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Review article

Emerging and re-emerging viruses of the honey bee (*Apis mellifera* L.)

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Abstract – Until the late 1980s, specific viral infections of the honey bee were generally considered harmless in all countries. Then, with the worldwide introduction of the ectoparasite mite *Varroa destructor*, beekeepers encountered increasing difficulties in maintaining their colonies. Epidemiological surveys and laboratory experiments have demonstrated that the newly acquired virulence of several viruses belonging to the family *Dicistroviridae* (acute bee paralysis virus, Kashmir bee virus and Israeli acute paralysis virus) in Europe and the USA had been observed in relation with *V. destructor* acting as a disseminator of these viruses between and within bee colonies and as an activator of virus multiplication in the infected individuals: bee larvae and adults. Equal emphasis is given to deformed wing virus (DWV) belonging to the *Iflaviridae*. Overt outbreaks of DWV infections have been shown to be linked to the ability of *V. destructor* to act not only as a mechanical vector of DWV but also as a biological vector. Its replication in mites prior to its vectoring into pupae seemed to be necessary and sufficient for the induction of a overt infection in pupae developing in non-viable bees with deformed wings. DWV in *V. destructor* infested colonies is now considered as one of the key players of the final collapse. Various approaches for combating bee viral diseases are described: they include selection of tolerant bees, RNA interference and prevention of new pathogen introduction. None of these approaches are expected to lead to enhanced bee-health in the short term.

honey bee / bee virus / paralysis / wing deformity

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1. INTRODUCTION

The agricultural revolution in Europe starting in the 17th century came with a flourishing of literature on agricultural practices. Among them, books on honey bee management, often written with a poetic touch and ornamented with nice pictures, are a delight. The constant progress in the knowledge of bee biology and in beekeeping has continuously fed the edition of new books on apiculture. Most of them claim that "*beekeeping is not difficult and its principles can be learned by anyone*"[1] and "*There is a large number of people who keep only three or four hives of bees to supply their own table, and [. . .] who get great pleasure from keeping them. . .*" [88]. Notwithstanding some sudden but generally transient outbreaks of mortality here and there, which were generally easy to counteract by means of hygiene rules, beekeeping was appraised as a beneficial activity by all European and North-American authors. However, beginning in the late 1980s, wild honey bee colonies demised great in number, small amateur apiaries became scarce and professional beekeepers started to encounter increasing difficulties in maintaining the population level of their colonies. How did we reach this situation?

2. HONEY BEE VIRUSES AND THEIR IMPACT

Since 1963, when Chronic bee paralysis virus (CBPV) and Acute bee paralysis virus (ABPV) were first isolated, a total of eighteen viruses have been identified and characterized from bees of the genus *Apis*. Most of these viruses may exist and even co-exist in honey bee individuals or colonies without provoking apparent symptoms. The development and application of sensitive molecular diagnostic tools have revealed that infections by one or several of these viruses are very common and therefore are considered as harmless [16, 19, 40, 64, 70, 122].

However, some of these "harmless" infections may have an impact that can only be revealed by specially designed experimental protocols. Bailey et al. [16] used traps at the hive entrance and uncovered an otherwise unseen mortality in 25 colonies co-infected by the microsporidian parasite *Nosema apis*, Black queen cell virus (BQCV) and Bee virus Y (BVY). This mortality observed in the UK was at its peak in May and June, a period when natural mortality should be low considering that most of the over-wintered population of workers have already died and that most of the adult bees are young. The losses, supposed to be related to the co-infection by the three pathogens, were concealed while the rapid production of young adults at this time of the year maintained a net growth of bees in most of the colonies. Considering that bees have a short life span, finding dead bees may be normal. Nevertheless, abnormal deaths may be preceded by undetectable symptoms and colonies with bees having a shortened life span can stay alive during the spring, summer and autumn, but fail to survive in the winter. Without provoking any easy-to-observe symptoms, several bee viruses provoke other detrimental effects including a lesser adaptation to cold and non-beneficial changes in brood care or foraging behaviors (review in [7]). In other words, in the absence of long term epidemiological surveys and specifically dedicated protocols, the possible emergence of viral diseases of bees and their impact may have remained unseen and impossible to assess.

[1] Haynie J.D., Murphey M., Beginning beekeeping. Agricultural Extension Service, Bulletin No. 171, Gainesville, Florida, July 1959, pp. 1–32.

Chronic bee paralysis (CBP) is one of the few viral diseases of adult bees with striking well-defined symptoms. In the laboratory, it can be serially transmitted by various routes and follows a classical dose-mortality model [8, 35]. It is also the only viral disease of bees that has been proven to follow the simple epidemiological model relating the frequency of outbreaks with host density according to two scenarios. One scenario is related to bad weather resulting in confinement and crowding of bees in the hive accompanied by light abrasion of the cuticle which in turn facilitates virus transmission and finally leads to disease outbreak [26]. The other is related to "*too many colonies per available foraging places*" [26] as shown diachronically and geographically by the correlation of the incidence with the density of apiaries [17]. In this context, the increase in CBP incidence for example in France [61, 62] could be considered as a direct consequence of a certain development in apicultural practices in this country [7]. Indeed, the increase in sun-flower mono-culture and the huge yield of honey it could provide has provoked a considerable crowding of colonies in these areas followed by an increase in colony losses with all the CBP symptoms[2]. As in this example, the increased incidence of CBP can be directly related to changes in the apicultural practice without any apparent change in the host-pathogen relationship or in the mode of transmission of the virus. Since CBPV incidence may be increasing here and there, only some geographical areas are concerned, and its occurrence seems to be determined by colony concentration as described in the past. For this reason, it cannot be considered as an emerging virus of bees. Other viruses, however, have recently developed new epidemiological pathways and are now responsible for large colony losses worldwide. These recently emerging phenomena are described in the following chapters.

[2] Aubert M., Faucon J.P., Chauzat M.P., Martel A.C., Recherches sur les mortalités d'abeilles et prévention des risques liés aux insecticides, Whether or not these losses were all due to CBPV, the alleged responsibility of imidacloprid that was used for sun-flower seed dressing is largely improbable, Bulletin Épidémiologique de l'Afssa (2006) 20:1–4.

