

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

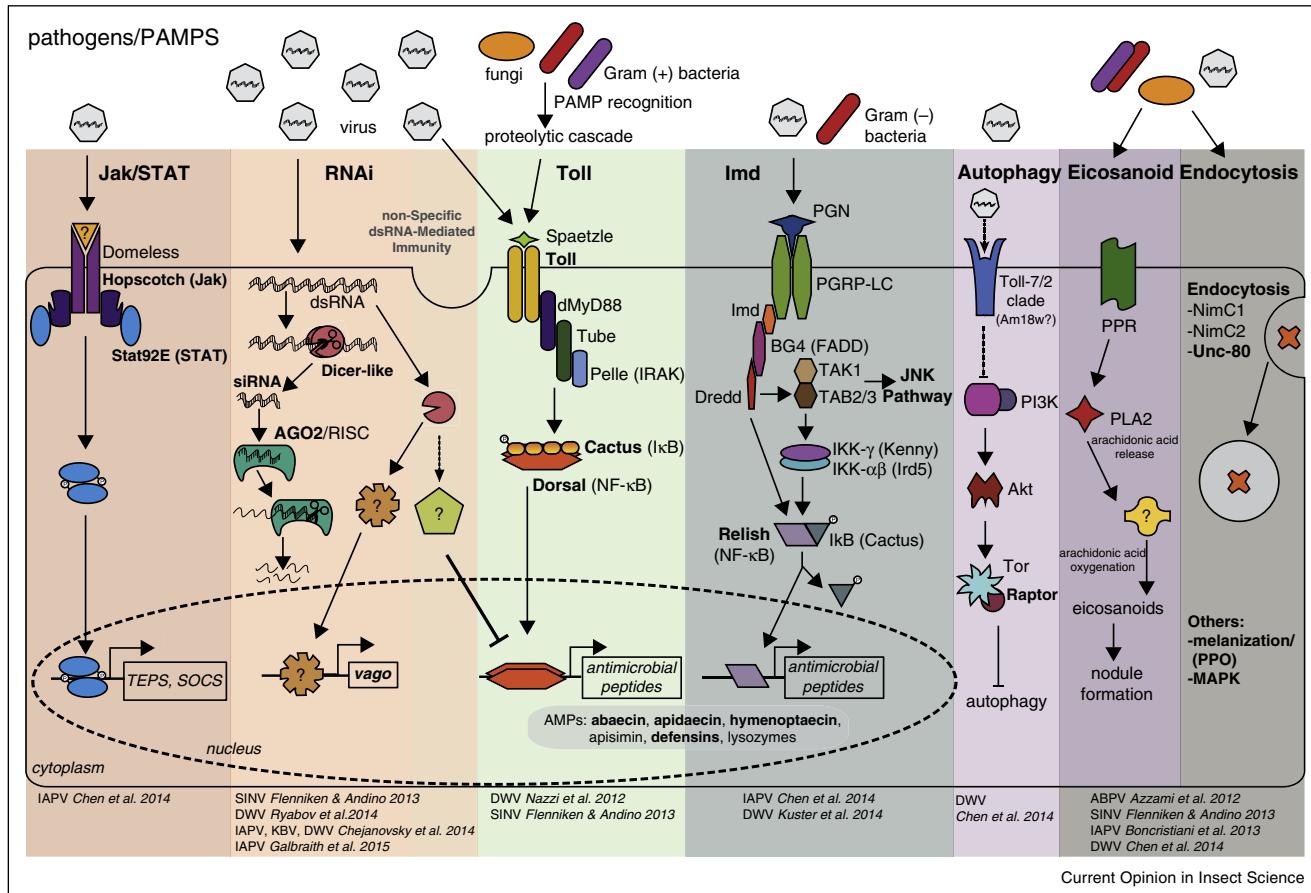
Honey bees (*Apis mellifera*) are fascinating insects that play a critical role in agriculture as pollinators of crops (U.S. value over \$15 billion/year) and plant species that enhance the biodiversity of both agricultural and non-agricultural landscapes [1]. Since 2006, honey bee populations in the 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], Varroa 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 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 Dipteran insects including fruit-flies and mosquitos approximately 300 million years ago [47,48**,49,50]. General aspects of

Figure 1



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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- κ B (Nuclear Factor κ B); 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 viral 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-Spaetze into mature Spaetze. The honey bee genome encodes two putative spaetze 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- κ B 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 ($I\kappa B$ 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 mosquitos (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 via injections with pure virus inocula, mutant-flies, or gene knock-down in mosquitos [59–63]. Likewise, field and laboratory based studies in *Apis mellifera* (Western honey bee) [64[•],65[•],66[•],67,68,69[•]] 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).

