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Honey bee colony losses and associated viruses

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Recent large-scale colony losses among managed Western honey bees (*Apis mellifera*) have alarmed researchers and apiculturists alike. Here, the existing correlative evidence provided by monitoring studies is reviewed which (i) identified members of the deformed wing virus and acute bee paralysis virus clades as lethal pathogens for entire colonies, and (ii) identified novel viruses whose impact on honey bee health remains elusive. Also discussed in this review is related evidence obtained via controlled experimental infection assays and RNAi approaches underscoring the damage inflicted by some of these viruses on individuals and colonies. The relevance of the ectoparasitic mite *Varroa destructor* acting as mechanical and biological virus vector for the enhanced virulence of certain viruses or mite selected virus strains is carefully considered.

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Introduction

Industrial management of the Western honey bee (*Apis mellifera*) has a long history with honey as a source of nutrition and trade for humans [1]. In addition to income from products directly harvested from honey bees, managed colonies contributed an average of €147 million to annual crop productivity from 2002 to 2007, as they have become an important supplement to pollination services provided by native pollinators across the globe [2^{••}]. Indeed, managed honey bees are the single most important global commercial insect pollinator [2^{••},3–5]. Despite some decreasing trends in parts of Europe and North America, the global number of managed colonies has risen by about 45% over the last 60 years [6,7]. However, this increase in the number of managed honey bee colonies

does not meet the steadily increasing need for managed pollinators in agriculture [6,8–10]. Hence, large scale managed colony losses, as experienced in the recent past in some parts of the world, exacerbate the shortage of pollinators and threaten human food security especially because wild pollinators are on the decline [11,12^{••},13].

Winter colony losses in general and CCD in particular

It is worth noting that the death of a honey bee colony due to natural causes, including disease, is within the scope of reasonable expectations for a living organism [14,15]. However, reoccurring unusually high winter colony losses at or above 30% in the recent past startled beekeepers and scientists alike [16–18], in part because simple, causal relationships remained elusive. The picture that emerged over the last decade is that such losses are multifactorial with weak Fall condition, starvation, queen failure, pathogens, parasites, pesticides, and climate all playing a role. The impact of each of these factors may differ on a case-by-case basis and even regionally [15,19,20]. Often when talking about colony losses, this general phenomenon is conflated with CCD [20]. However, CCD has a very specific case definition and is mainly characterized by the presence of a live queen and a lot of capped brood, indicative of rapid loss of adult bees. Additionally, the absence of dead bees in collapsed colonies and increased pathogen incidence are diagnostic of CCD [17]. CCD *sensu stricto* has so far only been reported from North America where it has been identified as one of many causes of winter mortality [21–23] and likely arises from multiple etiological agents. However, it has not been cited as a significant cause of winter losses since the winter of 2006/2007 [19]. In this review we refer to winter losses of managed *A. mellifera* colonies as the problem of interest, though summer losses may also be substantial. Primarily, we will concisely review the role that recently discovered and emerging viruses play in observed winter colony losses.

Viruses associated with honey bees

Till date 23 viruses have been reported to infect honey bees worldwide, primarily positive-strand RNA viruses in the families *Dicistroviridae* and *Iflaviridae* [24,25[•],26^{••}]. In the absence of the ectoparasitic mite *Varroa destructor* (hereafter referred to as *Varroa*) many honey bee pathogenic viruses only cause covert infections, which show no clinical signs and have no detectable impact on infected bees or colonies (for a definition of covert/overt virus infections see [27]). However, for acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV), black queen cell virus (BQCV), and sacbrood virus (SBV)