3. ABPV

3.1. Discovery

ABPV was discovered adventitious during laboratory work on another virus [8], but had never been directly associated with disease or mortality of bees in nature. Experimental administration of the virus to bees by feeding, spraying or injection provoked trembling, then paralysis of wings and bodies within 2–4 days, followed by death 1 to 2 days later [8]. These symptoms appeared earlier than those provoked by the administration of CBPV, hence the adjective acute versus chronic in the name given by Bailey to the new virus he had discovered.

Early field surveys had shown that ABPV did not cause such symptoms in nature: in sick or dead bees (adult or larval stages) sampled from colonies naturally affected with "paralysis", it was never ABPV but CBPV that could be detected at high concentration by serological tests [8]. While ABPV and CBPV both commonly occur at low levels in apparently healthy bees, only CBPV particles were numerous in diseased bees. With the absence of molecular diagnostic techniques at this time, ABPV could only be revealed by serial passages in bees from the same healthy colony (the "infectivity test" so-called by Bailey and Gibbs [9]). Bailey et al. [8] observed that "*bees infected with ABPV were common*", and they "*had not found a colony without some infected individuals*". For many years on, ABPV was shown to commonly exist in low concentrations as a covert infection (presence of virus in the absence of obvious disease symptoms; for definition of covert/overt see [51, 75]) in adult bees in Britain, especially during the summer [3, 9, 16] and also in bumble bees [9] but never producing outbreaks of paralysis. The virus was then isolated from healthy adult bees from most regions of the world: France, Italy, Canada [10], China [12], the USA [78], New-Zealand [4]. ABPV is known today to have a geographical distribution similar to that of *A. mellifera* [3, 55].

3.2. Acute paralysis: an emerging disease

However, shortly after the establishment of *Varroa destructor* in Europe, in contrast to the apparent low or null impact of ABPV before that time, large amounts of virus were detected by serological tests in individual adult bees and brood from *V. destructor*-infested collapsing colonies. The first emerging problems were documented in Russia and Germany by Batuev [27], Ball [20, 21] and Ritter et al. [107]. During the late summer, frequent cases of brood disease were observed in severely infested colonies, the symptoms resembling those of two bacterial diseases, American or European foulbrood depending on the stage at which the larvae or pupae died. Uncharacteristically, dead unsealed and sealed brood were often present at the same time, making diagnosis by symptoms difficult for the beekeepers, and the absence of bacteria difficult for the advisors to explain. In fact, it was shown that dead brood and adult bees contained as much virus as bees killed by injection of ABPV in the laboratory [21]. Similar observations followed in other newly *V. destructor*-infested countries: the Netherlands [22], Italy[3], former Yugoslavia [85], France [60], Hungary [28], Austria [29], Denmark [100], and the USA [78, 82].

Serological detection of ABPV in samples of dead adult bees from *V. destructor*-infested colonies in Germany and the Netherlands appeared closely related to the level of the mite infestation: the percentage of dead bees that were ABPV positive was 3%, 44% and 80% in colonies with low, medium or high infestation rates respectively. Moreover, a sharp decline in the adult bee population during the late summer coincided with a peak of ABPV incidence in dead bees [22]. At the same time, ABPV could not be detected serologically in samples of dead bees in the still non-infested UK[4]. Thus, acute paralysis caused by ABPV could be logically considered as an emerging viral disease whose etiological agent had always been present but has become more virulent in association with *V. destructor* infestation [3, 20].

3.3. ABPV and *V. destructor*

Like most of the RNA viruses of insects, ABPV belongs to the former group of Cricket paralysis-like viruses [43] but now form together with the Kashmir bee virus (KBV) and Israeli acute paralysis virus (IAPV) an unassigned group within the family *Dicistroviridae* [93, 94]. ABPV exhibits the typical genome organization of *Dicistroviridae*: The single-stranded RNA is positively oriented (i.e. it can be directly translated in infected cells) and contains two open reading frames (ORF) separated by an intergenic region and flanked by non-translated regions. The ORF in the 5′-half of the genome encodes the non-structural proteins, the ORF located in the 3′-half of the genome encodes the structural capsid proteins (for a recent review see [50]). Like most dicistroviruses [44, 124], ABPV commonly causes covert infections, i.e. the virus can be detected at low titers within the honey bee population in the absence of obvious symptoms in infected individuals or colonies. However, when injected into pupae or adults, ABPV is extremely virulent with less than 100 particles per bee required to cause death within a few days while, for observing the same effect by the oral route, 10^9 times more particles are necessary [8, 14, 106]. Considering the extreme virulence of this virus when directly injected into the bee haemolymph, it is not surprising that it started to cause problems as an emerging viral disease in the wake of the ectoparasitic mite *V. destructor* which is feeding on pupae and adult bees and in doing so transfers ABPV. When maintained for 6 h to 2 days on ABPV infected pupae then transferred on non-infected pupae, adult female *V. destructor* mites transferred the virus to the second ones in 50 to 89.5% of cases [23, 128]. The highest transmission rates were obtained with the longest period of feeding on the infected pupae and when the mites then fed several times on the same naïve pupa. The

[3] Carpana E., Vecchi M.A., Lavazza A., Bassi S., Dottori M., Prevalence of acute paralysis virus (APV) and other viral infections in honeybees in Italy, in: Ritter W. (Ed.), Proceedings of the international symposium on recent research on bee pathology, Ghent (Belgique), 1990, pp. 155–165.

[4] *Varroa* was first found in the UK on 4th April 1992 in Devon.

mite seems to solely act as a mechanical virus vector not allowing or supporting any virus replication: (i) there is no latent period in the mite between acquisition of the virus and its transmission, and (ii) the transfer efficiency decreases down to zero when the same mite is put successively onto 4 to 5 different naïve pupae [128]. In addition, no reports on ABPV replication in *V. destructor* can be found in the literature so far.

3.4. Towards a co-adaptation of the ABPV-varroa and honey bee complex?

While in the 1990s, acute paralysis was considered as "*a major cause of mortality in mite-infested colonies*" by some authors [3] or as "*a sporadic cause of adult bee mortality*" by others [28], during the following decades heavy mortality with acute paralysis seems to have been less frequently reported.

