viruses

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Review

Baculovirus Genetic Diversity and Population Structure

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Abstract: Baculoviruses can naturally regulate lepidopteran populations and are used as biological insecticides. The genetic diversity of these viruses affects their survival and efficacy in pest control. For nucleopolyhedroviruses, occlusion-derived virions and the occlusion body facilitate the transmission of groups of genomes, whereas this is not the case for granuloviruses. We review the evidence for baculovirus genetic diversity in the environment, in the host insect, and in occlusion bodies and virions. Coinfection allows defective genotypes to persist through complementation and results in the pseudotyping of virus progeny that can influence their transmissibility and insecticidal properties. Genetic diversity has marked implications for the development of pest resistance to virus insecticides. We conclude that future research is warranted on the physical segregation of genomes during virus replication and on the independent action of virions during infection. We also identify opportunities for studies on the transmission of genetic diversity and host resistance to viruses.

Keywords: nucleopolyhedrovirus; granulovirus; Lepidoptera; genotype interactions; bioinsecticide; pest resistance

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1. Introduction

Baculovirus structure and infection cycle

The *Baculoviridae* is a large family of insect-specific viruses distributed across four genera [1]. Within this family, nucleopolyhedroviruses (genus *Alphabaculovirus*) and granuloviruses (genus *Betabaculovirus*) lethally infect Lepidoptera and have an established history as the active ingredient for biological insecticides [2], and as biotechnological factories for protein production [3]. The genetic diversity of these viruses affects their survival, their evolvability, and their efficacy in pest control.

Baculoviruses comprise a circular genome of double-stranded DNA (80–180 kbp) within a rod-shaped nucleocapsid. The nucleocapsids are enveloped singly or in groups by a lipid and protein membrane to form occlusion-derived virions (ODVs) (Figure 1). Nucleopolyhedrovirus ODVs are occluded in groups within a crystalline matrix of polyhedrin to form polyhedral occlusion bodies (OBs) in the cell nucleus, whereas granulovirus ODVs are occluded singly in a matrix of granulin to form ovoid granule-shaped OBs in the cytoplasm and degraded nucleus of the cell [4].

Horizontal transmission mainly occurs when larvae consume foliage contaminated with OBs. During primary infection, the OBs dissolve, releasing ODVs that cross the peritrophic membrane and infect midgut epithelial cells [5]. Secondary infections are

mediated by budded viruses (BVs) that disperse in the hemocoel to infect the cells of other organs and tissues (Figure 2). Following replication, progeny nucleocapsids leave the cell as BVs early in infection but are retained later to form ODVs that are occluded into progeny OBs. Baculovirus replication has been reviewed in detail [6,7]. Nucleopolyhedrovirus-killed late-instar larvae can produce approximately 10^9 OBs (each containing dozens of virus genomes), whereas granulovirus-killed larvae produce 10- to 100-fold more OBs, although each OB contains a single-virus genome.

Insect populations are susceptible to epizootics of baculovirus disease when they reach high densities that favor efficient transmission of these viruses, particularly in ecosystems in which larvae feed on exposed plant structures close to the soil surface [8]. This is due to the facility with which OBs can move between the soil reservoir and the plant surfaces on which transmission can occur. OBs can persist in a viable state for months or years in the soil environment, which contrasts with a persistence of hours or days on plant foliage exposed to solar radiation [9].