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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*, *apidaecin*, 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, PKR, RIG-I, and MDA-5) that upon binding dsRNA, activate signal transduction

(Figure 1 legend continued) expression and/or apoptosis. In *Drosophila*, binding of vesicular stomatitis virus to the Toll-7 receptor promotes **autophagy**, likely by inhibiting the PI3/Akt/Tor (phosphatidylinositol 3-kinase/Protein kinase B/Target of rapamycin) pathway which suppresses autophagy. The honey bee genome encodes for one gene of the Toll-7/2 clade, *18-wheeler* (*am18w*), which shares ~49% aa identity with *Dm* Toll-7 and ~45% aa identity with *Dm* Toll-2. The role of *Am18w* protein in antiviral defense and autophagy in honey bees is unknown. In insects, **Eicosanoid biosynthesis** begins with the induction of PLA2 (Phospholipase 2) from signal cascades downstream of viral, fungal, or bacterial PAMP recognition. Activated PLA2 hydrolyzes arachidonic acid (AA) from cellular phospholipids. Eicosanoid production likely occurs via oxidation of AA by an unidentified enzyme. Eicosanoids are critical for nodulation and aid in phagocytosis, micro-aggregation, adhesion, and release of prophenoloxidase (PPO) from hemocytes. Experimental evidence also suggests **endocytosis**, **melanization**, and **MAPK pathways** are involved in honey bee antiviral defense.

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.

Gene name	Pathway	Accession number	Virus	Reference
abaecin	AMP	NM_001011617.1	SINV	Fleniken and Andino [73*]
apidaecin 1 (apid 1)	AMP	NM_001011613.1	SINV, DWV	Fleniken and Andino [73*], Kuster et al. [44**]
apidaecin 1 (apid73)	AMP	XM_006572699.1	SINV, DWV	Fleniken and Andino [73*], Kuster et al. [44**]
apidaecin type 22 (apid22)	AMP	NM_001011642.1	SINV, DWV	Fleniken and Andino [73*], Kuster et al. [44**]
hymenoptaecin	AMP	NM_001011615.1	SINV, DWV	Fleniken and Andino [73*], Kuster et al. [44**]
defensin-2	AMP	NM_001011638.1	DWV	Kuster et al. [44**]
apisimin	AMP	NM_001011582.1		
defensin-1	AMP	NM_001011616.2		
vago	antivir	XM_395092.4	DWV	Ryabov et al. [69**]
nimrod c1 (nimc1)	EGF Family	XM_006561053.1	SINV	Fleniken and Andino [73*]
phospholipase a2 (pla2)	Eicosanoid	NM_001011614.1		
unc-80/endocytosis	Endocytosis	XM_006558847.1	SINV	Fleniken and Andino [73*]
dscam	IG superfamily	-	SINV	Fleniken and Andino [73*]
relish (rel), var x1	IMD	XM_006562219.1	DWV	Kuster et al. [44**]
relish (rel), var x2	IMD	XM_006562220.1	DWV	Kuster et al. [44**]
relish (rel), var x3	IMD	XM_006562221.1	DWV	Kuster et al. [44**]
fadd	IMD	GB30399		
imd	IMD	NM_001163717.1		
ikkγ-kenny	IMD	XM_001120619.3		
ird5	IMD	XM_623132.3		
pgrp-lc	IMD	XM_392452.5		
dredd	IMD	XM_001120830.1		
tab, var x1	IMD	XM_001122664.3		
tab, var x2	IMD	XM_006565777.1		
tak1, var x1	IMD	XM_006572294.1		
tak1, var x2	IMD	XM_397248.5		
d-pias, var x1	Jak/STAT	XM_006561055.1	IAPV	Chen et al. [18**]
d-pias, var x2	Jak/STAT	XM_006561056.1	IAPV	Chen et al. [18**]
d-pias, var x3	Jak/STAT	XM_623568.4	IAPV	Chen et al. [18**]
hopscotch (hop), var x1	Jak/STAT	XM_001121783.3	IAPV	Chen et al. [18**]
hopscotch (hop), var x2	Jak/STAT	XM_006567688.1	IAPV	Chen et al. [18**]
hopscotch (hop), var x3	Jak/STAT	XM_006567689.1	IAPV	Chen et al. [18**]
hopscotch (hop), var x4	Jak/STAT	XM_006567690.1	IAPV	Chen et al. [18**]
stat92e-like	Jak/STAT	XM_397181.5	IAPV	Chen et al. [18**]
domeless	Jak/STAT	XM_003251652.2		
socs-5, var x1	Jak/STAT	XM_006570603.1		
socs-5, var x2	Jak/STAT	XM_624416.4		
tepb	Jak/STAT	XM_006570965.1		
tep7, var x1	Jak/STAT	XM_006565440.1		
tep7, var x2	Jak/STAT	XM_006565441.1		
tepa, var x1	Jak/STAT	XM_006571765.1		
tepa, var x2	Jak/STAT	XM_397416.4		
lysozyme 1 (lys)	Lysozyme	NC_007082.3		
lysozyme 2 (lys-2)	Lysozyme	NM_001120136.3		
lysozyme 3 (lys-3), var x1	Lysozyme	XM_393161.5		
lysozyme 3 (lys-3), var x2	Lysozyme	XM_006571783.1		
nimrod b (nimb)	Phagocytosis	GB12454		
nimrod a (nima)	Phagocytosis	XM_001120328.3		
nimrod c2 (nimc2), var x1	Phagocytosis	XM_006561040.1		
nimrod c2 (nimc2), var x2	Phagocytosis	XM_006561041.1		
nimrod c2 (nimc2), var x3	Phagocytosis	XM_006561042.1		
nimrod c2 (nimc2), var x4	Phagocytosis	XM_006561043.1		
pi3k, var x1	PI3K-Akt-Tor	XM_006570469.1		
pi3k, var x2	PI3K-Akt-Tor	XM_623894.3		
target of rapamycin (tor)	PI3K-Akt-Tor	XM_006566642.1		