Indeed, with the advent of molecular diagnosis, the large distribution of ABPV (or at least of its genomic components) in the honey bee became more spectacular and as a consequence, its impact in bee health has been questioned by some authors. Tentcheva et al. [122] determined the prevalence of ABPV (and 5 other viruses) in adult bees, pupae and mites, in 10 colonies from 36 apiaries located in several French regions. Whereas the sampled colonies of these apiaries were apparently healthy, ABPV was detected in adult bees at least once in 58% and in pupae at least once in 23% of the apiaries. Only a limited number of colonies were infected in the apiaries (mean: 15 infected colonies per 100 infected apiaries). The virus was detected in *V. destructor* in 36% of apiaries (mean: 10 infected colonies per 100 infected apiaries). The virus was more prevalent in infested colonies in the late summer and autumn, coinciding with the peak in the *V. destructor* population, and supporting the hypothesis that the mite contributes to virus transmission. However, since these authors used samples from healthy colonies only, no comparison between healthy and diseased colonies from the same region is feasible and no conclusion about the impact of this virus (and others) on the health status of the bees or the colonies can be drawn from this study. Two similar studies bear the same limitation in that in these cases only diseased colonies were sampled. In Austria, only problematic colonies suffering from symptoms of depopulation, sudden collapse, paralysis or dark colour, and *V. destructor* infestation were sampled to analyze the occurrence, prevalence, and distribution patterns of i. a. ABPV and *V. destructor* [29]. The mite infestation rate of the colonies analyzed was given at 100% by the beekeepers. ABPV could be detected via RT-PCR analysis of pooled bees in 68% of the colonies. The virus status of the mites was not analyzed, therefore, the presumed viral transmission by *V. destructor* and viral infection of the colonies cannot be correlated.

The study of Nielsen et al. [99] on Danish apiaries involved only apiaries with winter mortality but suffers from the fact that it does not become clear how many colonies per apiary were sampled and whether the surviving or collapsed colonies from these apiaries or both were analyzed. The ABPV infection rate of these diseased apiaries was 14%. This is very low compared with the ABPV infection rate of Austrian diseased colonies (68%) or French healthy ones (58%). The authors hypothesized that because most Danish beekeepers use organic acids (less efficient than amitraz) to combat *V. destructor*, their bees had to cope with a higher number of mites and that bees more susceptible towards ABPV may have been selectively eliminated together with the virus. However, although beekeepers in Germany use the same regime to combat the mite, ABPV has a much higher prevalence in this country according to a recent study by Siede et al. [118]. This study involving 110 colonies from 11 apiaries and conducted over a period of two years, revealed an ABPV infection rate of 73% in 2004 and 80% in 2005. The design of this study (prospective approach) allowed correlating colony winter mortality with ABPV infection rate and with the *V. destructor* infestation level in the previous autumn. A significant correlation between the pre-winter ABPV-infection and winter mortality could be demonstrated for the 2005/2006 season but not for the 2004/2005 season. ABPV load as determined by quantitative real-time RT-PCR positively correlated with the mite infestation level only in 2005/2006.

Mite infestation level and winter mortality were significantly correlated in both years. This study suggested that ABPV in association with *V. destructor* might be one of several causes negatively affecting overwintering of bee colonies. This conclusion can also be drawn from a comparable study from Hungary. Bakonyi et al. [19] demonstrated that at the *apiary* level, ABPV was equally frequent in apiaries that subsequently remained in apparent good health and in apiaries that reported colony mortality. However, significantly more *colonies* were infected with ABPV in one apiary that collapsed compared to the colonies from 12 apiaries without problem [7].

Therefore, while ABPV had originally been described as an economically irrelevant viral infection of honey bees [8] it can now be considered as an emerging viral disease with considerable virulence most likely due to the activities of *V. destructor*. The exact role of *V. destructor* in the increase in virulence of ABPV still remains elusive and needs to be analyzed experimentally.

In conclusion regarding the role of the ABPV-varroa complex in bee mortality, due to the absence of large scale and long term surveys on the impact of virus infection in bees, it is impossible to assess with certainty whether too much emphasis had been given to acute paralysis during the first years of its emergence. Alternatively, the less frequent reporting of the symptom "acute paralysis" may not reflect the actual infection situation. Rather than hypothesizing a recent evolution towards a less severe equilibrium between the ABPV-varroa complex and its host, we consider that the increase in ABPV incidence in pupae due to *V. destructor* and the fact that vectorially infected pupae die during their development [32] logically entail that colonies infested with *V. destructor* and simultaneously infected with ABPV are weakened and collapse more frequently, making the "acute paralysis" symptom less frequent in adult bees.

4. KBV AND IAPV

KBV and IAPV are two closely related members of the *Dicistroviridae* family which are also closely related to ABPV. Actually, these three viruses form a complex of genetically related species with similar routes of transmission, an apparent absence in the larval stages, and overt infections characterized by rapid adult mortality.

KBV was first described in 1977 as a contaminant of Apis iridescent virus preparations [13] isolated from the Asian honey bee (*Apis cerana*). When these preparations were fed to or injected into *Apis mellifera*, a contaminating virus, then called KBV, multiplied to high titers [14]. Infected adult bees died within six days when injected with purified KBV particles, but they seemed unaffected when they ingested KBV [14]. Therefore, from the very beginning it was evident that the pathology and virulence of KBV depended on the transmission route. KBV has been detected in *A. cerana* [3, 14, 15] as well as in *A. mellifera* populations [2, 3, 26, 34] but also in bumble bees and European wasps (*Vespula germanica*) [6] suggesting a rather promiscuous host range and making it difficult to identify the host origin of KBV. In the honey bee population, KBV is prevalent in North America and New Zealand [34, 78, 80, 82, 123] but rarely found in Europe [29, 116, 117, 122].

IAPV was discovered after inoculating healthy-looking bee larvae with the homogenate of a single dead bee collected in the course of studies related to severe bee mortality in Israel [89]. Bees from affected hives showed symptoms reminiscent of overt ABPV-infections, hence, the new virus was named Israeli acute paralysis virus. Due to its rather recent discovery, only a few studies on the host range and geographic prevalence of IAPV have been published implicating that while it is prevalent in the Middle East, Australia, and the USA [42, 45, 89, 105] it has been less frequently found in Europe [30].

