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<thead>
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<th>Collections</th>
<th>Subscription</th>
<th>One Time (perpetual)</th>
</tr>
</thead>
<tbody>
<tr>
<td>✔️</td>
<td>✔️</td>
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</tr>
<tr>
<td>Book Series</td>
<td>✔️</td>
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<tr>
<td>eHandbooks</td>
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<tr>
<td>Reference Works</td>
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<tr>
<td>Reference Modules</td>
<td>✔️</td>
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2015 Contents
The subject of insect science is divided into six major sections, each of which is reviewed once a year.

February
Insect genomics
Edited by Susan Brown and Denis Tagu

Development and regulation
Edited by Kenji Tomioka and Elzbieta Pyza

April
Ecology
Edited by Reta Gols and Erik H Poelman

Parasites/Parasitoids/Biological control
Edited by Bryony C Bonning

June
Pests and resistance
Edited by Fiona Goggin and Keyan Zhu-Salzman

Behavioural ecology
Edited by Jacintha Ellers and Cécile Le Lann

August
Vectors and medical and veterinary entomology
Edited by Nora Besansky

Social insects
Edited by Christina Grozinger and Jay Evans

October
Global change biology
Edited by Steven Chown

Molecular physiology
Edited by Alex Raikhel and Naoki Yamanaka

December
Neuroscience
Edited by Yehuda Ben-Shahar

Special section on insect conservation
Edited by Jeff Harvey
Editorial overview
201 Nora J Besansky
Editorial overview: Vectors and medical and veterinary entomology: From molecular biology to field ecology

Vectors and medical and veterinary entomology
Edited by Nora Besansky

14 Karima Zouache and Anna-Bella Failloux
Insect–pathogen interactions: contribution of viral adaptation to the emergence of vector-borne diseases, the example of chikungunya

59 Mara KN Lawniczak
Connecting genotypes to medically relevant phenotypes in major vector mosquitoes

83 Zainulabeuddin Syed
Chemical ecology and olfaction in arthropod vectors of diseases

90 Frank Criscione, David A O’Brochta and William Reid
Genetic technologies for disease vectors

98 Iliano V Coutinho-Abreu, Anderson B Guimarães-Costa and Jesus G Valenzuela
Impact of insect salivary proteins in blood feeding, host immunity, disease, and in the development of biomarkers for vector exposure

110 Igor V Sharakhov and Maria V Sharakhova
Heterochromatin, histone modifications, and nuclear architecture in disease vectors

118 Philip A Eckhoff, Caitlin A Bever, Jaline Gerardin, Edward A Wenger and David L Smith
From puddles to planet: modeling approaches to vector-borne diseases at varying resolution and scale

142 W Robert Shaw, Geoffrey M Attardo, Serap Aksoy and Flaminia Catteruccia
A comparative analysis of reproductive biology of insect vectors of human disease

156 Rosemary Susan Lees, Jeremie RL Gilles, Jorge Hendrichs, Marc JB Vreysen and Kostas Bourtsis
Back to the future: the sterile insect technique against mosquito disease vectors

Social insects
Edited by Christina Grozinger and Jay Evans

71 Laura M Brutscher, Katie F Daughenbaugh and Michelle L Flenniken
Antiviral defense mechanisms in honey bees

124 Christina M Grozinger and Gene E Robinson
The power and promise of applying genomics to honey bee health

133 Anthony D Vaudo, John F Tooker, Christina M Grozinger and Harland M Patch
Bee nutrition and floral resource restoration

204 David J Biddinger and Edwin G Rajotte
Integrated pest and pollinator management—adding a new dimension to an accepted paradigm

The cover
Global populations of honey bees and many other pollinator species are in decline. In this issue, we review the latest information about the status of pollinator populations and the factors underpinning declines, and demonstrate how basic research can be translated into effective strategies for conserving and expanding healthy pollinator populations. Image is of a honey bee (Apis mellifera) on a yellow hydrangea. Photo credit: Peggy Greb (USDA-ARS).
Editorial Overview: Social insects: From the lab to the landscape - translational approaches to pollinator health

Christina M Grozinger and Jay D Evans

Approximately 90% of flowering plants — corresponding to nearly three quarters of global agricultural crops — use pollinators to set seed and fruit [1,2]. However, populations of several species of pollinators are in decline throughout the world [3–5], threatening the stability of our ecosystems and productivity of our agricultural landscapes.

Three major events from 2006 to 2007 catalyzed global awareness of our dependence on pollinators and the severe and increasing threats to their populations. First, the publication of the US National Research Council’s report on the Status of Pollinators in North America [6] cataloged dramatic declines in several managed and unmanaged pollinator species, while highlighting our lack of understanding of the factors that are causing these declines and the surprisingly limited information available for the vast majority of pollinator species. Second, US beekeepers reported heavy and enigmatic losses of their honey bee colonies, a phenomenon soon called Colony Collapse Disorder (CCD) [7]. Third, an international coalition of researchers published the honey bee (Apis mellifera) genome sequence, providing critical new tools and approaches for studying honey bee health [8].

These events galvanized the scientific community, stakeholders, policymakers and the public to work together to conserve and expand pollinator populations. While there have been tremendous advances in our understanding of the status and health of global pollinator populations in the last 9 years, populations of many key species, including honey bees, continue to experience declines. Worldwide attention to the plight of pollinators continues to increase. Indeed, the US Pollinator Health Task Force recently released a sweeping set of US federal policy recommendations generated in response to President Obama’s presidential memorandum on pollinators [9], while the EU and member countries have funded a series of pollinator monitoring (e.g., www.COLOSS.org, [5]) and research initiatives.

In this special issue of Current Opinion in Insect Science, we have integrated the most recent scientific studies examining the state of global pollinator populations with the key factors impacting pollinator health. We have highlighted the remaining knowledge gaps and threats which must be addressed. Importantly, the reviews in this issue clearly demonstrate how basic scientific research has generated new approaches and recommendations to mitigate pollinator declines, and the critical need for ongoing research to create effective strategies to conserve and expand healthy pollinator populations.
The events of 2006–2007 made it clear that information about the status of most pollinator populations is woefully limited. Two of the reviews, led by vanEngelsdorp [10] and Meixner [11], discuss results of recent surveys which have dramatically improved our understanding of the status and epidemiology of honey bee losses in the US and Europe, respectively. In addition, Schwartz et al. [12] reviews the biotic risk factors tied to worldwide honey bee colony losses. The articles further discuss the importance of national and international monitoring of pollinator populations to both fully document pollinator declines and identify the associated drivers of these declines.

A consensus has emerged that honey bees and other pollinator species face acute risks from three primary stressors: exposure to anthropogenic chemicals, deficient food sources, and new or resurging parasites and pathogens. In addition, many unmanaged, wild pollinator species are vulnerable to natural habitat losses. Biotic risk factors for bee declines are discussed in this issue in reviews led by Flenniken (viruses, [13]), Genersck (bacteria, [14]), Evison (fungi, [15]), Vaudo and Winfree (nutrition and habitat, [16,17]). Abiotic factors, including the impacts of pesticides, pesticide formulations, and how they are applied in the field, are dissected in reviews led by Berenbaum [18], Mullin [19], Krupke [20], and Biddinger [21]. In all cases, the authors identify the key threats, describe the latest advancements in our understanding of how these impact pollinators, and provide recommendations for future lines of research as well as pollinator and land management strategies that can mitigate these threats.

Importantly, many of these stressors act synergistically, and thus it is critical to evaluate the effects of interactions among these stressors and within an ecological and genetic context. Reviews led by Schwarz [12], Evison [15] and Meixner [11] discuss the need to examine and account for interactions between symbiotic microbes, pathogens, parasites, host genetics and different environmental conditions, while DeGrandi-Hoffman and Chen [22] discuss the association between nutritional resources and disease. Moran [23] highlights exciting developments in the field of metagenomics — the application of new technologies and approaches to understand the impacts and evolution of microbes specifically adapted to their bee hosts.

In the case of social insects such as honey bees, examining the effects of different stressors in the context of social organization and colony-level population dynamics is critical to understanding how they can lead to the demise of social unit. The effects of stressors on social dynamics, colony collapse and overwintering success of honey bee colonies is discussed in reviews led by Barron [24] and Doke [25].

Several reviews discuss how recent advances in both techniques and knowledge can be harnessed to conserve and promote healthy pollinator populations. Biddinger and Rajotte [21] discuss the critical need to consider ‘Integrated Pest and Pollinator Management (IPPM)’ approaches to ensure growers can use an array of tools to sustainably enhance crop production while protecting the ecosystem services provided by pollinators. Advances in our understanding of bee breeding can lead to the generation of more resilient stocks of managed pollinators, as described in a review led by Niño [26]. With massive increases in genomic information for honey bees and other pollinator species, it is now possible to use genomic tools and resources to document population declines, study the factors impacting pollinator health, and develop more resilient stocks of managed pollinators, as reviewed by Grozinger and Robinson [27]. Finally, the new information and recommendations generated by the scientific community can only be effective if supported by social and regulatory frameworks produced by collaborative efforts across policymakers, stakeholder groups, and the public, as discussed by Suryanarayanan [28].

In the past decade, we have made great strides in our understanding of the status and health of global pollinator populations, and in the tools and resources available to examine these issues. These advances have revealed the complexity of conserving and managing species that are threatened by a myriad of interacting biotic and abiotic factors, and demonstrate the need to deploy a ‘systems approach’ to study pollinator health. Insights provided by these studies have already generated new strategies for better management of pollinator populations and their environments, and these strategies will be further refined and expanded as we address the existing gaps in our knowledge. Forging stronger connections across the scientific community, stakeholders, policymakers and public will ensure healthy agricultural and natural ecosystems through the conservation and expansion of pollinator populations.

References


Recent research has provided improved genome-level views of diversity across global honey bee populations, the gut microbiota residing within them, and the expanding pathosphere challenging honey bees. Different combinations of bee/microbiota/pathosphere genome complexes may explain regional variation in apiculture productivity and mortality. To understand this, we must consider management and research approaches in light of a hologenome paradigm: that honey bee fitness is determined by the composite bee and microbiota genomes. Only by considering the hologenome can we truly interpret and address impacts from the pathosphere, pesticides, toxins, nutrition, climate and other stressors affecting bee health.

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Influence of the bee genome on pathosphere susceptibility
Recent identification of significant positive selection on worker-specific honey bee genes compared to queen- or drone-specific genes [6*,7**] supports the theory that evolutionary fitness is largely determined by adaptations in the most ubiquitous caste, workers. Such adaptations include many traits associated with eusociality such as caring for young, foraging, defense against predators and competitors (aggression), and defense against the pathosphere (immune response, grooming, hygiene). Thus, the impact of selective pressures on workers, such as the pathosphere, can significantly affect the overall genome and phenotype of the honey bee population even though they themselves are a non-reproductive caste and do not directly transfer genetic information from one generation to the next. This fact justifies the importance and relevance of bee health research using workers and not just reproductive castes.

Although the full honey bee genome assembly is based on one lineage (U.S. domestic hybrid), a genome-wide single-nucleotide polymorphism (SNP) comparison across 140 lineages [7**] confirmed that honey bees are genetically diverse as a species and helped define relationships among all the lineages to four major groups, ordered from most to least genetically diverse: (1) Group A (Africa), (2) group O (Middle East), (3) group M (Western and Northern Europe) and (4) group C (Central Europe). Hybrid lines developed from these groups include Africanized (largely of group A ancestry) and U.S./European (largely of group C A. m. ligustica ‘Italian’ ancestry), which have equal or greater genetic capacity to respond to a diverse pathosphere and environmental factors compared to their progenitor groups.

Genetic comparisons among the four major bee groups [7**] revealed lineage-specific variation in immune genes that could be extrapolated as an explanation for differential susceptibility of certain bee lineages to the pathosphere.

Genomic frameworks to understand and improve bee health
Basic honey bee research clarifies factors affecting optimal colony performance and supports adaptive apiculture management practices to current problems and consumer demands. The recently updated honey bee genome and gene annotation [1] provides a significant research tool to understand how honey bees adapt and respond to their environment. Yet, overall honey bee health must be considered under a broader system that includes the bee genome in context with the genomes of their beneficial microbial symbionts (the microbiota) [2]. A myriad of parasites and pathogens (the pathosphere [3]) infect bees and are an additional genomic network challenging bees at all times in a multitude of combinations. To encapsulate this broader perspective, hologenome theory [4] argues that both the host and microbiota genomes adapt and evolve together as a unit of selection in response to pathogens and other environmental factors, both determining phenotype and overall fitness. We argue this emerging paradigm [5] is essential to refine our understanding of bee health. Toward improved understanding of the roles among these genomic frameworks, 18 strains of microbiota and 64 strains from the pathosphere have fully sequenced genomes currently available (Figure 1 and Table S1 for links to all full genome resources) with partial genetic information for many additional strains (not shown).
Data to support this may come from studies designed to directly contrast bee lineage susceptibility to pathogens [8,9] or by inference across multiple studies examining pathogen impacts. For example, reported virulence and colony collapses attributed to the presence of the microsporidian parasite Nosema ceranae [10], involved a particular lineage of group M (A. m. iberiensis). In contrast, group C and derived domestic hybrid lineages are largely less susceptible to this parasite [11,12], with no directly quantifiable correlation to colony collapses (summarized in Table S2). Nonetheless, N. ceranae are parasitic and damaging to bees in general [13] and synergistically (discussed in ‘Interactions among the pathosphere’). Compounding regional environmental factors and pathosphere composition likely exacerbate disease and are additional factors to consider.

Finally, genetic diversity within the major groups A, O, M, C and derived hybrids is pronounced [77] and is an important point for long-term management (see Nino and Jasper, this issue). Sustaining genetic diversity within hybrid stocks promotes adaptability to varied and changing pressures facing apiculture. From this genetic diversity, apiculturists can target particular genetic-based traits in any bee lineage using selective breeding for desirable traits to optimize resistance to the pathosphere by harnessing and expanding effective genomic features within a managed population. Initial identification of genetic underpinnings of such traits have been made for pathosphere-resistance to Varroa destructor mite [14,15,16], foulbrood (Paenibacillus larvae) [17], Nosema [18,19], and chalkbrood (Ascospaera apis) [20]. These and future research findings will enable genetic screening methods for refined development of disease-tolerant lineages, which should improve colony survival and productivity and reduce the use of pesticides as control measures.

Pathosphere contributors to colony collapses
Numerous honey bee surveys confirm that colonies typically face an increasingly diverse and dynamic pathosphere (see Box 1) [21–30]. Although each of these likely contribute a cost to its host, some may be more significant to colony mortalities (collapses). Summarized findings from multiple surveys [21,22,24,31–33] in Canada, Europe and the U.S. contrasting pathogen incidence and/or abundance from healthy and collapsed bee colonies highlight significant pathogens (Table 1). No
Box 1 The expanding honey bee pathosphere.

Expanding research aims and molecular diagnostics are uncovering an increasingly diverse honey bee pathosphere. *Protists*: Two species of trypanosomatids are now known to infect honey bees [64]. Molecular detection of what was widely presumed to be *Crithidia melliflora* (e.g. [23,26,31,32,65,66]) is actually a very different and novel species, *Lotmaria passim* [64], with at least two strains recognized from *A. mellifera*. Impacts of trypanosomatids are largely unexplored [47] but have been associated with colony losses (Table 1). *Fungi*: *Aspergillus* fungi are opportunistic pathogens that release mycotoxins when infecting hosts and can cause recognizable disease in honey bee larvae (stonebrood) but typically go unnoticed in adult bees. Recent work identified three virulent species to honey bee larvae (*A. flavus, A. nomius* and *A. phoenicis*) and adults (*A. flavus*, the only one tested) [67]. *Bacteria*: Two well-studied bacteria can cause fowlbrood disease in honey bee larvae, but variation in disease severity has only recently been linked to genomic variants of the etiologic agents *P. larvae* [68] and *M. plutonius* [69]. Full genome analyses identified substantial differences among two *P. larvae* strains that may help explain the hyper-virulence of ERIC II compared to ERIC I [89]. Many strains of *M. plutonius* originate from three major ‘clonal complexes’ and particular ones can predominate regionally, which may underscore virulent outbreaks in Northern Europe [89]. *Viruses*: At least five members of this pathosphere group can contribute significantly to colony collapses (Table 1). Genotype variants of the primary honey bee viruses are regularly reported including recently strains of IAPV (see Table S1), LSV [31,32], and DWV/VDV-1 hybrid strains [38**,70].

A single pathogen was consistently correlated with colony losses and many pathogens had no significant correlation (Table S2). However, several pathogens were repeatedly identified as significant and included: ABPV, DWV, KBV, *Nosema apis*, trypanosomatids and *V. destructor*. Varroa mites are undeniably bad pests that feed on the hemolymph of bees, leading to altered bee physiology via suppressed protein metabolism [34–36], and increased prevalence, titer, and pathology of certain viruses including KBV and DWV [28–30,37,38**,39] explaining the correlation of these three taxa with colony collapses (Table 1). Consequences of *N. apis* infection include midgit tissue damage followed by dysentery, precocious development [40], shortened life span [41*,42], impaired protein catabolism [43] and dysregulation of developmental, metabolic and sensory pathways [44]. The viruses (ABPV, DWV, and KBV) can be extremely virulent at high titer [45,46]. Assessed impacts from trypanosomatids are limited but include demonstrated [47] and predicted [3] metabolic and behavioral costs. Although we focus on addressing key pathosphere taxa with colony collapses here, this is a multifactorial issue with other significant contributors identified including nutrition quantity [33] and quality [48], colony size [25,33], and climate [49,50].

**Interactions among the pathosphere**

When parasites co-infect a host, facilitation or competition among them may occur and can change the nature and course of infection. Co-infection impacts on the host can by synergistic, enhancing damage to the host due to increased virulence (i.e. titer, lethality), skewed ability to regulate the infection (i.e. altered immune response), and increased susceptibility to subsequent infection by other species or strains [51]. Recent work documenting synergy among the honey bee pathosphere include increased virulence (defined by adult survival) during microsporidia and viral co-infections [52**], altered host immune responses during mixed microsporidia [44] and microsporidia/trypanosomatid infections [47], increased susceptibility (defined by co-prevalence) among black queen cell virus and sacbrood virus [29] and among *Spiroplasma* bacteria [49], and the mite-mediated increase in viral virulence discussed in the prior section.

The two microsporidian species in honey bees, *N. apis* and *N. ceranae*, occupy the same niche in their host (midgut) and provide a useful model of direct competition between parasites. These species compete with one another for access to their obligate tissue type for replication, midgut epithelial cells, via spatial occupation. The first species to establish in the midgut gains control and limits subsequent infection by the other species [53] but does not entirely block it [41*], allowing mixed microsporidia infections to occur with one species replicating predominately over the other. The reported predominance of *N. ceranae* over *N. apis* in many managed honey bee colonies supports that *N. ceranae* has a competitive edge in this dynamic. This may in part be due to higher spore production [54,55] and a somewhat stronger ability to establish over *N. apis* than vice versa [53]. Such *N. apis* and *N. ceranae* co-infections can be more virulent to the host bee (U.S. hybrids) than single-species infections [41*], although this is unsupported in smaller-scale studies [54].

Lastly, there is mounting evidence for within-host competition among populations of honey bee viruses (between DWV and both KBV and SBV) [29], and among DWV and *N. ceranae* shown both *in vivo* [56] and at the colony population level via non-overlapping prevalence patterns in Poland and Great Britain [27,57]. Thus, honey bee pathosphere taxa affect one another’s populations and cause greater overall harm to the honey bee synergistically as genomic diversity of the pathosphere increases.

**Contribution of the microbiota to the hologenome**

An important addition to this framework is the microbiota, which constitute a diverse second genome complex that can function hand-in-glove with the host genome (Figure 1). Insect microbiota can benefit their host in multiple ways including metabolizing food and toxins, nutrient supplementation, and protection against pathogens [58]. Phylogenotypes from the honey bee microbiota have largely been identified (see Moran, this issue) and can be separated into two broad ecological niches of the
adult gut, the crop (foregut) and the core gut (midgut through hindgut), which both have distinct phylotype compositions. Core gut microbiota may be particularly relevant in protecting bees from pathogens known to colonize/invade the gut and in enhancing nutrient availability. The crop microbiota may serve a unique role as a first line of defense against orally acquired pathogens or environmental toxins and chemicals as well as in maintaining the integrity of food stores within the hive [59,60], essential for survival during dearth periods (winter, dry season). Preliminary data support antagonistic roles of some Firmicutes strains (Lactobacillus spp.) [60,61] and improved larval survival with some Proteobacteria strains (Parasaccharibacter apium) [62**]. Largely, however, the specific contributions and importance of microbiota phylotypes to bee health have yet to be determined.

Apiculture management practices, including the common application of antibiotics to control bacterial pathogens that cause foulbrood disease, may unintentionally disrupt the microbiota diversity and density, thus altering the nature of the hologenome. Although the management intention is to target and kill pathogenic bacteria, non-target damage to the microbiota may cause side-effects such as nutritional stress, increased susceptibility to pathogens and an accumulation of antibiotic resistance genes [63]. As such, the applied use of prebiotics or probiotics: Pure cultures of individual honey bee microbiota isolates are enabling efficacy trials for probiotic applications. Uses may range from liberal supplementary application to more judicious use, such as following antibiotic treatment for foulbrood to quickly reconstitute the microbiota population and minimize risk of a pathogen breach or nutrient depletion. Further applications under development include paratransgenesis [71] to create hyper-beneficial probiotics, although this level of genetic engineering may be controversial. Before probiotics can be recommended for management purposes, careful and comparable assays that test the efficacy of microbial impacts under different host and pathogen genome combinations are required. Basic research on characterizing the honey bee microbiota will also enable development and testing of probiotics, supplements that promote the growth of microbiota already present, as another potentially valuable management tool.

**Box 2 Research applications to bee management**

**Probiotics**: Pure cultures of individual honey bee microbiota isolates are enabling efficacy trials for probiotic applications. Uses may range from liberal supplementary application to more judicious use, such as following antibiotic treatment for foulbrood to quickly reconstitute the microbiota population and minimize risk of a pathogen breach or nutrient depletion. Further applications under development include paratransgenesis [71] to create hyper-beneficial probiotics, although this level of genetic engineering may be controversial. Before probiotics can be recommended for management purposes, careful and comparable assays that test the efficacy of microbial impacts under different host and pathogen genome combinations are required. Basic research on characterizing the honey bee microbiota will also enable development and testing of probiotics, supplements that promote the growth of microbiota already present, as another potentially valuable management tool.

**Improved pathosphere diagnostics**: Apiculturists often want to screen their colonies for the presence/prevalence of certain pathogens to help management decisions (enact control measures or interpret colony collapses). Molecular methods are required to accurately diagnose some of the more common pathogen species (ex. _N. apis vs. N. ceranae, C. mellifera vs. L. passim_ and virulent pathogen strains (ex. _P. larvae_ ERIC I vs. II, DWV vs. VDV-1). Such molecular methods developed during basic research are increasingly optimized and available at bee diagnostics labs worldwide.

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### Table 1

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<th>Pathogen</th>
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<th>Incidence fold change&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Abundance fold change&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Location&lt;sup&gt;c&lt;/sup&gt;</th>
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<td>[21]</td>
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<td>124/1 year</td>
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<td>CCD</td>
<td>+1.07 ns</td>
<td>+6.67&lt;sup&gt;**&lt;/sup&gt;</td>
<td>US</td>
<td>124/1 year</td>
<td>[31]</td>
</tr>
<tr>
<td><em>IAPV</em></td>
<td>CCD</td>
<td>+1.53 ns</td>
<td>+2.14&lt;sup&gt;**&lt;/sup&gt;</td>
<td>US</td>
<td>76/1 year</td>
<td>[22]</td>
</tr>
<tr>
<td><em>KBV</em></td>
<td>CCD</td>
<td>+1.51&lt;sup&gt;**&lt;/sup&gt;</td>
<td>+14.26&lt;sup&gt;**&lt;/sup&gt;</td>
<td>US</td>
<td>124/1 year</td>
<td>[31]</td>
</tr>
<tr>
<td></td>
<td>Winter loss</td>
<td>+2.32&lt;sup&gt;**&lt;/sup&gt;</td>
<td>+14.26&lt;sup&gt;**&lt;/sup&gt;</td>
<td>Germany</td>
<td>~1200/4 year</td>
<td>[24]</td>
</tr>
<tr>
<td></td>
<td>CCD</td>
<td>+17.35&lt;sup&gt;**&lt;/sup&gt;</td>
<td>+5.49&lt;sup&gt;**&lt;/sup&gt;</td>
<td>US</td>
<td>51/3 year</td>
<td>[21]</td>
</tr>
</tbody>
</table>

nd, not determined; ns, not significant. ABPV, Acute bee paralysis virus; BQCV, Black queen cell virus; DWV, Deformed wing virus; IAPV, Israeli acute paralysis virus; KBV, Kashmir bee virus. Incidence: frequency of colonies with pathogen; Abundance: quantity of pathogen in colonies.

<sup>a</sup> Calculated from ratio of detection in diseased vs. healthy colonies.

<sup>b</sup> From Varroa no. mites/100 bees [24], total mite fall [25] or normalized qPCR data as \( \Delta \Delta C_T \) [31] or \( \Delta C_T \) [22].

<sup>c</sup> Bee lineages are presumed or given by reference to be U.S. or European domestic hybrids.

<sup>d</sup> Statistics as per the referenced study; significance levels shown are: *** \( P < 0.0001 \); ** \( P < 0.001 \); * \( P < 0.01 \); P \( \leq 0.05 \).

<sup>e</sup> Species combined (Noosema spp.) or species not confirmed (Trypanosomatidae).

<sup>f</sup> Fold change values estimated from graphs in this study.
probiotics to bolster or reconstitute microbiota communities as a management tool is an area of interest rapidly being enabled by basic research (see Box 2).

Conclusion

In addition to factors beyond the focus of this review (nutrition, epigenetics, pesticides and other abiotic factors), apiculturists must consider system-wide genome interactions as a framework central to managing bee health. We have highlighted the contextual importance of the hologenome jointly with the pathosphere genomes to better understand and fully contrast healthy, robust colonies with diseased and collapsing ones. Toward this, integrative research has provided new insights into the genetic complexity of bees, their microbiota, and the pathosphere as well as into interactions among them that will help address significant apiculture problems and lead to improved management solutions.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.cois.2015.04.006.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


This study provides strong support of the ‘superorganism’ theory for eusocial insects by showing evolution acts through positive selection on genes specific to a non-reproductive member of the colony, workers.


Important for contributing a large data set and genome-wide SNP analyses using 140 honey bee genomes from nine recognized subspecies and four hybrid lines (EU, US, Africanized). Supported by prior studies, this is the current culmination of genetic diversity within global honey bee populations.


These authors use a simple, well-designed study contrasting a naturally Varroa tolerant population of honey bees (meaning they can survive even when heavily infested with mites) to a nearby non-Varroa tolerant population to show viral titers (for two viruses) were significantly lower in the Varroa tolerant population, suggesting a strong antiviral response against certain viruses in this population is its key to success when facing Varroa infestations.


38. Ryabov EV, Wood GR, Fannon JM, Moore JD, Bull JC, Chandler D, Mead A, Burrows N, Evans DJ: A virulent strain of deformed wing virus (DVW) of honeybees (Apis mellifera) prevails after Varroa destructor-mediated, or in vitro, transmission. PLoS Pathog 2014 http://dx.doi.org/10.1371/journal.ppat.1004230. This study experimentally exposes developing bees to DWV alone or with Varroa to show Varroa drives DWV to high titers in some bees, which are

predominated by a particular virulent DWV genotype that successfully evades the bee immune response. Other pupae keep DWV titers in check despite Varroa, and contain many DWV genotypes instead of one predominant genotype. The authors also present phylogenetic analysis of sequence variants obtained from bees and mites, which form clades between putative ‘pure DWV’ and ‘pure DVV’.


41. Milbrath MO, van Tran T, Huang W-F, Solter LF, Tarpy DR: Lawrence F, Huang ZY: Comparative virulence and competition between Nosema apis and Nosema ceranae in honey bees (Apis mellifera). J Invertebr Pathol 2015, 125:9-15. Large sample size (n = 300/treatment group), multiple colonies (to account of intercolony variation), and a range of controlled doses and ratio combinations make this one of the more robust studies contrasting N. apis and N. ceranae virulence.


53. Natsopoulos ME, McMahon DP, Doublet V, Bryden J, Paxton RJ: Interspecific competition in honeybee intracellular gut parasites is asymmetric and favours the spread of an


The authors designate one of the most prevalent crop-specific bacteria (Alpha 2.2) here and perform in vitro studies supporting its association with larval food (royal jelly) and larval survival.


A detailed study that provides genetic data from the understudied *Aspergillus* community in bees and identifies dose-dependent virulence of some, particularly *A. flavus*, to larvae and adults.


These authors present a thorough, full-genome comparison for two strains of *P. larvae* with observed differences in virulence (ERIC I and ERIC II), which should provide strong foundation for future work with the important larval pathogen. A very helpful infection model summary graphic is also provided.


Uses multi-locus sequence typing (MLST) to assemble new and previous data for the largest population genetic study to date of *M. plutonius*. Data from this and other studies are available in a MLST database (http://pubmlst.org/mlst/) that should prove useful for other researchers.


Intersections between neonicotinoid seed treatments and honey bees
Christian H Krupke and Elizabeth Y Long

A growing understanding of the often subtle unintended impacts of neonicotinoid seed treatments on both non-target organisms and their environment have led to concerns about the suitability of current pest management approaches in large scale agriculture. Several neonicotinoid compounds are used in seed treatments of the most widely grown grain and oilseed crops worldwide. Most applications are made prophylactically and without prior knowledge of pest populations. A growing body of evidence suggests that these compounds become contaminants of soil, water, and plant products, including pollen and nectar. These unforeseen routes of exposure are documented to have negative impacts on honey bee health and also have potential to exert effects on a broader environmental scale.

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Seed treatments as crop protectants in agriculture
Concerns regarding the unintended consequences of pesticide use have recently received increased attention from researchers and regulatory bodies alike, particularly in the case of the neonicotinoid class of insecticides and their impacts on insect pollinators and ecosystems [1,2**,3,4**]. In the case of many of the principal agronomic crops grown worldwide (including maize, soybeans, wheat, canola, as well as cotton), neonicotinoids are routinely applied to seeds to guard against early season insect pests. In North America alone, these crops represent approximately 115 million hectares of production annually (94.5 million hectares in the United States and 21.5 million in Canada) [5,6]. Notably, this rapid adoption has occurred in the absence of any documented increase in pest threat [7]. The use of neonicotinoids as seed treatments began with the registration of imidacloprid in 1994, and it is now estimated that 60% of applications of neonicotinoid insecticides are delivered via soil or seed treatments [8], often in combination with protectant fungicides. The predominant neonicotinoids used in seed treatment formulations for grain and oilseed crops are thiamethoxam, its metabolite clothianidin, and imidacloprid. Although these formulations can provide crop protection, particularly from aphids and other sucking insects [9], the economic benefits associated with their use have been difficult to quantify in the major cropping systems where they are used, including maize [10–13] and soybeans [14,15]. These compounds also carry risks to beneficial insects and non-target areas surrounding fields both during and after planting. Chemical characteristics of these compounds that are frequently cited as beneficial for pest management include high water solubility that facilitates systemic movement through plant tissues and high persistence in soils. However, these same characteristics can enhance the potential for neonicotinoid active ingredients used in seed treatments to exert impacts on non-target areas and organisms within and beyond both the planted field and cropping season. In the sections below and the attached table, we outline the principal routes through which honey bees and other pollinators may encounter these compounds (Table 1).

Effects on honey bees & ecosystems
Exposure to residues via plant products
A wide range of pesticides (including several neonicotinoids) have been detected in honey bee hive resources including bee-collected pollen, stored pollen (or bee bread) and wax collected from honey bee hives located near commercial agriculture operations [16–21]. In most cases where neonicotinoids have been documented in honey bee or hive products, annual crops grown in the vicinity have been implicated as the likely source. This may be due to deposition of contaminated soil or planting dust upon bees, plants, or both. However, many crop plants grown from treated seeds express neonicotinoid residues in pollen or nectar, which poses exposure risks to honey bees via their food resources. Pollen loads from honey bee hives placed adjacent to oilseed rape grown from thiamethoxam or clothianidin-treated seeds in Poland have shown mean residue concentrations of these active ingredients in pollen to be 6.6 parts per billion (ppb) and 0.6 ppb respectively [22]. Imidacloprid concentrations ranging between 1.1 and 5.7 ppb have been detected in honey bee-collected pollen loads in France [16,18], while thiamethoxam and clothianidin...
concentrations ranging from 1.2 to 7.4 ppb and 3.9 to 88 ppb, respectively, have been detected in honey bee-collected pollen in Indiana, USA well after planting activities ceased [19]. Maize pollen grown from seeds treated with thiamethoxam and clothianidin contained 1.7 and 3.9 ppb respectively, and bees were shown to forage upon this pollen in the field [19]. In a 3-year study conducted in France, fifty-seven percent of 185 honey bee pollen loads exhibited imidacloprid contamination with an average concentration of 0.9 ppb [17]. The neonicotinoids thiacloprid, imidacloprid, and acetamiprid have been detected in 5.4%, 2.9%, and 3.1% of 350 pollen samples collected from North American honey bee colonies located in various cropping systems [20], although very few of these samples were collected from areas where neonicotinoid-seed treated crops were grown. Although the percentages reported in this study are low, individual detections of neonicotinoids included maximum values of 115 ppb for thiacloprid, 912 ppb for imidacloprid, and 134 ppb for acetamiprid.
There is further evidence that honey bees can be intoxicated by neonicotinoid residues in guttations, exuded water droplets, produced by maize seedlings grown from treated seed. Exposure in this case is the result of the systemic movement of active ingredients from treated seeds into the seedlings. Chemical analysis of guttations collected from field and laboratory-grown maize plants seed treated with imidacloprid, clothianidin, or thiamethoxam exhibit high concentrations ranging from 11,900 to 47,000 ppb in field-collected guttations and 82,800 to 110,000 ppb in lab-collected guttations [23]. Furthermore, honey bees fed the guttations from treated maize seedlings exhibited lack of coordination, irreversible wing paralysis and death shortly thereafter. Although honey bees are known to collect guttations from winter rape [24], the extent to which honey bees utilize water resources in the form of guttations from other treated crop species requires further study.

The range of concentrations listed above generally fall below acute toxicity levels (Table 2) and represent a chronic, sub-lethal exposure route for pollinators. Effects of ingestion of food containing sub-lethal doses of neonicotinoids have recently been quantified for honey bees and bumblebees. Although beyond the scope of this article, effects of these sub-lethal exposures have included impaired navigation and learning, impaired immunity and reduced colony growth and queen rearing [1,25,26–28,29**].

### Residues in dust from planting treated seeds

Neonicotinoid seed treatments are currently a focus of scrutiny for several reasons; but chronicling their unintended environmental impacts was first initiated by the deaths of large numbers of honey bees following the planting of neonicotinoid-treated seeds in several countries, spanning the period since these products were first widely adopted [19,21,30–32]. Initial investigations determined that seed-treatment coatings can abrade and fall away from the seed surface [21,30,33]. Investigations of these acute exposures suggested that some form of ‘operator error’ (i.e., below standard application of seed treatment pesticides) was responsible for the observed honey bee deaths during spring seed sowing [32]. However, despite improvements in pesticide formulations and the quality of seed coat applications, additional bee die-offs have been documented in the EU, Canada and the US [19,21,31]. It is now clear that during the course of normal planting operations, exhaust systems of modern pneumatic planters deliver seed treatment active ingredients into the air, where the dust can disperse and settle onto nearby vegetation or honey bees themselves [21,34**,35].

Efforts to quantify neonicotinoid contamination resulting from planter dust have documented the presence of residues in soil, grass, and flower blossoms following the sowing of treated seeds. Evaluations of environmental contamination by maize seed treatments containing clothianidin and thiamethoxam have found concentrations ranging between 2.1–9.6 ppb in soil samples and 1.1–9.4 ppb in dandelion blossoms collected from field margins [19]. Average concentrations of imidacloprid in grass and flower samples of 21 ppb and 32 ppb, respectively, have been documented [30], as well as higher concentrations ranging between 14–29 ppb in grass samples and 22–59 ppb in flower samples collected the day of, as well as several days following, the sowing of neonicotinoid-treated maize [33]. Variable clothianidin residue concentrations, some exceeding 40 ppb, have also been detected in flowers collected from untreated apple, dandelion, oilseed rape and other wildflowers [21]. The contamination of dew and guttation droplets by dispersing planter dust is another possible exposure route for honey bees. Evaluation of these water sources for contamination following the sowing of clothianidin-treated seeds revealed active ingredient concentrations ranging between 17.5 and 27 ppb, one hour after planting and concentrations between 6.5 and 12.5 ppb 24 h after planting [36]. Furthermore, the addition of seed

### Table 2

<table>
<thead>
<tr>
<th>Neonicotinoid</th>
<th>Honey bee (LD50)</th>
<th>Soil (Half-life)</th>
<th>Water (Half-life)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oral</td>
<td>Contact</td>
<td>LD50</td>
</tr>
<tr>
<td>Thiamethoxan [46,47]</td>
<td>5</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Clothianidin [48]</td>
<td>4</td>
<td>43.9</td>
<td>148–1155</td>
</tr>
<tr>
<td>Imidaclorpid [49]</td>
<td>3.7</td>
<td>59.7</td>
<td>40–124</td>
</tr>
<tr>
<td>Acetamiprid [50,51]</td>
<td>14,530</td>
<td>8090</td>
<td>2.6–133</td>
</tr>
<tr>
<td>Thiacloprid [52]</td>
<td>17,320</td>
<td>38,800</td>
<td>2.4–27.4</td>
</tr>
</tbody>
</table>

lubricants such as graphite or talc (a recommended practice for planting with most pneumatic planters) can exacerbate the abrasion of seed coatings in the planter, such that lubricants also become contaminated with active ingredients and further contribute to environmental contamination when expelled with exhaust air [19].

Direct contact with neonicotinoid-contaminated dust clouds has been shown to occur for honey bees foraging in and around fields during planting activities, and in fact individual foragers exposed to dust clouds during flight subsequently suffer mortality within hours, particularly in cases of high humidity [35–37]. Chemical analysis of bees following their exposure to planter-emitted dusts demonstrate that foragers may acquire 29–3661 ng/bee of imidacloprid and 118–674 ng/bee of clothianidin [37]; well in excess of concentrations sufficient to cause acute intoxication for honey bees (Table 2). Furthermore, the characteristic pubescence of honey bees causes them to become electrostatically charged during flight as a result of friction with air; this is generally an adaptive trait that increases the attraction of small particles like pollen to the body surface as bees visit flowers [38]. In conditions where insecticide-laden dusts are found, however, this same mechanism may render bees more likely to accumulate residues as they fly near areas where planter dust is present.

**Exposure to residues via contaminated water**

Several recent publications have documented contamination of water sources with neonicotinoids used in seed treatments [39**]. Sampling of surface waters in the US has revealed frequent contamination of stream waters with clothianidin, thiamethoxam, and imidacloprid. Of 79 water samples collected across 9 sites of high maize and soybean production in the US, 75% were contaminated with clothianidin, 47% with thiamethoxam, and 23% with imidacloprid [40**]. Furthermore, documented concentration fluctuations corresponded with planting of neonicotinoid-treated maize seed and subsequent rainfall. These findings implicate neonicotinoid-seed treatments as likely sources of contamination and also reflect the very high water solubility of these compounds [8]. Similarly, thiamethoxam was detected in groundwater samples collected from intensively-managed agricultural regions in Wisconsin, USA from 2008 to 2012 [41**]. In this case, leaching of thiamethoxam applied during potato planting was implicated as a key contributor to groundwater contamination in and around crop production areas, both in-season and beyond. Neonicotinoids were also frequently detected in water samples collected in a repeated sampling of 136 Canadian wetlands spanning the provinces of Alberta, Saskatchewan, and Manitoba with 36% of wetlands showing evidence of contamination with at least one neonicotinoid before seed sowing and 62% of wetlands exhibiting contamination following seed sowing [42]. Furthermore, the same study found that the percentage of wetlands contaminated with neonicotinoids increased to 91% before seeding in the following year, suggesting that movement of residues from seed-treated fields to wetland areas occurs via run-off from melting snow. Finally, imidacloprid concentrations evaluated in water samples potentially used by bees in urban, suburban, and rural areas of Maryland, USA have documented values between 7 and 131 ppb [43**]. A similar study in Quebec, Canada evaluated pesticide residue concentrations in field puddles during the planting of treated-maize seed and detected clothianidin and thiamethoxam at values between 0.01 and 63 ppb [44**], which can exert sublethal effects on honey bees.

**Quantifying impacts at the ecosystem level**

Although the levels of neonicotinoids applied to each seed are readily available, there is almost no knowledge about the efficiency of translocation (i.e., the uptake and circulation of active ingredients by seedlings from the treated seed) or the concentration of active ingredients in various plant tissues after germination and during the growth and maturation of crop plants. This represents a key gap in our understanding of the environmental fate of these compounds. The degree to which these compounds may remain in crop soils and later translocate into flowering weeds or subsequent crops in the same field is also unclear. The potential for abraded seed treatments to move across the landscape has also not been quantified. Given that these compounds are highly water soluble and act systemically, there is the potential for dispersing residues (e.g., in planter dust) to be absorbed by plant tissues or dissolved in surface or ground water. This is of particular importance in many North American crop fields, where fields are drained using a system of perforated, buried pipes that convey excess water to drainage ditches at field margins.

**Synthesis and future directions**

The additive effects of these various exposure routes are still being quantified. However, given the area devoted to production of crops grown from neonicotinoid-treated seeds, it is clear that a great degree of temporal and spatial overlap exists between neonicotinoids and pollinators and other non-target organisms. Exposure can take place through various matrices — including air-borne and stationary dusts, soil, plant products, and water. For honey bees, where most current research is focused, future estimates of individual and colony-level effects of these exposures should incorporate these multiple routes into assessments of risk posed by neonicotinoid residues. Of particular interest is the typical period of sowing of many annual crops grown from neonicotinoid-treated seeds, which corresponds closely with flowering of spring blossoms and the concomitant increase in honey bee foraging activity across the landscape [45].
References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


This article reviews the concerns raised over the widespread use of neonicotinoids, focusing particularly on risks posed to the environment as a result of the toxicity, persistence, and accumulation of neonicotinoids in soil, water, and plant products (nectar and pollen).


This article provides a comprehensive review of the risks posed to pollinators and pollination services by the widespread use of neonicotinoids. It highlights the sublethal effects of neonicotinoids on honey bees, bumblebees, and a few other bee species, as well as the synergism of neonicotinoids with other pesticides and biotic stressors that impact bee health.


This work provides novel insight into the relationship between method of neonicotinoid application and the contamination of plant products, like nectar and pollen, in a crucibit crop.


This article provides novel insight about the size and shape of neonicotinoid-contaminated dust clouds generated during planting operations and also evaluates the extent to which modifications to planting equipment might reduce the emission of contaminated dust.


This article provides a comprehensive review of the risks posed to aquatic organisms by exposure to neonicotinoid-contaminated water, with detailed information on the range of neonicotinoid concentrations reported in surface waters worldwide and the implications of these concentrations for aquatic insect species and communities.

40. Hladik ML, Kolpin DW, Kuivila KM: Widespread occurrence of neonicotinoid insecticides in streams that exist in proximity to agricultural areas where neonicotinoids are utilized extensively as seed treatments. This work also documents patterns of stream water contamination that implicate movement of contaminants from treated seeds into streams after heavy rainfall.


This work documents a connection between soil applied neonicotinoids and the contamination of groundwater as a result of neonicotinoids leaching from the area of application via irrigation water.


This article documents the in-frequent, but significant occurrence of sublethal levels of the neonicotinoid, imidacloprid, in various water sources sampled from urban, suburban, and rural environments.


This article provides novel information about the occurrence of neonicotinoid residues in water puddles following the planting of neonicotinoid-treated seeds. It further documents a trend whereby the concentration of residues in water puddles was greatest early in the season; implicating drift from treated seeds as a potential contributor to early season contamination of water puddles within fields.


Genomics of the honey bee microbiome
Nancy A Moran

The guts of honey bee workers contain a distinctive community of bacterial species. They are microaerophilic or anaerobic, and were not clearly delineated by earlier studies relying on laboratory culture of isolates under atmospheric oxygen levels. Recently, a more complete picture of the potential metabolism and functions of these bacteria have been possible, using genomic approaches based on metagenomic samples, as well as cultured isolates. Of these, most are host-restricted and are generally absent outside adult guts. These species include both Gram negative groups, such as *Gilliamella apicola* and *Snodgrassella alvi*, and Gram positive groups such as certain *Lactobacillus* and *Bifidobacterium* species. These gut bacterial species appear to have undergone long term coevolution with honey bee and, in some cases, bumble bee hosts. Prediction of gene functions from genome sequences suggests roles in nutrition, digestion, and potentially in defense against pathogens. In particular, genes for sugar utilization and carbohydrate breakdown are enriched in *G. apicola* and the *Lactobacillus* species.

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**Introduction**
The importance of gut-dwelling microbial communities in the health of animals, from humans to insects, has become widely appreciated only recently [1]. A key reason is that tools for studying microorganisms in non-laboratory environments have become much more powerful. Because most organisms in most environments, including guts, are not readily grown in laboratory culture, traditional culture-based methods gave an incomplete and often extremely misleading picture of microbial communities. Approaches based on DNA sequencing have enabled a more reliable picture, and the growth of nucleotide sequence databases has enabled routine classification of gut microorganisms characterized only by short fragments of sequenced DNA.

These new methods have revolutionized understanding of microbial ecology in general. They have now been applied to gut communities in honey bees (*Apis mellifera*), and have yielded insights into the dynamics of these distinctive communities and into potential effects of gut bacteria on bee hosts. Below, I summarize recent findings about the honey bee gut microbiota, mostly based on genomic methods.

**Who are they?**
Starting in 2003 with a study on honey bee workers from North America and South Africa [2], several novel rRNA gene sequences were obtained, pointing to the occurrence of characteristic bacteria in the guts of this species. Later, more intensive sequencing repeatedly retrieved these same distinctive sequences and a few others from honey bee workers from samples in Europe, North America, and Australia [3–6]. It became clear that honey bee guts harbor a specific set of bacterial species not retrieved from other environments. The guts of bumble bees (*Bombus* species) contain bacteria that are closely related to some of the honey bee associates [7] and that are classified as the same bacterial species [8]. These same distinctive groups are also found in other *Apis* species [9–11]. In contrast, bees outside of *Apis* and *Bombus*, whether solitary or social, usually lack these bacterial species [5,7,12*,13]. The exceptions are certain eusocial stingless bees (Meliponini) in Australia which share some of the *Lactobacillus* clusters with honey bees [14].