historical reports from the pre-*Varroa* era on overt disease outbreaks exist indicative of clinically significant infections in the absence of *Varroa* (Table 1; for historical references see [28]). However, we know from molecular data that even these four viruses can frequently be detected in seemingly healthy colonies [16,29–31], hence, also for these viruses covert infections are common. Most of the honey bee viruses might not even be honey bee specific because they infect a wide range of arthropod species [32*,33–35]. In the wake of *Varroa*, prevalence of virus infections has increased dramatically. This mite very quickly became established as a mechanical and biological vector of honey bee viruses as it spread through European honey bee populations since the 1980s (for a recent review see [36]). Augmented prevalence and virulence due to mite transmission was marked for members of the DWV/VDV-1 (*V. destructor* virus-1) and ABPV/KBV/IAPV (Kashmir bee virus/Israeli acute bee paralysis virus) clades [37–39].

The remainder of the review is divided into the following sections: correlative evidence (prevalence studies in symptomatic, asymptomatic, and not otherwise specified colonies), positive experimental evidence (infection assays),

and negative experimental evidence (RNAi approaches) for associations between viruses and colony losses with a final section on the role of *Varroa* in virus amplification and dissemination.

Correlative evidence for virus infections associated with colony losses

Recently, numerous prevalence studies have underscored the role of viral infections in honey bee colony losses [16,17,25*,26**,29–31,40–45,46*,47]. However, the design of many of these studies was rather weak. In most cases, the study period was one year or less, colonies were sampled once or twice, and whole bee extracts of pooled bee samples were analyzed by RT-PCR for the presence of six to eight known bee viruses (ABPV, IAPV, KBV, CBPV, SBV, DWV, VDV-1, and BQCV). Diagnostic results were then related either to the health status of the sampled colonies or to incidences of large scale colony losses in the sampled region. These studies substantiated the observation that covert virus infections are widespread and common in asymptomatic colonies [29–31]. At the same time they provided correlative evidence for members of the ABPV/KBV/IAPV and the DWV/VDV-1 clade being involved in colony losses, an interpretation

Table 1

This list gives a summary of the most common (designated with *) and recently emerging (designated with ») honey bee viruses, whether they are vectored by *Varroa*, and their symptoms. If *Varroa* has been shown to be a significant biological vector, the virus was designated with (+++). If the virus is frequently associated with *Varroa* but the mite has not been determined to be a biological vector, the vector status was designated with (++). Finally, if the virus is sporadically associated with tissues of the mite, or the vectoring status is in question but possible, it was designated with (+). The final column is a reference where more details can be found about the virus, including symptomatology, the evidence of its association with *Varroa*, prevalence data, association with colony collapse, among other things.

Virus	Family	<i>Varroa</i> vector status	Symptoms of overt infection	Reference
*ABPV clade	Dicistroviridae	++	– Paralysis – Darkened cuticle pigment – Impaired cognition and homing ability – Mortality (adult and immature bees) – Colony collapse	[77,82]
»ALPV	Dicistroviridae	Unknown	– Unknown	[25*]
*BQCV	Dicistroviridae	+	– Pale-yellowish, leathery cuticle of capped larva – Failure of larva to pupate – Sac-like appearance – Mortality (of larvae) – Deceased larvae and walls of cell turn black	[24]
»BRSV	Dicistroviridae	Unknown	– Unknown	[25*]
*DWV clade	Iflaviridae	+++	– Deformed wings – Learning deficits – Discoloring – Shortened and bloated abdomens – Mortality (adult and immature bees) – Colony collapse	[37]
»LSV1/2	Nodaviridae	Unknown	– Unknown	[25*]
*SBV	Picornavirales (super family)	++	– Pale-yellowish, leathery cuticle of capped larva – Failure of larva to pupate – Sac-like appearance – Mortality (potentially of adults, certainly capped larvae)	[24]
»TRSV	Secoviridae	+	– Dead larva becomes dark, brittle scale – Winter colony collapse? (correlative only)	[26**]