So far, studies on transmission and virulence of members of the ABPV-KBV-IAPV complex were conducted with ABPV and KBV less than with the recently discovered IAPV. However, due to the close genetic relationship especially between IAPV and KBV, IAPV may have been misdiagnosed as a strain of KBV during earlier studies on transmission and prevalence of KBV

[38, 40, 41, 122] since most of the primers used in these studies are not suitable to distinguish between these two viruses [50]. Like ABPV, KBV and IAPV can be detected at low titers in the honey bee population in the absence of obvious clinical symptoms at individual insect or at colony level [34, 42, 47, 78, 105]. As soon as elevated virus titers are reached, both viruses become extremely virulent [15, 45, 48, 89] as with ABPV [9, 14]. Such lethal viral titers have either been introduced artificially in the course of "virus induction experiments" and infection assays (injection bioassays) [5, 47] or they can be acquired naturally. Naturally acquired high viral titers seem to be related to or even depend on *V. destructor* acting as a vector or activator of the viruses [32, 79, 81].

Recently, the transmission routes of KBV (or IAPV?) were analyzed in detail [38, 113, 114] emphasizing the role of *V. destructor* in the lethality of KBV although the exact mechanism leading to pathology still remains elusive. One line of evidence suggests that the elevated viral titers in mite-infested pupae resulted from activated replication of endogenous virus infections [114]. In analogy to tick-borne pathogen establishment [129], it is proposed that parasitization by *V. destructor* suppresses the immune response of honey bees [131], leading to an activation of pre-existing covert viral infections. In this scenario with *V. destructor* acting as virus activator, direct transmission of KBV (or any other virus) through *V. destructor* is not a prerequisite for overt (symptomatic) infections. Another line of evidence suggests that *V. destructor* acts as a virus vector directly transmitting KBV to pupae since KBV could be detected in mite saliva [113]; in addition, acquisition of KBV by *V. destructor* and its transmission to bee brood by *V. destructor* could be demonstrated experimentally [38]. The same authors calculated a transmission efficiency for KBV to bee pupae through *V. destructor* of 70% and a mite-to-mite transmission or acquisition rate of 51%. Unfortunately, due to the experimental design, it was not possible to obtain any information on the health status of the pupae following vectorial infection since they were sacrificed for analysis after being exposed to the mites for only five days.

Considering the extreme virulence of KBV in injection bioassays [14], it is not surprising that the virulence of this virus in the field has been strongly associated with *V. destructor* infestation. *V. destructor* as a recently acquired ectoparasite of *A. mellifera* feeds on the hemolymph of adult bees and pupae thereby possibly "injecting" viruses. Indeed, KBV has been implicated in *V. destructor*-associated colony losses [15, 79, 81, 123] and IAPV virulence also seems to be associated with *V. destructor*. IAPV could be identified as a common marker of colonies in the USA which collapsed showing a set of distinctive characteristics, including the absence of dead bees in or near the colony and the presence of abundant brood, honey, and pollen despite vastly reduced numbers of adult workers [45, 103]. This syndrome was called colony collapse disorder (CCD) and has since been diagnosed in many other countries [125]. No common cause could be identified so far but viruses, especially IAPV, have been strongly implicated in CCD in the USA [45]. However, IAPV is prevalent in Australia without being linked to any CCD-like phenomenon, i.e. massive colony losses with IAPV as a marker pathogen [45]. A possible explanation for the increased virulence of IAPV in the USA is the honey bee parasite *V. destructor*, which is absent from Australia but present in the USA, again linking overt outbreaks of honey bee viral infections to simultaneous *V. destructor* infestation. Indeed, research on CCD in the USA [45, 83] and non-CCD winter losses in Germany [118] revealed an intricate relationship between the members of the ABPV-KBV-IAPV complex and *V. destructor* leading to increased virulence of these viruses in mite infested colonies.

In conclusion, in association with the ectoparasitic mite *V. destructor* IAPV and KBV can be considered as emerging viral diseases of honey bees. They are genetically and biologically closely related to the much better investigated honey bee virus ABPV. These three viruses differ in their geographical distribution [50] explaining why IAPV or KBV can be linked to colony losses in the USA and ABPV to colony losses in Europe.

Figure 1. Non-viable, adult bee (*Apis mellifera*) exhibiting deformed wings as clinical symptom of an overt DWV infection. A *V. destructor* individual is still clinging to one of the legs (picture taken by Michael Traynor). (A color version of this figure is available at www.vetres.org.)

5. DEFORMED WING VIRUS

5.1. Discovery and host range

When in 1975, Akratanakul and Burgett with a pertinent preview qualified *V. destructor* as "*a prospective pest of honeybees in many parts of the world*", they also mentioned without more details that emerging bees issued from infested pupae were "*deformed*" [1]. More precisely, infested emerging bees were undersized with deformed or atrophied wings. Since this deformed wing symptom (Fig. 1) had never been so obvious prior to the arrival of the parasite, it was attributed solely to the hemolymph deprivation of pupae by the mites [49, 84, 91].

However, since 1989, Ball related this symptom to the simultaneous infection by a virus she had discovered five years before in adult honey bees from colonies infested with *V. destructor* in Japan, and that she had thereafter identified in many countries as a cause of brood and adult bee mortality in infested colonies [18, 20, 23]. Ball named this agent Deformed wing virus (DWV) according to its pathology and symptoms [24] and the role of *V. destructor* in the transmission of DWV she had first demonstrated was then more deeply studied by several teams.

DWV is a member of the recently formalized picorna-like family *Iflaviridae*. The genome organization of DWV resembles that of picorna viruses and consists of a single ORF flanked by a long 5′-untranslated region (5′-UTR) and a short, highly conserved 3′-UTR and is terminated with a 3′ poly-A tail. Closely related to DWV – if not variants of DWV – are Kakugo virus (KV) isolated from allegedly aggressive *A. mellifera* worker bees from Japan [69, 109] and *V. destructor* virus-1 (VdV-1) isolated from varroa mites [104, 135]. The main host of DWV is unquestionably the European honey bee, *Apis mellifera*, where it has become globally distributed in the wake of *V. destructor* [3, 11, 16, 29, 36, 37, 64, 100, 106, 111, 122, 130]. DWV infections could also be demonstrated in the Asian honey bee (*Apis cerana*) and the dwarf bee (*Apis florea*) [3, 55] as well as recently in bumble bees (*Bombus terrestris*, *Bombus pascuorum*) displaying wing deformities [71]. In addition,

DWV has been detected in *V. destructor* [31, 39, 70, 100, 101, 112, 121, 133] and *Tropilaelaps mercedesae* [46, 65], two hemolymph-feeding, ectoparasitic mites of the honey bee. Both mites are involved in virus transmission, with *V. destructor* playing a key role in the transmission, pathology, and virulence of DWV [72, 114, 132, 133]. Furthermore, it was demonstrated recently, that DWV can infect the small hive beetle, *Aethina tumida*, a scavenger and vermin in honey bee hives rather than a parasite of honey bees [59].