Approximately eight bacterial species clusters dominate in the guts of *A. mellifera* workers (Table 1), making up over 95% of the bacteria in most individuals [10,15]. Each such cluster corresponds to a set of closely related bacterial strains. Because species designations are somewhat arbitrary within bacteria, some of these clusters are described as a single species, while others are described as multiple species. Three are newly described as species within the Gram negative bacterial phylum *Proteobacteria*; these are *Snodgrassella alvi*, *Gilliamella apicola*, and *Frischella perrara* [8,16], all restricted to the guts of *Apis* species and *Bombus* species, with *F. perrara* confined to honey bees. Strains within each of these three species show <3% sequence variation within the 16S rRNA gene but much more extensive divergence in their overall genomes, implying differences in metabolic capabilities [17*,18*]. Three similarly closely related clusters of Gram positive bacteria are also largely restricted to bee guts; these include two clusters within the *Firmicutes*.
phylum and specifically within the genus \textit{Lactobacillus}, and one \textit{Bifidobacterium} cluster within the phylum Actinobacteria. These have been called F-4, F-5 (or Firm4 and Firm5) and ‘Bifido’ [3–5]. Within the two ‘Firm’ clusters, strains have been given multiple species names within \textit{Lactobacillus} [19*]; likewise ‘Bifido’ corresponds to a species cluster that includes \textit{Bifidobacterium asteroides} from honey bee [20] and several species described from bumble bees [21–23] (Table 1). Two other species clusters are from distantly related clusters of Alphaproteobacteria, initially called Alpha1 and Alpha2 [4]. Alpha1 is a close relative of \textit{Bartonella} species, a group of specialized animal pathogens within the Rhizobiales, and is present and often abundant in about half of sampled workers [15]. Alpha2 consists of numerous strains of Acetobacteraceae, including Alpha2.1, which is a gut specialist, and Alpha 2.2 (\textit{Parasaccharibacter apium}) [24]. Among members of the typical honey bee gut community, only the Alpha 2.2 group, within Acetobacteraceae, appears to grow commonly in environments outside of bee guts. Closely related strains to Alpha2.2 are found in floral nectar, bee bread, and honey as well as both adult and larval guts [25].

Several other bacteria have been found at low abundance in some honey bee guts. These include a specific cluster from \textit{Bacteroidetes} [4,15] that has been retrieved from both European and American honey bee workers and from some bumble bees, but that is usually absent or at low abundance (<1%). In addition, several species of Enterobacteriaceae that are related to common insect pathogens are commonly present in low numbers (<0.1%), occasionally reaching higher frequencies in individual bees [15,26].

\textbf{Where are they?}

The honey bee adult worker harbors a large bacterial community in the gut, with roughly 1 billion bacterial cells in a mature worker [27,28]. Of these bacteria, ~95% are in the hindgut. The crop (honey stomach or foregut) contains bacteria [29], but studies that quantify cell numbers have shown that crop populations are very small [25,28]. The crop community is dominated by \textit{Lactobacillus kunkeei}, other environmental \textit{Lactobacillus}, and Acetobacteraceae (Alpha 2.2), taxa also present in the hive and in food, including nectar, dilute honey and bee bread [30]. Thus, the crop does not seem to harbor a specifically crop-adapted microbiota [25]. The midgut also contains relatively few bacteria, with most concentrated at the distal proventriculus region, adjoining the hindgut [28].

The hindgut is divided into two compartments, the anterior ileum, a narrow tube with six longitudinal invaginations, and the rectum, a larger, sac-like compartment;
each regions contains a characteristic community (Figure 1). The three main proteobacterial species, *G. apicola*, *F. perrara*, and *S. alvi* dominate in the ileum, forming a dense biofilm beginning at the junction with the Malphighian tubules and continuing along the length of the ileum wall [27,28]. *S. alvi* forms a layer directly on the cuticle lining the gut, and *G. apicola* is toward the lumen. Firm-5 is also present as small clusters in the lumen. *F. perrara* is typically less abundant and occasionally entirely absent, but often dominates ileum communities around day 8 of adult life, corresponding to the nurse stage [27]. The three dominant Gram-positives (Firm4, Firm5 and Bifido) dominate in the rectum, which contains a large bacterial community [27,28].

For adult worker guts, RNA and DNA samples give similar pictures of community composition, based on 16S RNA amplicons, indicating that these bacteria are alive and replicating within bee guts [15]. Likewise, metagenomic studies based on random sequencing of non-amplified DNA or RNA from guts indicate a similar taxonomic composition [31,32]. All of these studies point to a characteristic bacterial community that has evolved to specialize upon the honey bee adult gut as its sole ecological niche.

Although these specialized bacteria were first detected by non-culture based sequencing approaches, each of the species has since been successfully cultured in the laboratory [8,16,19,24,33]. In general, they grow on rich media, consistent with their host-associated lifestyle, and require anaerobic or microaerophilic conditions, that is, consistent with the likely lowered oxygen availability within the gut lumen, although some strains appear to grow at atmospheric oxygen levels [25]. Interestingly the *Bifidobacterium asteroides* present in honey bee guts retains capacity for aerobic respiration, unlike *Bifidobacterium* from mammalian guts, which are entirely anaerobic [20], reflecting the likelihood of higher oxygen concentrations in bee guts compared to mammalian guts. Culturing the bacteria enables full characterization of individual strains and facilitates progress in understanding the roles of the bacteria in bee health.

**How are they acquired?**

The characteristic gut bacteria in adult guts colonize during the first few days following emergence from the pupal stage [27]. When quantified, numbers of bacteria in larvae are small [28] and consist primarily of environmental Acetobacteraceae (Alpha2.2) and *Lactobacillus* [34]. Since these groups are also present in nectar and pollen, their presence in larvae may represent ingested bacteria present in the food, as suggested by phylogenetic analyses of strains from different sources [25]. Immediately following eclosure, adults lack gut bacteria. If pupae are removed from the hive and allowed to eclose within a clean dish in the laboratory, workers will remain nearly germ-free throughout adult life and will lack the typical gut bacterial species [27]. Colonization occurs through contact of newly eclosed adults with nurse bees and with
the hive environment. Oral trophallaxis alone is insufficient for normal community establishment, whereas exposure to live nurses or hindgut contents results in typical communities [27*]. Thus, at least some gut species, including those dominating in the ileum (G. apicola, S. alvi, F. perrara), are transmitted via a fecal-oral route. Adults that emerge in isolation in the lab can be experimentally colonized with known bacterial strains to determine effects on hosts [27*].

The worker gut community reaches its typical composition 3–5 days after eclosion [27*] and does not change appreciably when workers shift from within-hive activities to foraging [30]. Thus the adult gut seems to have a stable and distinct community that is not found elsewhere. Other bacteria are present in hive components, and these include a diversity of taxa such as L. kunkeei and Acetobacteraceae strains, [35**,36*]. These organisms occur in stored nectar and bee bread, where they appear to have a role in preserving but not in fermenting or digesting these stored products [35**].

What are they doing?

A metagenomic study of the worker gut community [31] contained an excess of carbohydrate-processing genes, particularly in strains of G. apicola. Pectate lyase, which can digest pectin present in cell walls of pollen grains, was present and functional in some strains of G. apicola, while absent in others. This indicates strain diversity in ability to use different dietary components, suggesting that the particular set of stains present in individual workers or in colonies might affect nutritional ecology of the bees or might act to neutralize dietary toxins. A study based on sequencing RNA from gut communities verified an excess of carbohydrate-processing genes actively expressed in the bee gut [32*]. Sequencing DNA from single bacterial cells from worker guts also indicated that G. apicola shows a striking enrichment for carbohydrate-processing genes and that gene sets in this functional category were highly variable among strains [18*].

Complete genome sequencing of cultured isolates of G. apicola and S. alvi shows that these two species have highly complementary metabolic capabilities [17**]. Whereas G. apicola strains contain a large number of sugar transporters and sugar utilization pathways, S. alvi cannot use sugar as a carbon source and instead must use carbohydrates produced as downstream products of sugar metabolism. This metabolic complementarity was found in all strains of G. apicola and S. alvi, suggesting a long coevolution as metabolic partners within the bee gut. In both G. apicola and S. alvi, individual isolates can vary in the presence of hundreds of genes despite near-identity of 16S rRNA sequences [17**]. Interestingly, the G. apicola strains from honey bee contain far more genes for use of carbohydrates including diverse sugars than did the bumble bee-associated strains [17**,18*], consistent with the expanded role of nectar-processing and honey in the ecology and nutrition of honey bees. The Lactobacillus and Bifidobacterium species also likely play a central function in carbohydrate catabolism and thus in nutrition of their hosts [32*].

Another potential role for the gut community is protection against parasites and pathogens. Genomic analyses have revealed many genes that produce toxins, potentially affecting bee parasites [17**,37]. The related gut bacteria in bumble bees have been shown to protect against infection by the trypanosomatid parasite Crithidia bombi [38], and variation in the gut microbiota underlies most observed variation in resistance to particular strains of C. bombi [39]. Community analyses of gut microbiota in three species of wild bumble bees in eastern North America revealed higher Crithidia incidence in individuals with low colonization by the core gut bacterial species S. alvi and G. apicola, consistent with a protective role by one or both of these bacterial species [40*]. Honey bees also can be parasitized by Crithidia species as well as by other parasites and pathogens. Some other bacterial symbionts of insects are known to lower infection rates by RNA viral pathogens of the hosts [41*]. Thus, honey bee gut bacteria plausibly can affect ability to limit proliferation of important bee diseases such as Deformed Wing Virus. Whether the characteristic gut bacteria play a role in resistance to these remains to be tested.

Some strains of bacteria associated with honey bees or bumble bees have antimicrobial properties, suggesting the possibility of inhibition of potential pathogens [21,42–46].

Probably gut communities are largely beneficial to their hosts, but this is rarely shown directly, even for well-studied mammalian systems. For bees, gut bacteria cannot be assumed to be entirely beneficial; possibly they confer a mixture of benefits and costs. For example, some strains of Escherichia coli in human guts contain a large locus (~50 kb) encoding a hybrid nonribosomal peptide-polyketide synthase pathway for production of a molecule called colibactin, which is implicated as a cause of colorectal cancer and tumors. Curiously, F. perrara, which is restricted to the honey bee gut ileum, encodes a closely homologous locus that produces a similar molecule, which has the same cytotoxic effects in cell cultures [37*]. The role of F. perrara and colibactin in honey bees is unknown, but one possibility is that F. perrara may have negative consequences for hosts, despite being found in guts of most workers.

Among genes that vary in presence among strains of G. apicola and S. alvi, some are likely involved in inter-strain competition. For example, S. alvi strains contain Type 6 Secretion Systems, mechanisms for delivering anti-bacterial toxins to other bacteria; the effectors are among the most variable genes sets in S. alvi [17**,18*].
Diversity — good or bad?
Although it is commonly believed that a diverse microbiota is a good microbiota [47], evidence for this is limited. In bees, species-level diversity is low, while strain diversity is high; however, whether more diversity is good or bad for hosts has not been experimentally addressed. Potentially, strain diversity provides more metabolic functions that benefit hosts; for example, *G. apicola* strains vary in ability to use particular sugars or other carbohydrates [17**,37**]. Some minor species that are erratically present are closely related to insect pathogens, such as *Serratia marcescens* and *Hafnia alvi*; these may represent opportunistic pathogens that can dominate if the typical gut community is compromised. For example, in the first few days following emergence and in workers deprived of normal routes of colonization, gut communities consist of erratic mixes of atypical bacteria, including some potential pathogens [15,27**]. Likewise, in bumble bees, individual workers sometimes have few of the typical *S. alvi* and *G. apicola* gut symbionts, and instead have higher representation of possible enteric pathogens [40**].

Implications of the bee microbiome for pollinator management and conservation
Very likely, the gut microbiota confers some benefits to honey bees, as genome sequencing suggests roles in digestion and nutrition, and experiments indicate protective effects in bumble bees. Some strains of bee-associated bacteria have antimicrobial properties, suggesting the possibility of inhibition of potential pathogens [21,42–45]. Given this suggestive evidence for a beneficial role of the gut microbiota, interfering with the normal gut community is likely to be detrimental. Long term antibiotic applications to colonies in the United States has resulted in ubiquitous presence of tetracycline resistance loci, present in most of the typical gut bacteria in US honey bees but absent from bees from countries where antibiotics have not been used [48]. Antibiotics lower diversity in the communities in which only some strains have resistance. Speculatively, the Food and Drug Administration approval of use of a novel antibiotic (tylosin) in 2005 played a part in lowering stress tolerance and in the unprecedented colony mortality that occurred in US honey bees during the winter of 2006–2007 [48]. Thus, it would seem prudent to avoid overuse of antibiotics, as this could have detrimental consequences for colony health, just as chronic use of antibiotics might affect human health by continually perturbing resident gut communities.

Another possibility is the use of honey bee gut bacteria as probiotics [17**,21**] or as agents for delivering gene products to the bee gut [49,50]. Potentially strains that are particularly useful in bolstering colony health under particular environmental or dietary conditions could be introduced to colonies. However, it is not clear that supplementing the typical bacteria already present will improve colony health. Potentially, the strains present in particular colonies have already been selected to perform well under local conditions.

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References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


The authors used a PCR-based approach to analyze the diversity of *G. apicola* and *S. alvi* in worldwide collections of a large set of bumble and honey bee species. Results show some phylogenetic congruence between each group of symbionts and their bee hosts, consistent with long term coevolution of corbiculate bees and gut symbionts. However, the congruence was not perfect, suggesting some incidence of interspecies-transfers of symbionts.


This paper reports the culture conditions and characterization for distinctive Gram-negative bacterial lineages within honey and bumble bee guts.


11. Disayathananowat T, Young JPW, Helgason T, Chantawannakul P: T-RFLP analysis of bacterial communities in the midguts of
Honey bee microbiota Moran 27


Several species of Lactobacillus are found in bees, but this survey shows that only honey and bumble bees are associated with host-specific groups. Other bees and ants contain Lactobacillus groups that are indistinguishable from strains found in diverse environments.


By amplification of single genomes of G. apicola and S. alvi, nearly complete genomes were obtained from individual cells, from a single honey bee colony. Even within a colony, each bacterial species shows considerable variation in genome content.

18. Olofsson TC, Alsterfjord M, Nilsson B, Butler E, Vasquez A: Lactobacillus cuminiperi nov. sp., Lactobacillus melissiger nov. sp., Lactobacillus melissventris sp. nov., Lactobacillus kimbladii sp. nov., Lactobacillus helsingborgensis sp. nov. and Lactobacillus kullabergensis sp. nov., isolated from the honey stomach of the honeybee Apis mellifera. Int J Syst Evol Microbiol 2014, 64:3109-3115.

This paper characterizes Lactobacillus strains corresponding to the host-restricted gut-dwelling Lactobacillus in honey bees, as well as some that are rare in bee guts and more typical in hive materials.


Experimental tests for different sources of the characteristic communities (hive materials, nurses, hindgut contents indicate that some of the typical community is transferred through a fecal route. Characteristic communities appear stable by day 5 following adult eclosion.


Guts of foragers communities similar to those in younger nurse despite their very different diets. Some Lactobacillus previously considered as crop associates instead consist of species that are found in the hive (such as L. kunkeei strains) or species that are largely confined to the hindgut.


The authors performed metatranscriptomic analysis based on RNA samples from bee guts and found an excess of transcripts associated with processing complex carbohydrates. These are associated primarily with G. apicola, and with Lactobacillus and Bifidobacterium groups.


Measurements showed a very low density of bacteria in harvested pollen and bee bread with numbers declining over time following pollen storage, but with no substantial change in the community composition. Thus, pollen stores do not appear to have specific communities selected for nutrient conversion; instead, food preservation is a likely function.


The Lactobacillus found in association with honey bees were shown to have a high performance on fructose. Communities in larvae and the hive environment were dominated by L. kunkeei, in contrast to communities in the adult gut.


Surprisingly, P. perrara, one of the honey bee-restricted symbionts, produces a complex molecule that interferes with normal division of eukaryotic cells. The role in honey bees is unknown.


Gut communities in three native bumble bee species were found to vary among species and not between habitat types or localities.


This commentary provides an overview of recent evidence that Wolbachia symbionts in insects provide protection against insect viruses, and provides an example of the unexpected roles in pathogen defense played by insect-associated bacteria.


Molecular pathogenesis of American Foulbrood: how Paenibacillus larvae kills honey bee larvae
Lena Poppinga and Elke Genersch

American Foulbrood caused by Paenibacillus larvae is one of the unsolved health problems honey bee colonies are suffering from. In the recent past, considerable progress has been achieved in understanding molecular details of P. larvae infections of honey bee larvae. This was facilitated by the development of molecular tools for manipulating P. larvae and by the availability of complete genome sequences of different P. larvae genotypes. We here report on several peptides and proteins that have recently been identified, biochemically analyzed, and proposed to act as virulence factors of P. larvae. For some of them, experimental proof for their role as virulence factor has been provided allowing presenting a preliminary model for the molecular pathogenesis of American Foulbrood.

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Introduction
American Foulbrood (AFB) is a lethal intestinal infection of honey bee (Apis mellifera) larvae caused by the globally distributed, Gram-positive, spore-forming bacterium Paenibacillus larvae (P. larvae) [1]. AFB is highly contagious under beekeeping conditions and, therefore, it is classified as a notifiable disease in most countries. Even though in many of these countries control measures include burning of diseased colonies and spore contaminated hive material, AFB has so far proved impossible to eradicate anywhere. Hence, this disease is one of the unsolved problems of beekeeping since hundreds of years.

This review will focus on AFB and P. larvae as the most relevant bacterial infection of honey bees though European Foulbrood (EFB) caused by Melissococcus plutonius may also be a serious problem in some regions. We will describe recent advances in genotyping of P. larvae, in elucidating the genomic differences within the species, and in identifying and functionally characterizing virulence factors of this honey bee pathogen. As a prerequisite for the development of novel treatment strategies, we will present a model for molecular pathogenesis of AFB based on these novel data.

Different genotypes within the species P. larvae
The correct classification of the causative agent of AFB was not trivial and it took several attempts [2–7] until it was eventually correctly classified as P. larvae [1] and shown to be represented by different biologically relevant genotypes. These genotypes were defined via repetitive element PCR (rep PCR) and enterobacterial repetitive intergenic consensus (ERIC) primers [8] and, therefore, they were called P. larvae ERIC I–IV [1]. The biological relevance of the ERIC-typing scheme was proven by exposure bioassays which revealed that the genotypes differed in virulence if the course of disease at larval level was analyzed and the lethal time (LT) was used as measure of virulence [1,9]. Strains of ERIC II–IV are fast killers: 100% of all infected larvae are already dead at day 6–7 post infection while ERIC I isolates need 10–12 days to kill 100% of all infected larvae. These differences in virulence at the individual larval level translate into differences at the colony level [10] which are relevant for the clinical diagnosis of AFB [11,12]. Recently, the ERIC-profile was additionally verified by state-of-the-art analytical approaches like Multi Locus Sequence Typing (MLST) [13] and MALDI-ToF mass spectrometry [14]. Both methods, though showing considerable heterogeneity within the species, confirmed the general clustering of the species into three to four groups correlating with the aforementioned genotypes ERIC I, ERIC II, and ERIC III/IV. Because P. larvae ERIC I and II are the genotypes which are isolated from AFB diseased colonies worldwide, research focuses mainly on these two genotypes and most of the results presented in the remainder of this review will refer to them.

Elucidating the genomic potential of P. larvae
Deciphering the complete genome of P. larvae turned out to be more complex than originally anticipated despite the availability of highly sophisticated and next generation sequencing tools. The first draft genome sequence published was based on a genome shotgun sequencing approach and consisted of 646 contigs [15], which were
later reduced to 388 contigs [16]. The subsequent attempt to provide an annotation of the *P. larvae* sequence was unfortunately based on the original 646 contigs sequence [16] and, hence, did not provide sound and reliable data. This became evident with the recent publication of two complete, manually curated and annotated *P. larvae* sequences representing the genomes of *P. larvae* ERIC I (strain DSM25719, 4 579 589 bp, 4868 predicted protein-coding genes) and ERIC II (DSM25430, 4 056 006 bp, 3928 predicted protein-coding genes) [17**]. These data provided significant progress in elucidating the genomic potential of *P. larvae* and allowed an in silico comparative genome analysis [17**] which confirmed considerable differences between the genomes of *P. larvae* ERIC I and II as previously suggested by suppression subtractive hybridization [18]. In both genomes, a large number of mobile genetic elements and prophage regions were found as well as genomic regions containing repeats and repetitive sequences. In addition, the ERIC II genome harbored a high copy number of mutator-type transposases. These features suggest frequent genome rearrangements and a high degree of genome plasticity in the species *P. larvae* and explain the difficulties encountered during sequencing [16,17**].

Not surprisingly was the high number of proteases identified in both genomes because proteases have been discussed as important virulence factors of *P. larvae* long since [19–23]. The genomes of *P. larvae* ERIC I and ERIC II harbor as many as 159 and 128 full or truncated protease genes, respectively, belonging to different families [17**]. This indeed points to a prominent and essential role of protein degradation during AFB pathogenesis. The proteolytic system of *P. larvae* is obviously highly redundant, most likely to avoid that the loss of a single protease results in insufficient degradation of the larval cadaver. Because total degradation of larval remains is a prerequisite for successful transmission of bacterial spores, insufficient degradation would pose a serious threat to the transmission success of *P. larvae*. However, this situation will also make it difficult to identify individual proteases as important virulence factors in case it is not the sheer number and diversity of proteases that is essential in the first place.

From descriptive to molecular pathogenesis of *P. larvae* infections

**Descriptive pathogenesis**

Honey bee larvae become infected by ingesting larval food which has been contaminated with *P. larvae* spores through adult bees [24]. The spores germinate in the midgut lumen and the vegetative bacteria massively proliferate there before they finally attack and breach the midgut epithelium thereby killing the larva [25]. The larval cadaver will be decomposed to a roty mass by the still thriving bacteria. Hence, the lifecycle of *P. larvae* in honey bee larvae can be divided into a non-invasive, commensal-like phase followed by an invasive and destructive phase. When nutrients become scarce the *P. larvae* population undergoes sporulation and the roty mass will dry down to a scale consisting of billions of spores which are distributed by adult bees within and between colonies facilitating spreading of the disease to healthy larvae and colonies (Figure 1). The success of *P. larvae* during pathogenesis and transmission depends on (i) the death of the infected larva and (ii) total degradation of the larval cadaver (iii) without saprophytes taking over to ensure that *P. larvae* spores are present and accessible for contaminating adult bees.

**Identification and role of secondary metabolites**

First insights into the molecular details of AFB pathogenesis came within reach not until (i) protocols for the genetic manipulation of *P. larvae* were developed [26*,27**,28] and (ii) the entire genomes of *P. larvae* ERIC I and II were sequenced and correctly annotated [17**]. Especially the latter facilitated the identification of giant gene clusters in the genomes of *P. larvae* ERIC I and II coding for non-ribosomal peptide synthetases (NRPS) and hybrid NRPS/polyketide synthases (PKS). The secondary metabolites produced by such multienzyme complexes can exhibit a wide range of biological activities, like antifungal and antibacterial but also cytotoxic activity [29,30]. For *P. larvae*, two purposes for such metabolites are conceivable during pathogenesis: (i) products with antimicrobial activity may help *P. larvae* to defend the niches ‘larval gut’ (Figure 2) and ‘larval cadaver’ against competitors originating from the larval microbiome and against ubiquitously present saprophytes; (ii) products with cytotoxic activity may help to breach the epithelium and to destroy the larval tissues.

Representatives of *P. larvae* ERIC II harbor an 11 kb gene cluster coding for a trimodular NRPS producing the tripeptide sevadicin (D-Phe-D-Ala-Trp) [31**]. The sevadicin synthetase was shown to be expressed during vegetative growth, but its product sevadicin had only weak antibacterial activity suggesting that it rather does not act as antibiotic during pathogenesis [31**].

Both genotypes also express an NRPS/PKS gene cluster responsible for the production of paenilaminins A and B, iturinic lipopeptides exhibiting strong antifungal activities [32*]. A role of these peptides in outcompeting fungal competitors during pathogenesis is likely but needs experimental proof.

Another NRPS/PKS gene cluster present in *P. larvae* ERIC II is about 60 kb in length. It encodes the biosynthesis machinery for the paenilaminics (Pam) which are rather complex peptide–polyketide hybrids with broad antibacterial and antifungal activity [33*,34**]. The biggest part of the cluster consists of five NRPS, two PKS, and two PKS/NRPS genes [33*]. Exposure bioassays
performed in the presence of the saprophyte *Paenibacillus alvei* with *P. larvae* gene disruption mutants for the pangen gene cluster (ΔpamA) in comparison to wild-type bacteria revealed that Pam is involved in preventing the growth of microbial competitors in the presence of *P. larvae* [34**]. This may explain the fact that larval cadavers regularly contain pure cultures of *P. larvae* [2,35].

The fourth already elucidated gene cluster is again present in both *P. larvae* genotypes. It is about 11 kb in length, consists of five genes, and shows strong homology to the bacillibactin gene clusters of *Bacillus subtilis* and the *B. cereus sensu lato* group as well as to the paenibactin gene cluster of *Paenibacillus elgii*, [17**,36**]. Mass spectrometric fragmentation analyses confirmed that the NRPS machinery is responsible for the production of the catechol-type siderophore bacillibactin (Dhb) in *P. larvae* [36**]. Production of Dhb by *P. larvae* could only be demonstrated in culture under iron limited conditions. Larval infection assays performed with gene inactivation mutants of *P. larvae* (ΔdhbF) and the corresponding wild-type strain did not give any hints for a role of Dhb during pathogenesis; neither total mortality nor disease progression were affected by the absence of Dhb production [36**]. However, the experimental design did not allow to test whether the lack of Dhb production had any effect on the growth of other bacteria like *P. alvei*. Since iron is a limiting factor for most bacteria under aerobic conditions, Dhb may be used by *P. larvae* as a means to create iron depleting conditions for competing bacteria rather than to compete with the host for iron.

**The role of PICBP49, a novel chitin-degrading enzyme**

The key step during pathogenesis, most likely marking the transition from the non-invasive to the invasive phase, is the degradation of the peritrophic matrix (PM) in the larval midgut (Figure 2). This step may be a prerequisite
Elucidated molecular details of pathogenesis of *P. larvae* infections. 1. Secondary metabolites (patterned hexagons) secreted by *P. larvae* help to outcompete microbial competitors and to set the stage for conquering the larval midgut lumen. 2. *P. larvae* secretes the chitin-degrading enzyme *P*ICBP49 (red stars) to digest the peritrophic matrix (PM, pink structure) which should actually protect the midgut epithelium (gray cells with blue nuclei) against pathogen attack. This step might also serve nutritional purposes and marks the transition from the non-invasive to the invasive phase of infection. 3.1. *P. larvae* ERIC I attacks the unprotected epithelial cells via secreted toxins (purple ovals) which interfere with cellular functions thereby destroying epithelial integrity and enabling bacteria to breach the epithelium via the paracellular route. 3.2. *P. larvae* ERIC II expresses an S-layer protein SplA (yellow edge) on its surface mediating direct attachment of *P. larvae* ERIC II to epithelial cells. This step seems to be important for the bacteria to breach the epithelium using the paracellular route via yet to be identified mechanisms.

for the bacteria to attack the epithelium by expressing additional virulence factors that are needed for further tissue disruption. The PM of honey bee larvae consists mainly of chitin and its functional integrity is essential for larval survival [37]. Degradation of the PM during *P. larvae* infection and the ability of *P. larvae* to metabolize colloidal chitin could both be demonstrated [37]. *P*ICBP49, a chitin-binding and — degrading enzyme expressed by both *P. larvae* genotypes, was shown to be crucial for PM degradation during larval infection and to mediate chitin-degradation via a metal-ion dependent, oxidative mechanism [38**]. *P*ICBP49 is a member of the auxiliary activity 10 (AA10) family of lytic polysaccharide monoxygenases (LPMOs) which are able to degrade recalcitrant polysaccharides [39]. Its role as key virulence factor of *P. larvae* could be convincingly demonstrated by larval infection assays using gene disruption mutants for *P*ICBP49 (∆*cbp49*) in both genotypes and the corresponding wild-type strains (wt). The mutant bacteria not able to express *P*ICBP49 could no longer degrade the PM and nearly failed to kill infected larvae, hence, nearly lost their virulence [38**].

**Identification and role of cytotoxins**

After the degradation of the PM, *P. larvae* genotypes ERIC I and ERIC II seem to follow different infection
strategies, as deduced from genome data [17**,18] and functional analyses of putative virulence factors (Figure 2). Comparative genome analyses revealed that several toxin loci are present in the ERIC I strain (DSM 25719) which are absent or disrupted by transposons in the genome of ERIC II (DSM 25430). No additional toxins replacing these non-functional toxin loci could be identified in ERIC II genomes so far. Furthermore, many ERIC II strains lack several proteases (enhancin-like protease, serine protease) present in ERIC I strains [17**]. These data clearly point to differences in molecular pathogenesis between the two genotypes, although both genotypes are equally lethal for infected larvae.

Once the PM is degraded and the epithelium is without further protection, P. larvae can directly approach and attack the cellular layer and prepare for invading the larval hemocoel. It has been shown that P. larvae uses the paracellular route for invasion [25]. Toxins are common virulence factors of bacterial pathogens aiming at disrupting host cellular functions thus enabling the bacteria to conquer and manipulate host cells. So far three toxins have been identified in P. larvae. The ERIC I-specific toxins Plx1 and Plx2 are novel AB-toxins which are putatively ADP-ribosylating yet to be identified cellular targets [40**]. Plx1 is a single-chain toxin. Together with MTX1 expressed by Lysinibacter sphaericus and piersinis-like toxins expressed by members of the family Pieridae (Lepidoptera), Plx1 comprises an enigmatic family of AB-toxins [41]. Plx2 is a binary toxin whose two entities are encoded by two genes, plx2A and plx2B [40**]. The A-subunit of Plx2 is similar to C3-like exoenzymes and most likely modifies RhoA thereby interfering with the integrity of the host cell actin cytoskeleton [40**]. This may lead to the rounding up of epithelial cells observed in the course of ERIC I infections which enabled P. larvae to move through the epithelium [25]. Several P. larvae ERIC I gene disruption mutants for both toxins (Δplx1, Δplx2A, Δplx2B, Δplx1Δplx2A) were tested in exposure bioassays in comparison to the corresponding wild-type bacteria and it could unambiguously be demonstrated that both toxins are important virulence factors for P. larvae ERIC I [40**].

The third toxin of P. larvae, C3larvin, has been identified via a bioinformatic strategy in the draft genome sequence of BRL230010 [42**]. C3larvin was shown to be a mART toxin with glycohydrolase and transferase activities targeting RhoA. Hence, it is the first P. larvae toxin with proven enzymatic activity and identified cellular target. C3larvin is part of the Tx7 toxin locus in DSM 25719 (ERIC I) and the TXIII toxin locus in DSM25430 (ERIC II). Both have originally been evaluated as rather non-functional mainly due to the interrupted B subunit gene (c04960 in ERIC I, c09280 in ERIC II) upstream of a gene encoding a putatively functional A domain [17**]. Hence, despite the extreme toxicity of C3larvin when expressed into the cytoplasm of yeast [42**], its role as virulence factor of ERIC I and/or ERIC II during P. larvae infection still awaits confirmation.

Based on larval feeding assays it has been suggested that low molecular weight metabolites present in P. larvae secretomes may possess larval toxicity although no further identification of the proposed cytotoxin was provided [43]. However, it was speculated that NRP or NRP/PK hybrid molecules are responsible for the observed effect [43]. In another study, the lipopeptides paenilarvins exhibited some larvicidal activity in feeding assays [32**]. In both studies control groups did not receive any control peptides but were fed normal larval diet [32**,43] and all secretome fractions were toxic to larvae [43]. Therefore, it cannot be ruled out that the observed larvicidal effects were just due to the added peptides per se rather than to a specific peptide because larvae are very sensitive to changes in the larval diet during artificial rearing [44]. The NRP/PK hybrid paenilacin, produced by P. larvae ERIC II, showed cytotoxic activity toward cultured lepidopteran cells [53**]. However, larval infection assays performed with P. larvae mutants no longer able to produce paenilacin (ΔpamA) and with corresponding wild-type bacteria did not show any difference in total larval mortality between the two groups arguing against a cytotoxic role of paenilacins during pathogenesis of ERIC II infections [33**]. In summary, Plx1 and Plx2, expressed exclusively by P. larvae ERIC I, are the only toxins with a proven role as virulence factor during pathogenesis so far and may be a hallmark of the pathogenesis strategy followed by ERIC I. Further biochemical and cell biological studies are necessary to unravel the role of Plx1 and Plx2 during pathogenesis in more detail.

The P. larvae ERIC II-specific S-layer protein SplA
A striking difference between P. larvae ERIC I and II is the expression of an S-layer protein SplA exclusively by P. larvae ERIC II [45]. Analysis of the splA genes in P. larvae ERIC I and II revealed that a point mutation, which could be detected in all ERIC I strains analyzed so far, renders splA non-functional in ERIC I by generating a premature stop codon [27**]. Gene disruption mutants (ΔsplA) were instrumental for the functional characterization of the role of this ERIC II-specific S-layer-protein [27**]. Cell culture assays demonstrated that SplA mediates adhesion of P. larvae ERIC II to pupal gut cells [27**] (Figure 2). Larval infection assays revealed that SplA is an important virulence factor for P. larvae ERIC II because total mortality of infected larvae was reduced by about 50% in the absence of SplA. However, the exact role of SplA in the ERIC II strategy for killing honey bee larvae still needs to be determined. Likewise, virulence factors of ERIC II manipulating or killing host cells to facilitate breaching of the epithelium still await identification.
Conclusions
This review integrates recent findings on molecular aspects of the interactions between *P. larvae* and honey bee larvae into a model of molecular pathogenesis of *P. larvae* infections. It focuses on the pathogen side during the infection process although the host side for sure also plays an important role. However, it seems that the infected larva has no means to successfully combat infection and to escape being killed by *P. larvae*. Hence, individual immune responses though elicited by infection [46] are obviously not effective leaving it to the social immune response to deal with the infection at colony level [10,47–51]. Breeding for hygienic behavior could, therefore, provide a solution to the AFB problem. But this will only be feasible in regions, where sustainable breeding programs can be established. The development of novel preventive and curative strategies is another option. Epidemiological studies taking into account the different genotypes of *P. larvae* will elucidate the spread and transmission patterns of *P. larvae* as starting point for programs to contain the infection. Virulence factors can be used as targets to develop inhibitory substances. For G3larvin, an inhibitor against its mART enzymatic function has already been identified [42***]. Such results give rise to optimism that more detailed knowledge of *P. larvae* virulence factors will make it possible to develop novel drugs against this deadly disease of honey bees.

Acknowledgements
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Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest


13. Morrissette BJ, Helgason T, Poppings L, Fünfhaus E, Genersch E, Budge GE: Biogeography of *Paenibacillus larvae*, the causative agent of American foulbrood, using a new multilocus sequence typing scheme. *Environ Microbiol* 2015, 17:1414-1424. http://dx.doi.org/10.1111/1462-2920.12625. This study describes the first MLST scheme developed for *P. larvae* which will be instrumental for the development of a state-of-the-art epidemiology of this important honey bee pathogen. The study also confirms the relevance of the previously developed ERIC-typing of the species *P. larvae* and provides valuable information on the world-wide occurrence of the *P. larvae* genotypes ERIC I and ERIC II.


17. Djukic M, Bruszkoziwicz E, Fünfhaus A, Voss J, Gollnow K, Poppings L, Liesegang H, Garcia-Gonzalez E, Genersch E, Daniel R: How to kill the honey bee larva: Genomic potential and virulence mechanisms of *Paenibacillus larvae*. *PLoS ONE* 2014, 9:e89094. This study provides the first complete, manually curated and annotated genomic sequences of the two most relevant *P. larvae* genotypes, ERIC I and ERIC II. It allows insight into the genomic potential of *P. larvae* and provides genetic proof for the hypothesis that *P. larvae* ERIC I and ERIC II developed different pathogenesis strategies for killing honey bee larvae.


In this paper, the authors described that allowed the expression of a heterologous protein in P. larvae, an organism that so far resisted all attempts of genetic manipulation.


A remarkable study experimentally proving that the P. larvae ERIC II-specific S-layer protein SpIA mediates adhesion of P. larvae to epithelial cells and is an important virulence factor.


Sevacin is a novel non-ribosomal tripeptide produced by P. larvae ERIC II through a multi-enzyme complex encoded by the sevacin synthetase gene cluster (sev-cluster). Sevacin exhibited weak antibacterial activity against Bacillus megaterium and might serve as signaling molecule during pathogenesis.


This study shows that P. larvae ERIC I and II produce paenilarins, iturinic lipopeptides with broad antibacterial and antifungal activity. Their role during pathogenesis needs to be established.


In this study, the gene cluster coding for the biosynthesis machinery for paenilarin produced by P. larvae ERIC I and II was genetically and biochemically characterized and first results concerning the biological role of this NRP/PK hybrid molecule are presented.


A remarkable study elucidating the chemical structure as well as the biosynthetic pathway of paenilarins, complex non-ribosomal peptide/polyketide hybrids with antibacterial and antifungal activity produced by P. larvae ERIC I and II. Co-infections assays with Paenibacillus alvei and both, P. larvae wild-type bacteria or gene inactivation mutants, which were no longer able to produce paenilarins, revealed that paenilarins are involved in outcompeting microbial competitors during pathogenesis.


This study describes the identification and biochemical as well as functional characterization of a siderophore produced by P. larvae.


This study presents PIBCP49, a novel chitin-degrading enzyme able to metabolize recalcitrant colloidal chitin as well as soluble chitin forms. Larval infection assays with gene inactivation mutants for PIBCP49 and the corresponding wild-type strains of P. larvae ERIC I and II revealed that in the absence of PIBCP49 expression, degradation of the peritrophic matrix is inhibited and larval mortality is nearly abolished. Hence, PIBCP49 is a key virulence factor of P. larvae.


This study identified and characterized the first two toxins expressed by P. larvae, Ptx1 and Ptx2. These toxins are specific for the ERIC I genotype. Larval infection assays performed with gene inactivation mutants for both toxins and the corresponding wild-type strain revealed that Ptx1 as well as Ptx2 are important virulence factors.


C3larvin is a toxin expressed by P. larvae ERIC I and ERIC II. It is a mART toxin and it was shown to be extremely toxic when expressed into yeast. Its role during pathogenesis of P. larvae infections still needs to be determined. Interestingly, an inhibitor for the enzymatic activity of C3larvin could be identified.


Honey bee surveillance: a tool for understanding and improving honey bee health

Kathleen Lee1, Nathalie Steinhauer2, Dominic A Travis3, Marina D Meixner4, John Deen5 and Dennis vanEngelsdorp2

Honey bee surveillance systems are increasingly used to characterize honey bee health and disease burdens of bees in different regions and/or over time. In addition to quantifying disease prevalence, surveillance systems can identify risk factors associated with colony morbidity and mortality. Surveillance systems are often observational, and prove particularly useful when searching for risk factors in real world complex systems. We review recent examples of surveillance systems with particular emphasis on how these efforts have helped increase our understanding of honey bee health.

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Detection, characterization, quantification of disease

Monitoring is a regular, repetitive and intermittent series of measurements designed to detect changes in the health status of a defined population (see Table 1 for examples). Apiary inspections are an example of monitoring as they have long been used to estimate disease in managed honey bee populations. These inspections quantify disease prevalence and range by sampling a number of ‘analytic units’ (individual bees, colonies, apiaries, or operations [1]) over a defined period of time and population. Traditionally, apiary surveillance was used to identify disease outbreaks in order to enforce regulations aimed at eliminating or containing disease spread. This approach is largely credited for reducing the incidence of the bacterial disease American foulbrood (Paenibacillus larvae) in the US [2]. More recently, disease surveys have expanded to include early detection of non-extant (or recently introduced) disease threats such as Tropilaelaps clareae mites in the US [6,7], small hive beetles (Aethina tumida) in Europe [8], or Varroa destructor mites (Varroa) in Australia [9]. Determination of disease free status for particular pests has implications for trade of bees and bee products [7]. Early detection of a new organism can permit containment efforts, such as the Australian effort to contain Apis cerana [10]. The utility of surveillance efforts in epidemiologic studies is dependent on numerous factors, including how samples are

Surveillance in honey bees

‘Observation sets the problem; experiment solves it’

Jean-Henri Fabre, (1823–1915)

Surveillance is an observation-based method of quantifying levels of ‘disease’ in a population. At their core, surveillance efforts quantify disease prevalence and incidence over space and time, which can help identify risk factors that contribute to disease incidence when coupled with other data. Data from surveillance efforts can identify or confirm risk factors that predict disease outcomes, and can guide the development of experimental approaches to demonstrate causation. Further, identification of risk factors can inform disease mitigation practices that can improve health at the population level [1,2].

Health and/or disease surveillance systems exist for most human and production animal health programs. When implemented sustainably, they help mitigate and prevent important diseases in populations. Considering the importance of honey bees (Apis mellifera) for pollination of agricultural crops [3,4,5], it is not surprising that many surveys have quantified health and disease burdens. Surveillance of non-apis species also exists, but is less developed compared to honey bees (Box 1). Surveillance system design is dictated by many factors, most importantly by the objectives of the study and availability of resources (Figure 1). Here we review examples of honey bee surveillance efforts, emphasizing their contribution toward understanding and improving honey bee health (summarized in Table 1).
Box 1 Non-apis bee surveillance

Non-apis bee species are major contributors to agricultural and natural pollination systems [4,57–59]. These species are largely unmanaged and have multiple different life histories, thus requiring specialized surveillance techniques.

Recently there have been several efforts to standardize survey effort approaches that document the abundance and diversity of non-apis species [60]. Application of standardized collection methods allows for ecological network analyses to help quantify the structure of bee-plant networks in various landscapes [61–64]. When standardization is not possible (such as in the case of comparing changes in abundance and diversity over time by using historical collections), statistical analyses can help elucidate important drivers of changing populations, including changes in agricultural policy and practice [65], ecological succession [66], landscape [67] and climate change [68].

Surveys of non-apis bee populations have been conducted to identify disease loads in populations [69–72], although generally these studies have concentrated on possible disease spillover from honey bees. Further surveillance on non-apis bees and their diseases is much needed.

Figure 1

Describes different approaches to honey bee surveillance and the corresponding relative degree of investment (time and monetary). **Purpose:** objective of the surveillance program. **Outcome:** measure of health under surveillance. **Passive:** approach: no intervention imposed on the regular management of the colonies under surveillance. **Active:** approach: implicates manipulation of the conditions experienced by (at least part of) the colonies under surveillance. **Questionnaire data:** interview or self-reported recollection from the stakeholder. **In field observations:** the overt symptoms expressed in the colonies under surveillance. **Biological samples:** clinical diagnostics from a physical sample collected from colonies under surveillance. **Retrospective:** collection of data regards exclusively past events. **Single point in time:** cross-sectional design where the collection of data (exposures and outcomes) are made at the same unique point in time. **Repeated measures:** the same colonies under surveillance are assessed repeatedly through time. **Convenience sample:** sample from the target population is only determined by the availability and willingness of the stakeholders. **At random:** selection of the sample from the target population is completely randomized, meaning all individuals from the target population have the same probability of being sampled. **Selection on disease status:** case–control studies comparing individuals classified as ‘diseased’ versus individuals classified as ‘disease-free’ for the disease of interest. **Selection on exposure status:** cohort studies comparing individuals classified as ‘exposed’ to individuals classified as ‘non-exposed’ for the risk factor of interest.

A notable monitoring program quantified disease load and colony mortality by inspecting randomly selected apiaries in 17 different European countries [8**]. By randomly selecting colonies and implementing a standardized inspection approach, the resulting data avoided selection biases inherent with many survey efforts. The ability to randomly select colonies from a known population is a central tenant of good survey design, but in practice is problematic as random sampling requires a near-complete description and access to the honey bee population, which is often difficult to attain or create.

Modified apiary inspections can be used to perform more directed surveillance for the discovery and characteriza-
tion of potential new diseases and/or pathogens, including virulence and distribution. Identifying new cases helps direct future monitoring or research to better understand if they contribute to colony mortality and morbidity. Several surveillance programs have utilized new molecular tools to discover new, possibly pathogenic, honey bee viruses, including Aphid Lethal Paralysis virus strain Brookings, Big Sioux River virus, four strains of the Lake Sinai virus, and the tobacco ringspot virus [11,12,13*].

Surveillance of known pathogens can shed light on the etiology of disease and support a hypothesis that is difficult to test experimentally. Examining the relationship between disease and other variables of interest at a single time point in a defined population can be done using cross-sectional studies (Table 1). In a cross-sectional study of Hawaiian colonies, Deformed Wing Virus (DWV) prevalence was correlated with the number of years *Varroa* was present on the island [14]. The observed DWV strain diversity was greatest in samples from *Varroa* free islands, while a single DWV strain replaced all others when *Varroa* was present for over three years. These findings imply that mite-mediated transmission of DWV favors certain, possibly more virulent, DWV strains [14]. This hypothesis is supported by survey results that demonstrate *Varroa* dramatically changes the viral complex in infested honey bee populations [15*], and experimental research showing one virulent DWV strain benefits from the direct injection route mediated by *Varroa* [16].

Generally, understanding the dynamics of ‘new’ host–parasite/pathogen interactions does not lend itself well to direct hypothesis testing, but benefits from surveillance efforts. A survey in Kenya documented the prevalence of *Varroa, Nosema* spp., DWV, Black Queen Cell Virus (BQCV), and Acute Bee Paralysis Virus (ABPV) [17*]. The surveyed pathogens did not appear to affect colony strength suggesting the presence of more benign diseases in the region, a more resistant host, or a combination of both. Data from a Swedish effort that looked at viral levels in ‘*Varroa* tolerant’ colonies seems to support the concept of host-based tolerance, as the study population has increased virus tolerance [18*] and an ability to reduce mite fitness [19].

**Identification of risk factors**

Surveillance can be used to investigate putative causes of unexplained disease states. Analyses of a limited number of disease samples can be reported as a case study: a detailed description and analysis of the occurrence of a particular health problem, its development and its outcome (Table 1). Although widely used in human and other animal health fields, case study reporting is rare for honey bees. A recent exception described efforts to determine the putative cause of two collapsed colonies [20], with the clinical disease symptoms ascribed to *Nosema ceranae*. On its own, a case study has limited utility in explaining population level health. However, if findings from other case studies make similar conclusions, the results can identify associated risks.

In case–control studies, colonies are first selected based on whether or not they have the disease/health status of interest, and then their exposure histories are obtained.

---

### Table 1

**Surveillance types. Without judging for the individual designs potential precision level, biases and confounding effects, we classified recent honey bee research according to the type of surveillance design to identify methodologies underrepresented in current publication trends.**

<table>
<thead>
<tr>
<th>Survey design (definitions from [73])</th>
<th>Samples (S)/questionnaires (Q)</th>
<th>Transversal (T)/longitudinal (L)</th>
<th>References</th>
<th>Objectives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monitoring</td>
<td>S/Q</td>
<td>L</td>
<td>[8**]</td>
<td>Early warning surveillance</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>L</td>
<td>[11,24,26,54]</td>
<td>Monitoring (colony, disease prevalence)</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>T</td>
<td>[12]</td>
<td>Identification of risk factors</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>L</td>
<td>[13,25]</td>
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<tr>
<td></td>
<td>S</td>
<td>T</td>
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<td></td>
<td>Q</td>
<td>L</td>
<td>[28,30,31,32,33,34,35,36,38]</td>
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<tr>
<td></td>
<td>S/Q</td>
<td>L</td>
<td>[45]</td>
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<tr>
<td>Cross-sectional</td>
<td>S</td>
<td>T</td>
<td>[43*,44**]</td>
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<tr>
<td>Case studies</td>
<td>S</td>
<td>T</td>
<td>[53**]</td>
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<tr>
<td>Case–control</td>
<td>S/Q</td>
<td>T</td>
<td>[20]</td>
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<td></td>
<td>S</td>
<td>T</td>
<td>[21]</td>
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<td>S</td>
<td>T</td>
<td>[22]</td>
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</tr>
<tr>
<td>Cohort</td>
<td>S/Q</td>
<td>L</td>
<td>[46**]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>L</td>
<td>[47,48,49*,50*,51,52**]</td>
<td></td>
</tr>
</tbody>
</table>

T (transversal), collected at one point in time.
L (longitudinal), more than one observation per replicate over time.
and compared to identify correlations between different risk factors (Table 1). One case–control study found apiaries with high rates of loss were less likely to have been treated for Varroa than apiaries with low rates of loss [21]. Another found higher pathogen loads and a different gut microbe community in collapsing colonies as compared to apparently healthy colonies [22].

