originating on both the east and west coasts of the United States and guts of bees from healthy colonies sampled before the emergence of CCD. Considerable variation in gene expression was associated with the geographical origin of bees, but a consensus list of 65 transcripts was identified as potential markers for CCD status. Overall, elevated expression of pesticide response genes was not observed. Genes involved in immune response showed no clear trend in expression pattern despite the increased prevalence of viruses and other pathogens in CCD colonies. Microarray analysis revealed unusual ribosomal RNA fragments that were conspicuously more abundant in the guts of CCD bees. The presence of these fragments may be a possible consequence of picorna-like viral infection, including deformed wing virus and Israeli acute paralysis virus, and may be related to arrested translation. Ribosomal fragment abundance and presence of multiple viruses may prove to be useful diagnostic markers for colonies afflicted with CCD.

microarray | picorna-like virus | ribosomal RNA

As the premier managed pollinator in the United States, the western honey bee, *Apis mellifera*, contributes more than \$14 billion to agriculture annually (1). Beginning in fall 2006, the American apiculture industry experienced catastrophic losses of unknown origin. The phenomenon, called colony collapse disorder (CCD), was identified by a set of distinctive characteristics, including the absence of dead bees in or near the colony and the presence of abundant brood, honey, and pollen despite vastly reduced numbers of adult workers (2). Losses were estimated at 23% over the winter of 2006–2007 (3) and at 36% over the winter of 2007–2008 (4).

Using metagenomics, Cox-Foster et al. (3) compared the microbial flora of honey bees in hives diagnosed with CCD and in ostensibly healthy hives, evaluating pathogens with respect to their association with diagnosed CCD. The most predictive pathogen was Israeli acute paralysis virus (IAPV), a picorna-like virus (Dicistroviridae) hitherto unreported in the United States. However, a later study found IAPV in U.S. bees before the appearance of CCD (5), discounting an exclusive causal relationship. The metagenomic analysis identified other pathogens associated with CCD bees, including the picorna-like viruses Kashmir bee virus (KBV) and deformed wing virus (DWV), and 2 species in the microsporidian genus *Nosema* (3), one of which, *N. ceranae*, was subsequently linked to collapses of colonies in Spain (6), although this may be unrelated to CCD in the United States. A high prevalence of multiple pathogens in CCD bees suggests that a compromised immune response may be integral to CCD.

Pesticides also have been suspected to play a role in CCD. Sublethal exposures leading to behavioral disruptions consistent with the failure of foragers to return to their hives have been associated with the neonicotinoid pesticides (7). The long-term use of combinations of in-hive pesticides for control of honey bee parasites also may have contributed to otherwise unexplained bee mortality (8).

To differentiate among possible explanations for CCD, we used whole-genome microarray analysis, comparing gut gene expression in adult worker bees from healthy and CCD colonies. We assayed the gut because it is the principal site of pesticide detoxification and an integral component in the immune defense against pathogens in *A. mellifera*. Samples from colonies varying in CCD severity were collected on the U.S. east and west coasts in the winter of 2006–2007 and compared with healthy (“historical”) controls collected in 2004 and 2005.

Results

Gene Expression Differences. The microarray contained oligonucleotide probes representing 9,867 different genes (with duplicate spots), based on gene predictions and annotation from the honey bee genome sequencing project; in addition, 2,729 probes specific for ESTs matching no existing annotation were represented on the array (9–11), including 21 probes derived from expressed sequence tags (ESTs) specific for rRNA [supporting information (SI) Table S1].

Of the 6,777 probes with expression above background level, 1,305 probes showed significant differences in expression in at least 1 of the 5 contrasts [false discovery rate (FDR), $P < .01$; and fold-change >2] (Fig. 1). The west coast versus historical comparison alone generated a list of 948 differentially expressed probes; however, 668 of these were not differentially expressed in any of the other comparisons.

There was little concordance in the lists of differentially expressed genes between the east and west coast comparison of guts taken from “severe” and “mild” colonies. Just 54 probes shared differential expression between the east and west coast severe versus mild comparisons, with 191 probes showing unique differential expression. That so many genes are differentially transcribed in the severe versus mild comparisons suggests these classifications may not be equivalent across geographical samplings.

Reflecting the strong differences in gene expression, hierarchical cluster analysis, using expression values for all probes,

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Data deposition: The data reported in this paper have been deposited in the ArrayExpress database, www.ebi.ac.uk/arrayexpress (accession no. E-MEXP-2292).