In *A. tumida*, replicating virus could be demonstrated [59] implicating an active infection going on in these beetles. However, to act as virus vector with biological significance, *A. tumida* must transmit DWV to living and surviving honey bees and the transmitted virus should then establish an infection in the newly infected host. In fact, the beetle has proven deleterious to *A. mellifera* colonies once entering a hive and reproducing in the colony [56, 57] making it less likely that any transmitted viral infection will have the opportunity to develop in the bee host. Not surprisingly, given the impact the beetle has on bees and colonies at the time being, vectorial transmission of DWV to honey bees via *A. tumida* has neither been proven experimentally nor demonstrated naturally so far. Actually, it is rather the other way around: the results presented by Eyer et al. implicate that *A. mellifera* can transmit DWV to *A. tumida* [59] as has obviously been the case with bumble bees, too [71]. It would be interesting to assess the impact of an active DWV infection on *A. tumida*.

In contrast, the evidence that detection of DWV in mites sometimes represents an active infection and that those DWV-positive mites can act as biological vectors is rather strong. DWV replication indicative for an active infection could be demonstrated in *V. destructor* and *T. mercedesae* by detecting the minus-strand RNA which is only produced during viral replication [46, 72, 133]. In addition, crystalline arrays of VdV-1, which is closely related to DWV [104], could be localized within *V. destructor* tissues also indicating active viral replication [134]. Still, the majority of the mites analyzed did not support or allow viral replication suggesting that these mites passively acquired and mechanically transmitted the virus [72, 133].

5.2. The triangular virus-vector/activator-host relationship: DWV, *V. destructor*, and *Apis mellifera*

In the absence of *V. destructor*, DWV is a rather benign virus causing true covert infections without any visible symptoms in infected bees [51, 75, 133, 134]. DWV is even vertically transmitted between bees via eggs and semen (Fig. 2) as well as between colonies via reproductive swarming [52, 134] indicating its low intrinsic virulence [67]. Occasional colony mortality has been attributed to DWV in Britain and South-Africa before *V. destructor* became established there [23] suggesting the possibility of mite-independent outbreaks of overt DWV-infections. Interestingly, although these outbreaks caused bee mortality, the occurrence of bees exhibiting wing deformities or any other obvious pathology aside from death was not recorded in these cases. With the establishment of *V. destructor* in the populations of the European honey bee, overt DWV infections became more and more prevalent [23, 24]. These overt infections are now characterized by bees emerging with deformed wings, bloated and shortened abdomens, and discoloration (Fig. 1). These bees are not viable and die within less than 67 h after emergence [132] causing the colony to eventually collapse [79, 81, 115, 120].

Due to this close association between *V. destructor* and overt DWV-infections characterized by deformed, non-viable bees (Fig. 2) affecting the vitality of the entire colony, the triangular relationship between the pathogen DWV, the virus vector/activator *V. destructor* and the host *A. mellifera* has attracted much scientific attention. A consensus exists that the vectorial transmission of DWV to pupae through parasitizing mites is a prerequisite for the manifestation of clear disease symptoms (malformed appendages, shortened and bloated abdomen, and miscoloring) in the emerging bee [22, 31, 114, 133]. However, the exact role of *V. destructor* in the development of clinical

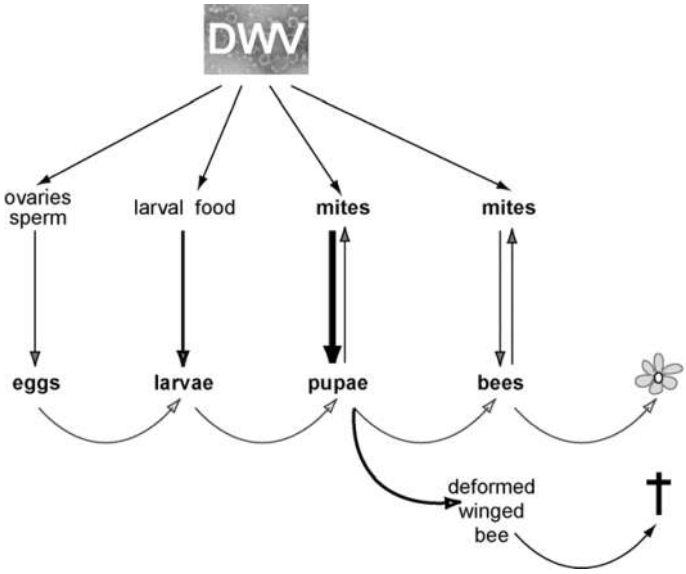

Figure 2. Transmission routes for DWV at the individual bee level within colonies. The vectorial transmission of DWV has been experimentally proven [52, 133]. Evidence for additional horizontal routes between nurse bees and larvae through larval food has also been provided [132]. The best analyzed transmission route for DWV is the vectorial transmission through the ectoparasitic mite *V. destructor*. DWV is transmitted by mites to parasitized pupae and during the phoretic phase to adult bees. The mites in turn acquire DWV when feeding on infected pupae and adult bees. Non-viable bees exhibiting deformed wings as the most prominent clinical symptom only occur when DWV transmission to pupae via *V. destructor* initiated an overt infection.

DWV symptoms is controversially discussed in the literature or even remains elusive when it comes to the underlying pathomechanisms.

5.2.1. Induction of endogenous DWV infections through V. destructor

For ABPV and KBV it was long since known that covert virus infections can be induced to overt lethal infections by repeated injection of biologically active substances into the haemolymph of adult or pupal honey bees [5, 47, 48]. Such repeated experimental injections were interpreted as a suitable model for mites feeding on the haemolymph of pupae and adult bees. It was suggested that during mite feeding, mite saliva is injected into the haemolymph non-specifically activating covert ABPV infections thus linking induced overt ABPV/KBV virus infections to *V. destructor* parasitization. Comparable experimental results proving that DWV is an "inducible virus" and that covert infections can be activated through injection of any biologically active substance are missing, although, recent studies proposed that *V. destructor* might "actively" contribute to viral induction or activation through immunosuppression of the host [131].