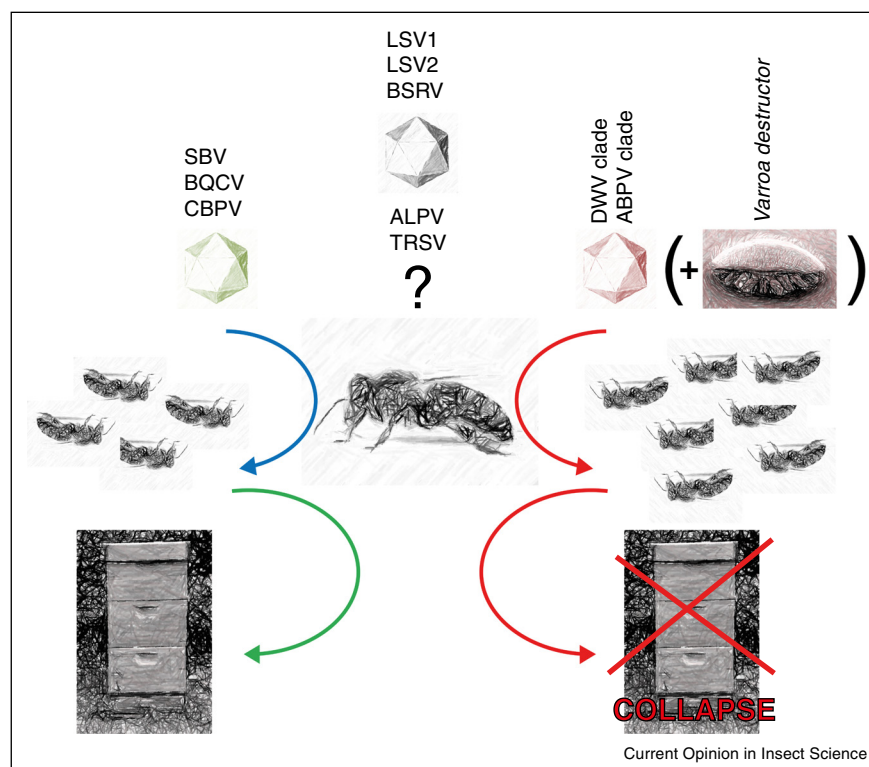
that was later validated by studies which have improved on these previous designs by increasing the length of the sampling period [16,40], improving screening precision by analyzing specific tissues [16,47], or even using advanced genomic techniques like next generation sequencing (NGS) or microarrays for detection of novel honey bee pathogens [25[•],26^{••},40,46[•],47].

At least two long term studies provided strong correlative evidence for IAPV and ABPV (both are members of the ABPV/KBV/IAPV clade) being involved in winter colony losses in the U.S. and Germany, respectively, [16,40] and DWV being a key factor for overwintering colony losses in Germany [16]. A recent survey of a large-scale migratory beekeeping operation in the U.S. identified for the first time a strain of aphid lethal paralysis virus (ALPV strain Brookings, *Dicistroviridae*) as a putative honey bee pathogenic virus (Table 1). However, its relevance to colony losses needs further investigation. [25[•]]. In addition, three novel viruses, Big Sioux river virus (BSRV), Lake Sinai virus 1 (LSV1), and Lake Sinai virus 2 (LSV2) were identified (Table 1). BSRV is a novel dicistrovirus, similar to the aphid-pathogenic virus *Rhopalosiphum padi* virus

(RhPV) but sufficiently divergent to justify a new species. LSV1 and LSV2 are two novel RNA viruses showing some similarity to CBPV and presumably belonging to the *Nodavirales* superfamily. Based on the ability of these viruses to replicate in adult honey bees and their abundance compared to other significant honey bee viruses, the authors suggested that they may play a significant role in colony health (Figure 1). A comparative study on pathogen loads of CCD and non-CCD colonies revealed additional LSV variants and suggested a potential association between LSV strain and CCD status [47]. However, the role of LSV or certain LSV variants in colony losses still needs to be established (Figure 1).