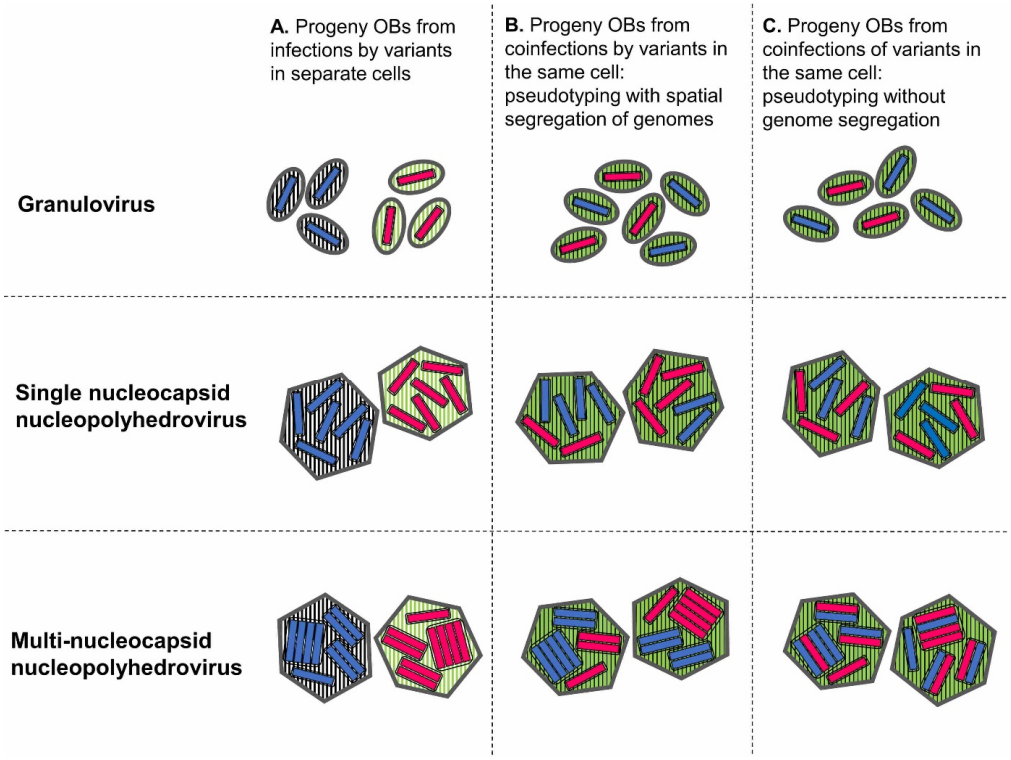

Figure 1. Occlusion body (OB) structure in relation to genetic diversity and the results of coinfection. Genotypic variants are represented as blue or pink genomes, encoding black hatched or green hatched proteins, respectively. If infections occur in separate cells, the content of each OB is homogeneous (column A). For granuloviruses, each genome is enveloped and occluded individually in an OB. Genotypic variants of granuloviruses that replicate in coinfected cells produce progeny with a mixed-variant pseudotype (mixed black and green hatching) but are occluded separately (column B, C). For single-nucleocapsid nucleopolyhedroviruses, single genomes are occluded in groups within each OB. Coinfection results in the pseudotyping of the progeny, with or without spatial segregation of genomes during replication and assembly (columns B, C). For multi-nucleocapsid nucleopolyhedroviruses, genomes are enveloped in groups comprising between one and many occlusion-derived virions (ODVs). Coinfection would result in the pseudotyping and segregation of virus progeny among ODVs if replication and assembly processes were spatially separated in the cell nucleus (column B), but this is uncertain. In the absence of segregation, progeny would produce genotypically diverse ODVs and OBs (column C).