However, conflicting results concerning the effect of *V. destructor* on the immune capacity of bees and their relation to DWV activation can be found in the literature. Yang and Cox-Foster [131] analyzed the expression of genes encoding three antimicrobial peptides (hymenoptaecin, defensin, and abaecin) and four immunity-related enzymes (PO, phenol oxidase; GOX, glucose oxidase; GLD, glucose dehydrogenase; and lysozyme) in response to microbial challenge (injection of *Escherichia coli*) in three categories of bees: (i) bees that

developed from mite parasitized pupae and displayed deformed wings (DW bees), (ii) bees that developed from parasitized pupae but did not show clinical symptoms of DWV infection (NW bees), and (iii) healthy looking bees that developed from mite free pupae (MF bees). Bees that emerged from mite parasitized pupae when exposed to microbial challenge (injection of *E. coli*) did not react with an increase in the steady-state levels of hymenoptaecin-mRNA, irrespectively whether they had deformed wings (DW bees) or normal wings (NW bees) while healthy looking bees that had not been parasitized by *V. destructor* during pupal development (MF bees) showed an up-regulated mRNA-level for this antimicrobial peptide. All bees increased their steady-state levels of defensin- and abaecin-mRNA in response to microbial challenge although DW bees reacted significantly weaker and NW bees significantly stronger than MF bees. Complex effects were also observed for the expression of the immunity related enzymes in response to *E. coli* injection. DW bees down-regulated their steady-state levels of all analyzed immunity-related enzymes, while in NW bees PO and lysozyme gene expression was unaffected upon *E. coli* injection. In addition, it was shown that *E. coli*-injected NW bees had much higher DWV RNA titers than saline-injected NW bees or MF bees. *E. coli*-injected NW bees reached the same DWV RNA titer as observed in DW bees indicating that the high DWV RNA titers seen in deformed bees can also be induced by injecting *E. coli* into healthy looking bees that had been suffering from *V. destructor* parasitization during pupal development. Hence, the authors provide correlative evidence of a partially impaired immune response towards exposure to *E. coli*, although not of a general immunosuppression, in adult bees that suffered as pupae from mite parasitism [130]. The authors concluded that *V. destructor* causes immunosuppression in parasitized bees thereby inducing DWV replication since they also found a positive correlation between mite infestation level of pupae and probability and, especially, the severity of wing deformity in the emerging bee. They also hypothesized that the more mites parasitize a pupa the higher will be the suppressing effect on the immune system and the more severe will be the clinical symptoms in the emerging bee [131].

Although *V. destructor* immunosuppressing its invertebrate host *A. mellifera* is a tempting analogy to ticks immunosuppressing their vertebrate hosts [129], subsequent studies have shed some doubt on this hypothesis. When transcript levels for genes encoding antimicrobial peptides (abaecin and defensin) were analyzed in pupae differing in the number of parasitizing mites, lower transcript levels were only found for pupae with low mite abundances. Heavily parasitized pupae actually showed higher levels of these immune effectors [74]. Similarly, a recent survey of honey bee immune-gene activity using microarrays [97] did not show a systematic change in the activity of predicted immune pathways. Although *V. destructor* parasitized pupae displayed high levels of DWV viral RNA, a decrease in transcript abundance of immune pathway members present on this microarray [58] could not be seen. However, the autophagic-gene 18 (Atg18) and the poly U binding factor 68 kD (pUf68), both presumably involved in innate immunity against bacteria and viruses, were down-regulated in parasitized bees. Based on their results the authors proposed a hypothetical pathway in which the down-regulation of Atg18 and pUf68 through *V. destructor* parasitism might induce the proliferation of DWV.

Although these studies suggest that *V. destructor* parasitism results in a decline in immune capacity which in turn induces DWV proliferation, the identified key players in this process are different. A possible explanation for this discrepancy could be the differential experimental design of these three studies. Yang and Cox-Foster [131] used adult bees challenged with *E. coli* injection and, therefore, analyzed the capacity of the adult bees' immune system to react properly. In contrast, the other two studies [74, 97] involved pupae differing in the number of parasitizing mites in the absence of any additional microbial challenge and, therefore, the base line immunity in pupae as a function of mite presence was analyzed. Hence, these three studies are not really comparable but they perfectly demonstrate that the

impact of *V. destructor* on DWV infection via manipulating the bee immune system is very complex and needs much more experimental work to become fully understood.

5.2.2. *Overt DWV infections through* V. destructor *acting as a biological vector*

Overt DWV infections characterized mainly by malformed appendages are closely associated with *V. destructor* parasitism. The ability of *V. destructor* to acquire and transmit DWV is unquestioned [22, 31, 114, 133] as is the fact that even in highly parasitized colonies with 100% DWV-transmitting mites the majority of infested bees still emerge asymptomatic [133]. Therefore, even if the transmission of DWV to pupae via *V. destructor* is necessary for the development of deformed wings it obviously is not sufficient. Authors in search of factors determining the outcome of vectorially transmitted DWV infections, have demonstrated in recent studies that the detection of replicative, negative-strand DWV RNA in mites is correlated with the occurrence of deformed wings in bees that had been parasitized by such mites during pupal development [133]. Therefore, overt outbreaks of DWV infection could be linked to the ability of *V. destructor* to act not only as a mechanical vector of DWV but also as a biological vector of DWV supporting or allowing DWV replication prior to transmission. These results were further supported by determining the DWV genome equivalents in mites that had parasitized pupae, which either developed an overt DWV infection (bees emerging with deformed wings) or emerged covertly infected, i.e. asymptomatic [72]. Mites that could be linked to the development of non-viable bees displaying deformed wings were shown to contain replicating virus and, hence, high viral titers (10^{10}–10^{12} genome equivalents per mite). In contrast, in mites that parasitized pupae emerging as covertly infected bees not showing any visible symptoms, no replication of DWV could be demonstrated and viral titers were significantly lower with a maximum of only 10^{8} genome equivalents per mite. Hence, according to these results, the development of crippled wings not only depends on DWV transmission by *V. destructor* but also on the replication and titer of DWV in the parasitizing mites. In summary, DWV replication in mites prior to vectorial DWV transmission to pupae seemed to be necessary and sufficient for the induction of a overt infection in developing pupae resulting in bees emerging with deformed wings as the clinical symptom. In contrast to what was shown by Yang and Cox-Foster [131], the study by Gisder et al. did not find any significant difference in mite infestation level between pupae developing into healthy bees and those that emerged with deformed wings [72]. However, even considering this last result, the chance for a pupa to meet a mite that contains replicating virus and, hence, induces an overt infection is positively correlated with the mite infestation level of the colony [72] again linking mite infestation level with the probability of overt DWV outbreaks and colony collapse.