The occurrence of ALPV, LSV1, and LSV2 in the honey bee population is obviously not restricted to the U.S., as similar viruses were also identified in a Spanish honey bee colony [46[•]]. Most recently, two independent studies reported Tobacco ring spot virus (TRSV) and Turnip ring spot virus (TuRSV), two plant pathogenic viruses of the family *Secoviridae*, to be associated with honey bees [26^{••},46[•]]. TuRSV was proposed to be passively present in bees due to the ingestion of contaminated pollen [46[•]].

Figure 1



Viruses affecting honey bees. Till date, 23 viruses have been found to infect honey bees. Although most bee viruses cause covert infections, some viruses do cause visible symptoms and death of individual bees (e.g. SBV, BQCV, and DWV). However, colony losses due to these viruses are rather rare. Members of the DWV and ABPV clade not only kill individual bees but have been related to the collapse of entire colonies, especially in the presence of the ectoparasitic mite *Varroa*. Recently, three novel bee viruses were discovered (LSV1, LSV2, and BSRV) and two known viruses were described for the first time to infect honey bees (ALPV, TRSV). The exact impact of these five viruses on individuals and colonies is still elusive (illustrated by the question mark) and awaits final experimental proof or disproof.

However, TRSV was shown to replicate in nearly every bee tissue and elevated virus levels were found in weak colonies [26**] (Table 1). This novel and surprising finding of TRSV replicating in honey bees caused some debate [48,49] although this is not the first example of a plant virus replicating in both plants and insects. So far, such viruses have been described from the RNA virus families *Bunyaviridae*, *Nodaviridae*, and *Rhabdoviridae* which are vectored by insects via persistent propagative transmission mode [50,51,52*,53]. However, TRSV is a member of the genus *Nepovirus* which is not transmitted through insects but through pollen [54]. It is long since known that honey bees mediate transmission of pollen-borne plant pathogenic viruses between infected and non-infected plants [55,56]. Hence, pollen collecting honey bees are exposed to plant viruses and it is conceivable that especially honey bee larvae fed with plant virus contaminated pollen might get infected and might then show infection in several tissues of the adult bee. The recent discovery of such an infection is likely for lack of looking for it. Hence, further investigations are necessary to unravel whether the TRSV-honey bee interaction is indeed another example of viral host range spanning the plant and animal kingdom and if so, it would be worthwhile to search for other similar relationships.

Experimental evidence for virus infections causing death of bees by infection assays (positive evidence)

Surveillance studies are at a disadvantage compared to controlled exposure bioassays because the former can only generate correlative evidence, as opposed to the causal relationship demonstrated by the latter. During the infancy of honey bee virology, around 50 years ago, it was via experimental infection that scientists identified ABPV and CBPV as etiological agents of bee paralysis [57]. Additionally, the authors describe not only plasticity of symptomology depending on route of transmission (injection versus ingestion), but they also noted the existence of covert infections caused by these commonly lethal viruses [57,58]. While years following led to discovery of novel viruses, it was not until *Varroa* began infesting the European honey bee that we began to see their potential lethality when contributing to Parasitic Mite Syndrome (PMS) [59–65]. In fact, it was in *Varroa* infested colonies in the 1980s that symptomatic DWV infections were noticed for the first time (i.e. crippled wings), driving the identification of this typically asymptomatic virus. However, as a factor within PMS, DWV has quickly become tightly associated with colony collapse (Figure 1), and is therefore one of the most well-characterized honey bee viruses [28,66].

This unique relationship has led to quite a few controlled experimental infections of bees of varying ages with DWV in order to elucidate its mechanism of virulence

and whether transmission route affects virulence. Indeed, it has been shown that mimicking mite-vectored transmission by injecting isolated DWV particles into virus-free white-eyed pupae, results in dose-dependent mortality of pupae and emergence of adults with deformed wings [67] which are also not viable [68]. Furthermore, it has been demonstrated that when a feeding *Varroa* mite is actively infected with DWV, and therefore acting as a biological vector, a bee is more likely to acquire a clinically significant DWV infection from the transmitting mite [69].