Surveillance efforts can also help validate experimentally identified risk factors. An experiment that measured effects of temperature on spore viability of Nosema apis and N. ceranae, found N. ceranae spores were more tolerant of higher temperatures while N. apis spores were more tolerant of colder temperatures [23]. This suggests N. apis should be more prevalent in colder locations, a finding documented by surveillance programs in Sweden, Germany, and Taiwan [24–26].

Surveillance efforts need not require field visits. Owner or caretaker observations can capture information about a population’s health, and have been used extensively to document colony losses [1,27]. Repeated surveys have shown that losses are highly variable between regions and over time [28–30], which may be explained by subgroups within a population. A consistent finding within US loss data is that beekeepers in northern states lose more colonies than those in southern states [28,31–36], suggesting winter temperature may explain some variability in loss rates. Indeed, winter loss rates in Pennsylvanian beekeeping operations were correlated to average winter temperature [31].

Although questionnaires are relatively easy to conduct, they are prone to biases: respondents may not be representative of the population, have poor recall, among others [27]. Further, when comparing results among different questionnaire-based surveillance efforts, consideration of different methodologies and definitions is essential. For instance, the timeframe for ‘winter’ loss calculations can be a set date [1,37] or self-determined by the responding beekeeper [30], making direct comparisons of loss rates difficult to interpret.

Properly designed questionnaires increase the ability to identify management practices correlated with increased survivorship. A consistent finding in several loss and management surveys has linked application of Varroa treatments with increased winter survival [30,38,39]. Despite the consistency of these findings across different surveys, this relationship remains correlative and should not be interpreted as causative unless data from experimental testing is considered [40–42]. However, resource availability can limit the ability to conduct experimental studies. In these cases, a combination of questionnaires and field surveys can be informative. An Argentinian study paired monitoring of Varroa loads with retrospective management survey data to identify factors that were predictive of above threshold Varroa populations at the time of sampling [43]. Another cross-sectional study, which also combined field sampling and questionnaires, found that acaricide treatments were 4.9 times more likely to fail when applied to colonies with Varroa infestations over a 3 mites per 100 bees threshold [44**]. Both these and other cases demonstrate a strong link between Varroa levels and colony mortality [45] and validate this method as a cost effective surrogate approach to more intensive surveillance.

Longitudinal surveillance (repeated sampling of the same colonies over time) is a powerful tool for connecting ‘risk factor’ exposure with disease outcomes. This approach can quantify the association between a risk factor and likelihood that a disease outcome will result in the future. Cohort studies are a form of longitudinal monitoring that compares the incidence of a particular health outcome between subsets of defined populations selected for having experienced a common exposure status (Table 1). For example, a US cohort study followed colonies in different migratory beekeeping operations and identified a close association with the occurrence of a queen event or the presence of ‘Idiopathic Brood Syndrome’ with subsequent colony mortality [46**]. Similar cohort studies have found relationships between Varroa (or its control), DWV, or a combination as being predictive of mortality [47,48,49,50**].

Longitudinal studies can also identify and quantify non-biotic factors that may predict disease outcomes. European researchers recently published a multi-year study conducted in 21 apiaries in 11 countries identifying effects of bee genotype and environmental factors on mortality and morbidity [51,52**]. They demonstrated that location strongly influenced autumn mite loads and viral (ABPV and DWV) prevalence. Location effects had a more pronounced affect on disease outcome than host genotype, suggesting disease thresholds likely differ by geography [52**]. Colonies in locations with a shorter active season (i.e. temperate regions) have longer lived winter bees compared to colonies with a longer active season [51], suggesting colonies managed in temperate regions require more vigilant pathogen control. Longitudinal data has also been paired with landscape data to develop a model, EcoBEE, that predicts optimal apiary sites [53**].

Multi-year longitudinal monitoring of colony health can identify region specific risk factors associated with colony mortality. Once identified, these factors can inform management and research priorities. Longitudinal trends in disease and/or risk factor prevalence may help predict future morbidity and mortality. Unfortunately, few of these long-term monitoring efforts exist, with some notable exceptions based in Europe [45,52**,54] and more recently in the US [39].
Conclusions and further directions

Honey bee health surveillance efforts quantify disease and disease risk factors in managed honey bee populations. Over time, these efforts provide data that can identify emerging threats and place disease measures in context by establishing baseline metrics. As surveillance-based studies are becoming more common, increased efforts to standardize approaches [55**] would foster greater comparison among studies and increase potential benefits. To maximize benefits and allow for comparison of studies across time and regions, several challenges need to be addressed: designs that ensure representative data are obtained, better coordination among efforts, and standardization of approaches [56]. The value of continuing and initiating other long-term surveillance efforts cannot be over stated. Surveillance data can be used to guide disease intervention methods and policy, hypothesis driven research efforts focused on discovering causes of disease, and, most importantly, measure the impact the application of this knowledge has on improving bee health.

Acknowledgement

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

● of special interest

● of outstanding interest


This recent review describes the various designs of surveillance systems applied to honey bee health, as well as how such designs can be evaluated and improved. It particularly defines the characteristics of a good surveillance system from sampling scheme to result outreach.


This reports the first harmonized surveillance effort on honey bee mortalities and disease levels at the European scale with the objectives to assess mortality levels, disease prevalence, as well as monitoring for emerging threats. This represents an unprecedented effort to unify sampling protocols across nations in a random-sampling longitudinal design.


This study is the first to document a trans-kingdom host alteration of a virus from pollen-borne plant virus into honey bees and raises awareness to a new reservoir of viruses that could emerge in honey bees.


This transversal study explored a naturally occurring range expansion of Varroa mites to characterize the interactions between honey bees, mites and viruses throughout the establishment process of the mites. This study pointed to the various dynamics of virus prevalence in response to mite invasion, resulting in a changing viral-landscape shortly after establishment of the mites as well as to an overall increase in multiple viral infections in honey bees.


This study represents the first East African survey of honey bees pests and pathogens since the introduction of Varroa mites, it is also the first attempt to correlate colony strength with the presence of those stressors in this region of the world, with results contrasting those typically found in Europe and the US.
This study investigates one of the rare occurrences of a honey bee population resistant to Varroa mite infestations. In addition to reducing the mite reproduction rate, this study documents contrasting viral infection curves between resistant and susceptible bee populations, hinting to a resistance or tolerance mechanism towards viral infections themselves.


This cross-sectional study combined both in-hive sampling and questionnaire data in order to identity risk factors associated with an increased autumn prevalence of Varroa mites. Though the study did not investigate effects on colony mortality directly, it represents a valuable effort in identifying management practices related to high Varroa mite levels.


This cross-sectional study investigated management and environmental confounders affecting acaricide treatment efficacy. It combined field-testing and questionnaire surveys in a well-replicated study design, focusing on colonies with no interruption of brood. It showed the importance of secondary management practices in improving treatment efficacy and the importance of keeping Varroa mite numbers under control throughout the season.


Death of the bee hive: understanding the failure of an insect society
Andrew B Barron

Since 2007 honey bee colony failure rates overwinter have averaged about 30% across much of North America. In addition, cases of extremely rapid colony failure have been reported, which has been termed colony collapse disorder. Both phenomena result from an increase in the frequency and intensity of chronic diseases and environmental stressors. Colonies are often challenged by multiple stressors, which can interact: for example, pesticides can enhance disease transmission in colonies. Colonies may be particularly vulnerable to sublethal effects of pathogens and pesticides since colony functions are compromised whether a stressor kills workers, or causes them to fail at foraging. Modelling provides a way to understand the processes of colony failure by relating impacts of stressors to colony-level functions.

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Introduction
Since 2007 the median annual honey bee colony loss rate in North America has been 29.6% (range: 22% in 2012 to 36% in 2008) [1,2]. Such high mortality rates are testing the ability of apiculturalists to maintain their bee stocks [1,3,4]. This period has also seen dramatic reports of mass deaths of bee hives, and cases of rapid colony depopulation with worker bees apparently disappearing from hives leaving just the queen, brood, and some food behind with no obvious cause of such a dramatic population collapse [5]. This phenomenon, termed colony collapse disorder (CCD), has galvanised research into why bee colonies are now failing at such high rates, and what might cause CCD. It is important to recognise that CCD is not the sole cause of the elevated honey bee colony failure rates [3] since in the majority of cases colony failures can be attributed to known stressors. The near global spread of the parasitic mite *Varroa destructor*, and its development of resistance to control measures has certainly driven up colony failure rates [3,6,7], but it is clear that neither CCD nor the general increase in incidences of colony failure can be attributed to any single cause. Both issues are massively multicausal. New research is examining how different stressors interact and synergise to impact bees, and the importance of sublethal effects of stressors that can cause colony failure by compromising individual and or colony function.

Causes
The list of pests, parasites and environmental stressors that have been linked to CCD is enormous [8]. There is now recognition that a stressor does not need to kill individual bees in order to contribute to colony failure. Any factor that compromises bees’ abilities to forage effectively or otherwise service their colony can drive a colony into decline [9,10]. This recognition has focussed attention on the social consequences of sublethal effects of stressors on bees.

There is a great deal of concern about the possible impacts of a wide range of pesticides on honey bees at sublethal doses [11**]. Here I pay particular attention to the neonicotinoid insecticides and organophosphate miticides on honey bees: both are in common use in agriculture and apiculture: the former as crop treatments to kill pest insects and the latter as in-hive treatments to control *Varroa* mite. Both target cholinergic neurotransmission in arthropods with potentially very wide-ranging effects on insect physiology and behaviour [12]. Both classes of agrochemical can interfere with signalling in the mushroom bodies of the insect brain at sublethal and field-relevant doses [13] and impair learning and memory in honey bees [14]. If neonicotinoids are damaging learning and memory (and possibly navigation) this may explain why sublethal neonicotinoid exposure reduces successful homing after foraging in bees [9,15–17]. Building on a simple demographic model of a honey bee colony proposed by Khoury et al. [18], Henry et al. [9] proposed that the forager losses they observed as a consequence of sublethal pesticide exposure could potentially cause colony failure. It now seems clear that sublethal neonicotinoid exposure can compromise colony function and may result in colony failure with symptoms resembling CCD [19].
Similarly, diseases do not need to kill individual bees to kill a bee hive: if they sufficiently compromise colony function this can cause colony failure. From the perspective of a colony maintaining its resource base and population it makes no difference if a pathogen kills worker bees out right, or simply prevents them successfully returning home from foraging. Both the gut parasite *Nosema ceranae* [20] and the Israeli Acute Paralysis Virus [21,22] reduce efficiency of foraging and increase the numbers of bees that fail to return to the hive from foraging trips. Nosema infections can kill colonies [23] with features similar to that considered diagnostic of CCD [24].

**Stressors interact to compromise colony function**

In the current apicultural setting a honey bee colony is rarely dealing with a single stressor in isolation, and stressors can interact in complex ways to alter worker physiology and colony function. Treatment with field-relevant sublethal doses of the organophosphate miticide coumaphos and the neonicotinoid pesticide imidacloprid in combination had a greater impact on bees’ odour learning and odour discrimination than treatment with either compound alone [14], even though there was no evidence of synergy between the two pesticides in a mortality assay [14]. Pesticides at sublethal doses can interact with complex, and even unpredictable, physiological effects that may not kill bees, but could reduce their performance and survival in a foraging situation. Field exposure of bees to a wide range of pesticides (including fungicides) sprayed on crops can also increase bees’ susceptibility to nosema infection, which (as described above) can impair foraging performance [11**,25].

The impacts of pesticides on bees vary with environmental conditions. Low temperatures and low protein diet both increased susceptibility of bees to nicotine poisoning [26,27], which may in part explain why the impacts of pesticides on bee colonies can vary seasonally. Colonies experimentally chronically treated with sublethal doses of the neonicotinoid pesticides imidacloprid and clothianidin progressed normally through summer and autumn, but failed to recommence brood rearing in late winter and hence failed just as control colonies were emerging from successful overwintering [19]. These experimental colonies showed some features of CCD in that no dead adult bees were found in the colony. Dively *et al.* [28], however, reported that effects of chronic imidacloprid exposure via pollen on overwintering survival of colonies were only seen at the higher end of the possible range of expected field contamination.

Bee diseases interact with each other and with season to intensify impacts on colonies [29*]. Heavy infestation during winter of either the varroa mite or deformed wing virus spread by the mite has been shown to be highly predictive of colony failure [30]. Deformed wing virus and other opportunistic infections spread by varroa significantly weaken workers immune systems and energetic reserves, which could seriously impair worker performance [30]. Co-infections may act synergistically to weaken workers and increase transmission of diseases in the colony leading to colony failure with CCD-like symptoms [29*]. In this discussion I have focussed on stressors of workers, but it should be noted that the loss of the queen is also a significant stressor for a colony, and the demographic interruption as colonies replace a lost queen can significantly increase the risk of colony failure [31].

**Death of the colony**

A honey bee society usually contains within it autoregulatory mechanisms that operate to maintain the functions of the society against external stressors: fully understanding colony failure will require understanding how these social systems have failed. Much of the work in this area has involved modelling of colony demographic processes, and this approach has proved useful for framing and exploring hypotheses of how a colony might react to stress.

Normally a bee hive contains a balanced division of labour. Worker honey bees segregate tasks by age: young adults specialise on brood rearing roles and older adults defend the hive and forage [32–34]. This system enhances colony efficiency by delaying exposing workers to the highest risk tasks until after they have contributed to colony productivity [35]. It is maintained by pheromonally mediated social inhibition whereby old foragers in the hive inhibit younger bees from becoming foragers [36–38] and in this way the colony maintains an appropriate balance of forager and hive bees. If the hive loses its foragers, however, social inhibition is reduced and younger bees are recruited to the foraging force to replace them [36–38]. Precocious foraging by young bees is a common response of individual bees to stressors: individual or colony starvation [39,40], pollen deprivation [41,42], disease [11**,24,35,43–45], and even wax deprivation [46] will all cause young bees to begin foraging precociously. This is an adaptive response to an acute stressor since it rapidly replaces any losses of foragers and shifts the colony to increased resource accumulation, but the reaction of bees to stress by foraging could be problematic in the face of a chronic stressor.

New data has shown that precocious foragers are markedly less effective than bees that begin foraging at the typical age of more than two weeks old [10]. Precocious foragers survived less long as foragers, completed fewer foraging trips and were less far more likely to die during their first few flights outside the hive than bees that commenced foraging at a typical age [10].
Perry et al. [10] constructed a simple compartment model of honey bee colony demography as a tool to explore how the reactions of individual bees to stressors (precocious foraging and poor foraging performance) might alter colony function (Figure 1) [18,47]. The model assumed that the age at which bees commenced foraging was regulated by social inhibition, and that foraging performance (in terms of food collection) and forager survival were both age-dependent and declined as bees began foraging at younger ages (Figure 1). The model was then used to examine the consequence of a colony suffering a chronic high rate of forager death, as might occur if foragers were impacted by disease or pesticide and becoming lost while foraging (Figure 2a–d).

In simulations if forager death rates were chronically raised above a threshold adult population decline was both rapid and markedly non-linear (Figure 2). Initially colonies buffered the consequences: populations stabilised for a period and brood rearing continued (Figure 2d). During this period, however, the foraging force became progressively younger and less effective until it could no longer sustain food levels in the colony, which triggered a very rapid terminal decline in the adult population. In the model the terminal phase saw a complete breakdown of division of labour with most bees becoming foragers and dying soon after, leaving just the queen, a few adult bees and abandoned brood in the colony.

It is notable that in this model colonies failed displaying several of the features of CCD [10]: an abrupt change from apparent health with successful brood rearing to total loss of the adult population, a decline in the average age of workers and in the ratio of nurse bees to brood in the colony, and colony failure leaving few adult bees but a queen and brood in the colony (Figure 2). If this model captures the demographics of a stressed bee colony then it may explain why colonies sometimes depopulate so rapidly and die.

The models of Perry et al. [10] and Khoury et al. [18,47] considered how a colony might respond to chronically elevated forager losses. Betti et al. [48**] built on the Khoury model framework [18,47] to consider how a contagious bee disease might interact with the colony’s demographic processes to damage colony function. In their model, highly transmissible diseases had a greater impact on colony function than diseases that were more lethal,

**Figure 1**

Schematic representation of key demographic processes operating in a honey bee colony considered as a compartment model by Perry et al. [10]. Workers develop as larvae, pupate as capped brood, emerge as adult hive bees and mature to foragers. Existing foragers inhibit hive bees from becoming foragers by social inhibition [36,56]. Blue lines represent consumption of food. Food also influences brood numbers indirectly since in periods of low food influx workers will cannibalise larvae (blue dotted line) [57]. Death rates during the transition from hive bee to forager, forager death rate, and the rate of food collection by foragers (indicated by red arrows) varied with the age at onset of foraging. Source: Figure adapted from Perry et al. [10] with permission.
 Outputs of the model of honey bee demography shown in schematic in Figure 1 and described in full in [10]. Responses of a colony to chronic stress shown as plots of populations of uncapped brood (dotted line), hive bees (dash-dot black line) and foragers (dashed line); food (solid line) and age at onset of foraging (dashed red line) against time for increasing rates of forager bee mortality. In these plots death rate is expressed as the ratio between death rate of the simulated hive and a healthy hive ($m_b$). (a) Plot for a healthy hive ($m_b = 1$); (b) $m_b = 1.6$; (c) $m_b = 1.9$ at this value the hive will collapse eventually but not when $t < 150$ days, (d) $m_b = 2.0$ (twice the death rate of a healthy hive). For this death rate, the forager population reached zero at about $t = 99$ days. At about 3 weeks before collapse the colony in (d) had 8250 uncapped brood items, 6780 hive bees and 1780 foragers, suggesting a colony could decline from an apparently healthy size and strong brood production to zero foragers in less than three weeks. The mathematics are such that modelling could not continue beyond the point of zero foragers. Note that (a) and (b) are on a different vertical scale to (c) and (d). In the model chronically elevated forager death rates caused a shift in the proportions of forager bees, hive bees and brood in the colony and resulted in a younger forager population. When death rates were chronically maintained at more than twice that of a healthy hive the colony rapidly collapsed.

Source: Figure adapted from Perry et al. [10] with permission.

since the former persisted in colonies for longer and infected bees when younger [48**]. In their model colonies were most at risk of failure if infected before winter since the infected colonies were unable to effectively resume brood production in spring. It is often observed that the winter/spring transition is when colony failure is most likely to occur [2,5,49], and this may be a point of particular vulnerability for colonies since they are attempting rapid growth having all but exhausted their food reserves [50]. Much of the challenge in understanding colony failure is that the interactions of stressors on individual bees with colony dynamics and environmental factors such as season and resource availability are complex, dynamic and layered. It is not easy to frame simple hypotheses for how best to intervene to improve colony health, but here the new modelling approach of Becher et al. [51] which considers colony performance and impact of disease in a landscape context could be a very powerful tool.

Models of honey bee colonies are important because they help clarify thinking of the demographic processes we imagine to operate within colonies, and they propose hypotheses of how and why colonies might fail [51]. Testing these hypotheses requires long-term studies of the changes in brood, food, adult population and foraging performance as colonies develop and decline. Such studies are now possible thanks to new sensor technologies such as continuous weight monitoring [52*] and RFID tracking of bees [10,53–55]. A very promising future direction will be combining the capacity of these new technologies to generate high-quality field data with new approaches to modelling honey bee hive performance [51]. Modelling can help frame and explore hypotheses for how to improve colony performance and prevent colony failure. New experiments then enable testing of these hypotheses and the provision of new field data to improve models.
Conclusions
Honey bee colony failure rates have increased, and in some circumstances colonies collapse rapidly and completely. The ultimate explanation of both of these phenomena may simply be that far too many honey bee stressors (environmental, agrochemical, parasitic and pathogenic) have increased in frequency and intensity, and honey bee colonies are too often dealing with too many chronic stressors.

A honey bee colony many be particularly vulnerable to sublethal effects of diseases and pathogens because colony functions are damaged to the same degree whether a stressor kills a worker outright, or simply causes her to fail in her foraging effort. Honey bee colonies have internal demographic processes to buffer against forager losses by recruiting young bees to the foraging force. This may be an effective response to restore colony function in the face of acute stressors, but modelling suggests it may accelerate colony failure and population collapse in the face of chronic stressors. Experimental analyses of the demographic processes of the bee hive combined with modelling have shed some light on what may happen when colonies rapidly fail. Field studies to test predictions of these models are now urgently needed to identify the best interventions to stop the process.

Acknowledgements
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References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of special interest


Xenobiotic detoxification pathways in honey bees
May R Berenbaum¹ and Reed M Johnson²

Relative to most other insect genomes, the western honey bee Apis mellifera has a deficit of detoxification genes spanning Phase I (functionalization), II (conjugation) and III (excretion) gene families. Although honeybees do not display across-the-board greater sensitivity to pesticides, this deficit may render them vulnerable to synergistic interactions among xenobiotics. Diet quality, in terms of protein and phychochemical content, has a pronounced influence on tolerance of toxic compounds. Detoxification gene inventory reduction may reflect an evolutionary history of consuming relatively chemically benign nectar and pollen, as other apoid pollinators display comparable levels of cytochrome P450 gene reduction. Enzymatic detoxification in the eusocial A. mellifera may be complemented by behaviors comprising a ‘social detoxification system,’ including forager discrimination, dilution by pollen mixing, and colony food processing via microbial fermentation, that reduces the number or quantity of ingested chemicals requiring detoxification.

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Introduction
The fortuitously timed release of the genome of Apis mellifera, the western honeybee, in 2006 provided novel insights into the biology of this agriculturally critical species just as apicultural losses associated with what became known as Colony Collapse Disorder reached crisis proportions. Among the most striking findings was that the A. mellifera genome is deficient with respect to its inventory of all gene families associated with detoxification [1], a pattern that has for the most part held as more insect genomes have been sequenced (Table 1A). The genome also provided timely novel tools for characterizing the biochemical mechanisms underlying the detoxification of natural and synthetic xenobiotics, critical for evaluating the possible involvement of nutrition and diet in honeybee decline [2].

In general, xenobiotic detoxification involves the conversion of lipid-soluble substances to water-soluble, excretable metabolites. In Phase I detoxification, the toxin structure is enzymatically altered and rendered unable to interact with lipophilic target sites. Such functionalization is effected primarily by cytochrome P450 monooxygenases (P450) and carboxylesterases (CCE), although other enzymes, including flavin-dependent monooxygenases [3] and cyclooxygenases can also contribute [4]. Phase II reactions typically involve conjugation of products of Phase I detoxification for solubilization and transport. Glutathione-S-transferases (GST) are the principal Phase II enzymes, although they can also function as Phase I enzymes. Other Phase II enzymes in insects include glycosyltransferases, phosphotransferases, sulfotransferases, aminotransferases, and glycosidases [4]. Nucleophilic compounds can be rendered hydrophilic by UDP-glycosyltransferases, although the natural substrates of these enzymes in herbivorous insects are unknown [5,6]. Phase III detoxification involves transport of Phase II conjugates out of cells for excretion. Among the proteins involved in this process are multidrug resistance proteins and other ATP-binding cassette transporters [7].

Phase I functionalization: cytochrome P450s
Functionalization of natural and synthetic xenobiotics in A. mellifera is attributable largely to the P450 superfamily [1], heme-thiolate enzymes that participate in both biosynthesis of endogenous signaling molecules and xenobiotic detoxification in insects [8]. Honeybees utilize P450s for detoxification of honey and pollen flavonoids [9], pyrethroid, organophosphate and phenoxypyrizole acaricides (fluvalinate, coumaphos, and fenpyroximate [10]), neonicotinoid insecticides [11], and mycotoxins (aflatoxin B1 and ochratoxin [12]). Despite this dependence on P450s, with 46 genes, the honeybee genome is reduced even in comparison with some other hymenopterans (Table 1B). The CYP2 and mitochondrial clans, with endogenous functions, are comparable in size with those of other insects whereas reductions in Clade 3 P450s, including the CYP6 and CYP9 families that carry out xenobiotic detoxification [8], are more pronounced. The greatest reduction is in the CYP4 clade; A. mellifera has only 12-14% of the number in other genomes (Table 1B). CYP4 genes in other insects have endogenous and exogenous roles [8]. This extreme reduction, also characteristic of the bumblebee Bombus terrestris [13] (Table 1B), is not easily explained without greater knowledge of CYP4 functions.
Relative to *N. vitripennis*, a non-social parasitoid wasp, the subfamily CYP6AS is more diverse in honeybees (Table 1B). CYP6AS1, CYP6AS3, CYP6AS4 and CYP6AS10 metabolize quercetin [9] and are upregulated by honey, pollen and propolis [14]. CYP6AS enzymes are not induced by their substrate quercetin [9], which is atypical of insect P450s [15]. CYP9Q1-3, however, are induced by their acaricide (tau-fluvalinate) and insecticide (cypermethrin and bifenthrin) substrates [15]. Genome-enabled surveys have identified P450 transcripts upregulated by xenobiotics, although corresponding enzyme activities have not yet been characterized in many cases (Table 2). *CYP6AS14* is upregulated by the monoterpene thymol, used in-hive as an acaricide against varroa mites [16]. Acaricides coumaphos and fluvalinate upregulated *CYP6AS3*, *CYP6AS4* and *CYP9Q1* in adult workers [17**]; these P450s are involved in xenobiotic detoxification, although the CYP6AS enzymes do not metabolize these acaricides when expressed heterologously [9]. Coumaphos exposure also led to a 3.4-fold increase in expression of *CYP350D1*, a CYP2-clan P450 of unknown function [17**].

Phase I functionalization: carboxylesterases (CCE)

Carboxylesterases have both endogenous and exogenous functions. That CCEs in *A. mellifera* participate in xenobiotic metabolism is suggested by induction of carboxylesterase activity by organophosphate, neonicotinoid, pyrethroid, phenylpyrazole, and spinosyn pesticides [18]. With only 24 members, the *A. mellifera* CCE inventory is reduced relative to other insect genomes [1] (Table 1A). Ten of 13 major CCE clades are represented; only eight, however, are in the class involved in xenobiotic detoxification. Toxicity of cyfluthrin and tau-fluvalinate is enhanced by CCE inhibitors, consistent with carboxylesterase-mediated cleavage of the ester bond [19]; moreover, CYP9Q1-3 convert tau-fluvalinate to a metabolite suitable for CCE cleavage [15], providing further evidence of CCE involvement in pesticide tolerance. Carboxylesterase gene GB10854 (esterase E4-like) involvement in xenobiotic metabolism is suggested by its upregulation in workers exposed to p-coumaric acid [20**] and coumaphos [17**].

### Phase II conjugation: glutathione S-transferases

Glutathione S-transferases contribute to Phase II detoxification of electrophilic xenobiotics via conjugation with glutathione (GSH), although they also participate in Phase I detoxification by binding and sequestering toxins (such as pyrethroids) directly [21]. Of the six major subclasses — sigma, omega, theta, zeta, delta and epsilon — delta and epsilon are largely restricted to insects and have been linked to xenobiotic detoxification. In these subclasses, the *A. mellifera* genome, with only ten ostensibly functional genes, is strikingly depauperate relative to other insects, but it contains comparable numbers of Sigma class GSTs. Inducibility by xenobiotics provides the only evidence of potential GST involvement.
in xenobiotic detoxification. The pyrethroid flumethrin induced GST activity in larvae, pupae and nurse bees but reduced activity in foragers [22] and workers consuming nectar and pollen of Ziziphus jujuba displayed elevated GST and P450 activities [23]. In the eastern honeybee Apis cerana cerana, the Sigma-class AccGSTS1 was upregulated by phoxim, cyhalothrin and ‘acaricide’ [24] and the Omega-class GST gene GSTO2 was upregulated by cyhalothrin, phoxim, pyridaben and parathane [25]. In both cases, upregulation may have been in response to oxidative damage caused by these pesticides. At the molecular level, coumaphos ingestion resulted in upregulation of GSTD, a Delta-class GST, which may, as in other insects, contribute to detoxification of this organophosphate [17**].

**Phase III transport: ABC/multidrug transporters**

Phase III transporters, primarily the ATP-binding cassette (ABC) proteins, hydrolyze ATP and move a diversity of hydrophobic or conjugated products across lipid membranes and out of cells for excretion. These proteins are not well known in insects; this gene family has been annotated in only seven arthropod genomes to date [7]. Of these, A. mellifera has the smallest inventory, with 41 (~40% of the inventory in the Tetanychus urticae genome). The ABCB-FT (full transporter) subfamily comprising P-glycoproteins, the ABCC subfamily comprising multidrug resistance-associated proteins, and the ABCG FTs comprising pleiotropic drug resistance proteins have all been implicated in insect xenobiotic metabolism. ABC transporter involvement in honeybee xenobiotic detoxification is suggested primarily by synergism studies utilizing inhibitors or by quantification of transcript levels. The toxicity of three neonicotinoids and two acaricides is synergized by the inhibitor verapamil, implicating multidrug resistance transporters in their detoxification. Prefeeding bees with oxytetracycline, an antibiotic frequently used in-hive to treat American foulbrood (Paenibacillus larvae), enhances toxicity of these acaricides, raising questions about beekeeping practices that entail simultaneous treatment for mites and bacterial diseases [26].

**Interactions between xenobiotics**

Anecdotal perceptions that honeybees are peculiarly sensitive to pesticides, bolstered by the genomic detoxification deficit, were dismissed by Hardstone and Scott [27], who compared published LD50 values for honeybees and other insects and documented the absence of across-the-board vulnerability. A more likely consequence of detoxification gene deficits may be increased susceptibility to interactions among toxicants, of particular significance in the context of recent documentation of massive contamination of hives by agricultural pesticides, averaging six pesticide detections per sample [28]. Indeed, workers
Table 3

Pesticide combinations with enhanced toxicity in *Apis mellifera* (underlined compounds detected in 10% or more of wax samples in [28]; references in brackets.

<table>
<thead>
<tr>
<th>Interactions between in-hive acaricides used by beekeepers</th>
<th>tau-fluvalinate</th>
<th>Coumaphos</th>
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Table 3 (Continued)

Interactions between neonicotinoid insecticides and fungicides

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<th>Interactions between herbicides and insecticides</th>
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<td>Attrazine</td>
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</table>

Sterol biosynthesis-inhibiting fungicides (which inhibit fungal P450s) synergize insecticides and acaricides, presumably by inhibiting P450-mediated detoxification [10**,30] (Table 3). In addition, tau-fluvalinate and coumaphos synergize other acaricides, possibly through competitive interactions at target sites or interference at P450 catalytic sites [30]; molecular models of CYP9Q1-3 show that both coumaphos and fluvalinate dock into the same catalytic pocket [15]. Combinations of fungicides, insecticides, acaricides and herbicides have all demonstrated some degree of interactive effects in bees [10**,31], though most of these interactions occur by undetermined mechanisms [30].

**Nutrition and detoxification**

Nutritional stress may be exacerbated in the presence of pesticides; upregulation of detoxification genes by imidacloprid is accompanied by down-regulation of genes associated with glycolysis and development [32**], indicative of an energy cost of detoxification. Additionally, nutrient quality can influence xenobiotic toxicity. Dietary protein quality and quantity are particularly important; pesticide sensitivity of adult bees depends at least in part on the quality of pollen consumed in the first ten days after eclosion, with bees consuming high-quality pollen displaying greater pesticide resistance than bees consuming inferior pollen or protein sources [33], especially with other stressors present (e.g., low temperature [34]). This mitigating effect of high-quality pollen explains in part seasonal differences in pesticide toxicity, with well-fed autumn bees less sensitive than early spring bees [33]. In a bioassay, workers consuming a pollen-based diet for 16 days displayed less sensitivity to chlorpyrifos relative to bees consuming sucrose alone [17**]. Pollen identity can also influence detoxification capacity, with different pollen diets associated with different levels of gut GST reared as larvae in brood comb contaminated with multiple pesticide residues experienced reduced longevity relative to brood reared in less-contaminated comb [29].
activity in bees with and without the gut parasite Nosema ceranae [34].

Beyond its nutritional content, the natural diet of honeybees contains phytochemicals that influence xenobiotic detoxification. A diet containing honey, pollen, or propolis added to sucrose upregulates CYP6AS genes, which encode enzymes that metabolize quercetin and other flavonoids, whereas a diet of sucrose or high-fructose corn syrup does not [14]. Constituents of honey, pollen, and propolis also upregulate CYP9Q genes, several of which encode enzymes that metabolize quercetin and acaricides [20**]. The most active inducers were p-coumaric acid, which in honey likely derives from pollen grains, and pinocembrin, and pinobanksin 5-methyl ether, which likely derive from the propolis lining cells in which honey is stored. Fourteen xenobiotic-metabolizing P450 genes and a γ-glutamyltranspeptidase-1-like gene are upregulated by p-coumaric acid (Table 2). Adding p-coumaric acid to a sucrose diet enhanced detoxification of coumaraphos by 60%, demonstrating its functional role [20**]. Schmehl et al. [17**] also showed that a pollen diet upregulates many of the same detoxification-associated transcripts upregulated by coumaraphos and fluvalinate, including CYP9S1, CYP9Q3, and GSTD1 (Table 2). The evident importance of dietary phytochemicals in regulating detoxification raises questions about beekeeping practices involving replacing natural foods (honey, pollen) with artificial substitutes [35].

‘Social detoxification system’?

Genome-level deficits in detoxification and immunity relative to other insects may be an evolutionary consequence of A. mellifera eusociality. In terms of immunity, bees display cooperative behavioral defenses against parasites and pathogens, including ‘social fever’ to kill temperature-sensitive bacteria, collection of antimicrobial plant resins for propolis, and removal of diseased brood, that collectively comprise a ‘social immunity’ system [36,37], which may have reduced reliance on genome-encoded physiological immunity. Whether social behaviors contribute comparably to xenobiotic detoxification, leading to or compensating for reduced detoxification gene inventories, has not yet received attention. Behavioral mechanisms that may reduce toxin exposure include selective foraging with detection and avoidance of toxins in nectar and pollen, dilution of toxins by mixing nectars and pollens, processing of nectar into honey, and culturing fungi and other microbes to convert pollen into bee bread. These behaviors may significantly reduce the number and concentration of toxins requiring enzymatic processing — a ‘Phase 0’ of sorts in the traditional scheme of xenobiotic detoxification (Figure 1).

The size and perennial nature of honeybee colonies necessitate nectar and pollen collection from a broad diversity of flower species; while bees encounter multidinous environmental chemical signals, the extent to which they detect and utilize these signals prior to ingestion to avoid toxicity remains uncertain. Bees do collect pollen contaminated with pesticides [28], suggesting an inability to detect or avoid these chemicals, yet the phenomenon of ‘entombed pollen’ (whereby bees seal cells containing pollen with high fungicide levels) suggests bees can recognize contaminants [38]. In terms of nectar, responses to nectar phytochemicals are idiosyncratic and dose-dependent, with bees avoiding certain substances but displaying paradoxical preferences for some neurotoxic compounds (e.g., caffeine [39]). Even if bees cannot taste and reject toxins, post-ingestive malaise allows bees to learn to avoid food cues associated with toxins with adverse physiological effects (e.g., amygdalin in almond nectar) [40**].

The diversity of floral resources utilized by A. mellifera also provides opportunities for mitigating nectar and pollen toxicity via mixing and dilution. This mechanism allows the generalist solitary bee Osmia cornuta, for example, to utilize toxic pollen of Ranunculus acris, which becomes essentially nontoxic if consumed in a 50% admixture of pollens from other species [41*]. Whether collecting nectars from multiple floral sources similarly dilutes potential toxins has not yet been assessed.

The degree to which toxins are degraded by honey and bee bread processing is also largely unexplored. Nectar phenolics decrease in concentration during honey ‘curing’ at hive temperatures of 35 °C [42] and incorporation of glucose oxidase into incipient honey may also reduce toxicity by converting glucose to gluconic acid and lowering honey pH. Beyond symbiotic gut microbes [43,44], bees benefit from mutualistic fungi in the hive in that bee bread is the product of fungal fermentation of pollen [45]. Aspergillus species dominate the fungal community during bee bread ‘ripening’ [46]. Many Aspergillus species are opportunistic plant pathogens that produce enzymes that degrade phytochemicals (e.g. [47]). In addition, glucose oxidase produced by Aspergillus has been linked to lignin degradation [48] and may thus be involved in phenolic detoxification. That associations with Aspergillus may be mutualistic is suggested by honeybee detoxification, rather than bioactivation (i.e., increase in toxicity after P450-mediated metabolism), of aflatoxins produced by these fungi [12]. Aflatoxin tolerance increases when bees consume honey rather than high-fructose corn syrup are also suggestive of adaptation to this mycotoxin [14]. As well, propolis, the resinous material used to line cells containing bee bread, induces xenobiotic-metabolizing P450s and enhances survival in the presence of aflatoxin B1 [12], also suggestive of adaptation to cohabitation.

During bee bread processing, bees may thus exploit the broad-spectrum detoxificative capacity of fungi, much like symbiotic yeasts associated with Lasioderma serricorne contribute to phytochemical detoxification [44]. Genomes of
insect hosts often lose genes associated with biosynthesis of amino acids provided by their endosymbionts; an association with a symbiont contributing detoxification enzymes may similarly lead to losses in detoxification gene inventories in host genomes. The genome of *Atta echinata*, a leaf-cutter ant with symbiotic fungi, for example, possesses fewer P450 genes than genomes of ants lacking such symbionts [49,50]. P450 gene deficits in *A. mellifera* may similarly reflect dependence on behaviorally mediated detoxification processes, including mutualisms [51]. If such is the case, the widespread contamination of beehives by fungicides may have profound implications for bee health beyond direct fungicide toxicity [52**].

**Implications for honey bee management**

How honeybee behaviors influence or complement enzymatic processing of environmental toxins is of considerable importance in insuring the sustainability of apiculture and, consequently, the overall agricultural enterprise in the U.S. The possibility that enzymatic detoxification in this eusocial species may be particularly vulnerable to synergistic interactions and may be complemented by behaviors, including forager avoidance of toxins that reduces intake and/or colony food processing activities that reduce toxicity, has profound implications for estimating risks of exposure and limits of tolerance for honeybees in U.S. agricultural landscapes, the vast majority of which are managed with the use of a diversity of pesticides.

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**References and recommended reading**

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


This study investigated the effects of pesticide exposure on gene expression. Extended exposure of adult bees to fluvanilate or coumaphos resulted in differential expression of over 1100 transcripts relative to bees that were not exposed to these acaricides, many of which were genes involved in detoxification, immune, behavioral maturation, and nutrition. In addition, these investigators examined whether the quality and nature of the protein source in the diet influenced pesticide sensitivity and found via bioassay that bees consuming pollen were more tolerant of exposure to chlorpyrifos, an organophosphate, than bees consuming an artificial diet. The protective effects of a pollen diet may be attributable to the fact that pollen ingestion results in upregulation of many of the transcripts upregulated in response to pesticides, providing additional evidence of the importance of considering impacts of phytochemicals characteristic of the natural diet of honey bees in evaluating effects of pesticide exposure.


Honey is known to upregulate detoxification genes in Apis mellifera but which specific constituents of honey were not identified until these authors fractionated honey extracts and identified three highly effective inducers that may not be derived from nectar per se — p-coumaric acid, which is a principal constituent of pollen cell walls, and pinocembrin and pinobankin 5-methyl ether, common constituents of propolis. RNA-seq with the most effective inducer, p-coumaric acid, revealed that this universal constituent of bee foods upregulates transcripts of every class of detoxification gene and, surprisingly, transcripts of immunity-associated genes. This "nutraceutical" function of p-coumaric acid and other phytochemicals suggests that apicultural practices involving replacement of honey with sugar substitutes, including high-fructose corn syrup, might compromise the ability of bees to contend with pesticides and pathogens.


28. Mullin CA, Frazier JL, Ashcraft S, Simonds R, vanEngelsdorp D, Pettis JS: High levels of miticides and acaricides in North


Although synergistic interactions arising from binary mixtures of (ostensibly) nontoxic fungicides, acaricides, and insecticides have been demonstrated previously, these authors tested for interactions in all combinations. In fact, the toxicity of the binary mixture of the acaricide flufenoxuron and the fungicide chlorothalonil is enhanced synergistically by the addition of the acaricide coumaphos. In addition, another striking finding reported in this paper is the toxicity of an ostensibly ‘innocent’ formulation solvent, N-methyl-2-pyrrolidone. Collectively, this study provides supportive evidence of the possibly heightened sensitivity of honey bee to combinations of chemicals as well as insights that should inform honey bee management practices.


These authors effectively used whole transcriptome sequencing (RNA-Seq) of larvae to compare gene expression in honey bee colonies exposed to or free from low levels of imidacloprid, a widely used neonicotinoid, for 15 days. Among the 300 differentially expressed transcripts in pesticide-exposed larvae were upregulated expression of genes encoding detoxification enzymes; that this upregulation was coupled with downregulation of genes in glycolytic and sugar-metabolizing pathways is compelling evidence of an energetic and developmental cost of chronic pesticide stress and detoxification. This work adds a new dimension to evaluating impacts of pesticide exposure on colony health.


The degree to which honey bees can assess nectar or pollen quality and avoid natural or synthetic toxins while foraging is a critical question, particularly in view of the increasing use of systemic pesticides that can contaminate nectar. That honey bees can detect and avoid certain natural toxins has been demonstrated but it is also known that there are toxins that bees apparently cannot detect. In this important study, Hurst et al. determined that honey bees can learn to avoid toxins even if they cannot be tasted if they induce physiological symptoms of ‘malaise’, including a toxin-induced locomotory deficiency. Although behavioral reactions to ‘malaise’ is well known to influence food choice in mammals, demonstrating its existence in honey bees clarifies the importance of associating olfactory conditioning in reducing consumption of environmental toxins.


This study provides the first evidence that pollen generalists can utilize toxic pollen by combining it with more suitable pollen from other floral sources. Previous studies have shown that bees can benefit from combining pollens from multiple sources by virtue of consuming more nutritious protein, this study shows that generalized pollinators can ameliorate toxic, behavioral means. Although the study was on the solitary megachlid Osymia cornuta, as the authors suggest, pollen mixing may in fact be a widespread strategy among pollen generalists.


By analyzing samples of bee bread from colonies pollinating orchards that differed in frequency of fungicide use, these authors documented not only fungicide residues but also the impact of those residues on the colonies. Honey bees depend on a diverse community of beneficial fungi for processing pollen into bee bread; fungicide contamination, however, was associated with reductions in the numbers of fungal isolates. The disproportionate impact of fungicide residues on *Aspergillus* may be particularly detrimental to colony health. There are indications that altering the fungal community may increase colony susceptibility to fungal diseases such as chalkbrood.

Chalkbrood is a fungal brood disease of the honey bee, *Apis mellifera*, caused by the parasite *Ascosphaera apis*. Considered as a stress-related disease, the severity of chalkbrood outbreaks depend on a multitude of interacting factors. The specific relationship between host and parasite in this disease is interesting because the parasite is both heterothallic and semelparous. Recent studies highlight that this specific host–parasite relationship is influenced by factors such as interactions with other parasite strains or species, and environmental perturbations. To understand how to protect pollinators most effectively, it is crucial that future research takes a more ecologically relevant approach by studying the basic biology of the host–parasite relationship in the context of the multi-factorial processes that influence it.

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**Glossary**

**Caste**: defines a subset of individuals within a social insect colony that have a functionally different role from other castes, for example the queen is functionally different from a worker.

**Emerging Infectious Diseases (EIDs)**: occur when a strain or species of pathogen infects a new host or spreads to a new population due to anthropogenic, genetic or ecological perturbations.

**Emergency queens**: are raised from the youngest larvae available in a colony of honey bees when the current queen is unexpectedly lost.

**Entomopathogenic**: is a parasite that can cause disease in insects.

**Drone**: larvae are the male offspring of the honey bee. Because honey bees have *Haplodiploid* sex determination (females are diploid and males are haploid), the males suffer from a lack of diversity in resistance alleles due to having only half the genetic material of females.

**Heterothallic**: species require two distinct, but compatible, mating types to produce their transmission stage, thus display sexual reproduction.

**Hygienic**: honey bees are colonies that display a genetically mediated defence mechanism against brood diseases, where worker bees detect and uncap cells containing brood that is dead or diseased.

**Idiomorph**: describes the differential gene sets found in the two mating types of ascomycete fungi that are responsible for sexual reproduction in these organisms.

**Negative frequency dependent selection**: is a rapid form of antagonistic coevolution between host and parasite where, when a parasite adapts to a common host genotype, this then favours a rare host genotype.

**Semelparous parasites**: rely on a single reproductive event before they die. They must kill their host before achieving any form of transmission; thus they are obligate killers that turn their hosts into their own transmission stages.

**Spillover**: is when a pathogen infects a reservoir of hosts that are not its co-evolved host, which provides further opportunity for re-infection of the host population, and exacerbation of EIDs.

**The Terrestrial Code**: sets out standards for the improvement of animal health and welfare and veterinary public health worldwide, including through standards for safe international trade in terrestrial animals (mammals, birds and bees) and their products.

**Introduction**

Chalkbrood is a honey bee disease caused by the entomopathogenic (see glossary, hereafter gl.) fungus *Ascosphaera apis* (Maassen ex Clausen) Olive and Spiltoir [1,2]. It exclusively infects honey bee (*Apis mellifera*) larvae, leaving them with a mummified chalky appearance (a. k. a. chalkbrood mummies), giving rise to its common name chalkbrood. As a brood disease, chalkbrood infestation weakens honey bee colonies and leads to reduced honey production through a loss of the workforce, and under certain circumstances can kill colonies, making it an economically important disease [3]. The persistence of the chalkbrood spores (up to 15 years viability [4]), combined with the international trade in honey bees and the migratory nature of commercial beekeeping on some continents, has led to a circumglobal proliferation of the disease [5]. Consequently, chalkbrood is not covered by *The Terrestrial Code* (gl.) [6], meaning there are no restrictions on international trade of honey bees carrying the disease. As a semelparous and heterothallic parasite (gl.), *A. apis* has a distinctive relationship with its host that affects both its virulence (i.e. within-host processes) and transmission (i.e. between-host processes) [7]. Several important factors can influence these processes (Figure 1), and thus impact on the epidemiology of chalkbrood. Here I explore these factors in the context of how a clearer understanding and consideration of them could help develop strategies for improved honey bee health.
Factors affecting disease establishment: within-host processes

The establishment of chalkbrood in a colony begins with ingestion of spores of *A. apis*, which are inadvertently fed to the larvae by adult bees via food provisions. The necessary anaerobic environment for spore germination is found in the closed hindgut of two–four day old larvae, where accumulation of CO$_2$ activates the spores, which swell and produce germ tubes that extend into hyphal growth [8]. Hyphae penetrate the gut epithelium through a combination of enzyme action and mechanical force [9,10**], and grow into the surrounding tissues, killing the host whilst utilising its tissue for growth. There are three important influences on these within-host processes that determine the likelihood of outbreaks of chalkbrood in a colony. Each of these could be a target for improved disease control: environmental stressors, the host resistance–parasite virulence relationship, and interactions with other parasite species.