Since the majority of bees even in highly infested colonies still emerge asymptomatic, the majority of mites should not contain replicative DWV. This might explain the results of a recent study analyzing DWV replication in mites by trying to localize DWV within the tissues and cells of *V. destructor* via immunohistochemistry [112]. The authors analyzed 20 mites collected from infested cells which were allowed to feed on white-eyed pupae injected with a semi-purified preparation of DWV. They only detected DWV in the midgut lumen of the mites analyzed and concluded that there was no evidence that DWV was replicating in the mites since no tissues showed specific antibody binding to DWV. The result presented by Santillán-Galicia et al. [112] suits perfectly with the above outlined hypothesis: a majority of mites indeed do not contain replicative DWV [72, 133]. For proving or disproving DWV replication in mites, the only suitable material would be mites that parasitize a pupa that subsequently develops into an overtly infected adult bee. For such mites, replication of DWV has been proven unequivocally using more sensitive and accurate molecular techniques [72, 104, 133].

In conclusion, DWV is currently among the most prominent honey bee viruses. Its global distribution has its origin most certainly in the close association of this virus with the ectoparasitic mite *V. destructor* which is strongly implicated in its transmission within and between

honey bee colonies. In some areas of Europe, depending on the season, up to 100% of the colonies can be found infected by DWV [29, 122, 133]. In the absence of *V. destructor*, DWV causes true covert infections without affecting the fitness of the host [52, 134]. Overt outbreaks of DWV infections are closely linked to *V. destructor* infestation and are mainly characterized by bees emerging as non-viable bees displaying deformed wings. A general presumption is that *V. destructor* acts as activator or vector of DWV thereby increasing its virulence. Hence, while DWV infections mainly went unnoticed prior to the dispersal of *V. destructor* in the 1970s and 1980s [3, 55], DWV has become an emerging viral pathogen of honey bees since then. Now, DWV is considered as one of the key players associated with the collapse of honey bee colonies infested by *V. destructor* and, hence, deformed winged bees are symptomatic of the final stages of colony collapse described as the parasitic mite syndrome [115].

Although it is not yet clarified how mites together with DWV (and other viruses) kill honey bee colonies, it is safe to assume that the mites do not serve as activators or vectors but that they rather do both, activating covert viral infections through suppression of an appropriate immune response and transmitting viruses biologically as well as mechanically. In any case, an increase in the virulence of bee viruses that were until then generally considered benign is the consequence.

6. THE FUTURE OF VIRAL DISEASES IN BEES

The long-term decline of managed honey bee hives in the USA and some European countries has become an issue of widespread interest and concern. As a consequence research projects aimed at identifying putative factors afflicting honey bees were initiated. Among the main culprits for these colony losses were *V. destructor* associated with emerging or re-emerging viruses [20, 25, 92] and indeed a metagenomic survey and comparison of colonies that collapsed with symptoms of CCD and that survived, revealing that virus infections (IAPV and DWV) could be linked to colony collapse [45, 83]. Nowadays there is no doubt that the impact of various syndromes implying bee viruses is a global threat for apiculture. Can we expect that naturally or artificially selected honey bee lines could alleviate this impact? Are the developments of new treatments against virus infections really promising? And can we still prevent undiscovered viruses (or other agents that favor their active multiplication) from disseminating worldwide? Answering these questions is forecasting the future of virus infection in the honey bee.

6.1. New developments to combat viral diseases in bees

The demise of bee colonies has stimulated research into several directions, including the following: the development of effective methods to combat or control *V. destructor*, the selection of *A. mellifera* strains more tolerant to *V. destructor*, and treatments against virus infections in honey bees.

Considering the scope of this review, we will only briefly mention the main results of the first two approaches. It has been well established that the mite population had to be controlled to avoid colony collapse (reviewed by [66, 110]). In addition, since emerging and re-emerging viral diseases of honey bees are associated with mite infestation, an effective treatment against *V. destructor* is the best way to also combat these viral diseases. In the absence of the mite, the here reviewed viral diseases will have no or little impact on honey bee health. Classical methods to control mite infestation levels in honey bee colonies have been reviewed recently [110]. In this review a compilation of chemical, biotechnical, and biological treatments currently in use or part of recent research activities are presented and evaluated.

Attempts to control mite infestation levels by breeding for mite tolerance or by selecting mite tolerant bees that developed "naturally" have been performed but are not satisfying so far. Fries et al. [68] monitored for six years 150 honey bee colonies infected with *V. destructor* without applying any acaricide treatment and letting them to swarm at will. As expected, winter mortality rate was very high: reaching up to

80% the third year, but decreased to 12 to 18% (of the remaining colonies) the last two years with 11 colonies only surviving the last year. In France, Le Conte et al. [86] followed for seven years a total of 82 honey bee ("resistant") colonies without treatment in parallel with control treated colonies. Over the period analyzed, the mean winter mortality did not differ significantly between "resistant" (non-treated) and control (treated) colonies, however, the honey production was 41% significantly lower in the non-treated colonies. It remains unknown if these developments occurred following an increased tolerance in the host, a reduced virulence in the parasite or were due to a combination of these factors. However, for the honey bee, this more tolerant status has been reached at a very high cost – not only in terms of colony mortality during the first years of the studies – but also in terms of honey production (much lower in surviving colonies). From these studies, the selection of more tolerant honey bee lines is hardly compatible with apiculture aims.