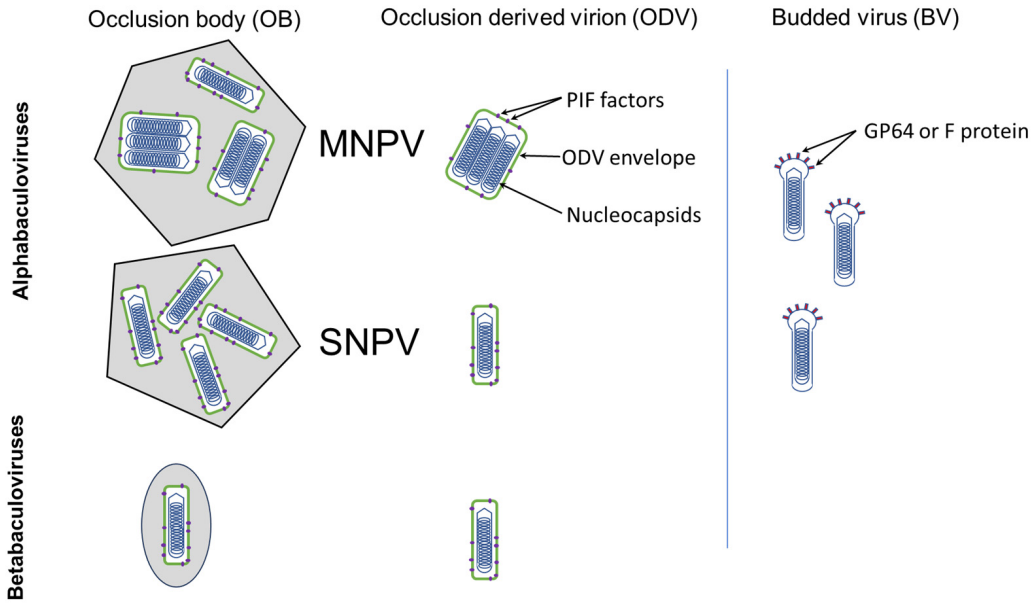

Figure 2. Structure of alphabaculoviruses (lepidopteran nucleopolyhedroviruses) and betabaculoviruses (lepidopteran granuloviruses). Following ingestion, occlusion bodies (OBs) dissolve and liberate occlusion-derived virions (ODVs). The ODVs infect midgut cells due to the presence of per os infectivity factors (PIF factors; purple ellipses) that link and fuse the virion membrane to the brush border of midgut cells. ODVs carry a single nucleocapsid in granuloviruses and single-enveloped nucleopolyhedroviruses (SNPVs). In contrast, each ODV can carry between one and several nucleocapsids in the multi-nucleocapsid nucleopolyhedroviruses (MNPVs). A different morphotype, the budded virus (BV), is produced in infected insects by the budding of individual nucleocapsids through the cellular cytoplasmic membrane. This BV carries fusion proteins of GP64 or envelope fusion protein (F protein), which allow infection of other cells in the larva (red spikes).

1.1. Variation in the Virus Genome

Nucleotide sequence variation mostly consists of indels (insertions and deletions), single-nucleotide polymorphisms (SNPs), and recombination events, all of which provide mechanisms for evolvability when viruses are faced with changes in their host or wider environment. This variation is not randomly distributed in the genome and affects regulatory regions and specific genes [10–19].

The DNA polymerases of baculoviruses have proofreading activity [20], resulting in ~1×10^{-7} substitutions per nucleotide in Autographa californica multiple nucleopolyhedrovirus (AcMNPV) [21]. However, due to the high number of genome copies, SNPs are frequently detected in baculovirus populations [22–24], ranging between 51 and 475 in Cydia pomonella granulovirus (CpGV) and 94 SNPs on average in the genome of AcMNPV [22,25]. Duplication events have also been described, both in coding regions [26] and in the *hrs* that act as transcription regulators and origins of replication. In addition, recombination has been observed during replication with coinfecting genomes of the same or different virus species [12,27–29] or with host genomic elements and transposons [30–32].

1.2. Variation in the Virus Population

Variation in genomic sequences is reflected in the appearance of "genotypic variants" in natural baculovirus isolates, but the observed variation largely depends on the methodology used. Cloning techniques, such as cell culture or in vivo cloning, do not allow all the variants to be amplified [33]. Replication in cell culture can also result in the production of variants with deletions in non-essential genes [34,35]. As such, studies on cloned variants may not accurately reflect the genotypic composition of a particular natural isolate [36].

Some of the variants present in field-collected isolates of nucleopolyhedroviruses may present large deletions in the genome [37–39], or disruption to genes involved in virulence [40], whereas others may be unstable during replication and represent a source of additional genetic diversity [41,42].

Restriction endonuclease analyses often demonstrate that isolates from diseased insects collected at the same location, or at different sites and times, are polymorphic for restriction fragment length and frequently present submolar bands that reflect their genotypic heterogeneity [43,44]. Such observations are more common in nucleopolyhedroviruses than in granuloviruses, although with the increasing use of next-generation sequencing, the presence of genotypic heterogeneity is also being recognized in granulovirus isolates [45].

1.3. Variant Interactions Affect Phenotype

Natural virus isolates almost invariably differ in insecticidal traits such as OB pathogenicity (measured as lethal dose metrics), speed of kill, and OB production when tested against a particular insect colony [39,46], across different host colonies [47], or in colonies of different host species [48]. Individual variants also show clear differences in their phenotype that influence their horizontal and vertical transmission [49], or their ability to replicate in a given host genotype [50,51].

Genotypic variants interact within the baculovirus population, and these interactions can increase or decrease the fitness of the population. For example, nine genotypic variants present in a Nicaraguan isolate (SfNIC) of the Spodoptera frugiperda nucleopolyhedrovirus (SfMNPV) were cloned, and each one was compared to the natural isolate. Significant differences were observed among variants in terms of OB pathogenicity and speed of kill. However, the frequency of each variant in the virus population was stable over time and generated a population phenotype that combined high OB pathogenicity but attenuated speed of kill, which resulted in high OB production [52].

A different interaction was detected in a commercial isolate of Spodoptera exigua MNPV (SeMNPV-SeUS2) [38]. However, in this case, the presence of one variant reduced the pathogenicity of mixed-genotype OBs, indicating that it was a cheater genotype that contributed negatively to virus transmission [33].