**Environmental stressors**

The likelihood of chalkbrood outbreaks increase when colonies have a weakened ability to care for brood, or to maintain an optimal brood temperature of 35 °C [11,12]. Brood chilling accelerates the infection process by inducing a stress response in larvae [13,14], as such, chalkbrood is often considered a stress-related disease [15]. Outbreaks are common during rapid colony growth due to a higher brood to worker ratio resulting in a drop in comb temperature and larvae receiving less care [11]. However, environmental stressors can exacerbate this and lead to more severe outbreaks. For example, agricultural intensification can reduce the quantity and diversity of flowering plants. In association with exposure to high levels of...
agrochemicals, adult honey bees experience increased stress and can become subsequently immunocompromised [16,17], leaving them less efficient at brood care. These stressors may have contributed to the severe colony losses witnessed in recent years [18**,19*]. The same stressors may disrupt the beneficial microbial community within the colony, weakening its overall resilience [20,21**]. Honey bee colonies that have a higher concentration of symbiotic microbes in the pollen fed to their larvae are less susceptible to chalkbrood [22], and there is some evidence to suggest the honey bee gut microbiota may have antifungal activity [23]. Considering this, it is clear that interacting environmental factors and microbiota composition affect chalkbrood susceptibility and require more attention.

The host resistance–parasite virulence relationship

Significant genetic variation in resistance to A. *apis* exists at both the colony [24] and individual level [25], and so resistance to chalkbrood is a candidate for selective breeding. The underlying transcriptional response by larvae to infection by *A. apis* is characterised by activation of the cellular machinery involved in a general stress response [26]. A working hypothesis that remains to be tested is that the stress response is primarily driven by nutritional deprivation due to a reduction in feeding rate, even though multiple pathways of the innate immune system are activated in *A. apis* exposed larvae [27]. Parasite exposed larvae also exhibit differential transcription of a chitinase-like enzyme [26], which could be linked to anti-fungal activity in the gut. Concurrently, production of fungal chitinases appears to be a key pathogenesis trait in *A. apis* [10**]. Variation in the host’s resistance appears to be based around single nucleotide polymorphisms in close proximity to a variety of genes, including host chitin biosynthesis and development [28], so the gut lining (composition and/or its products) could be acting as a point of defence against infection, and provides another target requiring further investigation. However, considering the significant genetic variation in resistance to different strains of *A. apis* [29] by different host genotypes [25], and a rapid rate of evolution in the parasite itself [10**], the host–parasite relationship appears to undergo negative frequency dependent selection (gf) [25]. Recent data suggests that in susceptible colonies, sublethal exposure to *A. apis* impacts on larval growth rate [30], probably as a result of the upregulation of immune signalling after infection being associated with a down-regulation of the major storage proteins involved in growth and repair [26]. Interestingly, sublethal exposure also lowers the chance that those larvae will be used to raise emergency queens (gf) [30]. Therefore, this parasite–mediated caste (gf) biasing could act as a mechanism of “social-control” of resistance genes, potentially speeding up the antagonistic co-evolution between host and parasite. Empirical evidence also suggests that coinfection by different strains of *A. apis* can influence the evolution of virulence in the parasite, depending on the characteristics of the strains involved [31*]. Thus, the potential for effective selective breeding of host resistance may be futile if the host–parasite relationship is rapidly evolving.

Multiple parasite interactions

As well as multi-strain infections, honey bees are often co-infected by a plethora of different species of pathogens and parasites. It is becoming increasingly apparent that the interactions between them are important stressors on overall colony health [18**]. The recent and damaging epidemics resulting from parasites switching hosts have taught us a lot about host–parasite dynamics in emerging infectious diseases (EIDs; gf) of honey bees (such as *Varroa destructor* [32] and *Nosema ceranae* [33**]). For example, the high virulence of the *Varroa* mite in *A. mellifera* is primarily attributed to their role as both a multiplier and vector of viruses that would otherwise be relatively benign to this host [32]. Increased DWV titres have also been associated with chalkbrood prevalence [34], which may be linked to the recent finding that multiple honey bee viruses, whose lethality is usually associated with *Varroa* infestation, can also replicate in the *A. apis* fungus [35**]. In Germany, outbreaks of chalkbrood appear to be positively correlated with outbreaks of both *V. destructor* and *N. ceranae* [15], suggesting that the interactions between these three parasites may be more important than we previously thought. In addition, the global spread of these EIDs are increasingly being found to spillover (gf) into other species of wild bee [36–38]. Even though chalkbrood infection is specific to honey bee larvae, non-host organisms apparently vector the spores. These findings together highlight transmission routes or reservoirs for chalkbrood disease that we might not have previously considered important.

Factors affecting disease spread: between host-processes

Certain characteristics of a social insect colony mean that the spread of disease in honey bees involves fundamentally different processes to those of solitary animals [39]. Honey bees in particular exhibit several behaviours that might increase between-colony transmission of the persistent chalkbrood spores, such as honey robbing and flower sharing [40]. Specific characteristics of *A. apis* also have important influences on between-host-processes, i.e. the transmission of the parasite, and could provide further targets for improved disease control: the parasite’s fitness, the social immunity mechanisms employed by bees in response to it, and ultimately interference from humans through beekeeping practises.

Parasite fitness

As a semelparous parasite (gf), the production of spores by *A. apis* is also influenced by the factors affecting the within-host processes because it must kill its host before it
can reproduce. However, the reproductive fitness of the parasite is what determines its transmission potential, and in *A. apis*, this is contingent on several factors. As a heterothallic fungus (gl.), spores are only produced through sexual reproduction after hyphal fusion of two opposite mating types [1]. Often only white, i.e. non-sporulating, chalkbrood mummies are produced from an *A. apis* infection, which has been suggested to be due to a single mating type infecting a host. However, as both mating type idiomorphs (gl.) are routinely isolated from white mummies [41], it is more probable that white mummies are simply an immature growth stage of an infection that will subsequently sporulate given the right conditions or host resources [42**]. Considering that fungal growth requires nutrients gained from the host’s resources, the ability to sporulate is intrinsically linked to the parasite’s virulence. A more virulent strain of parasite that kills its host quicker means the host larva will be younger, and so smaller, providing fewer resources for growth and reproduction by the parasite. Conversely, if virulence is too low it will lead to failure to infect; this is the basis to the trade-off hypothesis of optimal virulence [43]. There are multiple influences on this optimum, which will impact on transmission potential. These include the diversity of the strains infecting, and their interactions within the host during co-infection [31*], but host genotype and phenotype can also affect parasite fitness [44]. For example, chalkbrood infections in drone (gl.) larvae appear to produce more sporulating events, and provide a greater transmission potential for the parasite [45]. This is probably due to a combination of haploid susceptibility (gl.) [46], a higher feeding rate in drones leading to increased spore consumption, and that drones are usually reared within cooler regions on the edge of the comb [45].

**Social immunity**

The collective, group level defences employed by eusocial insects, termed social immunity, go some way towards mitigation of any individual level immunodeficiency in honey bees [47]. However, they also influence the establishment and transmission of *A. apis*. The prevalence of chalkbrood can be effectively reduced in hygienic (gl.) colonies of honey bees [48]. However, the efficiency of the hygienic behaviour against chalkbrood relies on early detection of larval death. If only late stage, potentially sporulating, chalkbrood mummies are removed [49], hygienic behaviour could increase rather than decrease transmission by exposing more individuals to the spores. A less appreciated mechanism of social immunity, that could be an alternative (or complementary) target for selective breeding, is food and nest sanitation by environmentally derived antimicrobials [50*]. For example, honey bees self-medicate by increasing their rate of resin foraging in response to chalkbrood challenge [51**]. However, the benefit of this behaviour could be acting on an evolutionary scale, by reducing investment in the inherently costly [52] individual immune responses [53], rather than simply acting to directly inhibit the viability of chalkbrood spores [54].

**Beekeeping practises**

As a managed animal, the spread of disease in honey bees is facilitated by human activities. Due to the persistent nature of the chalkbrood spores, lack of regulation by authorities, absence of effective chemical controls [55], and also the apparent natural reservoir of this disease in other pollinators [36–38], it is unlikely that we will ever eliminate chalkbrood as a disease of honey bees. However, there are specific beekeeping practices that can keep it in check. Management and sanitation practices should aim to reduce transmission [5], for example by keeping equipment clean and avoiding transfer of contaminated comb [56,57], or by managing drone production [45]. Ultimately however, enhancing natural resistance mechanisms is the most effective defence strategy beekeepers have; keeping hives well ventilated and avoiding nutritional limitation or chemical exposure boosts their resilience to the disease [58]. In the long term, selection for disease resistance is the goal of both scientists and beekeepers. To achieve this, a considered approach must be taken, for example, even though requeening to improve genetic stock is advised as a control strategy, maintaining genetic diversity is a crucial component in the overall health of honey bees [59] to keep ahead in the evolutionary arms race against parasites [60].

**Implications for pollinator management and conservation**

The biggest challenge for management of chalkbrood disease in honey bees is understanding the complex interactions in the multi-strain multi-species pathosphere in which they exist. Although the progress made in understanding the genetic basis to resistance and virulence in this host–parasite system has been extremely informative, it is crucial that we widen our study systems to incorporate the multiple interacting factors outlined here. Taking this more ecologically realistic approach to understanding the host–parasite relationship underlying chalkbrood disease is the sensible direction to focus research. Importantly, effort should be made to include more traditionally benign yet chronic diseases, such as chalkbrood, as an important factor interacting with the general health of pollinators.

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**References**


This paper annotates the genome of the A. apis parasite and provides important new hypotheses on its pathogenesis traits including how it penetrates the hosts tissue.


An overview of the multifactorial problem of pollinator losses identifies the stress factors that influence chalkbrood outbreaks as important outstanding questions in general honey bee health.


A thorough analysis of the complex interaction between sublethal pesticide exposure and diet revealing specific molecular mechanisms behind immunodeficiency in adults bees.


Study of the distinctive gut microbiota of social organisms suggests that the ecology of the host is a key driver of microbial diversity, and suggests how environmental perturbations influence this should be a key target for future research.


An empirical approach to understanding virulence evolution in the honey bee–chalkbrood relationship shows that genetically diverse infections could influence chalkbrood disease epidemiology through the effects of within-host competition.


Highlights the importance of considering ecology in studies of host–parasite interactions because the outcomes of within-host competition might negatively impact on disease epidemiology.


This study shows that multiple honey bee viruses can infect and replicate within the chalkbrood fungus, highlighting a potential novel transmission route for these viruses.


A comprehensive overview of our current understanding of chalkbrood disease, particularly the molecular mechanisms underpinning the infection process and host resistance.


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A review of external mechanisms of disease defence argues for integrated approaches to understanding immunity and disease.


A study on social immunity suggesting that self-medication through resin collection could mitigate the effects of individual immunodeficiency in adult honey bees.


Antiviral defense mechanisms in honey bees
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Honey bees are significant pollinators of agricultural crops and other important plant species. High annual losses of honey bee colonies in North America and in some parts of Europe have profound ecological and economic implications. Colony losses have been attributed to multiple factors including RNA viruses, thus understanding bee antiviral defense mechanisms may result in the development of strategies that mitigate colony losses. Honey bee antiviral defense mechanisms include RNA-interference, pathogen-associated molecular pattern (PAMP) triggered signal transduction cascades, and reactive oxygen species generation. However, the relative importance of these and other pathways is largely uncharacterized. Herein we review the current understanding of honey bee antiviral defense mechanisms and suggest important avenues for future investigation.

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Introduction
Honey bees (Apis mellifera) are fascinating insects that play a critical role in agriculture as pollinators of crops (U.S. value over $15 billion/year) and plant species that enhance the biodiversity of both agricultural and non-agricultural landscapes [1]. Since 2006, honey bee populations in the U.S., Canada, and in some parts of Europe have experienced high annual losses [2,3,4**]. An average of 33% of U.S. honey bee colonies die each year, and a fraction of these losses are attributed to Colony Collapse Disorder (CCD) [5,6*,7,8,9**]. Multiple biotic and abiotic factors contribute to colony health and survival (i.e., viruses, mites, microbes, bee genetics, weather, forage quality and availability, management practices, and agro-chemical exposure) [9**,10,11**,12**]. Understanding the most influential factors and potential synergistic effects on honey bee health is critical to developing pollinator management and conservation strategies that limit bee colony losses [13].

Several epidemiologic and temporal monitoring studies indicate the important role of pathogens in colony loss including viruses, bacteria, fungi, trypanosomatids, and mites [4**,9**,12**,14*,15,16*,17,18**,19**,20,21]. The majority of honey bee infecting pathogens are RNA viruses, including Acute bee paralysis virus [22], Black queen cell virus [23], Israeli acute bee paralysis virus [24], Kashmir bee virus [25], Deformed wing virus [26], Kakugo virus [27], Varoa destructor virus-1 [28], Sacbrood virus [29], Slow bee paralysis virus [30], Cloudy wing virus [31], Big Sioux River virus [17,20], Aphid lethal virus (strain Brookings) [17,20], Chronic bee paralysis virus [32] (reviewed in [33,34]) and the Lake Sinai viruses (LSV1 and LSV2 [20], LSV3 [12**], LSV4 [17], and LSV5 [35]). Honey bee virus infections may cause deformities, paralysis, death, or remain asymptomatic [33]. Bee viruses are transmitted via vertical and horizontal routes [36], including co-foraging with wild and managed bee populations [37–39]. The ectoparasitic mite Varroa destructor serves as a vector for several honey bee viruses [40–42] and causes colony loss by feeding on bee hemolymph and killing bee brood [43]. Several studies indicate that combinatorial effects of mites and viruses result in colony loss (reviewed in [34,44**,45**,46]). The relationship between colony health and pathogen prevalence and abundance is complex and dependent upon season, geographic location, pathogen strain, and both individual and colony level bee immune responses. Thus, temporal monitoring studies are key to understanding the relative impact of these variables on honey bee colony health.

The focus of this review is to summarize our current understanding of honey bee antiviral responses. Honey bees, like all other organisms, have evolved mechanisms to detect and limit virus infection. Knowledge of honey bee immune mechanisms is largely derived via comparison to the better-characterized immune responses in fruit-flies and mosquitoes. While comparative genomics is a useful approach for evaluating honey bee immune gene function, it is important to note that Western honey bees (Apis mellifera) are eusocial Hymenopteran insects, an order that diverged from the solitary Diptera insects including fruit-flies and mosquitoes approximately 300 million years ago [47,48**,49,50]. General aspects of
Honey bee immune pathways — highlighting genes implicated in antiviral immune responses. The honey bee genome encodes major members of insect immune pathways including: RNAi (RNA interference); Jak/STAT (Janus kinase/Signal Transducer and Activator of Transcription); Toll; NF-κB (Nuclear Factor κB); JNK (c-Jun N-terminal kinase); and MAPK (Mitogen-Activated Protein Kinases), as well as orthologs of genes involved in autophagy, eicosanoid biosynthesis, endocytosis, and melanization. Bold text indicates genes and proteins differentially expressed in virus-infected honey bees. Additional information including Apis mellifera (Am) gene accession numbers is provided in Tables 1 and S1. The first step in immune activation is host recognition of pathogen associated molecular patterns (PAMPs) including viral dsRNA, bacterial peptidoglycans, and fungal β-glucans. In general, the Toll pathway is involved in defense against Gram(+) bacteria and fungi and the Imd pathway is activated by Gram(−) bacteria, but specific host-pathogen interactions are unique. This is particularly true for host–virus interactions since data from fruit-flies, mosquitoes, and honey bees indicate differential activation of immune genes and pathways. The Jak/STAT pathway is activated via ligand binding to the Domeless receptor; while Drosophila melanogaster (Dm) express Domeless ligands (unpaired, upd, upd2, and upd3), a honey bee upd ortholog has not been identified. Following Domeless-ligand binding, Hopscotch Janus kinases are transphosphorylated, leading to phosphorylation and dimerization of STAT92E-like proteins. Activated STATs transcriptionally regulate antimicrobial effectors TEP7 (Thioester-containing protein 7), TEPA, TEPB, and the Jak/STAT inhibitor SOCS (Suppressor of Cytokine Signaling). The honey bee genome also encodes for D-PIAS (Protein Inhibitor of Activated STAT), another inhibitor of the Jak/STAT pathway. The RNAi-pathway is initiated by Dm Dicer-2 cleavage of virus dsRNA into 21–22 bp siRNAs; Am Dicer-like share ~30% aa identity with Dm Dicer-2. The siRNAs are then loaded into AGO2 (Argonaute-2), the catalytic component of the RISC (RNA Induced Silencing Complex). A single strand of the siRNA is retained in the RISC and used to specifically target cognate viral genome sequences for cleavage. In addition, Dm Dicer-2 serves as a dsRNA sensor that mediates a signal transduction cascade resulting in increased expression of Dm Vago and suppression of viral replication. Am Dicer-like may serve as a dsRNA sensor, and honey bees have a vago ortholog (Table S1), but the mechanism(s) of honey bee non-specific dsRNA-mediated antiviral responses require additional characterization. The Toll pathway is activated by a family of pathogen recognition receptors (PRRs) (e.g., peptidoglycan receptor proteins and Gram(−) binding proteins) that bind fungal and bacterial PAMPs. The Toll pathway is activated in some insect host–virus combinations, although the activation mechanism is unknown. Following PAMP binding, a serine protease cascade results in cleavage of pro-Spaetzele into mature Spaetzele. The honey bee genome encodes two putative spaetzele orthologs, which bind the membrane-anchored Toll receptor. Toll dimerization results in the recruitment of dMyD88, Tube, and Pelle. Pelle is likely involved in degradation of NF-κB inhibitors (e.g., Cactus-1, Cactus-2, Cactus-3), resulting in the release of transcription factors Dorsal-1A and Dorsal-1B. Nuclear translocation of Dorsal results in increased expression of antimicrobial peptides (AMPs). The Imd pathway is activated by Peptidoglycan recognition protein LC (PGRP-LC) binding to diaminopimelic-containing peptidoglycan of Gram(−) bacteria, followed by activation of the adaptor protein Immune deficiency (IMD), Relish phosphorylation by the IKK complex (IκB kinase), and cleavage of Relish by the caspase Dredd (Death-related ced-3/ Nedd2-like). Relish transcriptionally regulates expression of AMPs and other genes involved in antimicrobial defense. The JNK pathway is also activated by TAB (Transforming growth factor-activated kinase 1) and TAK1 (Transforming growth factor-activated kinase 1 binding protein), resulting in AMP
immunity, including detection of pathogen associated molecular patterns (PAMPs) and production of effector molecules are conserved in mammals, plants, and insects, and both plants and insects employ RNA interference (RNAi) as a major mechanism of antiviral defense [51,52,53]. These immune pathways provide a framework for understanding honey bee host–virus interactions.

**Insect immune pathways**

RNA interference (RNAi) is the major mechanism of antiviral defense in fruit-flies and mosquitoes (reviewed in [53,54,55,56,57,58]). RNAi is a sequence specific, post-transcriptional gene and virus silencing mechanism that is triggered by double-stranded RNA (dsRNA). Direct evidence of the antiviral role of RNAi in insects has predominantly come from studies in *Drosophila melanogaster, Aedes aegypti, and Anopheles gambiae*, which involved experimental infections with pure virus inocula, mutant-flies, or gene knock-down in mosquitoes [59–63]. Likewise, field and laboratory based studies in *Apis mellifera* (Western honey bee) [64–66,67,68] and *Apis cerana* (Eastern honey bee) [70] indicate that RNAi-mediated antiviral immunity is important in honey bees (reviewed in [71*]). In addition, dsRNA may serve as a non-sequence-specific virus associated molecular pattern (VAMP) that triggers innate antiviral immune pathways in fruit-flies [72] and honey bees [73,74], similar to the mammalian interferon response [75] (Figure 1, Tables 1 and S1).

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cois.2015.04.016.

Other insect immune responses include melanization, encapsulation, reactive oxygen species production, and activation of signal transduction cascades that result in the production of antimicrobial peptides (AMPs) and other effector proteins (Figure 1, Tables 1 and S1). These pathways include the Toll, Imd (Immune Deficiency) and Jak/STAT (Janus kinase and Signal Transducer and Activator of Transcription) innate immune response pathways (Figure 1) (reviewed in [52,56,76,77,78,79,80]). There are numerous orthologous proteins utilized in plant, insect, and mammalian immune defense mechanisms (reviewed in [51,81]), and discovery of the *Drosophila* Toll pathway led to the identification of a repertoire of mammalian Toll-like receptors (TLRs) (reviewed in [81,82]). The importance of the Toll, Imd, Jak/STAT, and other pathways in antiviral defense is variable and specific to individual virus–host interactions [76,80,83]. For example, the Toll pathway is involved in *D. melanogaster* and *Aedes aegypti* defense against *Drosophila* X virus [84] and Dengue [85], respectively, as *dif* loss of function mutants were more susceptible to virus infection. The *Drosophila* Imd pathway plays a larger role than the Toll pathway in limiting Sindbis virus [86] and Cricket paralysis virus (CrPV) [87], and the Jak-Stat pathway is critical to combating Drosophila C virus infection [88]. AMPs are small cationic peptides that penetrate microbial membranes, serve in innate immune signaling, and play additional uncharacterized functions (reviewed in [77,89]). While the role of AMPs in virus infection is not known, changes in AMP expression are used as indicators of immune pathway regulation. AMP induction in *D. melanogaster* varies, as some viruses induce expression (i.e., DXV and SINV) and others do not (i.e., CrPV and Rhabdovirus [90]). Numerous studies suggest the role of additional pathways in insect antiviral defense [72,80,88,90–92].

**Honey bee antiviral immune responses**

Bioinformatic analysis of the honey bee genome identified *A. mellifera* orthologs of insect immune genes and suggests that bees have fewer immune genes than *D. melanogaster*, *Ae. aegypti*, or *An. gambiae* [47,48,93]. The honey bee genome encodes the suite of genes required for RNAi including dicer-1, ago-2, r2d2, and dicer-like, which shares 30% nucleotide identity with *Dm* dicer-2 [47,94]. All the main components of the Toll, Imd, JNK, Tor, and Jak-STAT pathways have been identified (except *upd*), as well as immune effector proteins including AMPs (i.e., abaecin, hymenoptaecin, apidasecin, and defensin) and prophenoloxidases [48*]. RNAi, Toll, Imd, endocytosis, MAPK, and non-specific dsRNA-mediated immune pathways have been implicated in honey bee antiviral defense (Figure 1, Tables 1 and S1).

A distinguishing feature of virus infection is the presence of long, double-stranded RNA molecules in the cytosol of the infected cell. Since long dsRNAs are not typical products of eukaryotic gene expression, these molecules are recognized as PAMPs in hosts including plants, arthropods, insects, and mammals [95]. Mammals have several receptors (e.g., TLR3, PRR, RIG-I, and MDA-5) that upon binding dsRNA, activate signal transduction
**Table 1**

Honey bee immune genes. The *Apis mellifera* genome encodes major members of insect immune pathways including those depicted in Figure 1 and listed by gene name, pathway, and accession number in this table. Bold text indicates genes differentially expressed in virus-infected honey bees, and the specific virus and citation are provided for each. Transcript variants, the majority of which were predicted using Gnomon and the NCBI RefSeq Database, are listed although many have not been experimentally verified as expressed transcripts, nor been specifically implicated in antiviral defense. A list of additional honey bee immune related genes is provided in Supporting Table S1.

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Antiviral defense mechanisms in honey bees  

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* Note dscam has 104 transcript variants: NM_001014991.1; XM_006567003.1–XM_006567105.1.

...resulting in the transcriptional activation of genes involved in generating an ‘antiviral state’ including thousands of interferon stimulated genes (reviewed in [96,97]). Importantly, long dsRNAs also serve as the substrate for RNAi-mediated antiviral responses. The first step of the antiviral small interfering RNA (siRNA) pathway is cleavage of cytosolic dsRNA by the Dicer enzyme (Figure 1). Initial studies implicating the role of RNAi in honey bee antiviral defense demonstrated that feeding sucerue solutions containing IAPV-specific dsRNA resulted in increased bee survival, lower levels of IAPV [64*], larger colony size, and increased honey yields [67]. This also sparked commercial interest in dsRNA/RNAi-mediated antiviral treatments [67], and raised concerns regarding potential off-target effects and the use of RNAi-based insecticidal crops [98]. A subsequent laboratory-based study demonstrated that pre-treatment of larvae and adults with DWV-specific dsRNA prior to DWV-infection via feeding resulted in increased survival and decreased virus titers [65**]. Likewise Apis cerana larvae pre-treated with virus-specific dsRNA had reduced levels of Chinese Sacbrood virus following infection via feeding [70].

One of the hallmarks of RNAi-mediated antiviral responses in insects is siRNA production. Small interfering RNAs produced by Dicer-2 cleavage are 21–22 bp in length, with an approximately 19 bp double-stranded RNA core, 5’-monophosphate ends, and two-nucleotide single-stranded overhangs at the 3’-hydroxyl ends; the single-strand siRNA retained in the holo-RNA Induced Silencing Complex (RISC) is modified (2’-O-methylated) at the 3’-end (reviewed in [99]). The first molecular evidence of virus-specific siRNAs in honey bee samples was obtained by Northern blot analysis [64*,70]. Recently, Chejanovsky et al. evaluated siRNA populations isolated from bees obtained from either CCD-affected or unaffected colonies using high throughput sequencing and determined that there were more virus-specific (i.e., IAPV, KBV, and DWV) siRNA reads in CCD-affected samples [66**,69**]. These siRNAs were predominantly 22-nt long and distributed throughout the virus genome [66**], indicating that the dsRNA replicative intermediate form of the IAPV genome was the Dicer substrate (reviewed in [100]). Further analysis of the IAPV-siRNAs from CCD-affected samples determined that most were negative-sense, and may thus serve as guide sequences that target the (+)ssRNA IAPV genome [66**]. High throughput sequencing of small RNAs obtained from Varroa-infested, DWV-like, and VDV-1-infected bees identified a greater number of positive sense virus-specific siRNAs than negative sense siRNAs, and showed that DWV-like virus and siRNA...
abundance were proportional [69**]. Interestingly, pupae with low virus levels that were exposed to few *Varroa* mites had 5-times more siRNAs than viral genomes, suggesting that when mite-pressure was low, the honey bee RNAi-mediated defense system was able to overcome virus replication [69**].

Results to date indicate that honey bees utilize RNAi as an antiviral defense mechanism. Future studies that show increased virus copy number in response to experimental knock-down of *dicer-like* and/or *argonaute-2* would provide additional evidence of an RNAi-mediated defense strategy in honey bees. Likewise, demonstrating siRNA incorporation into the RISC by sequencing only 2'-O-methylated siRNAs would provide additional experimental support for honey bee antiviral RNAi. The relative contribution of RNAi and other immune mechanisms requires further examination in the context of specific viruses, in different developmental stages and castes, and in a range of colony health (i.e., weak, healthy, CCD-affected). Genome integration of IAPV also requires further examination [64*], since in *D. melanogaster*, both genome-integrated RNA viral sequences and RNAi are involved in limiting and maintaining persistent virus infections [63]. Together, these and other studies will reveal the relative role of RNAi in reducing or eliminating viruses in individual bees and colonies.

In *D. melanogaster*, Dicer-2 not only participates in RNAi, it also serves as a dsRNA sensor that upon binding results in the transcriptional activation of genes with antiviral function including *vago* [72] (Figure 1). Interestingly, Dicer-2 is a DEAD-box helicase motif containing protein, similar to the RIG-I-like family of mammalian cytosolic dsRNA sensors [72]. Recent evidence in honey bees suggests that dsRNA, regardless of its sequence-specificity, triggers an antiviral response that decreases viral burden [73*]. Also, *vago* expression was increased in pupae that were orally infected with DWV [69**]. Transcriptional profiling of Sindbis virus-infected and dsRNA-treated bees three days post-infection indicated that metabolic pathways were perturbed in both treatment groups. In addition, endocytosis and cecosanoid signaling pathways were differentially regulated in virus-infected bees, and dsRNA-treated bees differentially regulated genes involved in oxidative phosphorylation. The majority of differentially expressed genes were not involved in characterized innate immune pathways, albeit AMP expression was reduced (i.e., *apidaecin* and *hymenoptaecin*). Transcriptional changes in response to non-virus specific dsRNA (i.e., dsRNA-GFP) in developing honey bee workers were evaluated in a study aimed at investigating the off-target effects in RNAi-mediated gene knockdown experiments [74]. This study identified 1400 differentially expressed genes, and gene ontology analyses determined that the affected genes included those involved in development, metabolism, immunity, stress response, and RNA processing and transport [74].

Several transcriptional level studies in honey bees implicate the involvement of uncharacterized genes/pathways in antiviral responses [18**, 69**, 73*, 101**, 102**]. However, the roles of genes in the Toll, Imd, Jak-STAT, JNK, and RNAi pathways are the best characterized. Central players in honey bee immune signal transduction cascades include insect orthologs of a well-characterized mammalian transcription factor NF-κB, including Dor-sal-1A, Dorsal-1B, and Relish (Figure 1, Tables 1 and S1). Nazzí *et al.* determined that *dorsal-IA* expression is key in limiting DWV infection [102**]. Activation of NF-κB-family transcription factors results in the production of AMPs, which have unknown roles in antiviral immunity, and numerous other less well-characterized genes [19**, 48**, 103–105]. Symptomatic young bees experimentally infected with IAPV via feeding exhibited increased expression of Toll pathway members (i.e., *toll-6*, *cactus*, and *hymenoptaecin*) [101**], whereas transcriptional profiling of IAPV positive bees from naturally infected colonies did not implicate either the Toll or Imd pathways in antiviral defense [18**]. Young bees experimentally infected with Sindbis virus via injection and harboring very low levels of other bee pathogens expressed less *apidaecin* and *hymenoptaecin* than mock-infected controls [73*]. Similarly, neither ABPV-challenge nor ABPV and *E. coli* co-challenge via injection resulted in AMP production (i.e., Defensin-1, Abaecin, and Hymenoptaecin) in adults or larvae, indicating that ABPV may suppress bee immune responses [106*].

There are few general trends in the transcriptional response of honey bees to viruses due in large part to the relatively small number of studies performed to date and due to differences in virus-challenge methodologies (e.g., infection via injection, oral infection), experimental vs. natural infections, tissues examined, post-infection assay time, and developmental stage of the bee [107*] (i.e., IAPV [18**, 101***, 108], DWV [44**, 69**], SBV [70], CCD-affected [109], and Sindbis virus [73*]). Furthermore, variability between experimentally infected-bees may be attributed to differences in immune gene regulation between individuals within and between colonies, purity and strain of virus inoculum, varied microbiomes, and prevalence of pre-existing pathogens. In addition, there are relatively few predicted genes (~25%) that are involved in well-annotated pathways; 33% of the DEGs in naturally IAPV-infected adults had *Drosophila* orthogs and could be assigned putative function [18**]. That said, differential expression of genes in immune, endocytic, and metabolic pathways are common to several data sets, but the directionality of regulation varies between studies and bee developmental stage [18**, 73*, 101**]. Several investigations have focused on IAPV due to its association with colony health and the development of methods to
produce IAPV-augmented infectious stocks via passaging bee viruses in pupae [108]. In adult bees, IAPV abundance is highest in the gut and hypopharyngeal gland and low in hemocytes (insect blood/immune cells) and the fat body, a tissue involved in metabolic activities (insect liver) [18**,77,110,111]. Transcriptional profiling of IAPV-infected adults revealed differential expression of over 3000 genes [18**]. Functional analysis determined that genes involved in signal transduction and immune responses exhibited increased expression and that genes involved in metabolism and mitochondrial dysfunction had reduced expression [18**]. In addition, IAPV-infection resulted in increased expression of genes involved in the TCA cycle II, protein ubiquitination, and eIF2 signaling, and that IAPV-infection reduced expression of genes in the γ-glutamyl cycle [18**]. Chen et al. determined that IAPV-infection also perturbed expression of genes involved in insect immune pathways (i.e., oxidative phosphorylation, ABC transporter function, endocytosis, phagocytosis, TGF-beta signaling, Tor signaling, MAPK signaling, Jak-STAT signaling, and lysosomal degradation) [18**]. Specific immune genes with increased expression in IAPV-infected adult honey bees include Jak/STAT pathway members (i.e., cbl, stat, pias, and hopscotch), Tor pathway members (i.e., gbl, mo25, dmel, and elf4B), MAPK members (i.e., pointed, phi, and corkscrew), and genes involved in endocytosis (i.e., egfr, pastl, rabenosyn, and vacular protein sorting-associated protein 37B-like) [18**] (Figure 1, Tables 1 and S1). It is noteworthy that IAPV-infected larvae had a different suite of DEGs with little overlap in the adult dataset [18**]. Pupae infected with IAPV exhibited variable expression of ribosomal RNAs and increased expression of ribosomal protein S5a (RPS5), and glutathione S-transferase 1 [108]; bees from CCD-aFFECTed colonies also had increased RNA expression [109]. The transcriptional profiles of the fat bodies from young, IAPV-infected worker bees [101**] shared the most genes with IAPV-infected adult bees [18**], and had little overlap with DEGs in bees infected with either E. coli bacteria [112] or microsporidia (Vosma spp.) [113], indicating that honey bee antiviral responses are distinct from immune responses mounted against other parasites. Increased expression of argonaute-2 and dicer-like in response to IAPV-infection also supports the role of a distinct antiviral response involving RNAi, Toll, and Jak-STAT pathways [101**]. The research performed to date is informative, but additional studies are needed to better understand honey bee antiviral immune mechanisms at the transcriptional level (e.g., mechanisms of regulation of gene expression and the role of splice variants) and beyond.

**Viruses and other stressors**

The focus of this review is honey bee host–virus interactions, and honey bee antiviral responses, but honey bees live in a complex environment. The effects of viruses on bees, and the functionality of the bee immune responses, may be affected by the presence of other pathogens [12**,19**,20], the microbial context of infection (microbiome [114*,115*,116–117*]), environmental factors including agrochemical exposure [104,118,119*,120,121*], and adequate nutrition [122*,123*,124*]. Several studies indicate that bees infected with multiple pathogens have increased mortality and CCD-aFFECTed samples have a greater number of pathogens than control colonies [9**,12**,14*]. While it is widely accepted that mite infestation is detrimental to honey bee colonies and that mites also serve as virus vectors [40–42], the mechanisms of synergistic detrimental interactions have not been fully elucidated [34,44**,45**,102**,105*].

Nazzi et al. investigated the combinatorial effects of mites and virus in both field and laboratory settings from the colony to the molecular level [102**]. They determined that high mite infestation coupled with increasing levels of DWV from June to October resulted in increased colony mortality [102**]. Transcriptome (RNASeq) analysis of adult bees in these colonies revealed lower expression of 19 immune genes including dorsal-IA, pathogen recognition receptors (AmSCR, BR and B7 scavenger receptors, and C-type lectin 8), and immune signaling pathway members including hem, tak1, and sos [102**] (Figure 1). Bees from colonies with both high mite and DWV levels exhibited increased expression of other immune genes including genes involved in pathogen recognition (PGRP-S2, nimC2, eater-like) and serine proteases [102**]. Laboratory experiments confirmed that a combination of mites and DWV, but not mites alone, reduced dorsal-IA expression in adult bees [102**]. Also, larvae in which dorsal-IA expression was reduced by RNAi-mediated knock-down harbored a greater number of DWV genome copies [102**]. Recent studies by Kuster et al. demonstrated that DWV virus abundance increased up to 72 hours post experimental wounding or Varroa mite exposure [44**]. Assessment of the transcriptional responses to wounding and mite exposure at times ranging from 24 to 240 hours post-capping demonstrated increased expression of immune genes (i.e., abaeclin, apidaycin, defensin, hymenoptaecin, PGRPs, PPOact, and relax) and DWV infection (up to 72 hours) and reduction of mite numbers in conjunction with immune activation [44**]. Cluster analysis suggested co-regulation of defensin and relax, and apidaycin and hymenoptaecin, whereas abaeclin and PPOact were not associated with other immune gene regulation [44**]. Interestingly, results to date indicate that mite pressure, independent of transmission, results in increased levels of DWV-like viruses with a VDV-1 CP coding region [69**]. The interactions between the honey bee host, Varroa destructor, and viruses are not fully understood and require further investigation. Since honey bee colonies located in Newfoundland and Labrador, Canada [125], and several Hawaiian islands lack V. destructor [126], these populations provide unique opportunities
to examine the effects of viruses on colony health and immune regulation.

Two sides to the story — host vs. virus genetics
The genetic background of the host has implications on susceptibility to virus infection and disease severity. This is particularly relevant for honey bees as they live in colonies of ~30,000, the majority of which are sterile, genetic-half sisters, since queens typically mate with 12 drones [127]. Colony level diversity due to queen polyandry reduces the prevalence of honey bee diseases [128] and may result in varying transcriptional responses, variation between individual hemocyte populations, and differences in social immune mechanisms (e.g., grooming behavior, propolis production) [110,129]. Moreover, genetic diversity is not limited to the host, as the majority of honey bee viruses are RNA viruses with error prone polymerases that generate virus quasispecies over the course of infection [130]. Different virus variants within particular quasispecies populations may have greater or lesser pathogenicity in a particular host organism. In addition, different strains of honey bee viruses exhibit differential pathogenicity (i.e., DWV and IAPV) [18**,69**,126]. Recent studies determined that DWV strain prevalence was reduced in the presence of mites [126] and the recombinant strain of DWV, DWV*, is more virulent than other DWV-like viruses [69**]. A greater appreciation of the existing virus genomic diversity across the globe is needed to better evaluate the effects of distinct virus strains on colony health. The development of infectious virus clones that are amenable to mutation (reverse genetic systems) are needed to verify strain-specific virulence and determine mechanism(s) of enhanced virulence or increased tolerance. Honey bees may vary in their degree of virus tolerance [79**,131]. This should be explored at both the individual and colony levels, since the information gained may guide the use of virus susceptibility as an additional selectable trait in honey bee breeding programs [129,132,133]. In addition, further use and development of immortalized honey bee lines (i.e., AmE-711) [134**], long-term cell cultures [135], and primary cell cultures [136,137], are required to further the field of honey bee virology. Future use of immortalized cell lines and infectious honey bee virus clones will serve to normalize future studies and lead to a better understanding of honey bee antiviral defense mechanisms.

Conclusion
Investigating virus–host interactions throughout all domains of life has led to a greater biological understanding of fundamental cellular processes and host–virus coevolution. Honey bee host–virus interactions likely depend upon bee age or developmental stage, additional biotic and abiotic variables, and genetics of both host and pathogen. Only with additional research in laboratory and field settings at both the individual bee and colony level, will the mechanisms of honey bee antiviral defense be understood. Undoubtedly, continued investigation of honey bee host–virus pairs will lead to the discovery of evolutionarily conserved immune defense strategies, as well as reveal numerous unique co-evolved relationships that are specific to each host–virus combination. It is a critical and exciting time to investigate honey bee antiviral response mechanisms.

Acknowledgements
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References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


4. McMenamin AJ, Genersch E: Curr Opin Insect Sci 2015 http://dx.doi.org/10.1016/j.cois.2015.01.015. This paper reviews recent large-scale honey bee colony losses, and the field and experimental data that supports the association of viruses with colony loss.


This paper defines Colony Collapse Disorder (CCD), reviews other historical import large-scale bee losses, and provides a framework for the association of multiple parameters with colony loss.


This paper provides quantitative mass-spectrometry data on over 100 pesticides and metabolites associated with honey bees and hive matrices; it is the most thorough investigation of honey bee agrochemical exposure to date.


This is an excellent review of honey bee pathogens and analyses of pathogen abundance and associations in CCD and non-CCD colonies.


This paper describes the first metagenomic analysis of CCD-affected colonies.


This paper described results from a four-year monitoring project and identified several factors associated with over-winter losses including mitose infestation, DWV, ABPV, queen age, and weakness of colonies in autumn.


This paper is an important contribution to better understanding the role of IAPV/honey bee colony losses and honey bee host-IAPV interactions (i.e., honey bee RNA-mediated antiviral response, analysis of differentially expressed genes in IAPV-infected adults, and evidence for IAPV encoded suppressor of RNAi).


This is an excellent review of honey bee associated microbes, including pathogens.


42. Shen M, Cui L, Ostiguy N, Cox-Foster D: Intricate transmission routes and interactions between picorna-like viruses (Kashmir bee virus and sacbrood virus) with the honeybee host and the parasitic varroa mite. *J Gen Virol* 2005, 86:2281-2289.


This paper provides quantitative transcriptional level data of immune gene regulation in mite-exposed pupae at multiple time points and shows little support for mite-mediated immune suppression; the introduction and discussion are well-researched and very informative.


This short opinion article reviews factors associated with colony loss and provides a framework for understanding the combinatorial effects of mites and viruses.


The honey bee immune genes and pathways described in this paper are the basis for honey bee immunology.


This review article provides an excellent description of the immune mechanisms involved in insect innate antiviral immunity.


This is an excellent review of RNAi in insects, nematodes, plants, and vertebrates; it summarizes key concepts and fundamental studies.


This article provides an excellent review of RNAi-mediated insect antiviral immunity.


This paper provides initial evidence for the role of RNAi in honey bee antiviral defense.


This study demonstrated that pretreatment (via feeding) of both honey bee larvae and adults with dsRNA specific to DWV reduced viral burden, thus it provided additional evidence for the role of RNAi in antiviral defense.


Ultra-deep sequencing data was used to characterize siRNAs from bees obtained from CCD-affected and healthy colonies. Key findings from this study indicated that CCD-affected colonies had a greater number of 22-nt siRNAs than healthy colonies, siRNA sequences were distributed throughout the virus genomes, and that there were more negative-strand IAPV-siRNAs than positive-strand siRNAs.


68. Wang H, Xia J, Shreeve TG, Ma J, Pallett DW, King LA, Possee RD: Sequence recombination and conservation of *Varroa destructor* virus-1 and deformed wing virus in field collected honey bees (*Apis mellifera*). *PLoS ONE* 2013, 8:e74508.


This is an interesting report of a DWV strain that has enhanced replication in bee pupae, which is not observed in mites. In addition, this study identified differentially expressed genes in mite-infested and virus-infected pupae.


This review describes the siRNA pathway, provides phylogenetic comparison of Dicer-2 and Ago-2 from numerous bee species, reviews dsRNA-mediated gene silencing in both bumble bee and honey bees, and summarizes current experimental support for RNAi-mediated and non-specific dsRNA mediated antiviral responses in honey bees.


This study provides experimental evidence of a non-specific dsRNA-mediated antiviral response in bees infected with a model virus, and it describes differential gene expression in virus-infected and dsRNA-treated bees.


This is an excellent review of fruit fly antiviral mechanisms.


This is an outstanding review of fruit fly and mosquito antiviral mechanisms; it provides and comprehensive graphical representation of inducible immune pathways in Drosophila.


This study assessed the transcriptional profile of IAPV-infected young bees with high throughput sequencing (RNA-Seq). They found that 753 genes were differentially expressed in IAPV-infected bees, including a few genes in the RNAi and Toll pathways. This study did an excellent job assessing pre-existing/confounding infections and therefore ensured that the gene regulation observed in their well-controlled experiments was most likely due to IAPV-infection.


This study provides experimental evidence of a central role for Am dorsal-1A in limiting DWV infection in the context of mite infestation.


This study determined that ABPV-infected honey bee larvae did not produce AMPs, and suggested that ABPV may suppress bee immune responses, since AMPs were not produced by larvae co-challenged with ABPV and E. coli.

young adult honeybees masks their increased susceptibility to infection compared to older bees. Plos Pathog 2012, 8:e1003083.

This paper is an important step toward understanding age-dependent immunity in honey bees. They showed that older forager bees were more resistant to fungal infection due to age-related activation of immune pathways, whereas younger house bees had a greater number of differentially expressed immune genes in response to fungal challenge.


This study furthers our understanding of the honey bee microbiome, an important factor to consider in bee health and immune defense.


This study furthers our understanding of the honey bee microbiome, an important factor to consider in bee health and immune defense.


This work examined synergistic effects of agrochemical exposure and virus-infection in honey bees and determined that abacavir expression was reduced and DWV virus levels were greater in clothianidin treated bees. Overall this study indicated that the Toll pathway is important for antiviral defense, and that Toll signaling is reduced by clothianidin exposure.


This study provides evidence of the interaction between agrochemical exposure and pathogen load; honey bees from colonies exposed to sublethal amounts of imidacloprid had higher levels of Nosema than bees from control colonies


This study furthers our understanding of the honey bee nutrition, an important factor to consider in bee health and immune defense.


This study demonstrated that bees fed either protein supplement or pollen, in addition to high fructose corn syrup (HFCS) containing water, had lower DWV loads, than bees fed only HFCS-containing water. This controlled study is an important contribution toward understanding the role of nutrition in honey bee health immune defense.


This paper describes a honey bee cell line (AmE-711) derived from fragmentated honey bee embryonic tissue. Immortalized cell lines are important for investigating bee cell biology and honey bee host–pathogen interactions. Future use of these cell lines for honey bee virus-propagation will provide more uniform inoculum for virus–host interaction studies.


Anthropogenic change can have large impacts on wild bees and the pollination services they provide. However, the overall pattern of wild bee response to drivers such as land-use change, pesticides, pathogens, and climate change has been one of variability in both the magnitude and directionality of responses. We argue that two causes contribute to this variation. First, different species exhibit differential responses to the same anthropogenic drivers. Second, these anthropogenic drivers vary in type and magnitude that will drive variation in bee responses. For this second issue, we focus on land-use change, the most well-studied driver. We conclude by discussing how understanding species-level responses and the magnitude of land-use change can make bee conservation more effective.

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**Introduction**

Bees (Hymenoptera: Apoidea: Anthophila) are among the most ubiquitous and important pollinators of wild plants globally [1,2]. Furthermore, wild bee species, along with managed honey bees (Apis mellifera) and managed bumble bees (Bombus spp.), are important crop pollinators [3–5]. A few studies have shown regional [6] and local [7] declines in overall wild bee species richness. Declines in the best-studied genus, the bumble bees, is well-documented [8,9]. Given the ecological and agricultural importance of wild bees, these findings have raised concern among scientists, governments, and the general public. An array of anthropogenic drivers has been implicated in wild bee declines including pesticides, introduced pathogens, climate change, and land-use change [10–12]. Furthermore, multiple interacting drivers may have even stronger negative effects [12,13]. However, bee responses to anthropogenic drivers are far from universal, showing a range of magnitudes and directionalities [14–16]. For example, a recent review of bee responses to the largest global driver of species loss, land-use change, found that while a 42% of effect sizes showed a negative response, 45% were neutral and 13% were positive [17].