Table 1 (Continued)

Gene name	Pathway	Accession number	Virus	Reference
<i>akt-interacting protein-like</i>	PI3K-Akt-Tor	XM_625206.4		
<i>raptor</i>	PI3K-Akt-Tor	XM_624057.4	IAPV	Chen <i>et al.</i> [18••]
<i>phenoloxidase subunit a3 (ppo)</i>	PPO	NM_001011627.1		
<i>argonaute 2 (ago2)</i>	RNAi	XM_395048.5	DWV	Galbraith <i>et al.</i> [101••]
<i>dicer-like</i>	RNAi	XM_006571316.1	DWV	Galbraith <i>et al.</i> [101••]
<i>lysyl oxidase-like 2 (lox2), var x1</i>	Scav. Receptor A	XM_006560641.1		
<i>lysyl oxidase-like 2 (lox2), var x2</i>	Scav. Receptor A	XM_392090.4		
<i>nf-κ-β inhibitor cactus 1</i>	Toll/TLR	NM_001163712.1	DWV	Galbraith <i>et al.</i> [101••]
<i>toll-6</i>	Toll/TLR	XM_393712.4	DWV	Galbraith <i>et al.</i> [101••]
<i>dorsal, var a</i>	Toll/TLR	NM_001011577.1	DWV	Nazzi <i>et al.</i> [102••]
<i>dorsal, var b</i>	Toll/TLR	NM_001171006.1		
<i>dorsal-2 (dl-2), var x1</i>	Toll/TLR	XM_006565455.1		
<i>dorsal-2 (dl-2), var x2</i>	Toll/TLR	XM_395180.5		
<i>ikappaB kinase-like 2 (ik2)</i>	Toll/TLR	XM_396937.5		
<i>myd88, var x1</i>	Toll/TLR	NM_006560439.1		
<i>myd88, var x2</i>	Toll/TLR	XM_006560440.1		
<i>nf-kappa-β inhibitor cact1, var x1</i>	Toll/TLR	XM_006567107.1		
<i>nf-kappa-β inhibitor cact1, var x2</i>	Toll/TLR	XM_006567108.1		
<i>nf-kappa-β inhibitor cact2</i>	Toll/TLR	XM_394485.5		
<i>nf-kappa-β inhibitor cact3, var 2</i>	Toll/TLR	XM_625153.4		
<i>spatzle-like, var x1</i>	Toll/TLR	XM_003250921.2		
<i>spatzle-like, var x2</i>	Toll/TLR	XM_006566961.1		
<i>pelle, var x1</i>	Toll/TLR	XM_006565164.1		
<i>pelle, var x2</i>	Toll/TLR	XM_623999.4		
<i>traf6, var x1</i>	Toll/TLR	XM_006562507.1		
<i>traf6, var x2</i>	Toll/TLR	XM_624204.4		
<i>toll interacting protein (tollip)</i>	Toll/TLR	XM_624414.4		
<i>toll-1</i>	Toll/TLR	NM_006562720.1		
<i>toll-10</i>	Toll/TLR	XM_006562853.1		
<i>toll-8</i>	Toll/TLR	XM_393713.3		
<i>tube protein (tub)</i>	Toll/TLR	XM_001121229.3		
<i>18-wheeler (18-w)/toll like receptor</i>	Toll/TLR	NM_001013361.1		

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

cascades, 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 sucrose 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 eicosanoid 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 knock-down 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 Dorsal-1A, Dorsal-1B, and Relish (Figure 1, Tables 1 and S1). Nazzi *et al.* determined that *dorsal-1A* 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 *eIF4B*), MAPK members (i.e., *pointed*, *phi*, and *corkscrew*), and genes involved in endocytosis (i.e., *egfr*, *pastI*, *rabenosyn*, and *vacuolar 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 rRNA 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 (*Nosema* 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 mechanism(s) 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-1A*, pathogen recognition receptors (*AmSCR*, *B5* and *B7 scavenger receptors*, and *C-type lectin 8*), and immune signaling pathway members including *hem*, *tak1*, and *socs* [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-1A* expression in adult bees [102^{••}]. Also, larvae in which *dorsal-1A* 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., *abaecin*, *apidaecin*, *defensin*, *hymenoptaecin*, *PGRPs*, *PPOact*, and *relish*) 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 *relish*, and *apidaecin* and *hymenoptaecin*, whereas *abaecin* 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^v, 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.

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