2. Mechanisms and Processes Affecting Diversity

Both the physical structure and the infection cycle of baculoviruses influence the transmission of genotypic diversity. Nucleopolyhedroviruses and granuloviruses show marked differences in some stages of the infection cycle, which will be highlighted when examining each level of diversity.

2.1. In the Environment

The OB structure allows baculoviruses to persist for months or years in the environment outside their host. Virus diversity present in the environment is inferred by collecting field-infected larvae or by feeding plants or soil samples to laboratory-reared insects [53], but next-generation sequencing of environmental DNA has not yet been applied to the characterization of genotypic diversity in baculoviruses from environmental samples. Importantly, the presence of viral DNA in environmental samples does not provide information on the viability of those genomes, which can be determined only by bioassay.

2.2. In the Host Organism

Lepidopteran larvae that ingest nucleopolyhedrovirus OBs are often infected by multiple genotypes [36]. This may be due to the consumption of several OBs on contaminated foliage, by consumption of a single OB containing a diversity of genotypes [54], or by de novo generation of diversity from a single unstable variant [41,42]. In some cases, only

one genotype is detected in each larva, even in epizootic conditions in which each insect is likely to have consumed more than one OB, suggesting that selection for certain genotypes occurs very early in the infection process [55]. In granuloviruses, most OBs contain a single genome. Accordingly, multiple OBs have to be consumed to result in a genetically diverse infection, which is more likely at high OB densities or when the duration of the feeding period is extended [56]. The presence of more than one variant in a single larva has been observed in CpGV natural isolates from individual larvae [57] and in insects from virus-treated orchards [56].

In situations in which larvae consume multiple OBs, the probability of coinfection by different viruses increases. Natural coinfection of lepidopteran larvae by nucleopolyhedroviruses and granuloviruses is occasionally observed in field-collected larvae of various species of hosts [58], whereas natural coinfection by different nucleopolyhedroviruses is less common and limited to particular pathosystems, such as that of the multi-nucleocapsid and single-nucleocapsid viruses in *Thysanoplusia orichalcea* [59] and *Trichoplusia ni* [60] or the CfMNPV and the defective helper virus CfDEFNPV that infect *Choristoneura fumiferana* [61]. In other cases, distinct viruses have been isolated from the same host in a particular region, but natural coinfection has not been observed. One example is that of the nucleopolyhedroviruses that infect *Rachiplusia nu* in Argentina [62,63], possibly because coinfection is difficult to detect and is often overlooked in the absence of molecular studies or because one virus is capable of excluding or suppressing a second infection in the early stages of coinfection [64].

The midgut is an important filter for defective variants and acts as a genetic bottleneck of particular importance when insects consume low doses of OBs [65,66]. The number of OBs responsible for establishing an infection has been estimated at between 1.3 and 6.3 [67]. Accordingly, even low doses of OBs can result in multiple foci of infection, each comprising potentially different genotypes [68].

It is possible that covert infections of the host are also genotypically diverse, and this diversity might be transmitted vertically to the host's offspring [4]. There is, however, some evidence that vertically transmitted variants are genetically distinct from variants that are transmitted horizontally [49,69,70]. The genetic diversity of latent and sublethal infections has not been the subject of scrutiny in baculoviruses, but given the probable bottleneck that this route imposes, opportunities for the transmission of genotypic diversity seem likely to vary between viruses that transmit transovarially (within the egg) and those that adopt transovum transmission, by surface contamination of the egg [71].

2.3. In the Cell

A second bottleneck occurs during the secondary infection mediated by BVs in cells of the organs and tissues within the hemocoel of the host. Coinfection of cells by different BVs is tightly controlled [72]. Synchronous coinfection with two genotypes of AcMNPV reached 95% of cells, but this was reduced to less than 20% when a delay of 16 h was set between the initial infection and subsequent infection by other virions arriving at the cell surface, a process known as superinfection exclusion [73]. As a result, early in host colonization, when BVs are scarce, cells are likely to be infected by just one or two virions, whereas later in infection, when BVs are abundant, cells are infected by an average of four or five virions in the *Trichoplusia ni*–AcMNPV pathosystem [74]. In consequence, the dynamics of BV production is expected to have a marked impact on the diversity of variants present in progeny OBs. Recent studies also suggest that the efficacy of superinfection exclusion is sensitive to the cell cycle, at least in latently infected *S. exigua* cells (Se301) [75].

Importantly, genomes that replicate together in a cell compete for host resources, but also share a common pool of transcription products that can be considered as public goods.