The purpose of this essay is to explore the causes of variability in responses of bees to anthropogenic drivers. We have three main objectives. First, we highlight recent research that explores variability in species and species-group level responses of wild bees to anthropogenic drivers. Second, we examine how variation in an anthropogenic driver itself mediates wild bee responses. We focus on the most well-studied driver, human land-use change, which is also the leading cause of species loss globally [18], and describe how careful consideration of the magnitude and type of land use can reveal patterns of bee response that are otherwise obscured [17]. Third, we demonstrate how focusing on the responses of particular types of bee species can make conservation and management of wild bees more effective.

**Variation in species-level responses**

There are over 20 000 species of bees globally and they have a variety of life history traits and behaviors. It is therefore not surprising that different species show differential responses to the same anthropogenic drivers [19,20], and perhaps for this reason, also show different population trends over time [21**]. Recent species-specific studies move beyond simply assessing how aggregate wild bee abundance or species richness is affected by anthropogenic drivers (Figure 1), which had been the focus of the literature previously [17,22–24].

**Pesticides**

Pesticides are widely thought to be an important factor in wild bee declines [12], but bee species differ in their susceptibility to pesticides. A recent meta-analysis of lab-based, LD50 studies examined 18 bee species other than honey bees and found that species exhibit differential susceptibilities to direct application of insecticides [20]. These differences in acute susceptibility may be due to species-level differences in body size, hemolymph chemistry, and...
immune system function [reviewed in 20]. Different classes of pesticides have different levels of toxicity across bee species [20]. For example, Biddinger et al. [25] examined mortality of honey bees and Osmia cornifrons (Megachilidae) to five different pesticides and found bee species by pesticide type interactions. For example, the LD₅₀ was met after applying 4 µg/bee of Acetamiprid to O. cornifrons compared to 65 µg/bee for honey bees but for Imidacloprid the LD₅₀ was 3.8 µg/bee for O. cornifrons compared to 0.2 µg/bee for honey bees. The recent literature is increasingly finding sublethal effects as well, particularly for the systemic neonicotinoids [26]. Given the ubiquity of these insecticides, any species-level variation in susceptibility to neonicotinoids could have substantial ecological effects.

Behavioral and natural history variation among bee species will likely make species-level variability in response to pesticides even greater in field settings than in the lab, although few field studies have explored this yet. Different behaviors and natural histories will result in different likelihoods of exposures among bee species. For example, bee species that have flight times that overlap with pesticide applications, and species whose host plants are concentrated in the area of application will be most susceptible [27]. For systemic pesticides such as neonicotinoids, pollen typically has higher concentrations than nectar [28,29] and thus may have differential effects on species that are foraging for either resource. Landscape-scale studies are crucial to predict which bee species will be most susceptible to pesticides. In one of few such studies, Rundlöf et al. [30**] compared bee responses in landscapes with and without neonicotinoid-treated crops and found that bumble bee queen numbers and wild bee density was reduced with neonicotinoids while honey bee colony size showed no response.

Emerging pathogens from managed bees

The introduction of new diseases is a major concern for wild bees, with some species in decline likely due to pathogens, while others are unaffected. The best studied case concerns the bumble bee species in the subgenus Bombus sensu stricto which exhibited rapid declines in the midwestern United States [8]. This decline is associated with infection by the putatively introduced fungal pathogen Nosema bombi, which is commonly found in species in the subgenus Bombus sensu stricto. Spatially co-occurring species from other Bombus subgenera rarely host N. bombi, and these species are still relatively stable or increasing [8]. However, in the arctic and subarctic region of North America, some species in the subgenus Bombus show high N. bombi infection rates yet their populations are stable [31]. This example suggests that predicting which species will be most affected by emerging diseases will be challenging. Models with other insects suggest that diseases are most likely to affect species that are closely related to the hosts that harbor the new pathogens [32]. Thus given the ubiquity and global transport of honey bees, there is particular concern about their transmitting pathogens to others species of Apidae. Honey bees and bumble bees have been separated for over 70 million years [33], yet honey bee pathogens detrimentally infect bumble bees in laboratory settings [34–37] and vice versa [38*]. Honey bee pathogens have been found in multiple species of wild bees, but almost nothing is known about whether these pathogens have negative effects on wild species other than Bombus [39]. As domesticated bumble bees and honey bees are the most likely means by which these pathogens reach new locations, increased monitoring and control of these colonies is needed to protect wild bees [38*].

Climate change

Climate change will undoubtedly have differential effects on different bee species, as it does for other taxa [14]. A central focus of climate change research for bees has been potential asynchrony between bloom times and bee emergence [40]. Floral specialist bees could experience phenological mismatch differently from generalists. Two contrasting predictions have been made about this. First, some ecologists have predicted that specialist bees are at greater risk because if they emerge before or after their host plant blooms, they will be unable to forage [40]. However, some studies making this prediction are problematic as they confound rarity and sampling effects with true specialization [41]. Although there are well-known specialist (oligolectic) bee species [42], it is also true that species that appear to be specialists in a given study may not be specialists when more individuals are sampled, or when greater temporal and spatial scales are considered.
Second, it has been proposed that specialist bees will be buffered against climate change because they have been selected to use the same cues for emergence that their host plants do; thus while these bees might change their phenology with climate warming, they would remain synchronized with their host plants [44,45]. Generalist species will likely be little affected by asynchrony as they can visit numerous plant species as has been demonstrated in some studies [46,47].

Physiological differences among bee species might also make them differentially affected by climate change. For example, as temperature increases, species that overwinter as adults lose more body mass during winter diapause which is likely due to greater metabolic rates and energy expenditure during winter [48]. This suggests that these species are more likely to negatively affected as the climate warms. In the same experiment, Fründ et al. [48] found that these effects were more pronounced in earlier emerging bees, suggesting that, as is the case for other taxa, early spring species will be the most affected by climate change [49].

Land-use change
Land-use change is one of the most important and best-studied anthropogenic drivers of bee declines, and response to land use varies strongly across bee species. One group of bee species, the floral specialist bees, consistently shows stronger declines with land-use change as compared to more generalist species. Often the decline of specialist species can be linked specifically to declines of their host plants [7,50,51,52*,*53,54]. For example, in Europe, bee species that specialize on Fabaceae have experienced greater declines. This is due to changes in agricultural management, specifically a decline in planting Fabaceae as cover crops [52*]. Specialist bees have been shown to be disproportionately negatively affected in urban systems as well [53]. Conversely, populations of specialist bees can increase with land-use change if their host plants increase. In Europe, bee species that specialize on Rosaceae have increased along with increased plantings of Rosaceae crops [52**]. Some species have likely expanded their range following plantings of their host plant [55]. Therefore, while specialist species as a group are more negatively affected by land-use change than generalists [51], these responses are species-specific and dependent on changes in host plant abundance.

By contrast, other traits associated with species or groups of species do not strongly predict response to land-use change. No consistent patterns have been found for body size [53,56], nesting guild [7,53], or sociality [50,57]. This may be due to the statistical difficulty of separating the causal role of traits that are correlated across taxa [51]. For example, the well-studied genus Bombus (the bumble bees) are social, large, and polylectic, making it more difficult to determine which trait drives their responses to land-use change.

Variation in anthropogenic drivers: the case of land-use change
Another reason for the high variability in responses of bees to anthropogenic drivers is the variation in the anthropogenic drivers themselves. In this context, the relevant variation in responses includes not only that across bee species, but also in aggregate bee richness and abundance. We focus here on a single driver, land-use change, as this is the best studied of the anthropogenic drivers. Variation in land use has been found to influence the response of wild bee communities in two ways. First, the magnitude of the land-use change has strong effects on outcomes. For example, in systems where human land use is extreme (defined as some sites having \( \leq 5\% \) of natural habitat remaining), bee abundance and richness decline steeply [17]. However in systems with less extreme land use (all sites have >5% natural habitat), bee responses are mixed [17]. Importantly, in this review, 75% of the studies were done in systems with extreme human land use, which could bias the general impression that workers in this field have about how strongly bees are declining due to land-use change [17].

Second, bee responses vary with the type of land-use conversion studied. Bee abundance and species richness can increase with increasing urbanization and suburbanization [53,58]. Similarly, likely because many bee species are associated with open habitats, conversion of forest to more human-disturbed, open areas often increases bee abundance and richness [17]. Most studies of bee responses to land-use change have focused on conversion of natural habitat to agriculture, but within this broad category, different types of agriculture have differential effects [17]. For example in landscapes with red clover (Trifolium pretense L.), bumble bee queen densities were five time greater at sites with red clover than sites without [59]. By contrast to other studies that have demonstrated a negative effects of agriculture [e.g. 51].

Conserving wild bee communities
In this final section, we explore how the understanding of variability in species responses to anthropogenic drivers and variation in the drivers themselves can be applied to the conservation and management of wild bees. Understanding variation in species responses can provide valuable insight into an ongoing debate within conservation biology, which is: should conservation efforts focus on species that provide ecosystem services [61], or on the conservation of biodiversity per se, which generally emphasizes rare or threatened species [62]. A key question in this debate is whether the species that contribute ecosystem services such as pollination are also the important species driving patterns of biodiversity [63]. Recent studies suggest that only a small number of highly
abundant wild bee species do the great majority of crop pollination [64,65]. By contrast, rare species make up most of the biodiversity [65]. Specifically, a large synthetic study across 90 different field studies found 785 bees species visit crops yet 80% of pollination was provided by only 2% of those bee species, which were the more common species [64*].

Given that the important crop pollinating species and rare bee species might often be two distinct sets of species, it is crucial to understand how each of these groups responds to anthropogenic drivers as well as conservation actions. Few studies have examined whether these different groups respond differently to a given anthropogenic driver. Important crop pollinating bees have been shown to persist in agricultural landscapes [64*]. However, we know of no studies that have directly measured how well rare bees, as a group, persist in agricultural landscapes. It may be that common, crop-pollinating species that persist in agricultural landscapes are more robust to pesticides as these chemicals are ubiquitous on many farms. In support of this hypothesis, Brittain et al. [66] found that species richness declined with pesticide application yet pollination was not affected, suggesting that biodiversity and ecosystem services are not responding similarly to this anthropogenic driver. It is also important to determine whether conservation actions benefit both important crop pollinators and rare bees. The common conservation action of planting of pollinator habitat may benefit both important crop pollinators and rare bees [67]. However, no studies have examined whether conservation efforts that optimize conservation of one group results in trade-offs in the conservation of the other.

The variation in anthropogenic drivers themselves can have important implications for designing conservation actions, in particular to determining the most effective locations for conservation action. For example, the effectiveness of pollinator habitat plantings varies with the magnitude of land-use change in the larger landscape surrounding the planted site. When pollinator habitat plantings are placed in landscapes with very intensive human land use (<1% surrounding landscape in natural habitat within a 1000 m radius) few bees use the habitat plantings, likely because the potential colonist species pool is highly reduced [68**]. By contrast, in landscapes with extensive natural habitat (>20% surrounding landscape in natural habitat within a 1000 m radius) pollinator habitat plantings have little positive effect on bee communities because these landscapes already support diverse and abundant bees [68**]. The most effective location for pollinator habitat plantings are landscapes with intermediate levels of human land use (1–20%). In these locations, pollinator habitat plantings significantly increase bee abundance and richness [68**].

Conclusions
As global change intensifies, there is an increasing potential for negative effects on wild bees. The effect of this change will depend on bee species, and on the magnitude and type of anthropogenic driver. We have made significant progress in understanding how wild bee abundance and richness, in aggregate, respond to land-use change. However, few studies have directly addressed the impact of pesticides, pathogens and climate change on multiple wild bee species in field settings [but see 30**]. In addition, no studies have quantified the relative effects of anthropogenic drivers across species. Doing so will enable for more effective prioritization of conservation and management efforts. Finally, wild bees must respond to multiple anthropogenic drivers simultaneously and this may increase negative effects [12,13]. These more nuanced approaches will lead to more accurate assessment of bee responses and allow for more effective conservation of these ecologically and economically important organisms.

Acknowledgements
We thank members of the Winfree lab for helpful comments. All photographs in Figure 1 are licensed under Creative Commons 3.0. Attributions from top to bottom are Alveoxaspat (Bombus terrestris), Alveoxaspat (Anthidium florentinum), Gideon Pinyata (Megachile albiacuta), and Gideon Pinyata (Megachile incerta).

References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

19. Modest Current


45. Using over 30,000 museum specimens collected from a 140-year period across the northeastern United States, the authors found that only 3 of 187 species experienced dramatic declines. However, 29% experienced modest declines in relative abundance and 27% of species experienced increases. Species-level traits were only weakly predictive of decline.


65. Wild bee density, nesting activity of a native bee (Osmia bicornis L.), and bumble bee colony development was reduced in landscapes containing oil seed rape treated with neonicotinoids. However, honey bee colony strength was not reduced suggesting variation in species responses to this treatment. This study is a significant advance as the authors were able to compare bee responses among landscapes with and without neonicotinoid treatment.


83. Regulations in the UK are in place to limit the importation of bumble bee hives infected with parasites. Despite these regulations, 77% of hives were found to have microbial parasites with a number of these parasites demonstrating negative effects on bumble bee health.


Bees and plants were sampled in a desert community were sampled before and after a drought. Bee species exhibited differential responses to the drought and these responses tracked those of their host plants.


47. Forrest JRK: Plant–pollinator interactions and phenological change: what can we learn about climate impacts from experiments and observations? *Oikos* 2015, 124:4-13.


In this study, trends in species were determined using a national database with over 72,000 records across 98 years. Pollen loads were identified from museum specimens. The authors found that bee species that specialized on Fabaceae declined. This plant family has declined in the study region due to changes in farm management and loss of semi-natural habitat.


Using a dataset of 90 different studies across the globe, the authors found that 2% of wild bee species found on crops contribute 80% of total pollination contributed to wild bees. In addition, these common, abundant species were relatively robust to land-use change.


This meta-analysis tested whether landscape context influenced wild bee responses to agri-environment schemes across Europe. Wild bee abundance and richness exhibited the greatest increase when agri-environment schemes took place in simple as compared to highly cleared of complex landscapes. In addition, the benefit of the agri-environment schemes increased as the contrast between floral resources in agri-environment schemes the surrounding landscape increased.
The power and promise of applying genomics to honey bee health
Christina M Grozinger¹ and Gene E Robinson²

New genomic tools and resources are now being used to both understand honey bee health and develop tools to better manage it. Here, we describe the use of genomic approaches to identify and characterize bee parasites and pathogens, examine interactions among these parasites and pathogens, between them and their bee hosts, and to identify genetic markers for improved breeding of more resilient bee stocks. We also discuss several new genomic techniques that can be used to more efficiently study, monitor and improve bee health. In the case of using RNAi-based technologies to mitigate diseases in bee populations, we highlight advantages, disadvantages and strategies to reduce risk. The increased use of genomic analytical tools and manipulative technologies has already led to significant advances, and holds great promise for improvements in the health of honey bees and other crucial pollinator species.

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Introduction
The winter of 2006–2007 ushered in a new era in bee biology, with the simultaneous discovery of the devastating effects of Colony Collapse Disorder on US honey bee populations [1] and the culmination of a multi-year, international effort to sequence and analyze the Apis mellifera honey bee genome with a large series of papers in Nature, Science, PNAS and elsewhere [2]. As for other topics such as social behavior [3], the knowledge and tools that derived from the honey bee genome sequencing project were quickly deployed to address CCD [4**]. In the following years these resources, for honey bees and soon for other bee species, have formed the basis for new approaches to the study of bee health. This review summarizes the progress and challenges associated with applying genomics to understand the mechanisms by which abiotic and biotic factors undermine bee health and to develop novel strategies to mitigate the effects of these stressors (see Figure 1).

Comparative analyses of immune genes
Over the past several years there has been a steady increase in the availability of genome sequence information for a variety of insect species, including several bee species [5]. Additional sequencing and analyses substantially improved the A. mellifera genome in 2014, resulting in the identification of more than 5000 additional protein coding genes [6]. Sequenced genomes were recently reported or are underway for a managed Asian honey bee species, Apis cerana [7], a halictid bee, Lasiosglossum albipes [8], two bumble bee species, Bombus terrestris and B. impatiens [9], and several other bee species [10**]. In addition, transcriptomes for over ten bee species have been published (e.g., [11–13]).

Comparisons across a broad range of insect species have provided important insights into the molecular mechanisms regulating several traits of bees, including immunity. In the first such comparison, between A. mellifera and the only two other sequenced insect genomes at the time (Drosophila melanogaster and Anopheles gambiae) it was observed that though honey bees have a fully intact immune system with genes corresponding to all known branches of the immune response pathway, they appeared to have fewer of the canonical insect immune genes [14**]. However, as more genomes became available, this difference was not observed and honey bees are now thought to have a typical complement of canonical immune related genes [15,16**]. Comparisons across bee species suggest that these canonical immune genes are rapidly evolving, and thus may allow different species to adapt to species-specific immune challenges [13]. However, more recent studies suggest that this rapid evolution is not due to positive selection but rather relaxed selection [17**]. This might be the case because bees do not rely exclusively on canonical immune genes to fight infection, but can employ other genes and mechanisms to combat diseases and parasites, such as social immunity (behavioral mechanism to reduce disease load, [18]) or increased genetic diversity [19]. Consistent with this speculation, analyses of gene expression changes in response to immunostimulation revealed that honey bees...
Genomic approaches to bee health. (1) Sequencing the genome of parasites and pathogens can provide information about unique gene sequences that can be targeted by RNAi approaches, allow for the development of efficient molecular diagnostic tools, and characterize mechanisms for host-parasite interactions and virulence. (2) Genome sequences and quantitative genetic studies of bees can identify gene variants associated with resilience to different stressors, which can be used in breeding and stock improvement programs. (3) Metagenomic approaches can identify and characterize pathogenic and beneficial microbes. (4) Functional genomic studies to identify host and parasite gene expression changes (changes in the levels of RNA produced by a given gene) associated with infection or other stressors can help characterize mechanisms for host-parasite interactions and resilience. Graphical design by Harland Patch and Nick Sloff (Penn State University).

(and other insects) may employ a much broader array of genes than those identified as part of the canonical immune pathways, though the functional significance of these gene expression changes remain to be determined [20,21**].

Using genomic tools to investigate the effects of biotic and abiotic stressors on bee health
Many studies have used analyses of gene expression as a way to probe effects of various stressors on bee health (see Box 1). The underlying idea is that changes in gene expression can provide a sensitive indication of effects that will eventually negatively impact a variety of physiological systems. This approach also has provided insights into the mechanisms underlying tolerance or resistance to these stressors (see also [22,23], this issue). For example, viral infections in developing honey bee pupae led to changes in expression of genes encoding ribosomal RNA and proteins, consistent with viral impacts on protein translation [24]. Changes in expression of these genes were also found in gut samples from bees collected from colonies exhibiting symptoms of CCD [25], in accordance with the possibility that CCD, at least in some cases, involves infections with multiple viruses [26]. Viral infection caused upregulation of genes in the RNAi pathway in honey bee fat body tissues [21**], supporting previous studies demonstrating that the RNAi pathway plays an important role in mediating antiviral responses in insects [27]. Introduction of non-viral double-stranded RNA also can reduce viral titers in honey bees (likely by non-specific activation of the RNAi pathway, [28]), and thus may serve as a therapeutic tool to reduce viral infections in bee colonies (see below for further discussion). Exposure of young bees to neonicotinoid pesticides altered expression of a gene that regulates NF-κB-mediated antiviral immune responses, resulting in increased
Box 1 From the bench to the beehive: using genomics to improve bee health

(1) Identification and characterization of bee parasites, pathogens and beneficial microbes. Metagenomic sequencing of honey bee populations has identified several new viruses and demonstrated that a largely ignored honey bee parasite, Lotmaria passim (previously identified as Crithidia mellifica), is prevalent in honey bee populations and associated with colony losses [45,46,55,56]. Similarly, genomic approaches have demonstrated that honey bees host a multitude of species of microbes which may positively impact bee health (see [51], this issue). A comprehensive analysis of the parasites, pathogens and beneficial microbes circulating within bee populations is necessary for a complete understanding of bee health. Furthermore, since viruses and parasites apparently transmit between populations of bees and other insects readily, this screening should be performed on a broad array of species.

(2) Develop molecular diagnostics for rapid and inexpensive monitoring of bee diseases. It is impossible to fix what you do not see. Beekeepers need new, cost-effective tools to be able to rapidly diagnose their colonies for diseases, as well as information on treatment thresholds. This is the first step in an ‘Integrated Pest Management’ approach to bee diseases. At this point, beekeeper-accessible protocols for monitoring and treatment thresholds have only been developed for Varroa mites. All other viruses and pathogens require specialized screening using relatively expensive molecular or microscopy instrumentation, and treatment thresholds have not been well-defined.

(3) Develop molecular tools to control bee parasites and pathogens. Several studies have demonstrated that RNAi approaches can successful reduce parasite and pathogen loads in bees [87,88,90]. These tools are very promising, since they can specifically target genetic sequences in pathogens and parasites, thereby reducing off-target effects and potentially reduce the likelihood for selecting for resistant strains. However, there has been indication that off-target effects can occur (see text), and thus additional testing should be performed to ensure that these treatments do not cause unintended effects on bees. Finally, as in all treatments, it is important to develop an Integrated Pest Management approach, to reduce off-target effects, reduce the likelihood of resistance development, and reduce costs and labor.

(4) Identify factors that improve resilience which can be incorporated into management practices. Genomic approaches have provided considerable information about the types of genes that mediate the effects of stressors on bees, and genes that can underlie sensitivity and resilience to these stressors. However, breeding and maintaining genetic stocks of bees is challenging. Thus, these studies should be examined to identify management practices that can be easily employed to improve bee health. For example, recent studies demonstrated that both pesticides and rich (honey/pollen) diets impacted the same suite of genes, and, based on those results, the authors developed and validated the hypothesis that complex diets (pollen) reduced mortality in pesticide-exposed bees [36**].

viral titers in pesticide-treated bees [29**]. Finally, viral infection also caused changes in DNA methylation patterns in fat body tissue for a set of genes previously associated with antiviral responses in vertebrates but not insects [21**], and thus this may represent a heretofore undescribed genomic response to viral infections. However, it is important to note that most studies only show correlations between stressors and changes in gene expression or methylation levels, and detailed functional analyses of these processes must be performed.

Characterizing transcriptional responses to Nosema microsporidia infections has also helped explain the bewildering diversity of effects that Nosema has on bees. Nosema are gut parasites, and infections lead to increased hunger, accelerated behavioral maturation from brood care to foraging, reduced flight capabilities, and premature death [30]. Examination of genome-wide expression patterns demonstrated that the primary impact of Nosema in honey bee fat body tissue is on expression of genes in metabolic and nutritional pathways, which appears to subsequently lead to the transcriptional and physiological changes associated with accelerated behavioral maturation, altered immune function, and reduced longevity [31].

Global gene expression studies have also suggested that nutrition and diet can mitigate the effects of pesticides. Exposure to pesticides caused upregulation of detoxification genes in abdominal tissues, which should reduce the impacts of the pesticides, and altered expression of immune genes, which is consistent with studies demonstrating that pesticide-exposed bees are immunocompromised [32–35,36**]. Interestingly, the effects of pesticide exposure on gene expression in fat body tissue are similar to those caused by consuming a rich diet of honey and pollen (vs sucrose) [36**]. Honey and pollen contain a variety of chemically complex secondary plant compounds and thus may trigger similar ‘detoxification’ responses as pesticides. Indeed, feeding honey bees p-coumaric acid, a constituent of honey, or quercetin, found in both honey and pollen, caused upregulation of detoxification genes and improved detoxification abilities [37,38**,39]. However, while short-term feeding with pollen before pesticide exposure does confer some benefit in terms of longevity (consistent with a priming effect), long-term feeding is significantly more beneficial, suggesting that natural pollen/honey based diets result in improved overall health, which in turn improves responses to pesticides and other stressors [36**].

Do different stressors elicit common or distinct transcriptional responses in bees? Parasitization with Nosema and Varroa cause similar changes in brain gene expression, despite the fact that these parasites infect their hosts at different life stages (adult for Nosema, pupae for Varroa) [40]. Nosema parasitization, injection with E. coli bacteria, and exposure to pesticides all triggered similar changes in gene expression in fat body tissues [20,31,36**]. In contrast, gene expression changes induced by viral infection appear to be fairly distinct, and the effects of infection vary with the type of virus, developmental stage of infection, duration of infection, and tissue [21**]. Similarly, while pesticide exposure generally causes changes in expression of detoxification genes, the identities of these
genes can vary greatly across pesticides and studies [32,33,35,36**]. Comparing and contrasting the effects of biotic and abiotic stressors on gene expression in a variety of tissues is a promising avenue to understand how these stressors affect bee health.

**Genomic sequencing of bee parasites and microbial communities**

Genomic resources for honey bees have been used to generate great insight into the possible mechanisms underlying molecular and physiological responses to stressors, but it is also necessary to generate genomic resources for the parasites and pathogens infecting bees to fully understand and potentially mitigate the effects of the diseases they cause (see also [41], this issue). Recently, genomic information for several key parasites of honey bees has been produced, including a partial genome sequence for the parasitic mite *Varroa destructor*, a major cause for bee decline across the world [42]; whole genome sequences for the gut microsporian parasites *Nosema apis* and *Nosema ceranae*, which have been associated with increased mortality and colony loss [43,44]; and a draft genome sequence for the gut trypanosomatid parasite *Lotmaria passim* (previously identified as *Crithidia mellificae*), which has been linked to colony losses in Europe [45,46]. Additionally, genome sequences have been generated for two key honey bee brood parasites: *Paenibacillus larvae*, the bacterial species that causes American foulbrood, and *Ascosphaera apis*, the fungal species that causes chalkbrood [47–49]. Genome sequencing has been used to define and characterize variants of two major viral pathogens of honey bees, Deformed Wing Virus (DWV) and Israeli Acute Paralysis Virus (IAPV) [50].

Genomic information obtained from these sequences can be used to identify pathogen/parasite-specific gene sequences for RNAi-based control measures (see below), develop molecular diagnostic markers to efficiently monitor parasite/pathogen load and spread, or to generate insights into the mechanisms mediating host–parasite interactions and potentially host resilience to these disease agents. Furthermore, metagenomic approaches have allowed for characterization of the beneficial microbes found in honey bees, and the factors that may perturb these communities (see [51], this issue).

**Using genomics to reveal hidden diseases**

Genomic approaches have greatly facilitated the identification of previously unknown or uncharacterized pathogens and parasites in honey bee populations. However, it must be noted that if parasites and pathogens are very different from previously sequenced species, identification based simply on genomic sequence alone can be challenging. Furthermore, while genomics approaches can reveal an association of a parasite or pathogen with particular symptoms, additional testing is necessary to provide causation. For example, historically, 18 viruses were known to infect honey bees [52]. Metagenomic sequencing of control and collapsing honey bee colonies indicated that prevalence of a relatively understudied virus, IAPV, was higher in collapsing colonies [4]. It was subsequently shown that IAPV was present in the US before the occurrence of CCD [53], but more comprehensive longitudinal studies indeed demonstrated that colonies with high levels of IAPV are less likely to survive the winter [54].

High-throughput sequencing of RNA extracted from honey bees from colonies of US migratory beekeeping operations led to the identification of four additional viruses (Aphid Lethal Paralysis virus, Big Sioux River virus, and Lake Sinai viruses 1 and 2), some of which reached high levels of prevalence [55**]. Similar molecular screens later identified Lake Sinai viruses 3 and 4 in US and European honey bee populations [26,56]. Genomic and molecular diagnostic approaches also demonstrated that US and European colonies are frequently infected with the trypanosome *Lotmaria passim* (previously identified as *Crithidia mellificae*) [46,55**,56]. *L. passim* was not previously considered to be a significant threat to honey bee health, but more recent studies in Europe demonstrated that levels of *L. passim* are strongly associated with winter colony losses [56]. Recent studies also have suggested that a plant virus (tomato ringspot virus) can also infect honey bees [57], though further analyses are necessary to confirm replication with bee hosts and negative health effects.

Genomic approaches have also helped disentangle the complex pathogen-parasite-host interactions that have been observed between DWV, *Varroa* mites, and honey bees. DWV is found in nearly all honey bee populations, with *Varroa* mites both transmitting the virus and triggering elevated viral titers [58]. Interestingly, DWV titers also reach very high levels in bees when the cuticle is pierced by a needle [59,60**], and thus it may be the mechanical trauma from *Varroa* feeding that immunocompromises the bee, rather than factors introduced by *Varroa* during feeding. Recent studies have demonstrated that when DWV is introduced to a host bee by *Varroa* or injection, there is selective amplification of genotypically distinct, highly virulent strains of DWV, which in some cases correspond to a recombinant DWV-Varroa destructor virus strain [60**,61**]. Further studies are needed to determine the mechanisms by which these strains specifically amplify under these conditions, though it has been hypothesized that this represents a trade-off between anti-Varroa/melanization and anti-viral immune responses [62].

New diagnostic tools derived from genome sequences have facilitated our ability to detect pathogens and track their spread across bee populations. For example, *Varroa* mites were recently introduced into honey bee
populations in Hawaii, New Zealand, and Kenya, and molecular techniques allowed for the rapid analyses of these populations to determine the extent of the distribution of Varroa-associated viruses, and their impacts on bee health [61**,63,64]. Furthermore, molecular approaches have demonstrated that pathogens from commercial bumble bee colonies can spill over into wild bumble bee colonies [65] and may cause declines in wild species [66]. Similarly, pathogens and parasites of honey bees can infect populations of other bees and insects, likely via horizontal transmission by feeding on common flowering plants [67,68**,69–71].

Using genomic approaches to combat stressors

Ever since the discovery of honey bees resistant to the bacterial disease American Foulbrood [72], it has been known that there can be considerable heritable variation in the sensitivity of different bee genotypes to parasites and pathogens. These discoveries have fueled an interest in identifying the underlying genetic factors that drive this variation to breed more resilient stocks of bees. Breeding programs have generated stocks of bees that are more resistant to Nosema in Denmark, and this difference is associated with increased expression of immune genes [73] and inferred sequence variation in four locations in the genome [74,75]. Similarly, there is variation in resistance to Varroa mites in both natural and selected populations of honey bees. Resistance to Varroa is driven by multiple physiological and behavior traits, and different quantitative trait loci (QTLs) have been found that are related to these different traits, including grooming (where mites are removed from a nestmate’s body, [76]), hygienic behavior (removal of parasitized brood, [77]) and suppressed reproduction of female mites feeding on developing pupae [78]. Genetic differences in resistance to viruses have also been observed, though these have not been mapped to specific genomic regions [79]. Thus far, genetic differences in response to other major stressors of bees (pesticides and poor nutrition) have not been investigated.

Although it has been possible to identify several genomic regions associated with variation in resilience to different stressors, identifying the specific genes and using this information to breed and maintain improved stocks of bees can be challenging (for review, see [80], this issue). Variation in many of these traits is influenced by variation in many genes, thus setting up the possibility of many complex interactions among genes in determining phenotypic differences. In other words, a particular genetic variant that is associated with variation in grooming behavior or pathogen resistance in one population may not be causally relevant in a different population. Furthermore, honey bee queens typically mate with an average of 12 drones, always outside the hive [81]. Thus, beekeepers must use instrumental insemination or tightly controlled breeding yards to limit uncontrolled gene flow into selected stocks. In addition, there can be negative effects of inbreeding or low genetic diversity in a colony [19,82,83], requiring that stocks include considerable genetic diversity at non-selected loci.

There are exciting new technical developments that will greatly improve our ability to functionally characterize pathways involved in mediating bee health (rather than relying simply on correlations) and potentially breed more resilient bees. Recently, the piggyBac-derived transposon was used to transform honey bees and drive expression of an exogenous green fluorescent protein gene [84**], which, together with the development of general genome editing tools such as CRISPRs and TALENs [85], lays the groundwork for the development of transgenic bees with enhanced genetic resistance to different stressors. It should also be possible to generate transgenic strains of beneficial bee gut microbes [86], which could produce key nutrients, pesticide detoxification enzymes, or biotic factors targeting parasitoids or pathogens.

The use of double-stranded RNA (dsRNA, which activates the RNAi pathway and reduces RNA levels of target genes) has greatly enhanced our ability to study the function of genes involved in bee health [54,62], and is a promising new tool for mitigating the impacts of parasites and pathogens. Feeding parasitized honey bees with dsRNA corresponding to Nosema or Varroa genes reduces expression levels of these genes in populations of Nosema and Varroa collected from these parasitized bees, and reduces levels of both parasites in bees [87**,88**]. Importantly, in the case of the experiments using Varroa, there was bidirectional transfer of the dsRNA: dsRNA was fed to the bee, passed from the bee gut to the hemolymph, from the hemolymph to the Varroa, and then back to the bee. Similarly, feeding honey bees viral dsRNA can reduce viral titers, reduce mortality of individual bees, and have positive effects on colony health parameters [89,90**].

Using RNAi to reduce levels of pathogens and parasites has both advantages and disadvantages. dsRNA should in theory be highly specific for its target gene sequence, which should limit off-target effects [91]. However, if relatively long sequences are used to develop dsRNA constructs, they are likely to contain fragments that match the host bee’s genome sequence. Indeed, feeding bees with dsRNA corresponding to green fluorescent protein (a gene sequence not found in bees) resulted in altered developmental timing and significant gene expression changes, specifically in bee genes that had small regions matching sequences found in the introduced dsRNA [92**]. Furthermore, a previous study indicated that non-sequence specific dsRNA can trigger a general antiviral immune response [28], which can be both beneficial, since it can impact a broad range of viruses and viral
strains, and potentially problematic, if external, non-target dsRNA is introduced in large quantities for other applications, such as to control crop pests or weeds [93]. Though a second study found no effect of non-specific dsRNA on viral titers [90]**, further testing is needed to determine whether chronic exposure to dsRNA impacts bee immune function in a positive or negative way. Additionally, because most pathogens and parasites are broadly circulating in the environment, even if the dsRNA treatments are very effective, they will have to be frequently applied, or detailed studies will be needed to determine the most effective time period for treatment (e.g., treatments for Varroa are most effective in the fall, before the production of winter bees; for review see [94] this issue). As with all efforts to manage pests, parasites, and pathogens, an Integrated Pest Management approach should be employed, to reduce off-target effects, reduce the likelihood of resistance development, and reduce costs (see [95], this issue, for a discussion of IPM approaches to pollinator health).

Conclusions

The development of genomic resources and tools in honey bees has tremendously facilitated our ability to dissect the intricate mechanisms that regulate bee health. Furthermore, genomics has allowed us to make discoveries that have launched new fields of inquiry, including the identification of new parasites, pathogens, and genetic mechanisms that combat these. Finally, genomics is providing desperately needed tools to better diagnose and manage bee diseases. These tremendous advances have all been made possible with completion of the sequencing of the honey bee genome in 2006. With the development of the next generation of genomic tools and resources for a broader array of bee species, the next decade will bring even greater advances in our understanding and management of bee health and biology.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


Demonstrated the power of using a metagenomics approach to identify pathogens involved in declines of honey bees or other species.


Comparative genomic study of ten bee species.


Examined the repertoire of immune genes across multiple species.


Examined the repertoire of immune genes across also multiple social and solitary bee species.


Demonstrated that the rapid evolution observed in immune gene sequences of in social insects is likely due to relaxed constraint rather than positive selection, providing evidence that other immune mechanisms are playing a large role in social insect immunity.


Provided the molecular mechanisms linking pesticide application to increased viral titers in bees


Demonstrated a strong genome-wide association between genes involved in responses to pesticide and diet.


60. Ryabov EV, Wood GR, Fannon JM, Moore JD, Bull JC, Chandler D, **Mead A, Burroughs N, Evans DJ:** A virulent strain of deformed wing virus (DWV) of honeybees (*Apis mellifera*) prevails after Varroa destructor-mediated, or in vitro, transmission. PLoS Pathog 2014, 10:e1004230.

Used transcriptome sequencing to demonstrate the certain viral strains are preferentially transmitted by Varroa, and these strains are particular virulent.


Used molecular approaches to demonstrate that strain abundance in viral populations in honey bees change dramatically once Varroa mites are introduced.


Used molecular approaches to demonstrate that viruses are readily transmitted between populations of bees.


85. Demonstrated the feasibility of creating transgenetic strains of bees.


89. Demonstrated that dsRNA fed to honey bees could be transmitted to ectoparasites (Varroa) and reduce expression of the targeted Varroa gene and reduce Varroa levels.

Demonstrated that dsRNA fed to honey bees could be transmitted to endoparasites (Nosema) and reduce expression of the targeted Nosema gene and reduce Nosema levels.


Demonstrated that dsRNA fed to honey bees could be reduce viral titers.


Demonstrated that dsRNA from a non-honey bee gene can produce off-target effects in honey bees.


Bee nutrition and floral resource restoration
Anthony D Vaudo, John F Tooker, Christina M Grozinger and Harland M Patch

Bee-population declines are linked to nutritional shortages caused by land-use intensification, which reduces diversity and abundance of host-plant species. Bees require nectar and pollen floral resources that provide necessary carbohydrates, proteins, lipids, and micronutrients for survival, reproduction, and resilience to stress. However, nectar and pollen nutritional quality varies widely among host-plant species, which in turn influences how bees forage to obtain their nutritionally appropriate diets. Unfortunately, we know little about the nutritional requirements of different bee species. Research must be conducted on bee species nutritional needs and host-plant species resource quality to develop diverse and nutritionally balanced plant communities. Restoring appropriate suites of plant species to landscapes can support diverse bee species populations and their associated pollination ecosystem services.

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Introduction
A key factor driving pollinator declines is anthropogenic land-use intensification, which, among interacting factors such as pesticide use and introduced pests and pathogens, dramatically reduces the diversity and abundance of flowering plant species [1–5,6**,7**]. Bees (Hymenoptera: Apoidea: Anthophila), as a monophyletic group of ~20 000 species [8], depend entirely on nutrition derived from floral resources (especially nectar and pollen) obtained from diverse plant species [9]. Bees therefore experience nutritional stress when limited in their choices of host-plant species or when only suboptimal floral resources are available, both of which could result in reduced population sizes and pollination efficiency [1–5,6**,7**]. We propose a rational approach for restoring and conserving pollinator habitat that focuses on bee nutrition by firstly, determining the specific nutritional requirements of different bee species and how nutrition influences foraging behavior and host-plant species choice, and secondly, determining the nutritional quality of pollen and nectar of host-plant species. Utilizing this information, we can then thirdly, generate targeted plant communities that are nutritionally optimized for pollinator resource restoration and conservation. Here, we review recent literature and knowledge gaps on how floral resource nutrition and diversity influences bee health and foraging behavior. We discuss how basic research can be applied to develop rationally designed conservation protocols that support bee populations.

Bee nutrition
Adults and larvae of nearly all bee species depend on nutrients obtained from floral resources for development, reproduction, and health [9,10**]. Adult foragers are challenged with seeking out appropriate nutrients from the environment for developing larvae and/or nurse bees and queens confined to a nest [9]. At the simplest level, bee nutrition is partitioned between nectar and pollen: nectar provides bees’ main source of carbohydrates, whereas pollen provides proteins, lipids, and other micro-nutrients [11–13]. To obtain optimal nutrition, insects can balance their nutrient intake from complementary food sources, which is considered one of the most important factors shaping foraging behavior and insect fitness [14**].

Bee species likely have different quantitative and qualitative nutritional requirements, which are suggested by their differences in life history, brood size, social structure, and different distributions among plant species. Whereas most bees are solitary and oligolectic (a single reproductive female lays eggs and provisions brood; specializes on one plant family or genus), the majority of literature studying the nutritional needs of bees have focused on two species of long-tongued bees: honey bees and bumble bees, both of which are generalists (foraging on a wide range of plant species in different families) and social (living in colonies with cooperative brood care and overlap of generations) [8,10**,11,15]. The nutritional requirements of honey bees (colony, adults, and larvae) has been comprehensively reviewed [10**], and even though this level of detail does not exist for other bee species, we can assume that other species have similar macronutrient...
demands; the proportions of macronutrients required may be species-specific (as exemplified in other closely related insect species that share the same host-plants [14**,16]).

We can infer the general dietary requirements of bees from existing research. It is clear that both adults and larvae will starve without a constant carbohydrate, mainly nectar, source [10**]. Relatively immobile larvae do not require the amounts of carbohydrate needed by foraging bees and their limited carbohydrate demands can be met by a blend of pollen, which contains digestible carbohydrates, and nectar [17–19]. Protein concentration of pollen is positively correlated with larval development and adult reproduction (ovarian development and egg laying) in honey bee, bumble bees, and the sweat bee Lasioglossum sephyrum [20–26,27**]. Lipids are crucial for a variety of physiological processes in bees (e.g. egg production, wax production, secondary energy source) and contribute to larval and adult health, ontogeny, and diapause/overwintering [10**,27**,28–30]. Linoleic acid (omega-6), an essential fatty acid for most insect species, in collected pollen has been associated with higher worker production in honey bee colonies [31]. A second essential fatty acid for insects, linolenic acid (omega-3), is also obtained from pollen, but its specific importance for bees is still not described [28]. Sterols obtained exclusively from pollen are the precursors for molting hormones, making pollen essential for larval development [10**,27**]. Recent research indicates that both honey bee and bumble bee foragers regulate their intake of carbohydrates and proteins to high ratios [32,33], and bumble bees can simultaneously regulate their intake of carbohydrates, proteins, and lipids (Vaudou et al., unpublished). These studies reveal bees’ specific nutritional requirements, and potentially highlight how adults prioritize their foraging efforts between nectar and pollen for their nutritional components.

Information is lacking for the specific nutritional requirements of the vast majority of solitary oligolectic bee species, though bee taxa appear to have different requirements in nectar sugar composition (see section ‘Nectar’ discussion below). Even less is known of bees’ specific pollen nutritional requirements. For at least a few species of solitary bees, pollen quantity of brood provisions is linearly correlated to body size [34]. Additionally, some specialist bees do not survive well on non-host pollen [35], suggesting that either host-plant pollen is nutritionally optimal for specialists, or they cannot metabolize protective chemicals of non-host pollen. Because nectar and pollen quality varies considerably between host-plant species [11,12] and the bee community exhibits different host-plant visitation patterns over time [36–38], we can assume that different bee species have specific nutritional demands that may influence their host-plant foraging patterns [16].

Floral resource nutritional diversity and bee foraging behavior

Nectar

Nectar is the major carbohydrate source for most bee species [10**,39,40]. Bee larvae require carbohydrates for normal development often in the form of brood food (pollen and nectar mixtures), but the greatest quantity of carbohydrate-rich nectar is required for adult foraging [10**]. Nectar is an important floral reward and reinforcing stimulus for bee foragers, and profitable nectar sources can be learned and associated with floral characteristics such as scent and color [41–43]. Although nectar is a dynamic floral resource, varying by abiotic conditions and plant age [12,25,44–48], there are three relatively constant characteristics that influence bee host-plant choice for nectar: sugar composition, nectar volume, and nectar concentration [18,39]. Other characteristics of nectar composition undoubtedly play a significant role in nectar choice, such as amino acids, lipids, minerals, and secondary plant compounds [46,49–59]; however, research on these characteristics, perhaps with exception of amino acids (recently reviewed in Nepi [60]), has been limited and not systematic across bee species [59–63].

The three main sugars present in nectar are glucose and fructose (monosaccharide), and sucrose (disaccharide) [12,64,65]. Flowers of a given taxa vary in the relative amounts of these sugars and plant families show a characteristic pattern of sugar composition [12,48,64,65]. Early research found that long-tongued bees prefer high sucrose nectars and short-tongued bees prefer nectars with a higher percentage of monosaccharides [65]. Although the interpretation of these patterns has been questioned on many levels [12,66–68], it is likely that sugar composition of plant taxa is an important factor in determining pollinator host-plant choice [48,62,64,65,69–75].

Nectar concentration also determines patterns of pollinator host-plant visitation [12,76–79], limiting which pollinators can mechanically obtain the nectar, either by adhesion and capillary action or by suction. The rationale is that pollinators with long feeding apparatuses (long-tongue bees, moth/butterfly proboscis, long-tongue fly proboscis) will be limited to more dilute nectars. Although overall viscosity is affected by temperature (and sugar concentration) [80], patterns of preference are evident (reviewed by Willmer [81]) and therefore likely play a role in the evolution of plant-pollinator communities. For example, honey bees (a long-tongued bee species) prefer a concentration of 30–50% whereas short-tongued bees utilize higher concentration nectars of 45–60% [82].

It has been proposed that nectar volume, a third characteristic of floral nectar, is the result of an evolutionary tradeoff [83] between high volumes that are energetically costly (potentially influencing vegetative growth and
flower production) [84,85**] and volumes that are too low to attract pollinators. Ideally, nectar volume of a given plant species should be high enough to attract pollinators, but low enough to ensure efficient visitation to other conspecific flowers. Nectar volume, therefore, should be strongly associated with the primary pollinators of plant taxa [86,87]. In a classic study of Costa Rican plants and their pollinators, flowers producing high volumes of nectar, which also had large floral mass, were visited by larger bees in contrast to smaller flowers with lower nectar volumes, which were visited by small bees and wasps [44].

Pollen
Bees obtain the majority of their protein, including free and protein bound essential amino acids, from pollen, but protein concentration varies considerably between plant species, ranging from ~2 to 60% [88,89]. Although preference for high protein pollen has not been clearly demonstrated for honey bees [90,91], significant decreases in pollen protein in the colony result in higher pollen foraging rates [91]. It has been suggested that honey bees may prefer pollen higher in essential amino acids [92], or obtain a balance of amino acids by collecting a diverse pollen diet [89]. Increasing evidence exists that bumble bees do prefer and will increase foraging rates to pollen sources higher in protein or essential amino acid concentration [25,93,94,95**,96**]. Indeed, when foraging in the same habitat among the same host-plant species, bumble bees collect pollen higher in protein concentration than honey bees, which may be linked to different foraging strategies; bumble bees may preferentially forage for pollen quality, where honey bees may forage for quantity to meet the vast demands of the colony [97]. This tradeoff between quantity and quality likely exists in other bee species.

Pollen serves as bees’ main lipid source (including essential fatty acids and sterols), and lipid concentrations from different plant species can range considerably, from 1 to 20% [11]. Furthermore, the lipid-rich oily exterior of entomophilous pollen, the pollenkitt, is an important discriminative stimulus, phagostimulus, and digestible component for pollen recognition and bee nutrition [98–102]. Bees, therefore, may be cued by pollenkitt chemistry to recognize host-plant pollen quality, but research is sparse on how pollen lipid content and the pollenkitt influence bee foraging choice in the field.

Because protein and lipid concentrations between pollen species are variable and uncoupled [11,13] (Vaudio et al., unpublished), foragers may selectively collect pollen among plant species to regulate their intake of these nutrients, or, alternatively, collect from a large array of host-plant species to passively achieve a nutritional balance (this may apply to generalist and oligoleptic foragers alike). Research in other arthropod species, including beetles and spiders, indicates that they sense and regulate their intake of protein and lipids when choosing among food sources [103–106]. Bumble bees, for instance, appear to collect pollen diets from the field that are both high in essential amino acid and sterol content [96**]. Our recent research has demonstrated that ratio of protein:lipid concentration of pollen best predicted host-plant species preference of bumble bees; and when given multiple synthetic food sources, bumble bees indeed regulated their protein and lipid intake (Vaudio et al., unpublished). These results suggest that bees potentially analyze pollen quality in multiple nutritional dimensions. Furthermore, because bees may not be able to taste protein directly [18], pollenkitt lipid and amino acid chemistry could convey information on pollen quality to bees.