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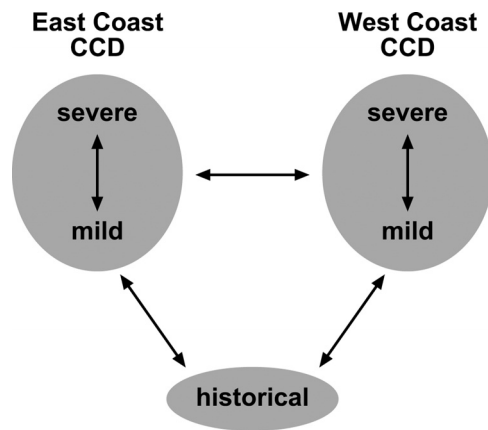


Fig. 1. Experimental design for microarray comparisons of CCD and healthy bees. East coast CCD includes bees collected in Florida and Pennsylvania, and west coast CCD includes bees from California. Guts from bees remaining in colonies that were classified as mild or severe were compared with each other and together, through a reference gut RNA sample, with guts of healthy historical bees collected in 2004 and 2005. A total of 22 microarrays were hybridized.

grouped the samples broadly by health status and geography (Fig. 2). All healthy bees clustered together, as did all CCD bees, which were further clustered according to geography, with west coast California bees forming one group and east coast Pennsylvania and Florida bees clustering together in another group. Differences in relative colony health at the time of collection, either mild or severe CCD, were not reflected in the cluster analysis.

Although geographic variation among the east and west coast CCD samples is evident, if CCD is a distinct phenomenon, then it should be possible to identify a list of CCD-related genes that show differential expression in both east and west coast CCD bees relative to healthy bees. But because these comparisons rely on the historical samples, which may carry their own geographic biases, we further winnowed the gene list by using only genes differentially expressed in the east coast severe versus mild comparison (Fig. 3). This comparison was informative; expression of 65 transcripts was consistently up or down when comparing relatively sick bees (CCD or severe) and relatively healthy bees (historically healthy or mild) (Fig. 2); 29 probes on this list correspond to annotated genes, 35 probes correspond to ESTs, and 1 probe corresponds to a pathogen, DWV.

Gene Ontology Functional Analyses of Gene Expression Differences.

Genes differentially expressed between groups were categorized according to Gene Ontology (GO) (12). FlyBase orthologs of bee genes that were differentially expressed were used for GO enrichment analyses (Table 1). In the cellular component ontology, genes associated with lipid particle (GO:0005811), cytosolic small ribosomal subunit (GO:0022627), and mitochondrial respiratory chain (GO:0005746) generally demonstrated reduced expression in west coast bee guts relative to historical samples.

In the molecular function ontology, genes related to transcription factor activity (GO:0003700) were more highly expressed in both east coast and west coast CCD bees and in severe CCD bees. West coast bees also showed reduced expression of both heme binding (GO:0020037) and carboxylesterase activity (GO:0004091) categorizations, which include cytochrome P450 monooxygenases (P450s) and carboxylesterases (COEs), the principal xenobiotic metabolizing enzymes. One P450, CYP4G11, was expressed at a lower level in all 3 contrasts tested.

In the biological process ontology, genes related to development (GO:0016348 and GO:0007509) and the peptidoglycan

catabolic process (GO:0009253) were overrepresented but did not demonstrate consistent directional differences. The peptidoglycan recognition proteins (PGRPs) contribute to immunity by detecting bacteria (13); PGRP-S2 was up-regulated and PGRP-S3 was down-regulated in CCD bees. Transcripts encoding sallimus, a large complex protein that mediates muscle elasticity (14), were more abundant in west coast bees. This protein also may contribute to immunity, because it contains Ig repeats and its transcripts are overexpressed in *Anopheles gambiae* mosquitoes after bacterial infection (15).

The gene *mbk-1* was more highly expressed in CCD bees; the *D. melanogaster* ortholog of this gene plays a role in hormone-triggered cell death (16) and larval midgut histolysis (17). Thus, overexpression of *mbk-1* may indicate apoptosis in the guts of CCD bees. However, of the 39 apoptosis-related (GO:0006915) genes on the microarray, only *mbk-1* and *GB14659*, which is similar to *apoptotic peptidase activating factor 1*, were more highly expressed in the guts of CCD bees.