The use of proteins from the shared pool has notable consequences. First, all the progeny viruses will have a shared pseudotype, irrespective of the genotype carried within their nucleocapsids (Figure 1, column B, C). This means they are likely to exhibit a similar infectious phenotype because they share the *cis*-acting proteins that are physically associated with virus particles and that have various roles in the early events of infection. Second, the production of essential factors by a complete genotype allows defective variants to acquire these factors for their own progeny, i.e., complementation. The transmission of defective genotypes is therefore dependent on frequent coinfection with complete genotypes.

2.4. In the Occlusion Body

The OB structure of nucleopolyhedroviruses is a clear example of a collective infectious unit [76], in which tens of virions are occluded in each OB [77]. Although the aggregation of virus particles in groups reduces the overall number of infectious units, this social transmission strategy can be advantageous in alleviating the genetic bottleneck during midgut infection [78]. In contrast, in granuloviruses, only a small fraction of the OBs may contain more than one nucleocapsid [79,80].

The first studies on the co-occlusion of variants inferred a physical association of variants [81,82]. More convincing evidence was provided by analyzing insects that consumed a single OB. Between one third and one half of insects acquired a genotypically diverse infection, involving between two and five variants from a single OB [54,83]. Recently developed methods, such as laser capture microdissection [84] and sequence-dependent genome autofluorescence [85], are likely to prove valuable for characterizing the composition of individual OBs.

2.5. In the Occlusion-Derived Virion

Single-nucleocapsid nucleopolyhedroviruses have one nucleocapsid and one genome wrapped within each ODV (Figure 1). In contrast, multi-nucleocapsid nucleopolyhedroviruses have ODVs that comprise between one and tens of nucleocapsids that are co-enveloped together within each ODV. The number of nucleocapsids per ODV varies in different nucleopolyhedroviruses and also across different isolates of the same virus species [86]. In AcMNPV, for example, 90% of the ODVs are of the multi-nucleocapsid morphotype, comprising 2–10 nucleocapsids per ODV and an average of six nucleocapsids per ODV [66]. The number or morphotype of ODVs occluded within OBs has been demonstrated to have a genetic basis [52,87–90], although cellular factors and cell physiology are also influential [91,92]. Despite its importance in the transmission of genetic diversity, this is an aspect of the insecticidal phenotype that is rarely considered in isolate characterization studies.

The simultaneous delivery of various nucleocapsids into midgut cells by multi-nucleocapsid ODVs may have direct effects on virus fitness. First, a fraction of the nucleocapsids can immediately be repackaged and exported as BVs without the need for virus replication [7,93]. It appears that single- and multiple-type ODVs have a similar probability of infecting midgut cells, but for the same number of nucleocapsids, envelopment in single ODVs creates far more individual virions. However, multi-nucleocapsid ODVs appeared to be capable of establishing secondary infections faster than cells infected by single-nucleocapsid ODVs, which favors the rapid establishment of a systemic infection that is independent of the fate of the primary infected cell [93]. Second, as virus genomes carry an arsenal of genes capable of blocking apoptosis and global protein shutdown responses of the host cell, the simultaneous delivery of multiple copies of these genes may increase the virus' ability to overcome the cell's innate immune response during the earliest stages of infection [94–96]. Third, among the virus nucleocapsids that migrate to the nucleus,

those with UV-damaged genomes would be able to recover viability through recombination with coinfecting genomes [91]. Fourth, SNPV and MNPV viruses can differ in their fitness depending on the host plant, as observed in the tussock moth *Orgyia pseudotsugata*, which feeds on various species of firs (*Abies* spp.) in North America. The single and multiple morphotypes coexist but differ in infectiousness, speed of kill, and in their ability to avoid detection by host larvae feeding on different species of OB-contaminated foliage [92].

Evidently, the diversity of genotypic variants present within ODVs depends on the number and diversity of the BVs that infected the host cell in which they were assembled. When cultured cells were inoculated with highly diluted suspensions of SfMNPV ODVs, mixtures of genotypes were detected at higher-than-expected frequencies, indicating that several genotypes were present in individual ODVs [54]. More recently, the presence of the genomes of different virus species were demonstrated to be co-enveloped within individual ODVs that were produced as a result of mixed-virus infection of a shared host [83,97].