Possible treatments against virus infections in honey bees have never been seriously considered before. One of the most promising developments in this field is the RNAi (RNA interference) approach that uses small interfering RNA (siRNA) to take advantage of the gene silencing pathway for the post-transcriptional regulation of gene expression present in every somatic cell of every metazoan eukaryote (plant and animal) [95, 96]. RNAi is triggered by double stranded RNA which also occur during the replication of RNA viruses. Hence, replicating RNA viruses are natural targets for degradation by the RNAi pathway and cells use this pathway to defend themselves against viruses [102, 126]. But of course, many viruses have evolved strategies to evade the immunity controlled by siRNA [87].

A recent study demonstrated that an RNAi approach directed against IAPV is successful in silencing IAPV infection and preventing bee mortality [90]. The authors concluded from their results that IAPV-RNA can be silenced in bees by ingestion of a segment of IAPV-dsRNA and that an RNAi-related pathway of silencing leads to viral RNA degradation. However, since siRNA does not easily cross membrane boundaries [127], the anti-IAPV (or in general: anti-viral) effect of orally administered siRNA will most likely be restricted to the cells lining the digestive tract, and less likely be effective against systemic infections like those observed for DWV following vectorial transmission by mites and involving infections of the brain and reproductive tissues [63, 133]. On the one hand, the final success of this new technique in combating viral infections in bees will depend on whether it will be possible to target the siRNA to all infected key organs and to prevent degradation of the siRNA during transport. On the other hand, this success will also depend on whether feeding viral nucleic acids – even if they are only 22–26 nt in length – to bees will find acceptance by the sensitive beekeeping industry and the critical consumer presumably afraid of any nucleic acid contamination in honey. In addition, considering the cost of this technology it may not even be affordable for beekeepers.

Prevention is better than cure. Considering that invasive species (such as *V. destructor*) are qualified as such when there is precisely no possibility to eradicate them, would it be possible to prevent their introduction?

6.2. Emerging diseases of bees and the international trade

Due to the lack of practical control measures, honey bee viruses do not currently form part of any statutory disease control programme anywhere and no virus disease of the honey bee is mentioned in the World Organization for Animal health (OIE) Terrestrial Animal Health Code.

The second reason for the restraint of the OIE in this matter is that the information on the geographical distribution of honey bee viruses and their respective impact is only emerging. Moreover, there is a postulate never mentioned but tacitly accepted by all: when the honey bee was introduced in geographical areas where it had never existed before, it carried with it all its viruses. As an example, the controversy on the native geographical origin of KBV illustrates that this postulate cannot be easily denied [33, 106]. Therefore, since all regions of the world are implicitly considered to be infected with all the

major bee viruses – and because no country has demonstrated by appropriate techniques and surveillance that it is free of any particular bee virus, no import limitation (such as quarantine measures) for hive products or bees aimed at preventing any undefined virus invasion would be legitimate. In reality, even in the limited scope of apoideae, there are many examples of unforeseeable transmissions of pathogens between introduced and native species: New Zealand native bumblebees are now hosts to a parasitic nematode and three mite species, all of which are thought to have come from the UK with the original introduction of bees [54] (see also the review by [73]). Conversely, in some parts of the world where the European honey bee is an introduced livestock, a still unknown or a benign pathogen of a native species may switch the host and invade the honey bee population with unforeseeable consequences. International regulation (reviewed by [33]) does not take into account and, therefore, is not suited to prevent this possibility, although this scenario was precisely the first step of the invasion of *V. destructor* from Asia into all continents (except Australia until now). The well-known consequences of this invasion were and still are huge losses of honey bee colonies due to mite infestation and associated health problems including emerging virus infections.

The small hive beetle (*Aethina tumida*), another major invasive pest, was first confirmed outside its sub-Saharan native range [76] in the eastern USA in 1996, then in Egypt in 2000 and Australia in 2002 [98]. Now it has been recorded in over 30 USA States demonstrating its potential to spread rapidly [77, 98]. Within its new range, it has caused considerable damages to colonies and honey-extracting facilities [108]. There are no control methods yet available except emergency short-term treatments of limited efficacy. The small hive beetle may also be a threat to biodiversity by attacking the nests of bumble bees and solitary bees [119]. It is noteworthy to recall that the invasion of an exotic species in an ecosystem is currently viewed as one of the most important causes of biodiversity loss and may lead to host eradication [53].

In the context of the BRAVE project (2005), worldwide specialists in bee pathology and virology observed that: "*Many thought that* Varroa *was the last great threat to world apiculture but then* Aethina *came to dinner. We must not take our eyes off the ball. The huge level of colony losses in Spain reportedly caused by* Nosema ceranae *is another example. Who would have predicted these?*" No doubt now that the risk of moving still unknown pests and diseases around the globe with potential disastrous consequences is serious. The most recent example might be the occurrence of severe colony losses in the USA due to CCD with IAPV as a marker and loosely linked to imported bees [45]. Unfortunately, the threat to bee health and bee survival imposed by the global trade of bees and hive products is not recognized as a legitimate reason for limiting the international trade of bees. Pathogens must be identified and described beforehand, their impact on the honey-bee well established, the free status of the country must be proven and continuously monitored. We are far from this level of knowledge and technical competence, thus we must live with an increasing global trade in bees whether we distrust it or not. Obviously, we must be prepared for more emerging bee pathogens including viruses.

7. CONCLUSION

The long-term decline of managed honey bee hives in the USA and European countries has become an issue of widespread interest and concern. Based on many research projects aimed at identifying all the putative factors afflicting honey bees, evidence is accumulating that one of the major causes – not to say, the major cause – is the association of viruses to these colony losses, which so far existed as covert infections in the honey bee population, with an invading parasite, *V. destructor*. This combination "*V. destructor* plus viruses" has triggered the emergence of overt viral infections with significant and sometimes fatal symptoms at both the individual bee level and the colony level. Nowadays there is no doubt that the impact of various syndromes involving *V. destructor* and bee viruses is a global threat for apiculture. Until now, the spontaneous or artificial selection of honey bee lines more tolerant to *V. destructor* infestation have produced poorly productive

colonies. However, no simple and economically acceptable treatment against virus infections are in view for replacing the heavy and not always efficient acaricide treatments which have already selected resistances in the target species. Repeating previously observed scenarios, the dramatic increase in emerging virus diseases in the honey bee may still be worsened by the continuing development of international exchanges and the potential dissemination of still undiscovered viruses or other agents that may favor their active multiplication.

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