Pathogens in CCD Bees. Twenty-two probes specific for 8 bee pathogens were present on the microarray [KBV, DWV, black queen cell virus (BQCV), acute bee paralysis virus, sacbrood virus, *Ascophæra apis*, *Nosema apis*, and *Paenibacillus larvae*] (Fig. S2), providing an opportunity to survey the samples for pathogens. In some cases, the number of colonies sampled was small with respect to standard pathogen surveys, so the results are best interpreted in a qualitative rather than quantitative manner. Three pathogens were detectable at different levels among the sampled bees. Chalkbrood (*A. apis*) RNA was more abundant in both east coast CCD bees and in severe CCD bees. BQCV RNA was more abundant in CCD bees, as was DWV, but DWV also was more abundant in east coast severe CCD bees. The DWV probe on the microarray also may have detected the closely related Kakugo virus or *Varroa destructor* virus 1 (18), with which it shares 91% identity. A probe specific for IAPV (19) was not included on the array and likely is not detected by the probe for KBV, with which it shares just 60% identity. The presence of *Nosema*, IAPV, and other viruses was surveyed with quantitative PCR (qPCR), indicating a generally higher viral load in CCD colonies (Table 3).

Validation of Microarray Results with qPCR. Expression changes of 8 transcripts were verified by qPCR. Five of 8 transcripts had similar expression profiles when measured using the same RNA samples analyzed by both methods (Table S4). Biological validation of microarray results was performed using qPCR on gut samples from healthy and CCD bees collected in California in 2007, a year after the samples used for microarray analysis were obtained. Differential expression of 3 of 8 transcripts was confirmed: CCD-associated up-regulation of the ESTs QW33 and *jdeC15*, corresponding to 28S and 5.8S rRNA subunits. DWV also was more abundant in CCD gut samples (Table 2).

Further qPCR validation of markers was performed with abdomens of bees from 147 colonies in healthy and CCD apiaries in 6 states. Only an rRNA-coding EST, QW33, was differentially expressed between CCD and healthy abdomens (Table 2). These CCD bees also were more likely to harbor both a larger number of viruses (as detected by these methods) and nonviral pathogens, as in the original samples (Table 3).

Discussion

No simple explanations for the cause of CCD emerge from the microarray analysis. Expression of detoxification and immune gene transcripts, which would indicate toxins or disease as the cause of CCD, was largely unchanged. However, considerable geographic variation existed among CCD bees, with west coast bees seemingly more severely affected. Transcripts of genes related to basic cellular processes involving ribosomal and

West / Historical East / Historical

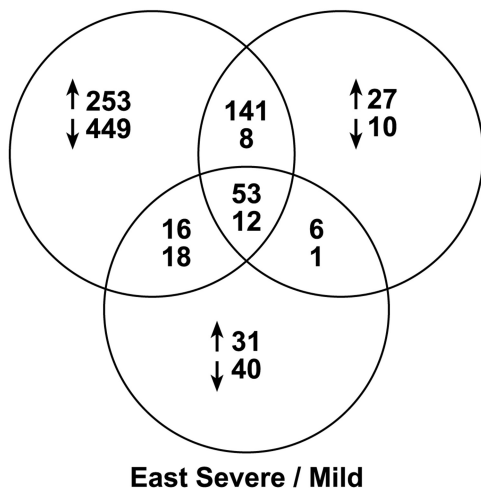


Fig. 3. Venn diagram showing the number of transcripts up-regulated and down-regulated in 3 comparisons: west coast (California) CCD versus historical, east coast (Florida and Pennsylvania) CCD versus historical, and east coast severe CCD versus mild CCD.

Although 88 known immune-related genes were included on the microarray, these also were mostly unchanged in expression in CCD bees. Given the association between pathogens and CCD (3), the finding that so few immune genes were differentially expressed in CCD bees was unexpected. Insect guts can produce antimicrobial peptides, including the apidaecins in honey bees (23), yet CCD-related changes in transcription of these genes were not detected. This lack of response does not rule out viral infection, however, because antimicrobial proteins are ineffective against viruses. In *D. melanogaster*, fungal or bacterial infection induces antimicrobial peptides, but flies infected with *Drosophila C* virus (DCV), another picorna-like virus, did not produce more antimicrobial peptides (24) and demonstrated elevated transcription of only a small number of immune genes relative to fungal and bacterial infection (25).