A study involving the use of recombinant viruses concluded that midgut coinfection was approximately ten-fold less common than expected assuming a random assortment of genomes in OBs [98]. Several factors may have contributed to this finding, in which a midgut reporter gene assay was employed to determine the presence of coinfected cells. The role of inoculum nucleocapsid repackaging and export may be particularly relevant given that the authors observed midgut infection foci at 72 h post-inoculation, which is notably later than the cell sloughing response to infection [94]. Alternatively, few cells may have suitably expressed reporter genes under the control of very late promoters. These authors argue that during replication in coinfected cells, nucleocapsids containing the genomes of different variants are spatially segregated into different ODVs, so that midgut infections originating from mixed-variant ODVs are uncommon [98]. These findings require confirmation and emphasize the need to better understand the physical arrangement of genotypic variants at the OB and ODV levels. In this respect, the recently developed single-cell and single-nucleus RNA sequencing technologies could provide valuable insights into the genetic composition of ODVs involved in the primary infection process [99].

It has been suggested that due to the high number of genomes produced in infected cells, a compartmentation mechanism could exist to limit contact between them [100]. This proposed mechanism would link genome replication and nucleocapsid formation, and perhaps membrane envelopment and OB condensation [7]. Under such conditions, the condensation of genetically diverse OBs would be limited.

3. Processes That Favor Genotypic Diversity

Genetic diversity in virus populations is selectively advantageous and has ecological and evolutionary benefits to these viruses. Diversity is maintained through four main processes.

3.1. Trade-Offs Between Components of Virus Fitness

Trade-offs between components of virus fitness mean that variants that differ in specific traits can be favored in different individuals (or different tissues) or when transmission opportunities vary. A well-recognized trade-off involves the negative correlation between the speed of kill and the number of progeny OBs that are produced in each infected host. A faster speed of kill could allow more cycles of horizontal transmission in a given period but results in the production of fewer infectious OBs in each infected host, which reduces the probability of transmission [101,102]. The environmental persistence of OBs was found to be negatively correlated with the transmission rate of 16 isolates of LdMNPV [103]. A trade-off was also observed between the transmission rate and variation in transmission, so that a strain with a lower and less variable transmission rate could coexist in a host population

with a strain having a higher but more variable transmission rate [102]. Indeed, trade-offs in virus phenotypes that affect transmissibility mean that phenotypically diverse virus strains are likely to provide better control of host populations than individual genotypes alone [104].

3.2. Interactions Between Virus Genotypes

The interactions between and among genotypes can involve *cis*- or *trans*-acting factors. The *cis*-acting factors require coinfection of cells, whereas the *trans*-acting factors do not. For example, the variants that produce enhancin factors that degrade the peritrophic matrix do so to the benefit of all variants present in the inoculum, which is an example of a *trans*-acting interaction that does not depend on a physical association of the variants. Such *trans*-acting interactions can even occur between different species of viruses [105].

Evidence for interactions between and among genotypes comes from two types of inocula, namely OBs from different sources that are mixed and used as inoculum, or mixtures of variants that have replicated in the same cell and become co-occluded in mixed-genotype OBs. When single-genotype OBs are mixed and used to inoculate larvae, the host mortality response, speed of kill, and progeny OB yields can be affected, although the mechanisms involved in these interactions are mostly unclear [37,106].

When OBs are produced in a coinfected cell, all progeny ODVs share the pool of viral proteins. In certain cases, virion pseudotyping can alter the infectivity phenotype and extend the host range of these viruses [83,97]. The factors that provide entry into host cells are *cis*-acting factors, physically associated with the virus particle, such as the per os infectivity factors (PIFs) that compose the fusion mechanism between the ODV envelope and the midgut cell membrane [107].

When mixtures of variants are co-occluded to produce mixed-genotype OBs, significant changes in the host mortality response, speed of kill, and progeny OB yield have been observed in several pathosystems [37,52,108–111]. Importantly, the direction and magnitude of these changes cannot be readily predicted from the phenotypic traits of the component variants, although in some cases, such as the *cis*-acting PIF-1 factor in Sf-NIC variants, manipulation of gene expression can provide useful insights into the mechanisms underlying variant interactions [112].

In theory, *cis*-acting factors might also act during systemic infection of the host, assuming that host cells are infected by several BVs. For example, CpGV-M does not kill the larvae of type I resistant codling moth, whereas CpGV-R5 is fully infectious and lethal. However, CpGV-M can infect and replicate in resistant insects previously infected by CpGV-R5 [113]. The "helper" effect of CpGV-R5 cannot be substituted by other baculoviruses that are able to replicate in codling moth. This finding brought into question the hypothesis of independent infection of variants and suggested the possible existence of a viral communication system, similar to the *trans*-acting arbitrium viral peptide produced following phage infection in bacteria [114]. Moreover, mixtures of OBs of both variants have higher pathogenicity to both susceptible and resistant insect colonies, when compared to each variant alone [115,116]. The mechanisms involved in this apparent cooperation remain uncertain.