Transmitting DWV to adult bees via injection, thus mimicking vectorial DWV transmission by phoretic mites, resulted in a systemic infection which included the nervous system when at least 10E+08 virus particles were injected [67] which may lead to cognitive impairment and learning deficits [70]. It is not clear, however, whether this latter effect was due to the virus or other components that may have been present in the whole bee extract used in this study. Therefore, this link between covert DWV infection and cognitive malfunctioning has yet to be adequately demonstrated. Recent studies have demonstrated the role of *Varroa* and the route of transmission in the evolution of virulence in DWV.

In 2011, Moore and colleagues began to clear up just how *Varroa* was augmenting the clinical severity of DWV infections in honey bees when they demonstrated that only certain variants of the DWV sequence space predominated in mite-infested colonies. Specifically, variants of the DWV quasi-species that had VDV-1 capsid proteins and DWV non-capsid proteins prevailed [71]. In 2014, Ryabov along with Moore and colleagues demonstrated that augmentation of DWV titers by *Varroa* mites is clearly due to facilitation of this specific virulent variant. A follow-up injection assay showed that it was the injection of the virus directly into the hemolymph, thus mimicking virus inoculation by the mite, that selected for this recombinant [72**]. It is conceivable, then, that these virulent DWV variants may be the precipitating factor for PMS.

It is possible that even oral transmission of DWV plays a significant role in increased mortality of larvae/pupae and the emergence of deformed adults, though the study did not evaluate this as no dose information was provided [73*]. However, the role of oral transmission seems to be substantiated by the infection of midgut epithelium, particularly in mite infested colonies suffering from PMS, which is conceivably DWV/VDV-induced rather than mite-induced [37,67]. In fact, highly infected brood are preferentially cannibalized by workers, who then are likely to become infected given that the pupae being cannibalized have even higher viral titers than deformed adults [74**,75]. Despite the restriction of infection in the

gut to the midgut epithelium [67], the threat that cannibalism of infected pupae poses to the adult must be further evaluated.

For members of the ABPV clade, especially for IAPV and ABPV, good correlative evidence exist that they are involved in large scale colony losses [40] or winter losses [16] (Figure 1). Like DWV, these viruses are also effectively transmitted by *Varroa* [59,60,76] (Figure 1). ABPV was first isolated and described 50 years ago after adult bees were observed darkening in pigment following infection with some unknown agent [57]. We now know that IAPV, first described in 2007, causes similar symptoms. However, it was also shown that IAPV-injected adult bees die within 4 days, while oral transmission resulted in death after only 10 days [77]. Two recent studies [78^{••},79[•]] aimed at a better and more detailed understanding of how IAPV infections damage honey bees.

Pupae injected with 10E+04 genome equivalents of IAPV showed a heterogeneous pattern of symptoms. Compared to controls (PBS (phosphate buffered saline) injected), IAPV injected pupae either stopped developing without any further symptoms or showed darkening of body parts [78^{••}]. It is uncertain however whether the darkening is due to virus induced tissue damage and necrosis, or melanization of tissue as a result of host immune response. Remarkably, studies suggest that heterogeneous pathology is dependent not just on IAPV strain diversity, but also on colony- dependent or patriline-dependent differences in susceptibility of the bees to IAPV infection [77,80^{••}]. As with DWV, IAPV has been found in healthy colonies despite being associated also with collapsing colonies [40].