Many of the transcripts differentially expressed in CCD bees were detected by probes based on ESTs matching no existing annotation (10, 11). Of the 2,729 geneless ESTs on the array, 28 were differentially expressed in CCD bees. Of all of the ESTs on which probes were based, 13 were discovered to contain rRNA sequence, and 5 of these were more highly expressed in CCD bee guts. These rRNA-coding ESTs were not identified as rRNA previously, because the *A. mellifera* rRNA sequence is not available in online repositories, and identification of these probes was accomplished only by manually transcribing the published rRNA sequence from printed text (26). Had these ESTs been previously identified as rRNA, the associated probes likely would have been excluded from the microarray.

The presence of rRNA on these microarrays is surprising, because both the EST projects on which the probes were based and the microarray hybridization rely on a polyadenylated 3' tail for the initial priming step in the reverse-transcription reaction. Ribosomal RNA is transcribed by RNA polymerase I, and the resulting transcript is not polyadenylated; as such, oligo(dT)-based reverse-transcription reactions should not reverse-transcribe rRNA. Yet transcripts of rRNA have long appeared in EST projects, and their reverse transcription has been attributed to poly(A)-rich internal sequences hybridizing to the oligo(dT) or to genomic DNA contamination (27). This differential expression of polyadenylated rRNA transcripts in insects cur-

rently defies technical explanation; differential expression of these transcripts associated with age and caste in a termite (*Reticulitermes flavipes*) EST project (28) suggests an underlying biological explanation.

One possible explanation for the presence of poly(A) rRNA sequences in bees is that they are degradation intermediates. Polyadenylation is a well-known marker of RNA degradation in bacteria and organelles (29), and poly(A) addition is known to contribute to eukaryotic rRNA degradation (30). Polyadenylated 25S rRNA comprises $\approx 0.02\%$ of all of the 25S rRNA transcripts in fission yeast *Schizosaccharomyces pombe* (31). Another explanation for these sequences is the "ribosomal filter hypothesis," in which conventional mRNA transcripts include tens to hundreds of nucleotides similar to rRNA sequence in either the sense or antisense direction (32). Transcripts of genes containing rRNA sequence can bind to ribosomal subunits and alter translation efficiency (33). Short sections of rRNA function as an internal ribosome entry site (IRES) to interact with the ribosome and alter translation. Indeed, functions have been assigned to some mRNAs containing large sections of rRNA sequence (34), although none of the predicted genes annotated from the honey bee genome contains sufficient rRNA sequence to hybridize to any of the rRNA probes on the array (Table S1). A related hypothesis posits that long sequences of rRNA-like transcript may aid in protein folding, taking on part of the enzymatic activity of rRNA (35).

The picorna-like virus DWV was more abundant in CCD bees analyzed in the microarray experiment and a broader sampling of bee colonies revealed that CCD bees carry a larger number of different picorna-like viruses (Table 3). Picorna-like viruses, including IAPV and KBV, that have been associated with CCD (3) initiate transcription of viral proteins by the ribosome through an IRES rather than through the 5'-methylation cap that initiates translation of most mRNA (36). The IRES sequences from both DWV and *Varroa destructor* virus 1 effectively enhance translation of a reporter gene (36). Picorna-like viruses in mammals also halt translation of host mRNA through cleavage of the translation initiation factor eIF4G and the poly(A)-binding protein (37), leaving the ribosomes incapable of binding host mRNA and giving the viral RNA little competition for its IRES-mediated binding to host ribosomes (38).

While there is no direct evidence that elevated poly(A) rRNA is a result of picorna-like viral infection, one consequence of the viral disabling of ribosomal function may well be increased ribosomal degradation. Picornavirus infection in mammals both reduces protein production and causes strings of translating polyribosomes to break down, and these idle ribosomal subunits may be more susceptible to degradation (39). Ribosomal degradation also could occur in CCD bees independent of viral infection; for example, starving bacteria demonstrate elevated ribosomal degradation (39). Regardless of the cause, the abundance of poly(A) rRNA may be a useful diagnostic marker for determining CCD status, although the presence of poly(A) rRNA does not in itself indicate CCD, because healthy bees, including those used to generate EST libraries, contain these fragments.