3.3. Differential Selection for Genotypes

Genotypic variants often vary in their capacity to infect and replicate in hosts. As a result, transmission experiments in homologous or heterologous hosts frequently result in changes in the prevalence of particular variants [117–120]. Indeed, quantitative PCR techniques are currently being developed to examine the transmission and persistence of genetic diversity in the SfMNPV Nicaraguan isolate [121].

Genetic drift was quantified in *S. exigua* larvae inoculated with an equal ratio of two AcMNPV genotypes and resulted in a roughly 100-fold difference in the ratio of genotypes that replicated in infected individuals, likely due to a combination of stochastic variation in the primary infection and variation in host susceptibility to infection [66]. Similarly, genetic drift caused by transmission bottlenecks and variation in virus replication within hosts was identified in a field-collected sample of 143 LdMNPV-infected *Lymantria dispar* larvae [122]. High levels of heterogeneity in host susceptibility to infection means that a fraction of the host population is highly prone to infection, which favors the transmission of inoculum with high genetic diversity, even at low inoculum concentrations [123].

Testing families of *L. dispar* against a range of LdMNPV isolates provided evidence for host genotype × virus genotype interactions, which promoted variation in both host and virus populations [124]. As no host family is resistant to all genotypes, and no isolate is highly infectious to all host families, genotype × genotype interactions may also promote negative frequency dependent selection, which could explain how rare virus genotypes are able to persist and seem to be a feature of baculovirus populations [120]. Such interactions are particularly apparent in the populations of *C. pomonella* that show different types of resistance to CpGV [125] and vary in their susceptibility to different CpGV genotypes [116].

3.4. Genotype × Environment Interactions

Variants perform differently in terms of transmission or persistence in distinct environments. For example, certain variants of SeMNPV were particularly prevalent in greenhouse soil substrate, even soils with an alkaline pH, suggesting that a fraction of the virus population may be better adapted to persist in the soil reservoir during periods when the host is absent [126]. Food plants can strongly influence virus transmission as plants affect the insect's immune response, virus interactions with plant chemicals, and the composition of the gut microbiota [127,128]. As a result, the composition of the virus population may reflect the host's feeding habits and the vegetation present in each locality [106,129,130].

4. Genetic Diversity in Biological Insecticides

As the genetic composition of insect populations fluctuates over time and space, their susceptibility to virus populations and individual variants varies [131], which poses a challenge in the choice of the most efficient isolate or variant for the production of biological insecticides. Mass production requires the continuous monitoring of the product quality, which is easier if the isolate is genetically homogeneous. Indeed, Lee and Miller [132] suggested that the use of cloned viruses or homogeneous isolates would facilitate quality control, but later, Lynn et al. [133] proposed the production of multiple independent clones that could be mixed in the final product to improve the efficacy of these products against genetically heterogenous pest populations. Currently, the selection of the active material of virus-based insecticides usually involves characterization of the insecticidal phenotype of a range of natural isolates. The most suitable isolate is then subjected to formulation and field efficacy tests and used to produce the desired insecticidal product [2]. In some cases, these products can comprise mixtures of different virus species, likely produced in different host species, to create insecticides with increased host range [134].

The use of recombinant DNA technology to improve the efficacy of virus-based insecticides has attracted considerable attention over the past few decades [135]. This requires the initial selection and cloning of a genotypic variant. However, the rapid demise of recombinant-infected larvae makes OB production challenging for these viruses [136], so that all commercial insecticides are currently based on unmodified viruses.

The spread of type I resistance to CpGV in codling moth populations highlighted the need for genetic heterogeneity in virus-based insecticides. To control resistant codling

moth, several of the currently available products appear to comprise mixtures of at least two genotypic variants, which increases their efficacy [115,116]. Accordingly, virus insecticides composed of various genotypic variants are likely to prove more sustainable as pest control products, but maintaining stable frequencies of each genotype is a challenge in their production. The methods required to produce uniform batches of genotypically complex OBs will vary according to the virus, the uniformity of the insect colony, and the type of interactions that can occur among variants.