Beyond proteins and lipids, pollen (and often nectar) is rich in micronutrients (e.g. vitamins and minerals) and phytochemicals (e.g. carotenoids, flavonoids, alkaloids and phenolics) that have antioxidant properties and antimicrobial activity [11,107–110]. High concentrations of secondary plant chemicals, however, as plant defenses, could be toxic to bees [54,111,112**]. Some specialist bee species do not survive well on exclusive non-host pollen, potentially because they cannot metabolize these chemicals [35]. It has been suggested that oligoleges of the genus Colletes specialize on pollen of the plant subfamily Asteroideae, while generalists of Colletes do not, possibly due to differences in their ability to cope with secondary plant chemicals of Asteroideae pollen [113]. A similar trend has been observed between larvae of closely related generalist Osmia species, having differing physiological abilities to survive on the same pollen diets due to pollen protective chemicals [114]. Therefore, bees could selectively collect or avoid host-plant pollen based on its phytochemical composition.

The importance of plant diversity for bee health
Large scale land-use that reduces floral abundance and species richness will negatively affect bee species populations through nutritional shortage in both quantity and quality of resources [1–5,6**,7**,115**]. For example, the recorded population declines of bumble bee and other bee species in Europe are associated with landscape-level reduction of host-plant availability [1–5,6**,7**,115**,116]. Although farmland of bee-pollinated crops may provide a large quantity of floral resources, these habitats may be insufficient at maintaining healthy bees because they may only present single-source pollen or nectar. Also, when the crop is not blooming, the landscape may have few flowering plants, affecting all bee species whose foraging periods do not discretely overlap with crop bloom. Without diverse foraging options and diets during critical periods of reproduction and development, bees may suffer negative health consequences. Additional intensification, such as agrochemical use, can further
Conceptual schematic presenting a holistic framework relating basic research and landscape application for bee conservation and habitat restoration. The essential research objectives are: (1) seasonal and daily phenology of bee and plant species, (2) bee nutritional requirements and the nutritional quality of nectar and pollen from commercially available host-plant species, and (3) bee species nesting requirements. These research areas provide the environmental criterion necessary for supporting bees’ annual life cycle: (1) timing of blooming that matches with bee active foraging periods, (2) nectar characteristics necessary for bee energetic needs, especially during foraging, (3) pollen characteristics necessary for bee reproduction and development, and (4) nesting habitat for bees to rear brood and spend periods of time in inactivity and dormancy. We can then rationally design conservation plant communities by selecting host-plant species (and natural habitat) that meet these criteria. These plant communities constitute a diversity of host-plant species optimized for bee nutrition. The outcome of a comprehensive conservation effort is that we provide a diverse group of bee species appropriate nutrition and exacerbate stress, negatively affecting bee foraging behavior [4,7*,117,118] and fitness [7*,119–121].

Bees should be given a range of diverse floral resources from which they can self-select their diet to meet their component nutrient requirements, which will sustain healthy populations that can endure disease and stress. For example, in bumble bees, the reproductive benefits of polyfloral pollen diets surpassed those of monofloral diets, even when lower in protein concentration [24]. Polyfloral pollen diets can provide a balance of essential amino acids and fatty acids, whose concentrations differ between species [89]. Exposure to single pollen sources, such as *Lupinus* crops, that contain plant defensive chemicals can be detrimental to bumble bee colony fitness [111]. Therefore, generalist bees may visit a variety of host-plant species to obtain pollen to dampen or nullify the harmful effects of pollen secondary metabolites [112**]. Appropriate nutrition is necessary for bee immunity (DeGrandi-Hoffman and Chen, this issue); diverse pollen diets can enhance bees’ immunocompetence and resistance to pathogens [122,123**] and pesticides [124].

**Applying bee nutrition to floral resource habitat restoration**

To alleviate the negative effects of reduced floral resource availability and interacting stressors of agricultural intensification on bee population health and crop pollination services, selective foraging habitats should be restored in sufficient quantity surrounding areas of land-change [7**,125**,126]. Thus, there is increasing demand and incentive based programs for farmers for application of agri-environmental schemes, including floral resource provisioning to support bee populations [7**,115**,127,128]. The development and design of these schemes have focused primarily on plant species that attract bee abundance and diversity. Because the bee community will visit different plant species throughout the day, season, and between years [36–38,129–131], floral diversity is the best way to attract and support multiple pollinator species over time. Furthermore, farmland in proximity to natural habitat and/or supplemented with floral resources will attract a wider species richness and functional-group diversity of bees that can result in higher fruit yield [132–138,139**,140], and economic benefit [139**].

However, plant species diversity alone is not sufficient to ensure pollinator conservation and thus the aim should be to provide nutritionally optimized floral resources. **Figure 1** provides a conceptual schematic relating research and application of criteria needed to support habitat that will stabilize their populations. Healthy and diverse bee populations will then be more effective pollinators of wild host-plant and crop species.
bee populations throughout their life cycle. While other factors (nesting habitat [141], structure of the pollinator community [131]) are important for developing pollinator plantings, for this review, we focus on the bee nutrition and the role it plays selecting appropriate plants that support a nutritionally balanced and diverse community. Foremost, plants should be chosen that present floral rewards in phenological succession throughout the day and season [129–131] spanning the active periods of bee species [139**]. Then, firstly, determine the nutritional value of the nectar and pollen of the agricultural crop, and commercially available native and, where advisable, non-invasive exotic host-plant species (exotic plants species should only be chosen that will not compete with endemic plant species and will promote plant-pollinator community stability [142]). These studies include analyzing nectar composition, concentration, and volume, and pollen protein, lipid, and micronutrient quality. Secondly, determine the nutritional needs of different bee species occupying the landscape, including those important for crop pollination. These studies can be conducted in field, semi-field, or laboratory settings correlating resource quality to nectar and pollen visitation data [94,129], or feeding assays using synthetic or supplemented diets [14**].

Integrating this information will allow us to select plant species that better meet bees’ nutritional needs. Rich nectar sources diverse in their quality and quantity will provide the differing carbohydrate needs of bees and other pollinators. Further, plant species that are attractive, but whose pollen are complementary (to each other and the agricultural crop) in their protein, lipid, and micronutrient quality will allow bees to self-select their diet to balance their intake of these nutrients to maximize their reproductive output and larval development/survival. Additionally, plant communities can be designed to match the changing nutritional needs of bees throughout the growing season. For example, with a strong understanding of pollen and nectar nutritional quality, we should be able to provide pollen sources early in the season to boost worker population growth for honey bee and bumble bee colonies [143,144], and late season nectar flow for honey bee overwintering and bumble bee gyno survival [115**,145] (SH Woodard, abstract 0406, Entomology 2014, Austin, TX). Finally, once pollen and nectar nutritional quality is better characterized, devised plantings should support wide generalists that collect diverse resources for quantity, or selectively for nutritional value. Because generalists visit the majority of host-plant species in local plant-pollinator communities [131], achieving diversity in our plant communities will also likely maximize attractiveness to solitary or specialist species that have limited foraging distances, shorter active periods, and narrower host-plant preferences.

Developing rationally designed floral provisioning schemes that optimize pollinator nutrition requires information about the nutritional requirements of pollinators, how these shape their foraging preferences, and the nutritional profiles of a range of the floral resources of native and agricultural plant species. Integrating this information will allow development of targeted, and simplified, plant communities, which can be used for conservation of a diverse range of bee species in a diversity of landscapes. These healthy and abundant bee populations will then sustain agricultural production in the face of increasing demands for food in a changing environment.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

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Using historical data from the mid-19th century until present, patterns of bee and wasp extinctions in the United Kingdom are highly correlated with periods of agricultural intensification policy and practice, including those that are associated with reductions in wildflower abundance and diversity.


This current review discusses the interacting and synergistic factors contributing to bee declines and potential pollination crisis, including loss of foraging and nesting habitat, monofloral diets, introduced parasites and diseases, pesticides, and phenological disruption; also included are suggestions for improvement such as restoring floral and nesting resources, reduction of pesticide use, and preventing the spread of non-native bees, parasites, and pathogens.


This essential and comprehensive review of honey bee nutrition throughout the lifecycle describes the variety nutrients obtained from nectar and pollen and their important effects on colony, worker, and larval behavior, development, and health; a key example of the detail needed for a variety of bee species to address their nutritional requirements.


This review highlights the history, theory, methodology, and results of how insect foraging behavior is adapted to prioritize and regulate macro-nutrient intake to reach an optimum target for fitness, and how reaching or modifying this target can offset deleterious effects of plant secondary chemicals and other stressors; also described are specific differences in intake targets between related insect species.


Using bumble bee microcolonies confined to monofloral pollen diets of varying protein and sterol quality, bumble bees collected all pollen equally, but increases in protein/aminic acid and sterols lead to higher larval weight, indicating healthier offspring that could lead to more productive adults.


By comparing local communities of alpine lotus at increasing distances from large bee apiaries and conducting a common garden experiment, the researchers found that honey bees place a significant selective pressure on plants causing adaptive trade-offs in floral characteristics: communities of plants nearer to apiaries exhibited reduced nectar volume, while increasing plant size, biomass, and number of flowers and seeds.


90. Van Der Moezel PG, Delfs JC, Pate JS, Loneragan WA, Bell DT: Pollen selection by honeybees in shrublands of the Northern


For the pollen generalist Osmis cornuta, larval survival, development time, and adult body mass are negatively affected by the proportion of unfavourable pollen in their diet, suggesting that generalists are able to dampen or nullify the toxic effects of chemical defenses by collecting pollen from a diversity of plant species.


In a lab-based study, when infected with Nosema, pollen quality (protein and lipid concentration) of monofloral and polyfloral diets was positively correlated to nurse honey bee physiology, immunity, and survival.


This review presents a conceptual framework for utilizing ‘ecological intensification’ to protect ecosystem services, including pollination, to meet the increasing demands for agricultural productivity and global food security.


Over four years, by planting large patches of native perennial wildflowers that bloom throughout the season adjacent to blueberry farms, wild bee and hoverfly abundance in blueberry fields increased annually, leading to higher blueberry fruit yield that lead to exceeding the cost of establishment and maintenance of the supplementary plantings.


Pesticides and pollinators: a context-sensitive policy approach
Sainath Suryanarayanan

I examine recent policymaking efforts in the United States (US) that seek to improve how risks posed by pesticides to insect pollinators are assessed and managed. Utilizing the case of ongoing honey bee die-offs, I argue for a context-sensitive policy framework. From a scientific perspective, this entails not ignoring the uncertain knowledge emerging from laboratory and field studies regarding the indirect effects of low levels of certain insecticides in combination with other factors. From a social scientific perspective, policy initiatives to build partnerships between growers and beekeepers toward mitigating exposure to pesticides are crucial, and need to acknowledge barriers to the adoption of best management practices as well as a historically-established asymmetry between growers and beekeepers in the pollination industry.

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Introduction
Roughly a decade after the first reports of Colony Collapse Disorder, the public continues to hear troubling echoes of concern from scientists and beekeepers about a ‘new normal’ of honey bee deaths being experienced by beekeepers in the US [1*]. Research points to a ‘complex’ set of causal factors, highlighting potential roles for parasitic mites, Nosema, multiple viruses, poor nutrition and pesticides — both beekeeper — and grower-applied [2*]. However considerable uncertainty remains about which factors are more influential, and how they might interact to cause the ongoing die-offs, exemplified by debates over the nature and extent of the role of neonicotinyl systemic insecticides (neonicotinoids), the world’s most widely used insecticides, whose usage in the US has risen dramatically since 2003, especially in the form of seed treatments [3]. An array of emerging laboratory and field studies suggest that relatively low and environmentally relevant levels of neonicotinoids, both alone as well as in combination with other factors such as microbial pathogens, negatively affect honey bees and other insect pollinators [4*,5–11]. These results stand in contrast to studies that purport to show that the effects of neonicotinoids on honey bees and other insect pollinators are negligible at field-representative levels [12–15]. The question of whether newer systemic insecticides are contributing to honey bee declines has developed into a scientific controversy with experts disputing results on multiple methodological grounds [16–18,19*,20*]. How are contemporary policymaking practices reflecting and responding to the scientific uncertainty and complexity that has come to mark this matter of public concern?

Policy responses in the US and the European Union
The dynamic and relatively uncertain situation about what is known and what is not known regarding the relationships between neonicotinoids and insect pollinator deaths has triggered divergent policy-level actions in the European Union (EU) and the US. Policymakers at the EU have declared a moratorium from 2013 to 2015 on the usage of neonicotinoids in pollinator-dependent crops [21*]. In doing so, they have taken seriously uncertain knowledge and suggestive evidence of harm from laboratory and field studies — a false-positive policy orientation that prefers to bear the costs of being wrong about the harm posed by these chemicals, rather than overlooking that harm [22]. By contrast, even though there has been considerable movement on this issue at the US Environmental Protection Agency (EPA), including new labeling requirements [23] and pollinator risk assessment guidelines [24], as well as likely restrictions on new outdoor uses of these chemicals in the absence of additional effects-data on developing honey bees [25], the EPA has to date refused to take action to prohibit or restrict the current use patterns of these chemicals in general, and in specific contexts of usage [26]. The EPA’s decision not to take such regulatory action in the absence of definitive knowledge of harm is a false-negative policy orientation that prefers to bear the costs of overlooking harm rather than being wrong that the chemicals are harmful. In justifying its policy position, the EPA calls into question studies reporting negative interactive effects of these pesticides in laboratory and field studies and cites the lack of certainty regarding the biological relevance of reported negative effects for actual field settings [26,27*].
Sources of uncertainty: ecological complexity

However, it is notoriously difficult to execute real-world field experiments of the effects of low levels of pesticides in combination with other factors on honey bee colonies [20**]. The practical challenges entailed in isolating the effects of the chemical in question from potentially confounding sources of environmental variability, require a high number of colonies, resources, and time to achieve sufficient statistical power [17,18]. As a result, field experiments tend to be relegated to measuring the direct, causal effects of individual chemicals [4**,13–15]. However, the observation that an active ingredient or pesticide product is not having any measurable direct effect (lethal or sub-lethal) at ‘field-realistic’ levels does not exclude the potential for significant indirect effects that come into play only in the presence of other factors in particular spatio-temporal settings. This scenario of cumulative and interactive multifactoriality is highly plausible [28], given that managed honey bee colonies encounter on a regular basis hundreds of pesticides, transgenic toxins, ‘inert’ ingredients and other synthetic chemicals, apart from ambient parasites, pathogens, nutritional and other abiotic and biotic variables in the particular landscapes in which they are situated and their multiple routes of exposure [11,29]. Furthermore, the complex structure of a honey bee colony may buffer it from the negative effects of neonicotinoids and other pesticides, to a greater extent than other pollinator species [30]. This may partly explain the mixed effects observed in studies of neonicotinoids and honey bees in comparison to the more consistent and robust effects seen in bumble bees and solitary bee species [5,6,19**,30].

While the EPA acknowledges on paper the complexity of ongoing honey bee deaths [2*], in practice the EPA’s policy belies an approach that privileges certainty about the direct effects of individual chemicals over uncertain knowledge about the more indirect effects of mixtures of chemicals and other factors. This is justified on the basis of field studies that are limited in their capacity to grapple with the multifactorial nature of honey bee declines. In effect, the EPA’s policy approach, in the case of honey bees and some other insect pollinators, ignores uncertain scientific knowledge pointing to the indirect role of newer systemic insecticides. A pollinator policy that ignores the ecological complexity in which honey bee colonies operate, even if scientific knowledge about it is highly uncertain, risks perpetuating a system in which honey bees, beekeepers and other insect pollinator species will continue to struggle.

Sources of uncertainty: social complexity

Uncertainty stems not only from the biological complexity of interactions between assemblages of plants and pollinators, but also from the multiplicity of values represented by those for whom and by whom the policy is made. When policy on pesticides and pollinators is considered, a range of values are invoked, including the benefits of pollination services, economic efficiency, protecting innovation, feeding the world’s growing population, environmental conservation, and sustaining future generations. The EPA incorporates valuation modeling through cost–benefit analyses with the goal of achieving the most cost-effective environmental regulation as mandated by the Reagan administration’s executive order 12291 in 1981: ‘regulatory action shall not be undertaken unless the potential benefits to society for the regulation outweigh the potential costs to society’ [31]. Cost–benefit analysis entails calculating ‘expectation values’ that are based on quantitative probabilities of expected benefits accrued to those whose lives are improved by a policy compared to expected costs to those whose lives are made worse off [32,33].

For example, as various neonicotinoids come up for registration review beginning in 2016–2017, the EPA’s latest cost–benefit analysis of neonicotinoid seed treatments in soybeans suggests that seed treatments provide negligible overall yield benefits to soybean production ‘in most situations’ and that ‘much of the existing usage on soybeans is prophylactic in nature’ [34]. ‘Independent’ analyses sponsored by agrochemical corporations counter the EPA’s cost–benefit analysis and highlight significant non-pecuniary benefits, such as ease of application, early planting, and reduced scouting for pests, which growers accrue from using neonicotinoid seed treatments on a variety of crops [35]. Such cost and benefit valuations make simplistic assumptions [33,36] about the dynamics of grower behavior and biophysical plant–pollinator relationships. Analyses of growers’ pest management decisions and perceptions are underpinned by the ‘rational-choice’ theory, which assumes that humans, growers included, are rational, calculating individuals who act to maximize their self-interests [32]. However, growers, like other people, do not always act in their own interests; they are also moved by their social networks, including other growers, crop consultants, extension agents, where shared norms and values regarding competition, size of operation, and specialization can influence preferences for certain pest management practices over others [37]. Related to this, growers also face constraints with regard to the available tools of pest control. For example, it is extremely difficult for US and Canadian growers to purchase Bt hybrid varieties without the seed being treated with a neonicotinoid–fungicide combination ([38], p. 5857). Similarly, the value of a pollinator valuation framework that does not take into account ‘variation in pollinator density, crop cultivars and growing conditions that exist in practice’ is debatable [39]. In the absence of comprehensive knowledge about the economic benefits of insect pollinators to various crops, and about the expected costs accrued due to pesticide-induced losses of pollinators and other beneficial insects, cost–benefit analyses of pesticides may tend to favor prophylactic use.
patterns. The contemporary regulatory process that renders the issues of pollinators in relation to pesticides in narrow econometric terms is the outcome of a much broader agenda of neoliberalization fueled by the Reagan administration [40], and here policymaking on pesticides has tended to systematically privilege the interests and values of agribusiness over others [41]. In this context, policymaking efforts to build coalitions between growers, beekeepers, conservationists, and other stakeholders take on an added significance.

Policy initiatives to building stakeholder partnerships

US policymakers are involved in a range of partially coordinated efforts at the federal, state, county and city levels to improve the plight of honey bees and other insect pollinators [42]. At the federal level, the intensifying debate over the role of pesticides in honey bee and pollinator decline has prompted the EPA to convene a Pollinator Protection Team dedicated to improving pollinator health, and to initiate a series of discussions with the US Department of Agriculture (USDA), pesticide manufacturers, beekeepers, and scientists toward improving the ways in which pesticide risks to pollinators are assessed and managed by regulators. In June 2014, President Barack Obama authorized the formation of a Pollinator Health Task Force to develop a comprehensive National Pollinator Health Strategy by 2015 in collaboration with governmental agencies, state partners, and industry stakeholders to recommend research to understand and recover pollinator losses, build pollinator-friendly habitat, raise public awareness and disseminate pollinator-friendly land management practices [43]. The National Pollinator Health Strategy serves to unite already ongoing efforts by various governmental agencies, and the President’s backing lends a welcome urgency and symbolic impetus to the entire issue. Based on the Presidential memorandum, several states have initiated the process to develop Managed Pollinator Protection Plans (MP³), which aim to ‘establish a framework for open communication and coordination among key stakeholders, including beekeepers, growers, pesticide applicators, and landowners’ toward arriving at mutually agreed best management practices (BMPs) that ‘both mitigate potential pesticide exposure to bees and allow for crop production’ [44*].

Such ‘bottom-up’ approaches have the potential to create the conditions for genuine dialog and innovative local solutions. Building education and awareness about pollinator issues among beekeepers, growers, and landowners and the challenges these different stakeholders face are important. However, it is also crucial to note that these federal and state policy initiatives are not binding; they rely on voluntary commitments and actions by involved stakeholders. Indeed, many of the recommended BMPs that the draft MP³’s outline for mitigating pesticide problems are mere repetitions of guidelines outlined more than half a century ago by university extension scientists and governmental agencies [45,46]. The central recommendations for minimizing exposure to harmful pesticides still entail giving beekeepers notice reasonably ahead of time of an imminent pesticide application, applying pesticides during times when bees are not foraging, and using ‘integrated pest management’ practices. The generally worsening plight of honey bees and other insect pollinators during this period suggests that doing more of the same education and outreach will not be enough. Even as US policymakers have initiated a range of discussions with scientists and stakeholders, newer systemic insecticides such as sulfoxaflor and flupyradifurone have entered our agroecosystems, beset by some of the very same issues of uncertainty and complexity that are at play in the controversy over neonicotinoids [47,48].

Efforts to build partnerships between various stakeholders to enhance pollinator health need to take into account historically established asymmetries in these relationships. Current policy recommendations treat pollinators and pesticides on equal terms, but in practice, the relationship between growers and beekeepers is fundamentally asymmetrical. Indeed, the development of a highly chemically dependent form of large-scale industrial agriculture in the US has constituted the very conditions in which honey bees and beekeepers have attained their commercial and societal status as valuable pollinators [49]. Here, pollinating honey bees and nomadic beekeepers are the ones who are working for, catering to, grower clientele [50]. And it is beekeepers that have had to adapt, by and large, to growers’ shifting patterns of cropping and pesticide use. They are expected to move beehives to some other place, to feed their bees with a battery of nutritional supplements and antibiotics in the nutritionally scarce environments that monocultures create, and ultimately cope with dying bees [49]. This ‘unfortunate incompatibility’ between growers and beekeepers has been justified as being an ‘essential’ part of ‘modern agriculture’ [14] — a ‘dilemma’ [51] that is somehow necessary to maintain in order to feed the world. Pollinator policy plans that ignore the historically established asymmetries in power and resources between the beekeeping industry and agro-industry risk amplifying the very same problem they are setting out to solve. Strategies to re-build trust between beekeepers and growers will be a key determinant of the success of these coalition efforts.

A context-sensitive approach

While there is no ready-made solution to this complex policy problem, an alternative context-sensitive approach would surely eschew any blanket ‘ban’ or ‘hands-off non-regulation’. It would entail moving to a more dynamic and nuanced approach, where, for example, modifications and
restrictions in pesticide use patterns are carried out in agroecosystem-specific ways. Also, the continued use of honey bees as the primary model system on which to base policy-relevant assessments of pesticide impacts on all pollinators would need to be reconsidered, since the relatively large colony size and complex, eusocial features of honey bees are not representative of most other species of bees and insect pollinators [30]. Significantly, a context-sensitive pollinator policy would also give weight to, and not ignore, uncertain scientific knowledge (Box 1).

A context-sensitive policy framework pertaining to pesticides and bees would also take into serious consideration beekeepers’ field knowledge of colony health. As deeply invested observers whose livelihoods depend on being able to accurately assess the health and strength of their honey bee colonies, commercial beekeepers take in situ and real-time approaches, and systematically utilize informal measures such as brood pattern [49]. While their approaches and measures in relation to bee health may not lend themselves to precise quantification and easy isolation of the effects of individual factors, they highlight biologically relevant phenomena (such as CCD) and shed valuable light on the multifactorial dynamics underpinning these phenomena [49]. It may be argued that the EPA’s National Portal for Incident Reporting, which allows beekeepers to file claims regarding pesticide-related incidents of bee-kills directly to the EPA, is an instance of beekeepers’ understandings being incorporated into pesticide risk assessments. However, the power to decide whether a beekeeper’s report of bee-kil death counts as an ‘incident’ or not is in the hands of governmental agencies, which significantly limits this mode of beekeeper participation in the regulatory arena of pesticides and pollinators [52].

To the extent that the diversity and abundance of insect pollinators are indicative of the well-being of the human and non-human ecosystems they constitute [53], documented declines in these populations should make policymakers pause and reconsider the evidentiary basis upon which pesticides are permitted to be released. In regulatory policy settings, science needs to reflect not only the values of the regulated communities of the pesticide manufacturers and users, but also affected communities of beekeepers spanning different economies of scale. In practice, EPA policy pertaining to pollinators and pesticides has to date tended toward a false-negative approach, even though, on paper, the EPA recognizes and provides risk assessors some flexibility to change their standards of statistical significance in field studies that involve high levels of environmental variability, and to consider biologically significant trends despite statistical non-significance [24]. The high stakes for the lives of pollinators and livelihoods of beekeepers and growers, as well as the uncertain state of knowledge around the issue of pesticides and pollinators, could be considered as a reason to question the relevance to false negative results in the arena of regulatory science. A potentially useful place to start may be to consider broadening the statistical threshold — the alpha-level — at which a particular result is deemed significant or not significant in regulatory scientific assessments of the effects of pesticides on ‘non-target’ organisms.

Precisely how we can adapt our systems of pesticide regulation to become more context-sensitive, and what would count in this framework as an acceptable threshold of evidence required for pesticide manufacturers to demonstrate that their products are indeed safe for insect pollinators, are major unresolved issues that need to be negotiated through carefully facilitated deliberations involving all key stakeholders and a broad range of experts, including beekeepers and their variety of expertise, as well as broader publics.

Conclusion
Neither what counts as good science nor what counts as a sufficiently reasonable level of certainty are set in stone; these assessments differ across different research fields and policymaking arenas [54,55]. Many of the experimental approaches and methods developed to assess the environmental hazards and risks of pesticides to pollinators are not based on consensus among scientists. Indeed, the EPA’s insistence on an inordinately high level of scientific certainty to justify its refusal to limit neonicotinoids may be construed as politics and bad science, because it ignores the accumulating state of scientific knowledge regarding the interactive and cumulative

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**Box 1 An example of uncertain scientific evidence of indirect harm posed by pesticides to bee health**

Petit et al.’s [7] cutting-edge study spanning field and laboratory experiments is an example of the kind of research that can shed valuable light on the subtler and less direct effects of neonicotinoids on developing honey bees. Honey bee colonies were fed with pollen patties containing varying levels of Admire® (imidacloprid) continuously over a period of 9 weeks in a field experiment. The 5 parts per billion (ppb) treatment had ‘negligible effects on colony health’, suggesting that imidacloprid at a level representative of residues in seed-treated crops is unlikely to be the direct cause of declines in honey bee health [13]. However, in a laboratory experiment [7], newly emerged (1-day old) honey bees from the field colonies that had been chronically exposed to the 5 ppb treatment were significantly more vulnerable to Nosema infection, compared to those from untreated colonies. This laboratory evidence of the indirect harm posed by low levels of a neonicotinoid in interaction with a microbial pathogen — also documented by others [9,10] — is suggestive and uncertain to the extent that it does not necessarily reflect the experience of whole colonies in field settings. While EPA regulators have chosen to give more weight to the assessment of direct over indirect harm [28], a context-sensitive approach would also take seriously studies such as Petit et al. [7], which adds to the accumulating evidence of the indirect harm posed by neonicotinoids to bee health.
effects of relatively low levels of neonicotinoids on honey bees and other insect pollinators — knowledge which conforms to the highest standards of peer-reviewed academic science, and has been taken seriously by the EU’s policymakers.

Banking on certainty in the regulatory policy context is even more problematic in the case of scientific disputes such as the one over neonicotinoids because of ‘the problem of experimenter’s regress’ [56]. In such high-stakes disputes over findings it is always possible to question experimental design. If there are problems with experimental design, then resulting data can be questioned. In this context, more research results obtained from the replication of experiments by themselves would not resolve the controversy, because the extent to which an experiment is actually replicated then becomes a new node of dispute.

While adopting a policy approach that gives the appearance of certainty and control by bracketing out uncertainty may shore up the authority of policymakers’ decisions, it risks eroding public trust by failing to attend to the methodological and epistemological limits of the knowledge which points to what is left not known [57]. In this sense, a context-oriented policy based on uncertainty is not a call for inaction, but is one for nuanced action, which responds to a situation where the current state of knowledge about interactions between pesticides and pollinators shows the possibility of multiple future outcomes.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


2. USDA: Report on the National Stakeholders Conference on Honey Bee Health. Agricultural Research Service; 2013. Several individuals from the Colony Collapse Disorder (CCD) Steering Committee, along with Pennsylvania State University, organized and convened a conference on October 15–17, 2012, in Alexandria, Virginia that brought together stakeholders with expertise in honey bee health. This important report synthesizes the current state of knowledge regarding CCD, bee pests, pathogens, and nutrition, potential pesticide effects on bees, and bee biology, genetics and breeding; facilitates the development and implementation of best management practices that stakeholders can realistically incorporate; and identifies priority topics for research, education and outreach to be considered by the CCD Steering Committee for an updated Action Plan.


This study investigated effects of sublethal dietary neonicotinoid exposure on honeybee colony performance. The authors implemented a fully crossed experimental design using 24 colonies, including sister-queens from two different strains, and experimental in-hive pollen feeding with or without environmentally relevant concentrations of thiamethoxam (5.3 ± 0.75 ppb) and clothianidin (2.05 ± 1.18 ppb). Honeybee colonies chronically exposed to both neonicotinoids over two brood cycles exhibited decreased performance in the short-term resulting in declining numbers of adult bees and brood, as well as a reduction in honey production and pollen collections. While colonies recovered in the medium-term and overwintered successfully, significantly decreased colony size of neonicotinoid-exposed colonies during the following spring was associated with queen failure.


19. van der Sluijs JP, Simon-Delso N, Goulson D, Maxim L, Bonnatin J-M, Belzunces LP: Neonicotinoids, bee disorders and the sustainability of pollinator services. Curr Opin Environ Sustain 2013, 5:293-305. This review highlights research suggesting that the neonicotinoids have adverse direct and indirect effects on pollinators at field-realistic exposure levels. The authors urge a transition to pollinator-friendly alternatives to neonicotinoids for the sake of the sustainability of pollinator ecosystem services.

20. Carreck NL, Ratnieks FLW: The dose makes the poison: have “field realistic” rates of exposure of bees to neonicotinoid insects been overestimated in laboratory studies? J Apicult Res 2014, 53:607-614. This relatively recent scientific review is beginning to be cited in arguments against increased regulation of neonicotinoids in relation to pollinator protection [58]. The authors review three key dosage factors (concentration, duration and choice) relevant to field conditions, and conclude that these have probably been overestimated in many laboratory-based studies reporting to show “field realistic” effects. For example, doses used in a United Kingdom (UK)-based study [6] were based on levels found in Canadian oilseed rape, which are treated at three times the recommended rate in the UK. It should however be noted that the levels of neonicotinoids used in studies such as Whitehorn et al. [6] are far below the no observed adverse effects concentration of 20 ppb published by Bayer. Furthermore, the authors do not apply this same critique of a lack of context-sensitivity to pesticide manufacturer’s studies for pesticide use registration. They also do not consider seriously studies that suggest indirect adverse effects of neonicotinoids, in combination with other factors, on bee health. Instead, they cite Creswell et al. [17], who take a parsimony-based approach, which assumes that the complex hypothesis of neonicotinoids acting in concert with other stressors needs to be considered only after the simpler case (of direct effects) is dismissed. Simplicity, however, does not imply accuracy, especially in the case of a complex superorganism such as a honey bee colony.


Also of interest in the draft guidance for MP3s is the ‘state flexibility’ clause, which seeks ‘to allow sufficient flexibility for state approaches to address pollinator health’. The extent to which ‘state flexibility’ will enable state agencies to circumvent potential federal restrictions on pesticide use patterns is a cause for concern, and indicates the challenges borne by the decentralized organization of US policymaking.


Improving the future of honey bee breeding programs by employing recent scientific advances

Elina L Niño and W Cameron Jasper

A recent abundance of studies investigating causes of honey bee (Apis mellifera) colony losses has led to enhanced recommendations in management practices with particular emphasis on breeding for resistant bee stocks. Here we review the latest advances in research which could improve the future of breeding programs. We discuss diversity in colonies particularly in breeding programs, giving special emphasis to recent improvement in cryopreservation of honey bee germplasm. We also review factors that affect the health and reproductive quality of queens and drones. We briefly discuss how techniques developed by scientists are finding more regular usage with breeders in the assessment of reproductive caste health and quality and in determining best management practices for breeding programs.

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Introduction
Honey bees are arguably one of the most important beneficial insects worldwide. Their positive impact can be measured by the value they contribute to the agricultural economy, their ecological role in providing pollination services, and the hive products they produce. Honey bees, however, are exposed to myriad of stressors including pests, pathogens, pesticides, poor nutrition due to monocropping and habitat loss leading to extreme colony losses [1–3].

Among the many recommended modifications to colony management practices in order to combat some of these maladies [4–6], selective breeding for parasite and pathogen resistant honey bee stocks represents an especially attractive and viable solution. For example, while the use of miticides has been effective in mitigating severe overwintering colony losses associated with Varroa mite (Varroa destructor: Anderson and Trueeman), selective breeding for increased Varroa resistance has the potential to reduce the use of in-hive pesticides as over-employment can produce pesticide-resistant mite populations and can negatively impact colony health (e.g., [7,8,9,10]). Additionally, there has been a recent rise in interest, particularly by backyard beekeepers, to breed for bees adapted to local microclimates. Credibility was given to these attempts by a recent European study which found that local genotypes fared better in their local environment ([11,12], for a more detailed discussion see [13], this issue).

Breeding programs are likely to be a sustainable, long-term solution for challenges facing honey bees. A few successful programs within the US have been established. The Varroa Sensitive Hygiene (also known as VSH) and Russian Honey Bee breeding programs have been developed by the United States Department of Agriculture laboratory in Baton Rouge, LA [14] and the Minnesota Hygienic line [15,16] developed by researchers at the University of Minnesota, St. Paul, MN. However, these are an exception as programs such as these are often plagued with issues including financial constraints, the length of time it takes to select and maintain stocks possessing the traits of interest, and potential difficulties with maintaining genetic diversity and high quality of reproductive castes [17]. Here we review recent research that has improved our understanding of the genetics and genomics of breeding in honey bees as well as health and quality of the honey bee reproductives which will certainly help support breeding programs across the globe. We also discuss how basic research tools have been or can be adapted to help breeders produce high quality honey bee stock with desired traits thereby improving breeding success and beekeeper satisfaction (Figure 1).

Genetics and genomics of honey bee breeding
Availability of the honey bee genome data [18,19] and development of various genetic tools opened the door for identifying novel biomarkers for pest and pathogen resistance to be incorporated into breeding programs via marker assisted selection. Quantitative trait loci (QTL) mapping, for example, allows for identification of DNA regions associated with genes regulating a quantitative trait of interest. Modern genomic techniques used for gene expression analysis (e.g., microarrays and RNAseq) are broadly expanding our understanding of how stressors affect transcriptional networks in honey bees (for a more detailed review and how these techniques can be utilized...
in breeding programs see [20], this issue). These techniques can easily find utility in breeding programs by zeroing in on candidate genes for developing parasite-resistant and pathogen-resistant honey bee stocks. This could lead to reduced time commitment and cost required for stock selection and maintenance by performing rapid genetic screens instead of labor intensive traditional stock evaluations.

A tremendous amount of effort has been devoted to identifying QTLs for resistance/tolerance to Varroa with specific emphasis on understanding genetic basis of hygienic behavior and more recently suppression of Varroa reproduction (for a review see [21]). Three new QTLs were identified as important in regulating suppression of Varroa reproduction [22]. However, their effect was epistatic rendering these loci informative but potentially problematic for implementation in breeding programs due to difficulties inherent in attempting to select for three versus a single QTL.

The genetic bases for the resistance to a microsporidian Nosema, a devastating bacterial disease American foulbrood (AFB, Paenibacillus larvae) and fungal disease chalkbrood (Ascosphaera apis) have also been explored. Nosema ceranae is a relatively recent honey bee association and it has been linked to colony losses. Mapping for resistance to Nosema, as marked by low spore counts, identified four QTLs and a gene of interest Aubergine involved in RNA interference [23]. Behrens and Moritz [24] identified a promising QTL on chromosome 1 for tolerance toward AFB containing genes involved in regulation of development. A fine mapping study identified a region on chromosome 11 associated with larval-mediated chalkbrood resistance [25]. This region included two candidate genes: single Ig IL-related receptor-like and juvenile-hormone-binding-protein-like hypothetical gene.

While genotype does play a robust role in resistance to pests and pathogens the effect of environment should also be taken into account when developing breeding programs. Thus far, there seems to be a limited effect of a standard colony environment on hygienic behavior suggesting that evaluations can be done at any time without much regard for colony size, presence of brood, food availability or time of year [26], however, the interaction effect between the last three factors was evident. In addition to identifying new resistant populations and genetic regions of interest, further research should also focus on potential environmental effects when testing for desirable traits in breeder colonies.

Promoting genetic diversity in breeding programs

Long-term management by humans and large colony losses due to various adversities have raised concerns about the genetic diversity of honey bee populations [17]. While there is a concern about decrease in genetic variability especially in Europe and North America (discussed in [27]), a recent investigation shows that genetic diversity of commercial honey bees in Europe and the United Stated is greater than that of European progenitor populations [28]. Despite these reassuring findings, breeders need to remain vigilant about promoting genetic diversity. Breeding programs often rely on the use of instrumental insemination or isolated mating yards which could lead to inbreeding. Breeders can greatly benefit from importation of new genetic material from foreign sources not only for preserving genetic diversity, but also to increase desirable traits. An effort is underway to import semen from Europe into the US and a slow but steady progress in developing methods for germplasm cryopreservation has made the importation and preservation of drone semen more feasible. Potential benefits resulting from the germplasm importation underscores the importance of effective cryopreservation technology.

Previously developed protocols for preparation and storage of honey bee germplasm have recently been modified to significantly improve sperm motility and viability. This was accomplished by using dialysis to minimize the damage dilution fluids can cause to the extracellular matrix of spermatozoa and optimizing wash procedures in order to minimize exposure to the damaging effects of cryoprotectants [29,30]. Field studies show that offspring
quality was not significantly different from that of queens inseminated with untreated semen [30].

An especially concerning consequence of reduced genetic diversity is the reduced viability of brood due to sex determination mechanism via complementary sex determining gene (csd). Gene csd has several allele forms. Female honey bees are heterozygous at this locus and normal haploid males are hemizygous while diploid homozygous males are non-viable and quickly removed by workers. Hyink et al. (2013) developed a simple and cost-effective PCR test which allows breeders to monitor csd allele diversity in their breeding lines and manage them accordingly. These methods have already been used by breeders in New Zealand and could be readily adopted worldwide.

Lastly, breeding operations (particularly those in the southern areas of the US) are vulnerable to introgression of genes from Africanized populations. Africanized colonies are not desirable as they are more aggressive and might exhibit high absconding and swarming rates. Wing morphometrics can be successfully used to distinguish between A. m. mellifera, A. m. ligustica and A. m. carnica [31,32] and can be successfully applied to Africanized honey bees [33]. This process could further be automated making the results rapidly available [34,35]. However, wing morphometric approach could be less reliable in areas of more recent invasion as is the case with southern USA [33]. Optimization of several molecular methods of assessing Africanization in honey bees via mitochondrial DNA [36,37] can therefore be easily utilized by breeders to ensure their stocks are not Africanized (see Box 1 for available services). These techniques are more reliable but do carry a higher expense that larger breeding operations are willing to sustain to ensure the quality of their breeding stocks (Niño, personal communication).

Mating success, health and quality of reproductive castes

In addition to resistant traits, the superior quality of reproductive castes is required to maintain the economic viability of breeding programs. Colonies led by highly polyandrous queens exhibit higher disease resistance [38–40], fitness and productivity (possibly through suppression of worker selfishness) [41,42], improved gut microbiota [43], and overall homeostasis [44]. Therefore, lack of genetic diversity within a colony due to poor queen mating success because of low queen quality or lack of healthy drones can disrupt colony homeostasis. This in turn could lead to economic losses due to beekeepers having to re-queen their colonies or ultimately colony loss [45,46]. As the mechanisms underlying the correlation between mating success and colony health are not fully understood, bee breeders can ensure the quality of reproductive castes only if these mechanisms are further elucidated.

The queen takes 1–5 mating flights during which she mates with an average of 12–14 drones [46] storing all the sperm she will use during her lifetime. After mating, queens undergo behavioral, physiological and transcriptional changes signaling their new role in the hive. Recent studies show that both seminal fluid components (such as proteins) and insemination volume trigger and maintain particular aspects of queen post-mating changes [47,48] which can in turn affect the entire colony [49].

While studies of honey bee reproductive health have historically focused on queens, drone health and quality have recently presented themselves as vital aspects of reproduction management. Large sets of seminal fluid proteins and, more recently, sperm proteins specific to honey bees have been identified [50*,51**]. Understanding the specific roles these proteins play in supporting sperm survival, mobility and regulating queen reproductive processes will provide opportunities to harness their potential for improving breeding protocols. For example, queen longevity could be extended by incorporating substances identified as crucial for queen life-span in the instrumental insemination fluid.

While many stressors are associated with colony losses, very few studies specifically address their effects on health and quality of reproductive castes. Gaining this

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Box 1 Maintaining high quality of breeding stocks

Commercial bee breeders are greatly concerned with maintaining high quality of the queens they sell. To achieve this they can now exploit various techniques commonly used in basic sciences. Molecular approaches such as standard PCR and Quantitative Real Time-PCR can be effectively used to determine absence, presence and relative levels of various viral, bacterial and fungal pathogens that can have profound negative effects on queen, drone and colony performance. Furthermore, QTLs and associated genes identified by researchers could eventually be used for stock selection in breeding programs by employing basic molecular techniques as a for-fee service offered by scientists. Molecular tools can also be used for detection of pathogens in breeding operations to help reduce the spread of diseases. For example, breeders can test the offspring of their breeder queens to determine if there are high levels of certain pathogens associated with that particular stock. This could guide their decision about terminating specific breeder stocks that show consistently high pathogen loads thereby minimizing spread of pathogens and pathogen-susceptible stocks to their customers. Pathogen detecting services are now being offered to beekeepers (e.g., North Carolina State University apiculture program, Raleigh, NC and USDA-ARS Bee Research Laboratory, Beltsville, MD). Molecular techniques are also being used for determining the level of Africanization in breeding populations, diversity of sex alleles and myotyping within colonies (e.g., offered by the North Carolina State University apiculture program). Queen mating success and sperm viability in drones or queen spermathecae can be quickly determined by the use of flow cytometry [74]. Interested breeders can also send hive samples for pesticide testing, the results of which could then be used to guide their management decisions (via LC–MS/MS and GC–MS; e.g., service provided by National Science Laboratories, Gastonia, NC).
knowledge is of paramount importance for developing successful and non-harmful management decisions in breeding operations. Breeders can use this information to create an integrated pest management program to reduce harmful parasites and pathogens while simultaneously minimizing addition of harmful chemicals. A few recent studies attempt to further elucidate the presence and the effects of various pathogens on queens and drones. Level and localization of viral infection and active viral replication in various queen tissues [52] can inform breeders of potential for vertical transmission which can facilitate the spread of viruses via sold queens. Viruses, such as chronic bee paralysis virus, can also directly affect queens by increasing their mortality [53]. Another study showed that while black queen cell virus had a limited effect, drones infected with a microsporidian N. ceranae exhibited higher mortality and lower body weight [54] suggesting a possible effect on semen quality and/or mating success, but these parameters were not quantified. Nosema infection can also alter mandibular gland pheromone production which could be associated with higher queen replacement rates [55]. Lastly, parasitization with Varroa during pupal stages can lead to significant reduction in drone weight [56], reduction in the number of spermatozoa and inhibition of drone mating flight attempts [57].

Research has also continued to address the potential effects of various pesticides on aspects of queen and drone health. Miticides used for the control of Varroa mite can accumulate in foundation wax, pollen and individual bees often with negative consequences. Most recently, coumaphos was found to negatively affect queen development, spermathecal weight and therefore sperm storage capacity [8]. Other agrochemicals collected by foraging honey bees can also accumulate in hive matrices and some have been shown to significantly reduce queen survival during development [7], increase the queen replacement rates [58], and reduce success of new queen rearing [59]. However, several commonly used miticides applied at sublethal doses did not seem to affect sperm viability in adult drones [60]. This result warrants further investigation of pesticide effects on semen quality in developing drones since the sperm production occurs during the pupal stage [61]. The interaction effects of various factors add yet another layer of complexity to maintaining healthy breeding stocks and should be further addressed. For example, exposure to certain agrochemicals can compromise the immune response of queens and allow for increase in viral titers [59].

Several other factors directly influencing semen quality in drones should be considered when maintaining drones in breeding operations. Rearing conditions (field, semi-field, laboratory), exposure of brood and adult drones to extreme temperatures, as well as drone age can affect seminal volume and sperm viability [62–65]. Rearing conditions can affect the number of spermatozoa and seminal proteins as well as ATP (adenosine triphosphate; main energy source for sperm motility) content and SOD (superoxide dismutase; an enzyme that protects cells from oxidative damage) activity [64]. Interestingly, while an immune challenge decreased sperm viability, pollen deprivation in adult drones had no effect [65]. However, a lack of pollen availability during drone development resulted in smaller drones less able to ejaculate [66]. Results of these and similar studies should be used to establish optimal conditions for drone rearing and semen collection for cryopreservation and instrumental insemination.

**Future directions**

While significant improvements in beekeeping are attributable to our greater understanding of the genetic bases of disease and pest resistance, better tools and protocols for increasing genetic diversity of the honey bee populations, and techniques available for considering queen and drone quality, there is still much to be gleaned from future research.

Novel tools to identify specific molecular signatures of parasite and pathogen resistance, such as bee-specific peptide array which characterizes and correlates phosphorylated protein profiles with Varroa resistance [67], should be developed and incorporated into breeding efforts. Varroa resistance may be conferred not only by increasing hygienic behavior but also by increased resistance to the specific viruses it vectors [68] thus highlighting the need to identify resistance-associated QTLs. Recently, striking differences in Israeli acute paralysis virus titers among infected pupae, indicated that there is likely a genetic variation in resistance to viruses which could be exploited in future breeding efforts [69]. Efforts to identify genetic markers for viral resistance and/or tolerance should be widely expanded especially considering the potential relevance of viruses to colony health.

**Box 2 Remote hive and individual tracking**

Use of remote sensing to track the state of the hive is gaining more traction in the beekeeping community and could easily be adapted for use in honey bee reproductive research and breeding programs especially as the cost of various technologies is declining. Portable hive scales are available for real time monitoring of the hive weights indicating the productivity of the colony particularly as it relates to honey production and food stores. Temperature, humidity and gases inside the hive can be monitored and could potentially provide information on the genetic diversity of the hive, while sound and vibration recordings could be used for disease detection. For a comprehensive review of currently available technologies for hive monitoring see [75]. Improvement in radio-frequency identification (RFID) technology to track flying individuals will also provide further information on the elusive mating behavior of honey bees [76].
Detailing individual drone contributions responsible for modulating specific reproductive processes in queens could be crucial for improving breeding success particularly when instrumental insemination is used. As such, this area of research should be expanded on and a recent success in integrating and expressing piggyBac-derived cassettes in honey bee queens [70][*] adds a valuable tool for genetic manipulation for such studies.