Although gene transcript analysis did not clearly identify a specific cause for CCD, our study documents several patterns suggestive of a causal mechanism. The reduced protein synthetic capabilities that would accompany ribosomal hijacking by multiple picorna-like viruses would leave bees unable to respond to additional stresses from pesticides, nutrition, or pathogens. Although any interpretation of the presence of these rRNA fragments is speculative, the reported interaction between bee picorna-like viruses and rRNA is suggestive of a possible root cause of CCD.

To establish a causal relationship, the quantitative association between multiple picorna-like virus infections and polyadenyl-

Table 1. GO analyses for differentially expressed genes in 3 contrasts

Comparison	GO ID	Term	Annotated	Significant	Expected	P value	Change in expression
Cellular component							
East vs. historical	GO:0035102	PRC1 complex	3	2	0.09	.003	+2/−0
	GO:0005576	Extracellular region	124	11	3.6	.008	+6/−5
West vs. historical	GO:0005811	Lipid particle	205	46	26.72	<.001	+6/−40
	GO:0022627	Cytosolic small ribosomal subunit	30	10	3.91	.003	+0/−10
	GO:0005746	Mitochondrial respiratory chain	53	14	6.91	.006	+4/−10
Molecular function							
East vs. historical	GO:0003700	Transcription factor activity	144	15	3.91	<.001	+14/−1
	GO:0008599	Protein phosphatase type 1 reg. activity	2	2	0.05	.001	+2/−0
	GO:0043565	Sequence-specific DNA binding	75	8	2.04	.001	+8/−0
	GO:0008745	N-acetylmuramoyl-L-alanine amidase	3	2	0.08	.002	+1/−1
	GO:0005102	Receptor binding	41	6	1.11	.003	+3/−3
	GO:0005001	Transmembrane receptor protein tyrosine phosphatase	4	2	0.11	.004	+2/−0
	GO:0042834	Peptidoglycan binding	4	2	0.11	.004	+1/−1
	GO:0005372	Water transporter activity	5	2	0.14	.007	+1/−1
West vs. historical	GO:0043565	Sequence-specific DNA binding	75	18	9.2	.003	+12/−6
	GO:0003700	Transcription factor activity	144	29	17.66	.004	+21/−8
	GO:0004091	Carboxylesterase activity	26	9	3.19	.008	+3/−6
	GO:0020037	Heme binding	49	12	6.01	.013	+1/−11
East mild vs. severe	GO:0003700	Transcription factor activity	144	10	2.92	.001	+4/−6
	GO:0004558	Alpha-glucosidase activity	7	2	0.14	.008	+2/−0
Biological process							
East vs. historical	GO:0016348	Imaginal disc-derived leg joint morphogenesis	2	2	0.05	.001	+2/−0
	GO:0045449	Regulation of transcription	274	17	7.5	.001	+16/−1
	GO:0007415	Defasciculation of motor neuron axon	3	2	0.08	.002	+2/−0
	GO:0007509	Mesoderm migration	4	2	0.11	.004	+2/−0
	GO:0009253	Peptidoglycan catabolic process	4	2	0.11	.004	+1/−1
GO:0035286	Leg segmentation	7	4	0.19	.007	+4/−0	
West vs. historical	None						
East mild vs. severe	GO:0006541	Glutamine metabolic process	4	2	0.09	.003	+2/−0

GO terms significantly enriched ($P < 0.1$, Fisher's exact test) in the 3 gene lists are presented. The number of differentially expressed genes and the direction of change in expression relative to the more distressed second group in each comparison are listed for each GO term.

ated rRNA fragment abundance merits further exploration. In addition, the consequences of viral infection and CCD on the function of ribosomes should be explored through assays of translational efficiency. Because of the potential for translational interference, studies on immune suppression should focus on bioassays or protein abundance rather than on immune gene transcripts.