Honey bee colonies might not only suffer from collapse when mortality rates of brood and adult bees increase due to virus infection but also when virus infections negatively affect bee behavior and, thus, colony performance. This aspect has been studied in adult forager bees injected with as little as 44 copies of IAPV and equipped with radio frequency identification (RFID) tags. While no mortality was observed, homing ability was significantly reduced at 2 and 3 days post-infection. Indeed, only between 2.3% and 0% of IAPV infected foragers found their way back, compared to 50% of those injected with PBS [79[•]]. It is reasonable to suspect that a considerable proportion of foragers not returning to the hive may have detrimental effects on the colony and contribute to weakening and eventually collapse of the entire colony.

Experimental evidence for the impact of viruses on honey bees by treatment with dsRNA (negative evidence)

RNA interference (RNAi) and Dicer, a multi-domain enzyme with RNase III activity, are key regulators in

invertebrate antiviral immunity and are crucial for suppressing viruses producing double-stranded RNA (dsRNA) intermediates. Dicer acts as a sensor of viral dsRNA. After binding and cleaving of virus-derived dsRNA molecules into siRNAs, it delivers these siRNAs to the host's RISC complex where they then can target viral RNA for inhibition [81]. Therefore, in honey bee virology RNAi approaches with experimentally delivered virus-specific dsRNAs can be used to investigate the relation between certain viruses and symptoms observed in individual bees and bee colonies.

There is only one study applying RNAi approaches to DWV infected bees. Honey bee larvae orally infected in laboratory experiments with high enough DWV titers either died during larval development or developed into adult bees exhibiting wing deformities [73[•]]. Preventive feeding of DWV-dsRNA revealed a more than 300-fold reduction in DWV levels in adult bees (21 days post egg hatching and infection) relative to bees developed from larvae that were fed with virus or with virus and GFP-dsRNA. In addition, significantly reduced frequencies of wing deformities relative to other virus-fed treatments were observed [73[•]] confirming that DWV is the etiological agent of the deformed wing syndrome.

For IAPV infections, data obtained through RNAi approaches at colony level are available. Maori and co-workers [82] used queen-right mini colonies with approximately 200 worker bees and experimentally infected these colonies by feeding IAPV in sucrose solution. By day 8 post infection, bee mortality in the non-infected control group was approximately 25% while around 80% of IAPV-infected bees had died. Preventive feeding of IAPV-specific dsRNA preparations (calculated as approximately 1 µg per bee) for three days prior to virus infection significantly reduced bee mortality in IAPV-infected bees to a level not significantly different from control mortality. Hence, ingesting IAPV-dsRNA obviously triggered an antiviral immune response in honey bees and prevented the bees from succumbing to a subsequently initiated IAPV-infection under laboratory conditions. This promising laboratory effect was then evaluated under natural bee keeping conditions using a total of 160 bee hives in Florida (USA) and Pennsylvania (USA) [83]. However, no significant correlation between increased colony or individual mortality and IAPV infection was observed in this study and, hence, it was difficult to conclusively assess the effect of dsRNA treatment on IAPV infection and colony survival. However, indirect measures suggested that IAPV-dsRNA treatment had some positive effect at the colony level.

A more recent study [80^{••}] suggested the existence of an IAPV-encoded viral suppressor of RNAi (VSR). In laboratory experiments with IAPV-infected caged bees it was demonstrated that silencing this putative viral interference

protein via RNAi through feeding of VSR-specific siRNA resulted in a remarkable reduction in IAPV replication within 24 hours post treatment. The authors concluded that the studies on IAPV inhibition through RNAi reinforce the therapeutic potential of carefully designed siRNAs for treatment of viral infections in honey bees.

Hence, the laboratory studies so far implied that there is a curative effect of virus-specific dsRNA on the severity and outcome of infections with the corresponding virus. However, a more recent study demonstrated that any dsRNA might serve as a viral pathogen associated molecular pattern (PAMP) in honey bees triggering an antiviral response that controls virus infection in general [84^{••}]. Therefore, approaches using virus-specific dsRNAs might not be suitable to prove the role of a specific virus or viral protein in a certain context but rather the involvement of viruses in a more general sense. Furthermore, the use of dsRNA as a management strategy for viruses in the field might rather not help to control a specific virus but may activate a nonspecific antiviral immunity resulting in a general positive effect at the colony level as observed by Hunter and co-workers [83].