Lastly, while there are many great protocols already available (see [71,72]), there is always a need for development of standardized, more efficient, reliable and cost-effective techniques for evaluating (I73; Box 2) and producing breeding stock. Determining how different stressors during various developmental stages impact reproductive potential will be crucial in providing accurate management recommendations for breeding programs.

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References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


9. Pettis JS, Lichtenberg EM, Andree M, Sitzinger J, Rose R: Crop pollination exposes honey bees to pesticides which alters their susceptibility to the gut pathogen Nosema ceranae. PLOS ONE 2013, 8:e70182.


A clear effect of local genotype on colony survivorship is demonstrated in a large scale study. This study lends credibility to micro-breeder attempts to breed for locally adapted queens. Varroa destructor is again identified as the primary agent associated with colony loss.


This study represents a significant advancement in terms of retaining the fertility of sperm following the cryopreservation process. Maintaining sperm interactions by keeping dilution to a minimum as well as decreasing exposure to cryoprotectants were important steps in improving fertility and stability of genetic material.

This study represents a comprehensive proteomics analysis of the seminal fluid proteome of the honeybee *Apis mellifera*.


The development of a bee-specific peptide array for kinase activity in a whole-body extract is highlighted. This array detects signatures of honey bee resistance to Varroa and could be adapted for application in breeding programs.


This is a pivotal study that has developed and successfully implemented for the first time a genetic tool allowing for the contextual manipulation of gene function in honey bees. The efficiency of transmission of this tool highlights its financial accessibility and potential use as a general method in reproductive studies and beekeeping.


Nutrition, immunity and viral infections in honey bees
Gloria DeGrandi-Hoffman1 and Yanping Chen2

Viruses and other pathogens can spread rapidly in social insect colonies from close contacts among nestmates, food sharing and periods of confinement. Here we discuss how honey bees decrease the risk of disease outbreaks by a combination of behaviors (social immunity) and individual immune function. There is a relationship between the effectiveness of social and individual immunity and the nutritional state of the colony. Parasitic Varroa mites undermine the relationship because they reduce nutrient levels, suppress individual immune function and transmit viruses. Future research directions to better understand the dynamics of the nutrition–immunity relationship based on levels of stress, time of year and colony demographics are discussed.

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Introduction
Honey bees and other eusocial insects comprise more than half of the insect biomass in the world making them one of the most ecologically successful insect groups [1]. Contributing to this success is the coordination of activities among members of a colony. Essential tasks such as thermoregulation, brood rearing and resource gathering are efficiently executed due to the architecture and organization of the nest and spatial proximity among individuals. However, crowded conditions, warm temperatures, high concentrations of resources and periods of confinement in the nest are ideal for pathogen invasion and transmission that can lead to epidemics [2,3]. The risk of disease outbreaks is mitigated by specialized group behaviors (social immunity) and immune systems in individuals.

Honey bees are important pollinators in undisturbed ecosystems, but are essential for the production of numerous high-value crops [4]. Over the past decades, the health of honey bees has been in steady decline especially with arrival of parasitic Varroa mites (Varroa destructor Anderson and Trueman). There has been considerable effort to identify parasites and pathogens that threaten the health and survival of honey bee colonies. Viruses have received much attention due to the significant loss of colonies especially over winter from Varroa mite and virus associations [5,6]. Greater attention also has been given to nutritional needs of colonies and how improvements in this area might reduce colony losses.

This review will focus on the role of nutrition in immune response to viral pathogens. We briefly describe the connections between nutrition and individual immunity, and speculate on the possible changing nutritional requirements of colonies throughout the year. These changes might revolve around trade-offs between colony growth and immune defense. Within this framework, we include the effects of parasitism by Varroa because when the mite is present, optimal nutrition alone might not be sufficient to keep virus levels low [7,8] (Figure 1).

Honey bee viruses
More than 20 viruses have been identified to infect honey bees worldwide [9]. The most common are: Deformed wing virus (DVW), Black queen cell virus (BQCV), and Israeli acute paralysis virus (IAPV) [10]. IAPV, Acute bee paralysis virus (ABPV) and Kashmir bee virus (KBV) often are referred to as the Acute–Kashmir–Israeli complex or AKI, and share similar characteristics [11] (Table 1). Viruses infect all developmental stages and castes [9,12]. Though always present in colonies, viruses often persist as covert asymptomatic infections. However, if colonies are under stress, virus levels can increase causing reduced worker longevity and brood survival and colony loss in winter or early spring [13–15]. Viruses such BQCV also can cause colony death by preventing the development and emergence of a new queen following queen loss.

A factor that has increased virus levels in managed colonies of European honey bees in the U.S. and Europe is Varroa. The mite weakens bees by feeding on hemolymph of larvae, pupae and adults. Varroa also can transmit viruses among nestmates and suppress host immunity thus leading to elevated virus replication [9,16–20,21]. In colonies with large Varroa populations, brood cells are invaded by multiple foundress mites causing higher DWV levels than in singly infested cells even in Varroa-resistant stocks [22,23]. Multiple infestations are
common in the fall because mite populations are peaking and there are fewer cells to invade. The combination of multiply infested cells and greater virus levels in autumn ultimately causes colonies to die over winter [5,6**,15].

In addition to the threat viruses pose to honey bee colonies, recent studies indicate that the viruses can cross the species barrier and infect non-Apis species (e.g., bumble bees) [24,25]. Bumble bees have experienced dramatic population declines, and might acquire viruses while foraging on flowers previously visited by infected honey bees. Therefore controlling viral diseases in honey bee colonies is vital for stopping the spread of viruses among wild pollinators [26*].

**Honey bee immune system**

The risk of disease outbreaks is reduced in colonies of honey bees and other social insects by group-level behaviors (‘social immunity’) and individual immunity. Together these provide multiple levels of disease prevention and responses to challenges from pathogens and parasites.

**Social immunity**

The collective defense against parasites and pathogens that emerges from the behavioral cooperation among individuals in colonies is ‘social immunity’ [2,3*]. With social immunity, many individuals do small tasks that collectively have a colony-wide impact on reducing the spread of parasites and pathogens. For example, workers remove adults that die in the colony (undertaking or necrophoric behavior) and brood that are diseased or parasitized (hygienic behavior). Adults that die outside the nest also contribute to social immunity if they have high pathogen loads [3*]. Thermoregulatory behaviors also are a type of social immunity particularly when worker bees generate a behavioral ‘social fever’ against heat-sensitive pathogens such as chalkbrood fungus (Ascosphaera apis) [27].

In addition to group behavior inside the nest, bees collect plant resins (propolis) and use them to create a water and airtight antimicrobial and antiviral envelope around their nest [28–32]. Some compounds in propolis such as p-coumaric acid up-regulate immunity genes [33]. Other compounds might limit the growth of Varroa populations because they have miticidal properties [32,34].

**Individual immunity**

At the individual level, honey bees have several lines of innate immune defense against foreign pathogens. Physical and chemical barriers including the exoskeleton cuticle and the peritrophic membranes lining the digestive tract are a first line of defense that prevent pathogens from adhering to or entering the body [35]. If a pathogen breaches the physical and chemical barriers, honey bees can protect themselves from infection with cellular and humoral immune responses which represent a second line of defense [35,36]. The activation of the innate immune responses involves recognition of the highly conserved structural motifs on the surface of pathogens, termed Pathogen-Associated Molecular Patterns (PAMPs), by Pattern Recognition Receptors (PRRs) that are germline-encoded proteins [8]. The binding of PAMP by PRRs triggers signaling cascades that lead to the activation of hemocyte-mediated cellular immune response including phagocytosis, nodule formation and encapsulation of the invading pathogens, the initiation of phenoloxidase cascade that regulates coagulation or melanization of hemolymph, or the synthesis of antimicrobial peptides (AMP). Several AMPs such as abaecin, apidaecin, hymenoptaecin, and defensin have been identified in the hemolymph of honey bees upon induction of microbial infections [37–40].
Table 1
Viruses commonly detected in honey bee colonies.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Transmission</th>
<th>Lifestage infected</th>
<th>Symptoms</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute bee paralysis virus (ABPV)</td>
<td>Horizontal primarily through feeding, Varroa parasitism</td>
<td>Brood and adults</td>
<td>Paralysis, trembling, inability to fly, darkening and loss of hair on thorax and abdomen</td>
<td>[9,11]</td>
</tr>
<tr>
<td>Black queen cell virus (BQCV)</td>
<td>Horizontal primarily through feeding, Varroa parasitism, possible vertical transmission through eggs</td>
<td>Brood and adults</td>
<td>Dead queen larvae or prepupae sealed in queen cells with dark brown to black walls</td>
<td>[12]</td>
</tr>
<tr>
<td>Chronic bee paralysis virus</td>
<td>Horizontal primarily through feeding and contact, possible transovarial</td>
<td>Adults</td>
<td>Trembling inability to fly, bloated abdomens, black hairless bees</td>
<td>[12]</td>
</tr>
<tr>
<td>Deformed wing virus</td>
<td>Horizontal primarily through feeding, venereal, transovarial, transpermal, Varroa parasitism</td>
<td>Brood and adults</td>
<td>Deformed wings in emergent bees, premature aging of adults</td>
<td>[12,17,21*]</td>
</tr>
<tr>
<td>Israeli acute paralysis virus</td>
<td>Horizontal primarily through feeding, transovarial, venereal, transpermal, Varroa parasitism</td>
<td>Brood and adults</td>
<td>Similar to ABPV. Also, reduced mitochondrial function, and possible disturbance in energy-related host processes.</td>
<td>[10,18]</td>
</tr>
<tr>
<td>Kashmir bee virus (KBV)</td>
<td>Horizontal primarily through feeding, transovarial, Varroa parasitism</td>
<td>Brood and adults</td>
<td>Weakening of colonies but no clear field symptoms</td>
<td>[9,16]</td>
</tr>
</tbody>
</table>

There are several signaling pathways including Toll, Imd, Jak-STAT as well as JNK, that have been experimentally demonstrated to control the expression of many AMP genes in Drosophila in response to virus infection [41–43]. While a study reported that honey bees infected with ABPV did not trigger either cellular immune or humoral responses [44], a more recent study showed that a diverse range of signaling pathways implicated in the cellular innate immune responses are regulated in IAPV infected honey bees [10**].

Recent studies indicate that RNA interference (RNAi) is the major antiviral innate immune response in insects [45–49]. This innate antiviral pathway is triggered by the detection of exogenous double-stranded RNA (dsRNA), an intermediate generated during RNA virus replication. The response includes an RNase III-like enzyme called Dicer 2 (Dcr2) that recognizes virus dsRNA as a PAMP and cleaves long stretches of it into short interfering RNAs (siRNAs) that are 21–23 nucleotide-long duplexes. The resultant siRNA duplex, in association with Dcr-2 and the dsRNA-binding protein, is loaded onto RNA Induced Silencing Complex (RISC) which comprises multi-subunit effectors with Argonaute 2 (Ago2) as the catalytic core of this complex and degrades the passenger strand of siRNA. The guide strand of the siRNA remains bound to RISC and guides the RISC to cognate viral RNAs that are sliced by the endonuclease activity of Ago2 at the point of complementarity, thereby restricting viral replication (reviewed in Brutscher et al., 2015). The honey bee genome encodes the core components of the RNAi pathway including Dicer enzymes, Argonaute endonucleases, a Drosa homologue, dsRNA-binding proteins Loquacious, R2D2, Pasha [50] and homologue of systemic RNA interference defective protein (SID-1), a gene essential for transporting of dsRNA between cells and the systemic spread of RNAi signals [51].

The role of RNAi in mediating dsRNA-induced antiviral response in honey bees was confirmed in several studies. IAPV is a widespread RNA virus of honey bees that was initially linked with colony collapse disorder (CCD) [52]. Deep-sequencing analysis of honey bee workers from CCD-colonies revealed abundant siRNA matching the nucleotides of IAPV and other viruses associated with colony losses, indicating the activation of RNAi pathway in CCD-colonies for combating viral infections [53]. Injection and feeding of dsRNA corresponding to a segment of the intergenic region (IGR) and a segment of gene encoding the capsid structural protein can reduce the intensity of IAPV infection in honey bees [54,55]. Feeding siRNA targeting an Internal Ribosomal Entry Site (IRES) of IAPV required for protein translation can confer antiviral activity in bees [56]. Additionally, feeding dsRNA that is specific to DWV can lead to reduction in DWV infection in DWV-inoculated bees [57]. dsRNA-mediated non-specific antiviral response was demonstrated by a study showing that the administration of dsRNA, regardless of sequence could trigger an antiviral response that controls virus infection in honey bees [58]. More recently, a study of the global gene expression in both IAPV infected and uninfected bees indicated that RNAi pathway had increased activity in the virus infected bees, further confirming the role of RNAi in antiviral immunity. The study also showed alterations in DNA methylation patterns in response to viral infection, suggesting that honey bees may possess parallel epigenomic and transcriptomic mechanisms to respond to viral infection [59].
These findings and those reported by others are very encouraging for RNAi development as a tool for managing virus diseases in honey bees.

**Nutrition and immunity**

Honey bees meet all their nutritional needs with nectar and pollen. These resources are collected in quantities that exceed colony demands and are stored for periods of dearth as honey and beebread. Nectar and honey contain carbohydrates and are the energetic fuel for all stages and castes. Pollen and beebread provide protein and nutrients required for physiological processes such as brood rearing, growth and immunity [60*,61].

The connection between nutrition and immunity has been demonstrated in numerous organisms where immune function is affected by caloric restriction [62**,63]. Dietary protein (pollen) provides essential amino acids needed for the synthesis of peptides in immune pathways [64,65] including components of AMP [66**]. Carbohydrates (nectar and honey) provide energy for metabolic processes associated with innate humoral and cellular immune reactions, and can provide secondary plant metabolites that have antimicrobial properties [67].

The relationship between nutrition and immunity is compromised when bees are parasitized by Varroa. Workers that are parasitized during development emerge with lower protein levels that cannot be raised even if sufficient pollen is available [68]. Varroa-infested pupae also can have significantly lower protein content, elevated free amino acid levels, and lower emergence weights than uninfested pupae suggesting that protein synthesis, and ultimately growth, are inhibited by Varroa [69].

Recently, nutrigenomic studies have revealed the effects of both carbohydrate and protein sources on transcriptional profiles of adult bees. Constituents in honey up-regulate detoxification pathways in the gut [33] and genes associated with protein metabolism and oxidative reduction [70]. These effects were not found in other carbohydrate sources commonly fed to bees in managed colonies (e.g., sucrose solution or high fructose corn syrup). Pollen activates nutrient-sensing and metabolic pathways, and influences the expression of genes affecting longevity, immune function, the production of certain AMP [7] and pesticide detoxification [71]. However, if bees are parasitized by Varroa, there is a decrease in protein metabolism, inhibition of certain immunity genes and increased virus levels that cannot be reversed by pollen feeding. Thus, there are limitations to the benefits of diet on immune function in Varroa parasitized bees [7,8,69].

**Conclusions and future directions**

Honey bee nutrition is one of the most rapidly expanding research areas in bee biology largely due to colony losses from malnutrition and the accompanying pathologies. Though honey bee nutrition has been investigated for many years, molecular tools and the availability of the honey bee genome are enabling more comprehensive studies on the role of nutrition in honey bee health. To this end, we suggest several areas for future investigations. The first is a comprehensive evaluation of the nutritional value of pollen and nectar within the context of the nutritional needs of colonies throughout the year. An underlying assumption in comparing the nutritional value of pollens and in the development of protein supplements is that the nutritional needs of colonies are constant, and the relationship between diet and immunity is simply driven by energy consumption rather than specific nutrient blends that are key in determining an individual’s immune response. Honey bee colonies go through yearly cycles. Brood production and colony demographics change throughout the year, so it is reasonable to assume that so do nutritional needs. As demonstrated in other insects, diets that are optimal for growth are not necessarily optimal for immunity [62**]. Thus, colonies that are building in the spring may require nutrients geared toward growth while in the fall when brood rearing is reduced and colonies are preparing for overwinter confinement, nutrients needs may be directed at supporting immune function. Nutritional analyses of pollen collected at different times of year in combination with nutrigenomic studies examining the effects on metabolic and immune gene expression could broaden our perspective on the nutritional needs of colonies and how they are met by the seasonal pollens bees collect.

The second area needing greater study is the role of the microbiome in nutrient processing and immunity. The composition of nutrients obtained from food influences microbial communities in the gut [72,73,74**]. The communities could affect immune function by providing essential nutrients, inducing host immune responses or reducing the growth of pathogens [75–79]. While there is evidence for these benefits in other organisms, the role of microbial communities as extensions of social and individual immune systems has only begun to be explored in honey bees.

Though improved nutrition can optimize colony growth and immune responses to virus, Varroa parasitism might undermine any benefits that nutrition might offer. Abundant resources stimulate brood rearing and population growth throughout the spring and summer. However, as the colony grows, so does the Varroa population. In the fall, when less brood is available the large Varroa population generates high parasitism rates [13,14,80]. Going into winter, the colony will be comprised of a majority of adults that were parasitized during development and harbor virus [5]. Colonies such as these have high overwintering mortality rates. A final research area that needs further study is the role of...
nutrition on Varroa reproductive success and virus transmission. If improvements in bee nutrition affect either of these factors, then the relationship between nutrition and immunity could be re-established even when Varroa are present.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

• of special interest

•• of outstanding interest


A comprehensive overview of all facets of social and individual immunity including properties of propolis that can reduce pathogen loads.


Reports a trend of rising viral titres in colonies with Varroa over the course of a season from spring to autumn even in colonies receiving miticide treatments. The combination of virus and Varroa was a major cause of winter colony loss with AKD and DWV playing a major role.


This is a comprehensive and in-depth analysis of IAPV and the effects of the virus on honey bee health.


Describes how DWV levels in developing bees are low in the absence of Varroa but generally high following mite exposure. The study complements observations of the effects of Varroa introduction in Hawaii where it was associated with a dramatic reduction in DWV variation and the emergence of dominant strains.


Reports a link between pathogens in managed honey bees and the occurrence in bumblebee populations using infection experiments and landscape-scale field data.


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Effects of genotype, environment, and their interactions on honey bee health in Europe
Marina D Meixner¹, Per Kryger² and Cecilia Costa³

There are several reports of honey bee populations in Europe which survive without treatment for Varroa. However, when evaluated outside their native area, higher survival and resistance traits were not observed in colonies of a survivor population. Varroa infestation is strongly influenced by environmental factors, probably affecting threshold levels on a European scale. In a Europe-wide experiment colonies of local origin survived significantly longer than colonies of non-local origin, clearly indicating the presence of genotype–environment interactions. Transmission by Varroa selects for virulent strains of DWV, but it is currently unknown how these may interact with different genotypes of bees. The distribution of Nosema ceranae is significantly affected by environment, but there is at least one Nosema-resistant population.

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Introduction
There is now agreement among scientists [1] that multiple factors contribute to the frequently reported colony losses of honey bees (Apis mellifera). Recently, increased efforts are noted to analyze this phenomenon using a holistic approach, by investigating interactions between the multiple factors, such as parasites and pesticides, that are known to affect honey bee health [2–4]. Indeed this approach is the most promising, because it reflects the complex situation of a honey bee colony which is closely linked to the environment it lives in [5].

However, one aspect relevant for colony health has received less attention than pathogens and parasites, or pesticides: the interactions between a colony’s genotype and its environment. It is worth noting that the natural range of Apis mellifera extends from Northern Europe to South Africa and from Iberia to Central Asia [6]. Within this large distribution range there is considerable environmental variation, both in relation to weather patterns and flowering seasons, and to occurrence and variation of parasites and pathogens. Ten of the approximately 26 subspecies are of European origin, including A. m. carnica and A. m. ligustica, which are preferred by beekeepers worldwide [7–9]. Breeding activities have focused on commercially desirable traits, often using inter-subspecies crosses and mass reproduction from limited stock, leading to hybridization or replacement of the original honey bee population in many places [7,10–12]. Substantial numbers of queens are traded across the borders of Europe, especially A. m. ligustica from Italy, A. m. carnica from Austria and Slovenia, and the Buckfast hybrid mainly from central European countries [7]. In consequence, the natural distribution of the autochthonous European honey bee populations has changed very much. Other human activities, such as agriculture and urbanization have also affected the distribution and status of European honey bee populations, negatively influencing genetic diversity and density of honey bee colonies [13]. The arrival of the invasive parasitic mite Varroa destructor in Western Europe in the 1970s [14] all but wiped out the wild honey bee population [8,9]. Management practices changed to include regular application of acaricides, thereby helping to secure the survival of colonies. In consequence, though, mite-susceptible colonies are given the chance to propagate and transmit their susceptibility traits to the next generation. In the long term, this practice leads to an increasing dependency on medication and prevents the establishment of mechanisms of mite-tolerance, as detailed in a recent study [15*]. In spite of the availability and regular application of Varroa control measures, higher than usual colony losses were noticed in Europe from 2002 to 2003 onwards [16–18].

The question of adaptation of genotypes to rapidly changing environments on honey bee health in Europe has only recently received scientific interest. In this paper, we review the impact of two invasive parasites, Varroa destructor and Nosema ceranae, known to affect honey bee health, under the aspect of environmental influence and genotypic variation, with a special focus on genotype–environment interactions.
**Varroa resistant bees — genotype effects or local adaptation?**

Numerous surveys and experimental studies have identified the invasive parasitic mite *Varroa destructor* (hereafter referred to as *Varroa*) as the most prominent factor affecting honey bee health and contributing to colony losses, both in Europe and worldwide [16,19–21]. Reports of honey bee populations which survive without any *Varroa* treatment are known from the literature, resulting from selection in both natural and experimental situations [22–25]. In some cases the phenomenon was ascribed to development of avirulence of the mites [24], but in many of these instances it is attributed to the genotype of the bees that have developed specific mechanisms to counterbalance mite population growth [26–29]. For instance, when comparing colonies descending from the two European survivor populations ‘Gotland’ and ‘Avignon’ [23,25] to non-resistant colonies in each respective environment, a recent study [30] found that although both populations were able to reduce the fitness of the mites by limiting their reproductive success, they differed in their mechanisms to achieve this. While mites in the Avignon population showed higher levels of infertility, likely achieved by *Varroa*-sensitive hygienic behaviour of the bees (VSH [27,31]), mites in the Gotland population showed signs of delayed egg-laying, which the authors of the study interpreted as result of potential inhibition or delay of egg-laying, maybe through pupal volatiles [30].

Interestingly, however, when colonies descending from the Avignon survivor population were tested outside their native environment in a Europe-wide experiment [32], neither their *Varroa* infestation rate after one year without treatment nor their survival outperformed that of colonies descending from non-selected genotypes tested at the same locations, showing that the resistance traits are strongly influenced by genotype × environment interactions [33**,34***]. Similarly, in the same experiment the mite infestation rates and survival data of genotypes originating from ‘Varroa-tolerance breeding programs’ [29], did not differ significantly from non-selected genotypes, when tested at locations outside their native habitat. Indeed, the most important result of the Europe-wide experiment was a significantly higher survivorship of the local genotypes compared to the non-local ones, which, according to the authors, clearly indicates the presence of genotype–environment interactions originating from specific local adaptation of the honey bee populations in the study [34***]. These were considered ‘local’ when the strain had been bred or simply kept in that area for at least 25 years, with no introduction of foreign stock. The term ‘local adaptation’ includes traits such as disease susceptibility, colony development, and behavioural traits in regard to all environmental factors, including colony management practices, but the experimental design did not allow the authors to further differentiate among these factors.

While data from one single experimental apiary [35] suggested that mite infestation rates between colonies of local origin may be significantly lower compared to those of non-local origin, this trend could not be confirmed over all locations in the Europe-wide experiment, because in many of the test apiaries too few colonies survived long enough to allow meaningful statistics.

**Varroa infestation rates by environment**

Results of the Europe-wide experiment also strongly indicated that environment had a highly significant and much stronger effect on *Varroa* infestation rates than the genotype of the bees [33***]. Mite levels after one year without treatment varied considerably across regions, with several magnitudes difference between the highest and lowest infestation rates. Nonetheless, at each individual location colonies with infestation rates below the location median survived significantly longer than colonies with higher mite levels, suggesting that *Varroa* infestation thresholds may vary considerably across Europe. However, there is a surprising lack of studies investigating *Varroa* damage thresholds under different environmental conditions, and currently only few data are available on a European scale [15**,16].

The mite population in a honey bee colony strongly depends on the colony development itself, which is closely linked to environmental conditions [36,37]. As recently demonstrated [38*], the population dynamics of colonies differs significantly between the north and south of Europe, with a much lower ratio of adult bees to brood in the south, but a higher life expectancy of bees in the north. These differences may result in less dramatic consequences of *Varroa* infestations for colonies in south European climates [33***]. A study conducted in four different regions of Italy [39] also found that regional effects exerted a notable influence on the increase of mite infestation over the season. However, they could not deduce a clear trend from their data, as apiary effects, such as reinfestation from untreated colonies with high mite levels, may have been interfering. Recently, [40] it was demonstrated that high colony density and colonies with high mite levels in the surroundings of an apiary could lead to considerable mite reinfestation rates.

**Interactions with viruses**

Among the factors hypothesized as potentially responsible for colony losses, the viral pathogen Deformed Wing Virus (DWV) in association with *Varroa* mites is currently discussed as one of the prime suspects by numerous authors [41–43]. DWV infections are known to prevail widely in all regions where honey bees live [44,45*]. However, in the absence of *Varroa* mites, infection with DWV rarely leads to overt disease [46]. It has been shown [44] that a *Varroa*-free apiary can harbour a very diverse DWV population with numerous different strains, but low-level infections. In the presence of the mite,
however, the number of strains appeared to decrease with time until only one single strain could be found [44], which was hypothesized to be the most virulent one. In support of this hypothesis, experimental transmission of DWV from Varroa-infested bees to the hemolymph of bees from a Varroa-free colony has recently been shown to select for a characteristic virulent virus strain [47]. Experimental evidence also indicates that the virulence factor of DWV may be connected to a specific capsid protein characteristic for the DWV strain VDV-1 [48]. However, there are currently no data available on the geographical distribution of the many varieties of DWV, including VDV-1 and Kakugo Virus. In addition, data are lacking on potential variability of DWV virulence factors and how these may be related to honey bee genotypes. For instance, in South Africa where colonies were never treated against mites since their arrival in 1997, Varroa is not considered a significant factor for losses [49,50]. Yet, in a recent comprehensive study of pathogen prevalence in honey bee colonies in South Africa, of all DWV varieties only VDV-1 was found [51].

Comparing the Gotland Varroa-resistant population to mite-susceptible colonies over one year at the same location, a recent study [52] found that although autumn mite infestation rates of the resistant colonies were significantly lower, their autumn DWV titres were similar to those of the mite susceptible colonies. Nonetheless, the Varroa-resistant colonies survived the following winter, while the susceptible colonies vanished. The authors interpret this observation as result of tolerance against DWV as part of the bees’ Varroa resistance. In contrast, autumn levels of Sachbrood Virus (SBV) and Black Queen Cell Virus (BQCV) of the resistant colonies were reduced, and the authors discuss lower colony size and smaller broodnest size as adaptive traits to cope with pathogen levels.

Results of the detailed case study within the Europe-wide experiment [35] indicated that, beyond a lower Varroa mite infestation rate, the level of all pathogens investigated, including the titres of DWV and ABPV, were consistently lower in colonies of local origin.

**Distribution of Nosema species influenced by environment**

A century ago, Nosema apis was noted as a serious pathogen of honey bees in Central and Northern Europe [53], but due to improvement of management practices in the past decades, colony losses due to N. apis are nowadays not frequent. Instead, the invasive N. ceranae, originating from the Asian A. cerana, has recently been perceived as threat to honey bee health [54] although it has been shown to be present in Europe at least since 1998 [55]. Linked to the poor survival of N. ceranae spores at low temperatures [56], the development of this parasite in the honey bee colony and its prevalence in different regions of Europe is strongly dependent on environmental conditions: N. ceranae has been reported as a severe problem in Southern Europe, especially in Spain [54]. It is also the most frequently found Nosema species in honey bee colonies in Italy [57], Greece [58] and the Balkans [59], affecting very different genotypes of bees. In contrast, in Central and Northern Europe the species N. apis is still present, both in single and in co-infections with N. ceranae [33,60–62]. High levels of polymorphism of N. ceranae can be observed within a single bee colony, which were hypothesized to be linked with varying levels of virulence of the parasite [63–65]. However, it was recently demonstrated that levels of diversity do not vary significantly across the range of N. ceranae, with the variation within single colonies by far exceeding the proportion of genetic variance that can be assigned to different geographic areas [66,67]. These results support the hypothesis of recent colonization and expansion of N. ceranae into the A. mellifera range, but render the existence of more virulent strains unlikely.

**Susceptibility of honey bee strains to N. ceranae?**

Investigating whether honey bees belonging to different evolutionary lineages exhibited differential susceptibility to N. ceranae and N. apis, it was recently reported from Spain [68] that colonies of the mitochondrial M-lineage were infected to a greater extent by N. apis. It is known that the frequency of the M-lineage in Iberia decreases following a NE to SW cline [69–71]; in NE Spain, the climatic conditions are more similar to Central Europe, with lower temperatures and higher rainfall. The authors thus conclude that the significant relationship between honey bee lineage and infection by the specific Nosema type could be due to adaptation of both the parasite and the host to the different climates of the Iberian peninsula. On the Canary Islands, an increase of the presence and distribution of N. ceranae during the last decade has been observed in parallel with a higher frequency of foreign queens [72]. In the Europe-wide experiment [33], where different genotypes were assessed both within and outside of their area of origin, no significant effect of genotype or of G × E on colony susceptibility to Nosema was noticed. Nonetheless, the successful selection programme for Nosema-tolerance in Denmark [12] provides an indication of a genotype effect on susceptibility to Nosema. Analysis of the phenomenon at the individual level [65] showed that the mechanism is linked to an ability to survive high infection levels, possibly connected to up-regulation of the immune response.

**Conclusions**

Considerable variation of pathogen loads and pathogen strains exist across different environments, but also across bee genotypes. Varroa-resistant populations of bees are known, but recent research suggests the presence of a highly significant environmental effect on
Varroa infestation rates which may be stronger than the effect of the bees’ genotype. Colony life histories, driven by environmental conditions, have a significant influence on Varroa infestation rates; in consequence, mite infestation thresholds probably vary considerably across Europe. While interaction between Varroa mites and viruses is being discussed as one of the main drivers of colony losses, recent research demonstrates considerable variation within DWV with dramatic changes of diversity following the spread of Varroa, suggesting a virulence shift. While there are some data reporting variation in geographic distribution of virus strains and varying susceptibility of bees against virus variants, comprehensive evidence regarding these aspects is still lacking.

Distribution and prevalence of the gut parasites *N. ceranae* and *N. apis* seem to be strongly affected by environmental conditions. Recent research reports substantial genetic

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**Figure 1**

Genotype-environment interactions result in local adaptation of honey bees, including adaptation to locally prevalent pathogens. Different populations may have developed their own specific resistance mechanisms tailored to match the challenge of the environment they are located in, including the locally prevailing combination of pathogens. These mechanisms may not work as effectively in a different environment.
variation within *N. ceranae*, but there is yet no evidence for differential virulence or pathogenesis in relation to host variation.

Different honey bee populations may have developed their own specific resistance mechanisms tailored to match the challenge of the environment they are located in, including the locally prevailing combination of pathogens and pathogen variants. These mechanisms may not work as effectively in a different environment (Figure 1). Recent evidence demonstrates that local populations of bees show better survival in the presence of pathogens than introduced bees, indicating the presence of genotype–environment interactions. In consequence, genotype–environment interactions should be considered in research efforts to develop sustainable solutions for the improvement of honey bee health in Europe (Box 1).

### Conflict of interest
The authors declare that there are no conflicts of interest.

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### References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


This study describes the development of a sustainable treatment strategy against Varroa destructor which is adapted to common beekeeping situations. Although conventional control measures can be used, selection pressure towards increased mite tolerance is exerted. Based on a feasible treatment threshold, death of highly infested colonies was mimicked by treatment and requeening of heavily infested colonies. Although one third of the colonies were routinely left untreated, it was possible to decrease mean mite infestation levels and maintain a stable bee population.


This study investigated parasites and pathogens in colonies of different genotypes in different environments across Europe. The results indicated a significant influence of location on the occurrence of all pathogens investigated (Varroa, Nosema and viruses). Varroa infestation rates varied extensively across locations, yet at each location colonies with below-medium infestation rates survived longer. Although the occurrence of pathogens in local and non-local colonies did not differ significantly, colonies of local origin survived significantly longer.


This study describes an experiment comparing the survival and performance of honey bee colonies, representing five subspecies and 16 different genotypes, in 20 apiaries across Europe. No chemical treatments were applied during the two and a half years of the study. On average, colonies with queens from local origin survived significantly longer compared to non-local origin, demonstrating strong genotype by environment interactions.


In the context of the Europe-wide genotype-environment interactions experiment, this study gives a detailed description of the development of parasite and pathogen levels in one selected apiary over more than two years. The results show that among the four genetic lines tested at this location, levels of Varroa mites and Nosema spores were consistently lower in the local bees compared to the non-local strains, as also observed for viral titers of DWV and ABPV. Six of ten local colonies survived until the end of the experiment, while all non-local ones except two (from two different strains) vanished.


The study analyse the development patterns of colonies belonging to 16 different genotypes and placed in areas grouped within six environmental clusters across Europe over a period of two years. Colonies placed in Southern Europe tend to have lower adult bee populations compared to colonies placed in colder conditions, while the brood population tends to be smaller in the North, thus reflecting the shorter longevity of bees in warmer climates and the shorter brood rearing period in the North. Both genotype and environment significantly affect colony development.

To investigate the efficacy of different treatments against Varroa mites, mite levels in four different regions of Italy were monitored over one season. Notable regional differences in mite population growth were observed and are discussed.


Varroa destructor plays a key role in honey bee mortalities, since uncontrolled infestation inevitably results in the death of the colony. The pathological effects of Varroa infestations are partly attributed to the association of the mite with several honeybee viruses, primarily deformed wing (DWV). The 10-year history of the expanding front of Varroa infestation in New Zealand offered a rare opportunity to assess the dynamic quantitative and qualitative changes in honeybee viral landscapes in response to the arrival, spread and level of Varroa infestation.


This study investigated virus infections in relation to Varroa mite infestation in a naturally mite-resistant honey bee population on the island of Gotland. From the observation that colonies of the mite resistant population are able to overwinter in spite of high autumn DWV infection titers the authors conclude that they probably also developed resistance mechanisms against DWV.


References Box 1: G×E


Overwintering honey bees: biology and management
Mehmet Ali Döke, Maryann Frazier and Christina M Grozinger

In temperate climates, honey bees (Apis mellifera) survive the winter by entering a distinct physiological and behavioral state. In recent years, beekeepers are reporting unsustainably high colony losses during the winter, which have been linked to parasitization by Varroa mites, virus infections, geographic location, and variation across honey bee genotypes. Here, we review literature on environmental, physiological, and social factors regulating entrance, maintenance, and exit from the overwintering state in honey bees in temperate regions and develop a testable model to explain how multiple factors may be acting synergistically to regulate this complex transition. We also review existing knowledge of the factors affecting overwintering survival in honey bees and providing suggestions to beekeepers aiming to improve their colonies’ overwintering success.

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Introduction
Honey bees (Apis mellifera) live in a wide geographic range occupying various climatic regions and facing different challenges in different parts of the world and times of the year [1]. Winter is the greatest challenge to the honey bee colonies in temperate regions. The honey bee is one of the few insect species that is adapted to survive winter conditions without becoming completely dormant, entering a distinct physiological and behavioral state [2]. Before Varroa and tracheal mites were introduced, overwintering colony losses were ~10% [77]. However, an average loss of ~30% winter loss was reported by the US beekeepers in surveys since 2006, compromising sustainability of commercial beekeeping operations [37,49]. Honey bees provide critical pollination services for natural and agro-ecosystems world-wide. Successful overwintering of honey bee colonies is critical to meet the pollination needs of early spring-blooming crops such as almonds, apples and cherries. Indeed, the February/March California bloom requires 1.7 of the 2.5 million US honey bee colonies to pollinate 860 thousand acres of almonds, and this demand is projected to continue to rise. This demand could be met, at least in part, by improved overwintering: a 10% reduction in overwintering loss could provide an additional 250,000 colonies for early spring pollination.

The overwintering state of a honey bee colony is characterized by changes in the behavior and physiology of individual bees, including reduced individual activity, changes in endocrine profiles, increased nutrient stores and increased longevity, as well as changes at the colony level, including cessation of brood rearing and most remarkably, formation of a thermoregulating cluster. In non-temperate parts of the world, there are seasonal factors other than winter (i.e. dry, rainy, or hot periods with little forage available) seem to trigger a broodless state followed by absconding of the nest site in search of a new one [78]. While certain aspects of the two life cycle events are similar (i.e. lack of forage, decreased/halted foraging, and cessation of brood rearing), overwintering provides a unique challenge for the honey bees to overcome; extreme cold.

Previous studies have demonstrated that levels of Varroa mites, viruses, geographic location, and genotype are correlated with winter colony losses [4–9]. However, the effects of these factors on overwintering bees at the molecular, physiological and behavioral level have not been comprehensively studied, making it challenging to develop better management approaches to improve overwintering success. Here, we describe our existing knowledge of the environmental and physiological factors regulating entrance, maintenance, and exit from the overwintering state in honey bees in temperate regions. We then discuss factors associated with overwintering losses. Finally, we synthesize this information to provide recommendations for beekeepers to maximize overwintering survival and highlight areas of future investigation.

Overview of the annual colony cycle in temperate regions
Honey bee colonies exhibit distinct seasonal states (see Figure 1) [2]. In temperate climates, brood rearing starts in winter (when the average maximum ambient temperature is as low as 4 °C), peaks in spring, decreases through summer, and ceases in early fall [6,10,11**,76]. Brood build-up in the spring typically leads to swarming, where the majority of the workers leave the colony with the old queen in search of a new nest site, leaving behind a new
Overwintering behavior and physiology of worker honey bees

The behavior of individual bees in the colony also changes dramatically as the colony moves through the different seasons [17]. In late spring, summer, and early fall, workers are short-lived (~30 days) and exhibit an age-based division of labor. The youngest bees, generally <10 day old, perform nursing tasks, middle age bees between 10 and 20 days old engage in tasks such as comb building, food storage, guarding and undertaking, while the oldest bees in the colony serve as foragers [18]. In the fall, as brood rearing declines, the long-lived (up to 8 months) bees that will survive the winter are produced (hereafter referred to as ‘winter bees’) [19**]. These winter bees form the thermoregulating cluster when temperatures drop. Once brood rearing re-initiates in late winter/early spring, the division of labor resumes among overwintered worker bees [10].

In addition to these profound differences in behavior, honey bees also exhibit dramatic physiological changes across the seasons. Levels of juvenile hormone (JH), vitellogenin (Vg), and hemolymph proteins as well as the size of hypopharyngeal gland (HPG) are correlated with and regulate each other, and vary significantly between nurse, forager, and winter bees (see Figure 2). Briefly, JH levels are low in nurse and winter bees, and higher in foragers. In contrast, levels of Vg and hemolymph proteins are significantly higher and HPG is larger in nurses and winter bees than foragers.

Though the physiological differences in nurse bees, forager bees and winter bees are well documented, few studies have examined changes in physiology of bees over the course of the winter. Flur et al. [19**] marked newly emerged bees in fall (beginning of September) and then sampled them in January and February to compare levels of JH, Vg, total protein, and HPG weight. Interestingly, there were no significant differences between the bees in fall and mid-winter or late-winter. These results suggest that bees produced in the fall are already in the ‘winter’ physiological state, and this state remains stable throughout the winter. Using a broader range of timepoints, Huang and Robinson [20] found that the JH biosynthesis rate decreases from early October to mid-November, reaches its lowest level in mid-January, and then steeply increases in February and March. The early spring rise in JH titers correlate with a decrease in Vg levels, hemolymph protein levels, and a HPG size; thus, bees that overwintered return to the forager bee physiological state in spring [19**].

Factors that trigger transition to and exit from the overwintering state

Several environmental cues associated with seasonal changes, such as photoperiod, temperature, and nutritional state, have been examined to determine if these trigger
seasonal changes in worker behavior and physiology and overall colony behavior. Cherednikov [21] found that artificially shortening the day length in late spring and summer causes several changes in treated colonies versus control colonies, including: cannibalization of brood and eggs, increased collection and consumption of nectar and pollen, greater accumulation of fatty tissues in workers, increased resistance of workers to cold and starvation, and aggregation at ambient temperatures as high as 18–20 °C. Fluri and Bogdanov [22] confirmed that shortened photoperiod causes an increase in both lipid and protein content of the worker bees’ fat body (the tissue which primarily regulates metabolism and nutrient storage), resulting in a more ‘winter-like’ physiology in worker honey bees. However, artificial shortening of photoperiod did not result in the appearance of long-lived winter bees in Fluri and Bogdanov’s study, and Cherednikov did not measure this variable. Since decreasing the daylight hours would also result in restricting foraging behavior (which is likely an important factor in triggering the production of winter bees, see below), it would be valuable to test the effects of artificially lengthening daylight period to determine if this delays the timing of production of winter bees in the fall.

Temperature has also been investigated as a factor that can trigger overwintering changes in bees. Moving a summer colony to a cold room resulted in a significant decrease in both biosynthesis rates and titers of JH in foragers within 8 days [20]. However, it was not explored if this was a direct result of temperature and/or photoperiod cues (these colonies were also kept in the dark) or an indirect effect due to associated decreases in nutrition, brood rearing, or restricted foraging activity. It is unlikely that a rise in the ambient temperature serves as the environmental cue to commence the brood rearing after the winter break, since brood rearing stops in October and November but restarts in the much colder December and January [23]. However, it is possible that different factors serve to trigger the production of overwintering bees and the exit from overwintering in the spring. Again, experiments that uncouple light and temperature cues and monitor impacts on the timing of winter bee production can greatly improve our understanding of the regulation of this process.

Changes in availability of nutritional resources can shift the timing of the entry to and exit from the overwintering state. Increasing pollen stores through supplementary feeding or restricting pollen stores by placing pollen traps across the colony entrances will, respectively, delay or accelerate the timing of the production of long-lived winter bees relative to control colonies [24**]. Supplementing colonies with pollen in the spring also stimulates brood production [25**]. However, though nectar and pollen can vary widely in nutritional quality and diverse floral resources are optimal for honey bees and other pollinators (Vaudo et al., in this issue), the effects of nutritional quality on overwintering behavior and success remain to be determined.

These effects of nutrition may be due to indirect effects on brood production. Pollen serves as the primary source of protein and lipids, which are critical for brood rearing [14]. Honey bees adjust the rate of brood rearing according to the availability of protein resources and cease reproduction when they are completely deprived [24**,26,27]. When broodless winter colonies were transferred into a flight room and fed pollen, the queens immediately initiated egg-laying and workers activated their HPGs in 3–4 days [28]. Moreover, workers in free-flying colonies in the summer will physiologically resemble winter bees when the colonies are made broodless [19**,28–30]. Thus, lack of brood alone can induce colonies to transition to their wintering physiological state, regardless of photoperiod, temperature, or availability of floral resources.
It is unclear how brood triggers the winter-like physiological changes in worker bees. Bühler et al. [31] demonstrated that when a micro-climate resembling the brood nest (35 °C, 1.5% CO₂) is artificially created in broodless colonies, JH titers of workers rapidly rise. Additionally, the presence of brood can change the circadian rhythm of worker bees; broodless colonies exhibit a prominent circadian rhythm in their activity, while colonies with brood are less rhythmic [32,33]. Developing larvae also produce brood pheromone, which elicits a multitude of behavioral and physiological responses in worker bees and could be a key factor in overwintering behavior (reviewed in Grozinger, in press). Brood pheromone triggers pollen foraging by forager bees, which, as noted above, will in turn stimulate brood rearing and the production of brood pheromone [34,35].

Brood pheromone and its components can accelerate maturation and the transition to foraging in middle aged bees [36,37]. If bees are reared in the absence of brood or brood pheromone, they have higher levels of Vg when they are middle-aged (7–20 days old), initiate foraging later, and live longer, even under winter-like conditions [38**]. Thus, it seems likely that brood pheromone exposure prior to the production of overwintering bees or after brood rearing is initiated in the spring would be beneficial (because it stimulates brood production and colony growth), while brood pheromone exposure during the middle of the winter may trigger early maturation of the winter bees and be detrimental.

Foragers also release a pheromone (ethyl oleate) which, like brood pheromone, impacts behavioral maturation of young bees. However, ethyl oleate will slow down the transition to foraging, and maintain bees in the nursing state longer [39,40]. Restricting the flight of foragers so they remain in the colony will slow behavioral maturation of young bees, presumably by increasing their exposure to ethyl oleate [41,42].

Integrating the available information, we suggest that the entry and exit to the overwintering state is mediated by interactions between environmental nutritional resources, brood/brood pheromone levels, forager pheromone, and potentially temperature and photoperiod cues. During the fall, there is a decrease in available foraging resources which, in combination with days getting shorter and colder, results in a decrease in foraging effort in colonies. This should result in more foragers staying in the colony, and an increase in the exposure of young workers to ethyl oleate, which should slow their behavioral maturation. Additionally, the reduction in pollen foraging should decrease levels of brood production and brood pheromone, which also slows the behavioral maturation of workers. With reduced brood pheromone, foraging is further reduced, further amplifying the effects of ethyl oleate. In the late winter/early spring, day length increases sufficiently to trigger the production of a small amount of brood. The presence of brood pheromone stimulates behavioral maturation in some of the worker bees, results in the generation of a foraging force reversing the process that has happened in fall. Brood pheromone also stimulates the collection of pollen by these foragers, once temperatures are high enough. The influx of pollen into the colony further stimulates brood production and facilitates rapid colony growth in spring.

Factors impacting overwintering losses

Multiple factors can impact colony survival during the winter [73]. Based on surveys of US beekeepers, reasons for winter colony losses vary by operation size: backyard beekeepers generally blame weak colonies in fall, starvation, queen failure and Varroa mites, while commercial beekeepers blame queen failure, Varroa mites, pesticides and colony collapse disorder [43]. Furthermore, colony loss during winter is not uniform through US, with some geographic regions experiencing greater losses than others. However, the different average winter losses among states cannot be fully explained by latitudinal differences [43]. Below, we review recent research studies that sought to examine several of these factors and their correlations with colony loss, and recommend management practices to mitigate the effects of these factors.

Large-scale assessments of beekeeper-managed colonies in Canada [44] and Germany [6] found that levels of Varroa mites most strongly correlated with overwintering losses (also see [74]). Varroa mites are ectoparasites of honey bees which feed on the hemolymph of developing pupae [45]. Varroa feeding negatively impacts many of the physiological changes associated with overwintering, including decreasing Vg titers [46], reducing abdominal protein and carbohydrate levels [47], and reducing lifespan in both summer and winter bees [48]. Smaller experimental studies found a significant reduction in overwintering losses when colonies are treated with miticides to reduce Varroa levels [7–9]. Self-reporting by beekeepers indicated that beekeepers that employed Varroa mite control products had ~40% loss while those who did not experienced ~60% loss [49]. However, miticide use to control Varroa can also negatively affect bees [50], and thus we recommend using an ‘integrated pest management’ approach to regulate Varroa mite populations and improve overwintering success (see recommendation 3).