Our results also indicate an unappreciated variation in gene expression patterns and pathogen loads with geography. This variation provides insight into the different stresses facing bees and clearly demonstrates that diagnostic surveys must sample extensively across numerous bee populations. Colony surveillance via assay of rRNA-like transcript abundance may provide

Table 2. Comparison of fold-change expression differences as measured by microarray and qPCR

Transcript name	Entrez ID	Fold-change microarray			qPCR	
		W vs. H	E vs. H	S vs. M	Gut	Abdomen
Deformed wing virus	AJ489744.2	4.05	5.34	7.33	1.36*	3.00
Bee brain EST	BI515001	1.21	0.95	0.68	—	−1.21
mbk-1	NM_001011629.1	3.97	1.84	2.69	−1.51	—
Cytochrome P450 (CYP4G11)	NM_001040233.1	−4.01	−1.82	−7.44	−0.3	4.45
Peptidoglycan recognition prot. S3	XM_001123180.1	−1.94	−1.15	−2.47	−0.48	2.47
28S rRNA 401–469 (EST jdeC12)		4.67	3.58	3.94	0.72	—
5.8S rRNA 18–60 (EST jdeC15)		5.29	3.15	2.34	0.61*	—
28S rRNA 1838–1903 (EST QW33)	BG101565	2.92	2.80	3.99	1.92*	2.47*

Microarray: W vs. H, California 2006 CCD vs. historical; E vs. H, Florida and Pennsylvania CCD vs. historical; S vs. M, severe CCD vs. mild CCD. QPCR: gut, California 2007 CCD vs. California 2007 healthy ($n = 60$); abdomen, pooled abdomen samples collected from healthy and CCD bees in Pennsylvania, Florida, and California and healthy bees collected in Massachusetts and Illinois during 2006–2008 ($n = 147$) (see Table S3). Transcripts showing similar trends in abundance in microarrays and qPCR samples ($P < .05$, one-tailed Mann-Whitney test) are indicated by * and using pooled abdomen samples collected from healthy and CCD bees in Pennsylvania, Florida, and California and healthy bees collected in Massachusetts and Illinois during 2006–2008 ($n = 147$).

*Significance ($P < .05$).

Table 3. Proportion of colonies with detectable levels of viral and microsporidial pathogens, as measured by qPCR

Year	Status	State	Colonies	ABPV	KBV	IAPV	DWV	SBV	Number of viruses	<i>N. apis</i>	<i>N. ceranae</i>	Number of Pathogens
2006	Healthy	MA/PA	14	0%	14%	7%	64%	36%	1.21 ± 0.80	0%	50%	1.71 ± 0.99
	CCD	FL	24	38%	38%	25%	46%	8%	1.54 ± 1.67	8%	42%	2.04 ± 2.03
	CCD	CA	57	51%	21%	21%	58%	30%	1.81 ± 1.41	40%	60%	2.81 ± 1.74
2007	Healthy	CA	14	86%	7%	14%	29%	7%	1.43 ± 1.02	7%	86%	2.36 ± 1.00
	CCD	CA	16	69%	44%	25%	44%	6%	1.88 ± 0.72	0%	94%	2.81 ± 0.83

Total number of different viruses and pathogens detected are summarized (\pm SD). More pathogens were detected in bees from colonies identified as suffering from CCD in both 2006 and 2007 ($P < .05$; one-way Mann-Whitney test), and CCD colonies overall contained more viruses ($P < .05$; Fisher's combined probability test).

an earlier indication of CCD status than has hitherto been available and allow beekeepers to take actions to reduce losses.

Materials and Methods

Biological Material and RNA Extraction. Remaining adult bees in colonies diagnosed with CCD at apiaries in Florida, California, and Pennsylvania were collected during winter 2006–2007. CCD colony health was scored at the time of collection as either severe or mild, depending on the apparent strength of the colony. Historical bees, collected before the appearance of CCD and hence ostensibly healthy, were collected in 2004 and 2005 from colonies set up on new equipment with no miticide treatments in apiaries of Pennsylvania State University near State College, Pennsylvania (Table S3). Bees for the reference (ostensibly healthy) sample were collected from 5 hives near Urbana, Illinois in July 2007. To date, there have been no confirmed cases of CCD in central Illinois. All bees were immediately frozen on dry ice and kept at -80°C until dissection.

Dissection, RNA extraction, microarray, statistical and qPCR validation analyses are described in SI Text. Pooled samples of RNA from 6 guts collected from each CCD colony were hybridized to 2 arrays, incorporating a dye swap (Fig. S1). Two separate pools of RNA from 6 guts each were created for both historical samplings, and each was hybridized against the reference on a single microarray. Experiments were designed to meet Minimum Information About a Microarray Experiment standards, and all microarray data obtained in these studies were deposited at ArrayExpress [www.ebi.ac.uk/arrayexpress (accession no. E-MEXP-2292)].

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