Impact of *V. destructor*

It is without question that *Varroa* infestation poses the most serious threat to the Western honey bee colonies and that this is related to the mite's ability to vector virus infections or to exacerbate preexisting infections [39]. Virus infections of honey bees became a serious health problem for entire colonies only after *Varroa* started to infest honey bee colonies. *Varroa* theoretically can take up any virus present in bee hemolymph and, hence, can mechanically vector any virus back into the hemolymph when feeding on the next bee or pupa. Therefore, it is not surprising that since the introduction of *Varroa* the prevalence of honey bee viruses and infections increased. However, for most viruses, conclusive evidence linking them to colony losses is still lacking despite the nearly ubiquitous presence of the virus vector *Varroa* in the honey bee population. Only members of the ABPV clade and the DWV clade are thought to play a major role in colony losses in the presence of mite infestation.

Overt DWV infections, the development of the deformed wing syndrome, and colony collapse due to the PMS are closely associated with the vectorial transmission of DWV by *Varroa* (Figure 1). It is widely accepted by now that *Varroa* serves as a biological vector of DWV, and thereby plays a crucial role in the virulence of DWV. The selection of an especially virulent variant of DWV through *Varroa* has been anticipated and discussed for some time. Primarily, because it became obvious that DWV replication in the mite is necessary and sufficient for enhanced virulence of DWV as well as the development of deformed wings [69,85]. A recent study in Hawaii found that

the introduction of *Varroa* to a naive population greatly reduced the strain diversity of DWV while increasing the prevalence of infection [86]. These results supported the hypothesis that *Varroa* facilitates the dominance of certain strains which might be associated with increased viral virulence. Some hallmarks of such a virulent variant may have been identified recently [71,72^{••}]. The described variants were characterized by the capsid protein coding region of VDV-1 (a member of the DWV clade originally isolated as virus replicating in *Varroa* [87]) and the coding region for the non-structural proteins from DWV. However, the question of whether this variant was selected by amplification in the mite or by transmission route was not fully solved. Both of these studies further our understanding of the molecular mechanisms that underlie the increased virulence of DWV in the presence of *Varroa*.

Although ABPV and its close relatives are also associated with *Varroa* and colony losses, a similarly fatal triangular relationship between virus, *Varroa*, and honey bees could not develop. Members of the ABPV clade, when injected into pupae by mites, rapidly kill the infected pupa thereby preventing the developing bee from emerging and interrupting the reproduction of the mite which is trapped in the capped cell. Therefore, although mite transmitted ABPV/IAPV/KBV infections are lethal and contribute to colony losses (Figure 1), mite transmission of this virus clade is a dead-end hampering any further virulence evolution [38].

Conclusion

This review is not an exhaustive review on colony losses but rather collects on different levels recent evidence for the involvement of viruses in colony losses (Figure 1). Hence, we did not include all factors involved in the collapse of honey bee colonies such as non-viral pathogens, metazoan parasites other than *Varroa*, pesticides, malnutrition, climate, and beekeeping practice although we are aware of the relevance of these factors. It is important to point out, that THE colony loss or THE honey bee decline does not exist. Instead, the phenomenon of colony losses, although globally observed and reported, has different dimensions, reasons, and key players varying by region and time. It is also important to note that large scale colony losses are not happening for the first time but rather similar losses have been described in the historical past. This recurrence far from detracts from the importance of studying honey bee health, but rather increases the interest from the perspective of not only agriculture, but also basic science and ecology. Indeed, this is the first time in history that honey bee colony losses have attracted so much attention which has in turn boosted scientific interest and research in the field of honey bee diseases. Much of this research is interesting in its broader applicability in terms of potential control strategies for viral infections.

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