In studies in which viral infections were monitored, infection with deformed wing virus (DWV), Israeli Acute Paralysis virus (IAPV), and acute bee paralysis virus (ABPV) were also strongly correlated with colony survival [6,7,9,51,75]. Varroa mites transmit viruses and reduce their host’s immunocompetence, resulting in increased viral loads [52–54]. Thus, it remains to be determined to what extent the correlations between Varroa loads and
colonies losses are due to the effects of *Varroa* or viruses. Viruses alone, however, have been shown to have dramatic impacts on honey bee physiology and behavior, including accelerated maturation, though effects on nutritional stores, Vg levels, and other aspects of overwintering physiology have not been fully examined ([75], reviewed in McMenamin and Generisch, in this issue; Flenniken et al., in this issue). To reduce viral titers and protect immunocompetence of honey bees, beekeepers should aim to minimize *Varroa* mite populations and reduce exposure to other stressors, such as pesticides, which can increase viral titers (see recommendations 3 & 5) [55].

The genetic background (genotype) of the colony may also influence overwintering success. In a large study spanning 16 different genetic stocks and 20 apiaries across Europe, survival was significantly impacted by both location and genotype [56**]. Furthermore, colonies headed by local queens survived an average of 83 days longer than colonies headed by non-local queens. Thus, the results suggest there can be local adaptation of genotypes to their environments. However, while there is evidence for distinct populations/subspecies of honey bees in Europe [57], US honey bee populations are likely more genetically heterogeneous, given extensive migratory beekeeping practices (more than 60% of US colonies are moved to California for almond pollination, for example) and the nationwide shipping of thousands of queens and package bees produced in the southern US and California. Recent studies from our group have not found any evidence for local adaptation of honey bees stocks to winter conditions (Doke, Frazier and Grozinger, in prep). However, studies from a SARE-funded farmer grant conducted in Maine, compared 50 colonies established from package bees, half requeened with local survivor stock while the other half were not requeened. Eighty percent of the requeened colonies survived the winter while only 28% of those that were not requeened survived. These results are consistent with two previous studies by the same group [58]. However, it is unclear if the effects are due to local adaption of the survivor stock, or simply that queens produced by local beekeeping operations are of higher quality than those produced by large-scale commercial rearing operations. Indeed, there can be substantial queen effects on the entry into the overwintering state and colony winter survival as requeening in mid-summer effects when the winter bees are first observed in colonies [11**]. Thus, if beekeepers need to requeen their colonies, they should consider requeening during summer using queens from high quality stocks and breeders (see recommendations 1 & 2).

Colony size and nutritional stores are also expected to impact colony survival. Larger colonies use their stored food more efficiently as *per capita* food consumption is lower in large colonies compared to small ones [59] and colonies which are larger entering the winter are more likely to be successful exiting the winter and ultimately produce greater annual honey yields (Farrar, 1952 as reviewed in [6]). Indeed, recent studies from our group showed that fall weight was an important indicator of winter survival; 90% of colonies ≥22.5 kg survived, while only 27% of those ≤18 kg survived (weights expressed here exclude the weight of woodenware associated with hives; Doke et al., unpublished data). Interestingly, supplementing or restricting colonies’ access to pollen in fall do not impact the number of winter bees produced, their physiological nutritional stores, or their performance [27,60**], and thus factors other than fall nutrition (perhaps genotype and/or parasite/pathogen loads and/or resource availability throughout the season) seem to regulate the population size and performance of winter bees [73]. Thus, beekeepers should facilitate brood rearing throughout the spring, summer and fall by maintaining colonies in nutrient rich locations and/or supplementing nutrition, and combine small colonies early in the fall. Additionally, when colonies are treated with synthetic brood pheromone in fall, they consume more protein supplement and brood production and colony growth increase [61]. Year-long brood pheromone treatment of colonies results in increased honey production, colony size and overwintering success [62]. Thus, if it is not feasible to support the colonies through these means, brood pheromone treatment may be an option to facilitate colony growth and increase chances of successful overwintering (see recommendation 1).

Honey bee exposure to pesticides has recently been better documented ([63–65]; Mullin, 2015, Berenbaum, in this issue; Long and Krupke, in this issue). Mullin et al. [63] found miticides used for *Varroa* control to be the most prevalent residues in wax, enhancing the chances of resistance in *Varroa* against these chemicals while these and other identified agrochemicals increase the potential for pesticide contamination of hive products. However, fungicides were found at the highest levels in pollen and residues of all pesticide classes were found in samples of wax and pollen. The impacts of pesticide (active ingredients and formulation materials) exposure have been associated with numerous sublethal effects including reduced longevity, impaired immune function, learning and memory, orientation, foraging and motor coordination [66–70]. While these impacts likely compromise the ability of colonies to successfully overwinter, little work has been done to examine pesticide effects on overwintering success. Nonetheless beekeepers should attempt to minimize honey bee colony exposure to pesticides (see recommendation 5).

Conclusions and future directions

Overwintering in honey bees is a complex process, which integrates multiple environmental cues, social cues and interactions within the colony, and physiological and molecular changes in individual bees. Using the available
information, we have developed a model which explains how the entry, maintenance and exit from overwintering may be regulated by these factors (see Figure 3), but further studies are necessary to comprehensively test this model by uncoupling and individually testing these factors, many of which are closely correlated. Furthermore, it remains to be determined whether ‘winter bee’ development is triggered during larval stages or adult stages, or both. We have also developed recommendations that should improve overwintering survival, some of which are well tested (integrated pest management approaches to *Varroa* control), while others remain to be fully explored (effects of brood pheromone exposure and pesticides). With the development of genomic tools and approaches that will allow us to understand how and when the genome of individual bees responds to seasonal cues and conditions (Grozinger and Robinson, in this issue), our understanding of this complex process can be vastly improved, and with it our ability to better manage honey bee colonies.

**From the bench to the beehive: science-based recommendations for best management practices for beekeepers**

Efforts to reduce overwintering losses should focus on enhancing colony strength and food stores in fall, improving queen quality and protecting bees from *Varroa* mites, pathogens and pesticides.

1. **Only strong colonies with large populations of workers and abundant food stores should be overwintered.** Weak colonies, showing no symptoms of disease, should be combined early in the season. To build strong colonies that are both productive and well-positioned to survive winter, select apiary locations with abundant and diverse sources of pollen and nectar throughout the season. Sufficient stores of honey and pollen are vital and quantities needed for overwintering vary depending on geographic location. If honey stores are inadequate, colonies can be fed a 2:1 (granulated sucrose: water) syrup in early fall to bring them up to a desired weight while minimizing the energy spent by the workers for removal of moisture, which would be the case if a less concentrated syrup was preferred (e.g. 1:1). High-quality pollen substitutes are also available if pollen is in short supply. However, diverse, pesticide-free, natural pollen is considered optimal.

2. **Only colonies with young, high-quality queens should be overwintered.** Poor-laying or old queens should be replaced with young queens, ideally reared from *Varroa* resistant or survivor stock, prior to winter.

3. **Varroa mites should be monitored and controlled using Integrated Pest Management (IPM) tactics.** Resistant or survivor stocks can be considered a first line of defense. Other techniques such drone brood removal and screen bottom boards have delivered mixed results but are likely advantageous when integrated as part of an overall IPM approach. Monitor mite levels throughout the season using alcohol or soapy water wash, sugar rolls or sticky boards and keep mites below an established threshold. Thresholds will vary depending geographic location, beekeeper tolerance for chemical use as well as risk of losing colonies. In general, if mite levels reach a threshold of ≥5–10 mites per 300 bees in mid-late summer, a control chemical should be considered, before the winter bee population is produced. There is evidence of widespread resistance to coumaphos [71] and fluvalinate [72], for this reason beekeepers are encouraged to consider other alternatives.

4. **Wind breaks and insulation can be beneficial as long as proper ventilation is provided.** Honey bee colonies require varying degrees of protection because winter conditions vary dramatically across the US. Wind breaks are advantageous in locations with prominent cold winter winds. In geographic regions with long harsh winters, overwintering can be improved when colonies are wrapped, insulated or overwintered indoors [79]. Other considerations include providing good hive ventilation to reduce moisture build-up and condensation and entrance screens of ¼ in. hardware cloth to keep our invading mice.

5. **All beekeepers, but particularly those engaged in crop pollination, should take steps to protect bees from pesticide exposure, including those used for mite control.** If colonies are used for pollinating multiple crops, allow them to recover on high-quality forage between pollination contracts or consider a rotation scheme where half are pollinating while half are
allowed to recover, then reverse. Feeding a high-quality pollen substitute during crop pollination may dilute the effects of pesticide-tainted pollen. Good communication with growers and pesticide applicators is critical to minimize pesticide exposure. Additionally, reducing the excessive use of Varroa control chemical and eliminating use of off-label products will minimize their build-up in wax. Lastly, all beekeepers should have a comb-culling program in place to minimize the build-up of pesticides and pathogens.

Efforts to improve overwintering success of honey bee colonies will require the attention of beekeepers from the time colonies come out of winter in early spring until they are ‘put to bed’ for winter. Additionally, these efforts will depend upon on-going research to develop a deeper understanding of the complexities of honey bee overwintering behavior and physiology.

Acknowledgements

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

● of special interest

●● of outstanding interest


Authors demonstrated that many of the physiological characteristics of winter bees resemble those of nurse bees, and these winter bee characteristics are already found in bees reared early in the fall.

24. Mattila HR, Otis GW: Dwindling pollen resources trigger the transition to broodless populations of long-lived honeybees each autumn. Ecol Entomol 2007, 32:496-505. This study demonstrated that timing of transition from summer bees to winter bees can be influenced by pollen availability.

This study demonstrated the importance of protein supply and the negative effect of limited protein on the rapid increase colony size of honey bees in spring.


This study demonstrated that brood pheromone exposure alone can reduce the longevity and reduce ‘winter bee’ physiological characteristics.


This study demonstrated substantial effects of genotype (stock) and environment on colony survival, and demonstrated that, at least in Europe, there appear to be locally adapted stocks that have improved performance.


Mattila HR, Otis GW: Manipulating pollen supply in honey bee colonies during the fall does not affect the performance of winter bees. Can Entomol 2007, 139:544-563.

This study demonstrated that pollen influx in fall does not affect colony and growth in the consecutive spring.


Mullin CA, Frazier M, Frazier JL, Ashcraft S, Simonds R, vanEngelsdorp D, Pettis JS: High levels of miticides and...


Honey bees are sensitive to widespread co-formulants used in agrochemicals, and evaluation of the role of these ‘inerts or inactives’ in pollinator decline is only in its formative stages. Lack of disclosure of formulation ingredients in major products and lack of adequate methods for their analysis constrain the assessment of total chemical load and agrochemical exposures on bees. Most studies to document pesticide effects on honey bees are performed without the formulation or other relevant spray adjuvant components used to environmentally apply the toxicant. Formulations are generally more toxic than active ingredients, particularly fungicides, by up to 26,000-fold based on published literature. Some ‘inactive’ candidates for future risk assessment for pollinators include the organosilicone surfactants and the co-solvent N-methyl-2-pyrrolidone.

Introduction

Over 150 different pesticides have been found in beehive samples from apiaries worldwide [1–4], most frequently miticides to control Varroa destructor Anderson & Trueman, crop fungicides and the neonicotinoids. While pesticides have acute, chronic and sublethal effects on bees, no correlation has been found between residues of any one pesticide and pollinator decline [1–6]. However, pesticides are not introduced into the environment as pure active ingredients. There is mounting evidence that inert ingredients can also be toxic to pollinators, and formulations often have higher levels of toxicity than active ingredients. Common ‘inactive’ ingredients or co-formulants are more generic in their multiple uses among formulations and tank mix adjuvants. The role of these agrochemical ‘inerts or inactives’ in the ongoing investigation of pollinator decline has only begun to be investigated [7**].

Modern agrochemical formulations including seed treatments and spray tank additives comprise an average of 10 ingredients including the active ingredient (AI) and other components termed inerts, co-formulants or adjuvants [8,9] (JD Fowler, abstract ACS AGRO 384, 13th IUPAC Congress of Pesticide Chemistry, San Francisco, CA, August 2014). Numerous studies have found that pesticide AIs elicit very different physiological effects on non-target organisms when combined with their formulation ingredients [7**,10]. These formulation surfactants, penetrant enhancers, spreaders, stickers, and co-solvents serve to optimize the pest control efficacy and stability of the AIs. Typical formulations (Figure 1) contain less than 50% AIs, combined with newer technologies including polyethoxylated tallow amines, organosilicone ethoxylates and co-solvents such as N-methyl-2-pyrrolidone (NMP) [7**].

While pesticides and associated agrochemicals are generally recognized as contributors to pollinator decline, the uncertainty to what extent they are involved is due to lack of reliable information on all the agrochemical residues and chemical participants to which bees are exposed. Lack of disclosure of formulation ingredients in major products and lack of adequate methods for their analysis constrain the assessment of total chemical load and agrochemical exposures on bees. Members of the agrochemical industry are usually unwilling participants in publicizing the needed data [8,11]. Regulators, in turn, are constrained by the liabilities associated with open access to what is deemed proprietary information in the course of registration of inert ingredients and their incorporation into confidential statements of formulations from registrants used to support approval of agrochemical products [11]. Concerning bee declines and pesticides, we need to think holistically about all the chemical ingredients and their combined effects.

Formulations can have greater toxicity than active ingredients

Dramatic impacts of formulators on the bee toxicity of AIs for agrochemical products have been documented (Table 1). An orally ingested formulation (Vertimec 18 EC) of the insecticide abamectin was 8970-times more toxic to the stingless bee Melipona quadrifasciata and 709-times more toxic to the honey bee than the topically applied AI in acetone [12]. Similar, although less extreme, adult bee toxicity differences were found between a formulation and AI of the pyrethroid deltamethrin (Decis 25 EC) and the organophosphate methamidophos (Tamaran BR). A direct topical or oral comparison using the formulation alone would have aided the assessment
Co-formulant disclosure will optimize future for modern agrochemicals. MSDS details for an older and more recent formulation for glyphosate are shown. The revealing of NMP and ethoxylated tallow amines in the newer formulation will guide risk assessment for pollinators and other sensitive non-target species such as aquatic organisms. Knowledge of amounts of co-formulants in products will stimulate development of tools to environmentally monitor residues and determine their relative hazard to bees. Chemical structures are from ChemSpider (http://www.chemspider.com).

[12°]. This is rarely performed since industry does not usually provide a formulation blank lacking only the AI because it is proprietary [personal communications]. The Institute of the Control of Agrochemicals, Ministry of Agriculture, China also has published extraordinary results (Table 1) that honey bee oral toxicity of the common fungicide tebuconazole can be equivalent to the most bee-toxic insecticide known, enamectin benzoate

Table 1

<table>
<thead>
<tr>
<th>Formulation/active ingredient</th>
<th>Exposure route</th>
<th>LD50 (µg ai/bee)</th>
<th>Fold-difference</th>
<th>Pesticide class</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vertimec 18 EC</td>
<td>Oral</td>
<td>0.011</td>
<td>709</td>
<td>Insecticide</td>
<td>[12°]</td>
</tr>
<tr>
<td>Abamectin</td>
<td>Topical</td>
<td>7.800</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decis 25 EC</td>
<td>Oral</td>
<td>0.85</td>
<td>132</td>
<td>Insecticide</td>
<td>[12°]</td>
</tr>
<tr>
<td>Deltamethrin</td>
<td>Topical</td>
<td>11.20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tamaron BR</td>
<td>Oral</td>
<td>3.7</td>
<td>110</td>
<td>Insecticide</td>
<td>[12°]</td>
</tr>
<tr>
<td>Methamidophos</td>
<td>Topical</td>
<td>408.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taktic EC</td>
<td>Oral</td>
<td>28</td>
<td>&gt;4</td>
<td>Miticide</td>
<td></td>
</tr>
<tr>
<td>Amitraz</td>
<td>Oral</td>
<td>&gt;103</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Undisclosed formulations most toxic/least toxic

<table>
<thead>
<tr>
<th>Formulation/active ingredient</th>
<th>Exposure route</th>
<th>LC50 (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tebuconazole 25% EC</td>
<td>Oral</td>
<td>0.039</td>
</tr>
<tr>
<td>Tebuconazole 5% SC</td>
<td>Oral</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Buprofezin 65% WP</td>
<td>Oral</td>
<td>7.00</td>
</tr>
<tr>
<td>Buprofezin 25% SC</td>
<td>Oral</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Enamectin benzoate 1% EC</td>
<td>Oral</td>
<td>0.09</td>
</tr>
<tr>
<td>Enamectin benzoate 1% WP</td>
<td>Oral</td>
<td>0.90</td>
</tr>
<tr>
<td>Enamectin benzoate 25% SC</td>
<td>Oral</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Enamectin benzoate 5% SC</td>
<td>Oral</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Enamectin benzoate 1% WP</td>
<td>Oral</td>
<td>0.09</td>
</tr>
<tr>
<td>Enamectin benzoate 1% WC</td>
<td>Oral</td>
<td>0.90</td>
</tr>
</tbody>
</table>
able typically bee ity such published spray ed of Intercida (LD50 = 196 [13]. Current formulations, for example, intended for use on crops such as corn, provide little or no protection to bees. The general toxicology of glyphosate and other commonly used insecticides is well documented [14].

Less dramatic or even neutral toxic effects of formulations relative to AIs on bees have been noted (Table 1). Examples include the low toxicity of neonicotinoid insecticides such as imidacloprid and the fungicide chlorothalonil when used in 1.6F formulations [15]. However, a comprehensive reanalysis of this equivalency claim should be undertaken.

Most studies to document pesticide effects on honey bees are performed without the formulation or other relevant spray adjuvant components used to environmentally apply the AI, most often due to lack of such required tests for product registration [14]. The highest pesticide AI residue found in honey bee pollen to date was recently published [17]. In an enclosed tunnel experiment, a soluble concentrate formulation of glyphosate isopropyl amine sprayed at a single-application field rate on flowering Phacelia resulted in glyphosate acid equivalent residues in trapped pollen of foraging honey bees of up to 629 mg/kg (ppm). This is much higher than other reported pollen residues for other pesticide AIs [14]. The authors then conducted feeding studies on honey bee colonies with only the AI using equivalent doses in sucrose, and compared its effects with a fenoxycarb formulation as a toxic insecticide reference, and reported no significant effects of the herbicide [17]. The parallel study with the glyphosate formulation (Figure 1) was not compared. The formulation, although undisclosed, would typically be about 50% of glyphosate acid equivalent (~300 ppm) as the MON 0818 tallow amine cationic surfactant [18]. While technical glyphosate has virtually no toxicity for honey bees, common formulations such as Weathermax® do [19]. Indeed, glyphosate has negligible ecotoxicity and systemic movement without tallow amines and other adjuvants [20]. Glyphosate toxicity on human cells is similarly dependent on these co-formulants [21].

For glyphosate, the number one pesticide used globally, the distinct requirements for its trace analysis at high cost precludes its inclusion in routine multi-residue analyses [18]. This insufficiency in publically available residue data hinders efforts to refute claims of its association with widespread human mortality from conditions such as chronic kidney disease [22]. Glyphosate and its combinations with 2,4-D or dicamba will continue to dominate pesticide loads on crop ecosystems in new formulations that match the stacked-herbicide tolerant genetically modified corn and soybean technologies now being commercialized. These new binary herbicide formulations will include NMP and tallow amines (Figure 1), supplemented by tank mixing with organosilicone adjuvants to improve pesticide application and product efficacy.

**Toxic and behavioral effects of formulation inerts or spray adjuvants on honey bees**

Considerable recent progress has been made on surveying the prevalence of pesticide AIs within hives [1–3] and on improving the active-ingredient risk assessment for honey bees [23,24]. However, little work has been done to examine the safety to pollinators of agrochemical formulations and spray adjuvants, and to measure their relevant residue concentrations in bee environments. Some nonionic, organosilicone and other surfactant sprays and adjuvants in 0.1% aqueous solutions have been shown to deter or kill honey bees, and can be used alone to manage unwanted feral or Africanized honey bees [25–28]. While the organosilicone adjuvant Break-Thru fed to nurse bees at 200 ppm did not indirectly impact queen-rearing [29], much higher toxicity occurs when honey bees are fed directly on related organosilicone surfactants in 50% sucrose, with oral LC50s for pure commercial trisiloxane surfactants ranging to below 10 ppm, and significant mortality down to 100 ppb [7**] (J Chen et al., unpublished). Inadequate formulation stickers/lubricants present in neonicotinoid seed treatments have led to toxic airborne dusts during planting and concomitant bee kills in European countries, US and Canada [30,31**]. The penetration-enhancing formulation solvent NMP has high oral toxicity to honey bee larvae down to a concentration of 0.01% (100 ppm), after 4 days feeding on a royal jelly diet [32]. The adult honey bee is much more acutely susceptible than the adult Japanese hornfaced bee, Osmia cornifrons (Radoszkowski) to a formulated neonicotinoid imidacloprid (Provado 1.6F), while the opposite trend was found with another neonicotinoid acetamiprid (Assail 30SG) [33]. These
dissimilarities in topical toxicities may reflect intrinsic differences in species susceptibility, but distinguishing this from a more direct role of the other formulation ingredients in enhancing neonicotinoid penetration of the bee cuticles will require further testing. Bumble bees are highly susceptible to 1 ppm of a strong surfactant perfluorooctylsulfonic acid [34]. Clearly, hazards of agrochemicals to pollinators cannot be predicted if technical ingredients are tested alone without the formulation ingredients and if tested only on a single species.

Given the complex associative learning, communication and navigational skills required in bee foraging, sublethal and chronic effects of agrochemicals are especially important [35]. Classical conditioning proboscis extension reflex tests were conducted on adult bees after ingestion of 20 μg adjuvant in 50% sucrose [36]. All organosilicone surfactant adjuvants impaired learning more than other nonionic adjuvants, while all crop oil concentrates were inactive. Organosilicone surfactants are used up to 1% (10,000 ppm = 10 μg/μl) of the spray tank mix [36]. Thus organosilicone adjuvants at low concentrations independent of active ingredient(s) can impact the olfactory learning required for foraging. Organosilicones cause a greater reduction in surface tension than both nonionic surfactants and crop oil concentrates, making them extreme surfactants and super-penetrants and this action may lead to learning impairment in adult bees [36]. This potent surfactant activity can drive the stomatal uptake of even large bacterial-sized mineral particles [37], and thus may aid movement of pathogens into bee tissues.

Organosiloxane, nonyl-phenol and octyl-phenol polyethoxylates are widely used as nonionic surfactants around honey bee hives or in their foraging areas as spray adjuvants or additives in agrochemical formulations. Trisiloxane surfactants were detected in every wax sample and 60% of the pollen samples, while no trisiloxane surfactants were found in honey [38]. Total trisiloxane surfactant concentrations were up to 390 μg/kg (ppb) in wax and 38.8 ppb in pollen. Samples analyzed had no known association with bees foraging on crops sprayed with organosilicone adjuvants. Nonylphenol more than organosiloxane and octylphenol polyethoxylates were found in wax samples, while pollen and particularly honey residues were lower [39]. The wide occurrence of substantial amounts of alkylphenol ethoxylates and organosiloxane surfactants in US bee hives calls for a renewed effort to investigate their consequence to bee health and the ongoing global bee decline.

Billions of pounds of synthetic organic chemicals from all uses are released into US environments as formulation ingredients such as NMP and organosilicone or nonylphenol polyethoxylates, which are currently classified as GRAS, have no mandated tolerances, and their residues in the environment are largely unmonitored [7**]. Estimated global production of organosilicones in 2008 was greater than 10 billion pounds, of which 1.3 billion pounds were the surfactants, and their yearly use continues to increase [40]. These agrochemical ‘inerts = inactives’ found also in drug and personal care products represent an important component of the chemical landscape to which bees [7**] as well as humans [41] are exposed. The broad use of organosilicone surfactants as formulation adjuvants [42,43] suggests a high potential for environmental harm. These super-surfactants should readily move across membranes, become systemic in plants and animals and be degraded to silica [44] causing silicosis in sensitive tissues of exposed organisms.

Gaps in our knowledge

We have found that honey bees are poisoned by widespread co-formulants used in agrochemical formulations and spray tank adjuvants. Effects include learning impairment for adult bees and oral toxicity for adults and larvae. Relevant pesticide risk assessment for pollinators and other non-target species cannot be addressed solely by evaluating the actives without the concomitant formulation ingredients. Toxicity synergisms from agrochemical blends in the beehive often start from annual miticide choices made for Varroa control, and the co-occurring formulates based on products chosen will predictably influence bee poisoning outcomes [45*]. In-hive or foraged pollutants in pollen, nectar, and water will include proprietary adjuvants that greatly influence pesticide fate [46] in bee environments. We have found 100% of co-formulants analyzed for in beehive samples, while only 70% of pesticide active ingredients searched for have been detected [7**]. Documenting the formulation and spray adjuvants used to enable bee exposure to an active ingredient from field applications should facilitate analysis to identify specific pesticides associated with current bee declines.

Multiple factors including pesticide exposure, diseases, parasites and malnutrition are most often invoked to explain recent pollinator decline [5,47–50]. Part of the difficulty in assigning major factors for bee loss is the lack of defined benchmark criteria for a healthy bee. Being pesticide free, virus free, Nosema free, or not immunosuppressed is insufficient as starting points in risk assessment, since levels that define ‘free’ are constrained by sensitivity of the analytical method and its limit of detection. Defining a benchmark or reference dose, particularly for bee viruses [51], for what can be tolerated or considered acceptable to maintain bee health (or health of the bee ecosystem) will allow the development of risk assessment tools of adequate sensitivities to explore individual and combined factor interactions in endpoints that predict colony health or death. This is easier to address with pesticides and co-formulants since more precise measures (LD₅₀, ED₅₀) of defined lethal and sublethal endpoints can be determined in the laboratory and used for
predicting consequence of measured exposures or the combinatory effects of multiple agrochemical residues. A confounding factor for a bee disease such as *Nosema*, that is good nutrition can negate the effects of a high spore load [52]. Total agrochemical formulation exposures co-occur with relevant risk levels of other stressors including *Varroa*, viruses, *Nosema* and poor nutrition [5,47,50–54] require further evaluation.

More industry and regulatory agency disclosure of the chemical identity of agrochemical formulation components and their total production would greatly aid academic research to assess the safety for use around pollinators. Publically available bee toxicity data that includes all the presently used formulations [55] and recommended adjuvant technologies and not only the active ingredients would catalyze risk assessments by independent researchers. Amounts of specific named formulations and tank adjuvants applied by calendar date to the various crops of the US landscape would greatly aid in assessing chemical exposures and potential toxic burdens for honey bees and other generalist pollinator species. Honey bees as a model terrestrial indicator organism reveal that the formulation and not just the dose makes the poison. A needed goal is to continue exhorting industrial and regulatory scientists, and ecotoxicologists to include all formulations, and not just active ingredients, in studies to document the safety and low risk for pollinators and other non-target species of their agrochemical products prior to registration and use. Providing the ecotoxicological data used in risk assessments publicly to academic and other independent researchers would help foster consumer confidence for a safe outcome to their use.

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References


This is the first general review of impacts of presently recognized “inert or inactive” formulation and adjuvant ingredients on honey bees and other pollinators.

12. Del Sarto MCL, Oliveira EE, Guedes RNC, Campos LAO: Differential insecticide susceptibility of the neotropical stingless bee *Melipona quadrifasciata* and the honey bee *Apis mellifera*. *Apidologie* 2014, 45:626-636. This documents the huge increases of bee toxicity by oral exposure of formulations respectively of the active ingredient using the standard topical bioassay on adult bees.

Here a better approach to determining actual bees exposures from sprayed pesticides is presented, which demonstrates that the standard factor used in previous risk assessment models was too low by at least an order of magnitude. Secondarily reported is a strong correlation between mortality after spraying and mortality based on topical toxicity of pesticide active ingredients at the equivalent dose, suggesting a lack of formulation effects. However, the correlation did not include the practical use of non-toxic actives by topical assay since a dose could not be calculated. Most of the formulation-dependent impacts of actives on bee toxicity in the literature involve low toxicity pesticides, particularly fungicides.


This is a meritorious and typical example of industrial disclosure of impacts of agrochemicals on honey bees. While the largest pollen and nectar residues known for any active ingredient, in this case glyphosate, are revealed; impacts of the herbicide are only explored using the active ingredient. No residues of the highly ecotoxic formulant fatty acid amines are reported or is a standard formation of glyphosate evaluated for toxicity to honey bees though the positive toxic control used in these tests.
was another pesticide formulation. Tests to evaluate product toxicities to non-targets based only on the active ingredient are not sufficient.


   Reports the toxic effects of a glyphosate formulation on honey bees at field-relevant doses.


31. Bonmatin JM, Giorio C, Girolami V, Goulson D, Kreutzweiser DP,  
   This review reveals by citing patent literature that non-systemic actives such as fipronil can be rendered plant systemic dependent on co-formulants, exceedingly increasing its environmental consequence to bees. Formulations compared to actives also increase the leaching potential of neonicotinoids from soil into water.

   This is the first study to show toxic effects of a major, often undisclosed formulation solvent, NMP, on honey bee larvae.


   The general wide occurrence of the organosilicone surfactants (CAS Nos. 67674-67-3, 27306-78-1 and 125997-17-3) as residues in bee-hive samples was shown for the first time. This is the first recorded presence of the latter acetyl-capped organosilicone surfactant in the environment.


45. Johnson RM, Dahlgren L, Siegfried BD, Ellis MD: Acaricide,  
   A very good example of the potential for synergistic interactions among Als within in-hive treatments for routine mite and disease control, in addition, a formulant synergist, piperonyl butoxide, and some fungicides present in pesticide formulations applied where honey bees forage were also evaluated for toxicity interactions with the miticides.


47. Cox-Foster DL, Conlan S, Holmes EC, Palacios G, Evans JD, Moran NA, Quan PL, Briese T, Hornig M, Geisel DM et al.: A metagenomic survey of microbes in honey bee colony collapse disorder. Science 2007, 318:283-287.


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This agrochemical-industry sponsored assessment of a major-use organophosphate is commendable in that formulations, and not just the active ingredient, are evaluated. However, it does not disclose data on toxicity of all the modern formulations used in crop pest control worldwide. For example, microencapsulated (the more bee toxic) formulations are not assessed and citations for honey bee acute toxicity of formulations are mostly older, unpublished reports.
Integrating pest and pollinator management — adding a new dimension to an accepted paradigm

David J Biddinger and Edwin G Rajotte

In this chapter we argue that while pesticides can be harmful to pollinators, when they are used in an integrated pest and pollinator management (IPPM) context, both pest management and pollinator protection may be achieved. Our growing knowledge of the impacts of pesticides on honey bees as well as bumble bees and solitary bees allows us to use the latitude we have in pest management including non-pesticidal pest management practices, changing pesticide types and incorporating other, less susceptible pollinator species into commercial practice. Pollinator health should be a central component of integrated pest management research, education and extension to produce viable IPPM approaches.

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Pesticides and the current pollinator crisis
Insecticides, by definition and design, kill insects including pollinators if sufficient dosage and exposure levels are met. Plant systemic neonicotinoid insecticides in particular may affect bee health and may contribute to the decline of some species [1**]. In 2013, the European Commission imposed a two year moratorium on the use of some of these compounds [2] based on laboratory studies that demonstrated sublethal effects on honey bees or bumble bees [3**]. Polemics for and against neonicotinoid bans concentrate on extremes, but the solution lies somewhere in between. Neonicotinoid types are not equally toxic [1**,4*], and not all bee species are equally susceptible [5**]. Rather than banning neonicotinoids (or other pesticide types) as a class, we argue that we should modify pest management practices to include considerations for pollinator health.

Although laboratory-based studies can provide some information [6], appropriate field-realistic concentrations and formulations, as well as evaluating relevant short-term and long-term exposures and impacts are the best indicators of pollinator impacts [1**,3**,5**]. Acute exposures of only a few days also conflict with growing evidence for more subtle, sublethal effects on growth, reproduction and behavior from long-term chronic exposure at low doses [7**]. For example, the chitin inhibitor, novaluron, applied during bloom in almonds is not toxic to adult bees, but has sublethal effects on Osmia and honey bee reproduction [8]. The ‘field relevancy’ of some of the laboratory studies that led to the European Union ban of some neonicotinoids is hotly debated [3**,9**] and is difficult to gauge because most studies use a single dose rather than a range of doses to generate a response curve [9**]. Other considerations should include synergy of insecticides with fungicides and other mixtures [5**,10**] and the impact of acaricides and antimicrobial drugs used against hive pests. It is these products which are the most frequently found contaminants in honey bee hives and have also been shown to affect bee reproduction and health [11**].

Importance of neonicotinoids
Any pesticide has benefits and costs associated with it. Neonicotinoids were developed partly because of the Food Quality Protection Act (FQPA), which restricted organophosphate, organochlorine, and carbamate pesticides on public health grounds [12]. The generally vertebrate-safe neonicotinoids also contributed to pesticide resistance management by offering a different mode of action. But actions to ameliorate the perceived costs, such as the well-intentioned EU neonicotinoid ban, were based on the Substitution Principle (one set of compounds is replaced by newer, safer alternatives [13]), furthers the ‘pesticide treadmill’ [14], and could force growers to revert to the remaining older compounds, which have largely unknown pollinator impacts. Used judiciously, targeting pests at critical timings as in the following example in apple production, neonicotinoids can be effective while sparing pollinators as well as other beneficial organisms in conservation biological control programs [15].

Integrated Pest Management and pollinators
Integrated Pest Management (IPM) is a long standing, science based, decision making process whose ecological roots lie in the use of multiple biological, cultural, physical, and chemical tactics to protect crops in a way that
minimizes economic, health, and environmental risks. IPM can address any pest complex (insect, disease, weed, vertebrate, among others) and can be adapted to any agricultural production goals including conventional, sustainable and organic. In addition, IPM can evolve to meet new production demands such as pesticide use reduction, incorporation of ecosystem services and food safety [16]. Likewise, IPM can be adjusted to protect pollinator health just as it is adjusted to protect other beneficial organisms such as predators and parasitoids. Indeed, the IPM paradigm, already understood by growers, will facilitate adoption of pollinator protection practices.

Although well understood, relatively easy to maintain, mobile in large numbers, and can rapidly communicate food source locations [17], honey bees are not the best pollinators for all crops [18,19]. Sole reliance on honey bees can be risky. North American beekeepers lost 1/3 of their colonies due to Colony Collapse Disorder (CCD) [20] and other factors [21] including a general 40% decline since 1947 [22]. Recent colony scarcity increased rental costs three-fold prompting consideration of alternative pollinators in Pennsylvania tree fruit [23]. So, the IPPM challenge is integration in two dimensions: Integrating alternative pollinators into crop production and integrating the welfare of all pollinators into the IPM crop protection programs, which often include pesticide use.

The importance of pollinators in apple production
In southern Pennsylvania, apple farms are nestled in the rolling Appalachian Mountains among a patchwork of forest land and diverse agriculture. All cultivars require cross-pollination to ensure commercial, fresh market crops in which size and shape of the fruit is as important as yield, in contrast with other crops (e.g. almonds) where yield is maximized [24]. Unlike some apple production regions, mid-Atlantic U.S. apple pollination needs can be met by native bee species that occur in the landscape, and over half of Pennsylvania and New York apple growers do not rent honey bees [25,26*].

Reliance on non-honey bee, wild pollinators requires multiple species to provide the biological insurance for sustainable pollination. However knowledge of species’ distributions, pollination effectiveness, nutritional requirements, alternate food sources and nesting sites is needed in order to modify usually oversimplified agricultural landscapes. Of the 3500 bumble bee and solitary bee (or pollen bee) species in the US that are potential crop pollinators [27,28], the value of pollen bees alone is at least $3 billion annually [27]. Wild and managed pollen bees can supplement or replace honey bees, with each wild pollinator species within a crop pollination guild having its own life history traits, flower preferences and pollination usefulness. The early bloom of tree fruits (e.g. plum, apple, among others) requires species that overwinter as adults.

For apples these include univoltine, solitary species (e.g. *Osmia*) and multivoltine species (e.g. *Bombus*) [26*]. Of the 371 known bee species in Pennsylvania [29], over 180 occur in orchards during the growing season and 52 pollinate apple. Some such as *Osmia* can be 80 times more effective than honey bees [30].

Roles of landscape and floristic diversity in support of apple pollinators
The reliability of wild pollinators depends on habitat suitability, both in the orchard and in the surrounding countryside [31*]. In the Pennsylvania Appalachian mountains, orchards have steep slopes, well drained soils and a landscape matrix of approximately 8% orchards, 24% arable and pasture land, 9% developed area and 56% forests [32] and a continental climate [33]. The average two to four ha orchards are bordered by undeveloped scrub, forest, or fence rows where floristic diversity is correlated with pollinator communities. The forest edge/orchard border is the most species rich (169 out of 228 plant species recorded) and a significant predictor of bee species richness and abundance in the orchard [31*,34–36]. This floristic diversity is also attractive to beneficial predators and parasitoids [37].

IPM, pesticides and pollinators in apple
During the apple growing season, more than a dozen insect and mite pests [38,39], 8–10 fungal and bacterial diseases, and several vertebrate pests can attack the fruit and the trees [38]. Pennsylvania tree fruit IPM is an efficient and profitable combination of host plant resistance, biological control, sophisticated pest monitoring, and model-based pest predictions resulting in specifically-timed management practices [38]. Apple IPM has been modified to protect living IPM tools (biological control agents) [40–42,43*,44], and can be further modified to protect pollinators giving rise to IPPM.

Pesticide applications may include multiple types of insecticides, fungicides, bactericides, herbicides, surfactants and others, each having a toxicity profile and impact on various insect species. Bee health is affected by field exposure to pesticides. Most studies consider only short-term acute contact exposure to adult bees in the laboratory using technical product in acetone [5**,7**]. Lab assays can be poor predictors of field performance [45,46,66**]. Up to 100-fold toxicity differences were found comparing commercial formulations in water with technical products in acetone [5,4*]. In addition, pesticide combinations sometimes add unexpected pollinator mortality [5**,47–49,45].

Importantly, neonicotinoids control multiple sucking pests and are safer to biocontrol agents [38]. Not all neonicotinoids are equally toxic to bees [4*]; specific active ingredients can be toxic to a particular pollinator species or not and may become more toxic when mixed
with fungicides [5**]. Eliminating neonicotinoids would necessitate using less effective alternative pesticides, increase secondary pests and production costs and aggravate pest resistance problems. Neonicotinoid insecticides can be used to manage pests in apples, and through an IPPM approach, pollinators can be protected from them.

Since insecticides are not applied during the short apple bloom, direct contact of surface residues by bees is not likely. The mostly likely route of exposure is through the ingestion of contaminated pollen and nectar from systemic insecticides and fungicides applied before bloom. Ingestion bioassays are rare for bees other than the honeybee and some Bombus species, as are studies of exposure levels when bees encounter low doses in multiple flower visits over time [1**,4**]. The neonicotinoid, thiamethoxam, sprayed at the pink bud stage of apple at a typical 100 ppm field rate is reduced by translocation in the plant tissues; thus 5 days after a pre-bloom application, only 1–4 ppb is present in the nectar and pollen at 25% bloom [50]. Bee consumption rates of nectar and pollen are important in determining toxicity, but except for the honeybee, such consumption rates are largely unknown.

Pesticide recommendations in apple IPM to protect pollinators

An important advantage of IPM is that the pest management practices can be adjusted to accommodate new factors such as pollinator protection. Information on pesticide effects on non-honeybees is for the most part lacking, but certain groups such as the megachilids (including Megachile and Osmia) appear less susceptible on average than honeybees [5**,**51]. This varies among species and pesticides even within the same pesticide class [5**]. Using the honeybee as proxy for all pollinator species (as is presently done) is not an accurate predictor for other species like Osmia [5*], leafcutter bees, or bumblebees, since susceptibility varies by bee species and pesticide [51]. For example, the Japanese orchard bee (Osmia cornifrons) was 26 times less susceptible to imidacloprid than the honeybee, but 12 times more susceptible to acetamiprid [5**]. Present tree fruit IPM recommendations for pollinators are based on minimizing pesticide impact on honey bees rather than protecting wild pollinators. Pesticide recommendations for honeybees include no insecticide applications during bloom or when hives are present (except for insecticides that are non-toxic to honeybees (e.g. lepidopteran insect growth regulators [26*,**45]), and apply bloom fungicides at night or early morning. Pesticide restrictions are lifted when hives are removed; sometimes well before the end of bloom and without regard to wild bees that may still be foraging.

Although systemic pesticides are regarded as biocontrol-friendly since the pesticides are absorbed into the plant tissues where they were accessible only to plant feeders [52], the potential movement into the nectar and pollen from pre-bloom sprays may make them toxic to bees. Although neonicotinoids have harmed bees in some agricultural systems [53,54], these pesticides may be integrated into agriculture to preserve their pest management aspects as long as precautions are taken to minimize their impacts on non-targets such as pollinators and other beneficial insects. For example, the rosy apple aphid (Dysaphis plantaginea), which is resistant to organophosphates, carbamates and pyrethroids, and for which no alternative control methods (including biological control) exists [55], is killed by a neonicotinoid application made just before bloom. Simply, adjusting the pesticide’s application time to 10 days before bloom controls the aphids and drastically reduces pesticide residues in nectar and pollen (Biddinger, pers. comm.). Solutions like this should be investigated in all crops serviced by pollinators. A complete ban of this pesticide class would cause (a) a reversion to the older, more toxic compounds they were meant to replace, (b) exacerbation of pest resistance by removing a rotation partner, (c) a switch to broad spectrum pyrethroid sprays which would destroy existing IPM programs by eliminating most biological control agents, or (d) the complete loss of control of pests like the rosy apple aphid.

Even fungicides, long thought relatively harmless to pollinators and therefore safe to spray during bloom, are now indicted as potential pollinator threats [11*,56]. Bloom-sprayed fungicides break crop disease cycles early in the season reducing many sprays later in the season. Although most fungicides alone still appear safe, the simultaneous application of some fungicides (ergosterol biosynthesis inhibitors, DMI) may synergize neonicotinoid toxicity [5**,10**] and possibly that of other insecticides such as the pyrethroids [57]. A single lab study [10**] using a technical neonicotinoid product dissolved in acetone, found synergism of 105–1141-fold to honeybees by contact with acetamiprid and thiacloprid when mixed with two different DMI fungicides. However, synergism of other neonicotinoid insecticides with DMI fungicides was not found. Formulated versions of acetamiprid and imidacloprid with field rates of a formulated DMI fungicide in water tested on both honeybees and Osmia cornifrons revealed synergism that was barely significant at a 5-fold level with acetamiprid, and insignificant for imidacloprid in the lab [5**]. Field trials with formulated product of both the insecticide and fungicide showed similar results [58]. Rightly or wrongly, almost all fungicides, except the older contact fungicides, are considered bee-safe even in combinations [38,49,55,59]. These older fungicides have some insecticidal properties that can affect larval development through chronic exposure during nectar and pollen feeding [59,60]. Even fungicides acceptable in organic agriculture, sulfur and lime sulfur, are restricted during bloom because the odor is repellent to bees for up to 48 hours [61,62].
IPM recommendations for conserving wild pollen bees for tree fruit pollination

Tree fruit IPM programs can be adjusted to provide both pest control and pollinator health protection in an IPPM framework if we can better understand the relative levels of susceptibility of various bees species to both acute and chronic exposures to pesticides and the sources and levels of exposure in the field over time. With this information, we can inform farmers on how to adjust their spray programs to choose pesticides that are less toxic to bees while still controlling pests, or how to adjust the timing of toxic pesticides to minimize exposure levels just as we have done for over 40 years in biological control programs to conserve predatory mites and other beneficial arthropods. Since neonicotinoid residues in plant tissue does not carry over the winter (Biddinger, pers. comm.), only prebloom applications have to be adjusted in tree fruit. In addition, while wild bees have great pollinator potential, they are also susceptible to pesticides and other factors such as lack of alternate forage and nesting sites. These other factors can also be part of the overall IPPM approach by expanding orchard management to include the surrounding landscape as well as siting nesting sites and hive placement in orchard interiors to accommodate species-specific foraging ranges [63]. This same approach can be applied to other crops.

The recognition of honeybee decline and a rising reliance on wild, pollen bees has started to be included in public policy, providing increased funding for research and education on this topic. The Food, Conservation, and Energy Act of 2008 (aka The Farm Bill) acknowledged the great importance of pollen bees for agriculture by providing funding for farmers to increase and protect pollinator habitat on farm land. Farmers are encouraged to seed strips of wildflowers along their property to encourage bee visitation to their crops, or to leave part of their property fallow to increase pollinator habitat [64,65]. USDA/NRCS provides conservation payments that underwrite pollinator-friendly farm practices.

Although some may say that we are in a pollinator crisis mainly based on honeybee declines, we assert that in addition to honey bee protection, we need to encourage and make use of the myriad other pollinator species and, by properly adjusting crop IPM practices to create IPPM that will protect all the pollinator species. Going forward we need to:

- Include pollinator protection in IPM education.
- Expand pesticide toxicity testing to include other pollinator species in addition to honey bees.
- Encourage federal and state agencies to fund research and extension programs that integrate pollinator health into IPM, yielding IPPM.
- Encourage USEPA and other regulatory bodies to include pollinator protection in pesticide use regulations.
- Encourage conservation bodies such as USDA/NRCS to incentivize farm practices that preserve pollinator health.
- Work with the private sector (commodity groups, agricultural input companies, food retailers, among others) to incorporate pollinator protection into their businesses.
- Provide public education so consumers can choose products produced with good pollinator protection standards.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


A key review of the recent literature of neonicotinoid insecticide lethal and sublethal effects on bees in agriculture, residue levels within plant tissues and evaluation of a risk assessment scheme for systemic pesticides.


Review of what a ‘field-realistic’ dose of neonicotinoid insecticides is an how to relate laboratory studies with field evaluations.


A general review of the literature surounding neonicotinoids for the public that categorizes by crop and application type on various pollinator species.

5. Biddinger DJ, Robertson J, Mullin C, Frazier J, Joshi NK, Vaughn M, Ashcraft S: Comparative toxicities and synergism of orchard pesticides to Apis mellifera (L.) and Osmia cornifrons (Radoszkowski). PloS One 2013, 8:e72587 http://dx.doi.org/10.1371/journal.pone.0072587.

Examines the acute contact toxicities of neonicotinoid other insecticides and fungicide mixtures as formulated product in water to better simulate field applications on both the honey bee and Osmia cornifrons.


Discussion of the banning of some neonicotinoids in the European Union and the issue of pesticide replacement.


A review of the literature on the effects of neonicotinoid insecticides on pollinators in support of the European Union ban on several of these products.


One of the few studies examining differential contact toxicity of various neonicotinoid insecticides to the honey bee and demonstrating high levels of synergism with mixtures of fungicides.


Examines the effects of various pesticides and drugs used within the honey bee hive to control various pests.


Identification guide to apple pollinators with notes on alternative forage and a ranking of pesticide toxicity.


Meta-analysis of many studies worldwide demonstrating a strong corre- lation of habitat diversity and pollinator diversity.


A review of integrated pest management in US tree fruit and the transition to reduced-risk pesticides that include the neonicotinoids.


An easy to read manual for entomologist outlining the proper procedures for conducting, analyzing and interpreting pesticide bioassay data.