A recent approach to improving virus-based insecticides has focused on producing laboratory-designed mixtures of genotypic variants with desirable insecticidal properties. This involves the coinfection of larvae with mixtures of variants in varying proportions to produce mixed-genotype OBs with increased pathogenicity compared to natural isolates or the component genotypic variants [137]. This approach has been applied to the development of SeMNPV [138] and HearNPV [109], whereas co-occlusion of mixtures of variants did not improve the pathogenicity of AgMNPV or SfMNPV OBs over that of natural isolates [37,109]. Serial passage of co-occluded preparations in larvae can affect the prevalence of genotypes in the mixture, resulting in an additional increase in OB pathogenicity [109]. Although increased pathogenicity has been the primary objective of these studies, the same approach could be applied to optimizing speed of kill or OB production characteristics [27].

The concept of co-occlusion was taken a step further with the observation that in some cases it was possible to co-occlude mixtures of different nucleopolyhedroviruses. For this, a shared host is required in which both viruses can replicate. A fraction of the viruses that coinfect and replicate in the same cell are enveloped together in mixed-virus ODVs [97]. For example, mixtures have been produced for AcMNPV mixed with SfMNPV or MbMNPV, SfMNPV in mixtures with MbMNPV or SeMNPV, and mixtures of the single-nucleocapsid HearNPV and the multi-nucleocapsid HearMNPV, which are not closely related and show divergent host range characteristics [83,97]. The mixed-virus OBs produce lethal infections in both the original hosts, but the replication of each virus in the heterologous host is marginal in the systems studied. This suggests that following application of the co-occluded preparations in the field, a heterologous virus would likely be eliminated from mixed-virus OBs within a few cycles of insect-to-insect transmission, thus ensuring the biosafety of co-occluded insecticides. In a separate laboratory study, SeMNPV OBs were found to harbor co-occluded iflaviruses. The ingestion of these OBs resulted in the transmission of both types of viruses and was associated with significant variation in their insecticidal properties, although the degree to which this phenomenon occurs in nature remains unclear [139]. Nonetheless, when applied to mixtures of nucleopolyhedroviruses, the co-occlusion technology could pave the way to new virus insecticides with a host range based on the needs of growers of crops attacked by combinations of lepidopterous pests [137].

Risks of Resistance

Heterogeneity in insect susceptibility to virus infection means that repeated application of high doses of viral OBs over large areas selects for resistance in pest populations. This has been demonstrated in laboratory colonies of insects exposed to repeated challenges of granuloviruses [140], or nucleopolyhedroviruses [20,141–144], with resistance ratios of approximately ten-fold to many thousand-fold, depending on the host species and the number of generations exposed. Resistance can also appear in natural insect populations during epizootics of disease, but to a lesser extent [36]. No notable resistance issues arose during two decades of widespread use of genotypically diverse AgMNPV-based products in soybean crops in Brazil [145], whereas resistance was observed in laboratory colonies challenged with a single isolate of this virus [141].

Insecticides based on CpGV for control of the codling moth in Europe were all developed using the CpGV-M isolate originally collected in Mexico [146], which presents undetectable variability and may be considered as clonal. Beginning in 1988, the use of CpGV-M-based products in European countries progressively increased to >100,000 Ha/year, but failures in pest control from 2003 were due to the development of resistance [147,148]. Today, CpGV genotypic variants are classified in seven phylogenetic groups [25,149], and a total of five types of resistance to CpGV have been identified in *C. pomonella* populations [125]. CpGV variants are able to bypass one or more types of resistance mechanisms but are fully or partially blocked by others [25,150].

Possible approaches for resistance management in codling moth involve either the sequential use of products containing a single variant, or the use of products comprising a mixture of variants. Selection of the appropriate control strategy needs to consider not only the independent action of each isolate, and the fitness cost of resistance to each isolate for the host, but also the result of the interaction between isolates. The observed increase in efficacy of genotypically diverse CpGV suggests that the second approach might be more sustainable [115,116]. However, as coinfection by granuloviruses implies ingestion of more than one OB, this will require increasing the dose of OBs applied in the field, with an associated increase in production costs.

5. Future Issues

The growing recognition of the importance of genetic diversity in baculovirus populations has highlighted four principal issues that merit the attention of researchers in virology and bioinsecticide development.

5.1. Physical Segregation of Variants

The spatial and physical processes within the nucleus that determine the production of nucleocapsids, their envelopment into ODVs, and OB condensation are not well established. Clearly, these processes are likely to influence the composition of ODVs and OBs that transmit genotypic diversity from insect to insect. The cell environment [19] and mutations in the viral DNA polymerase [151] have been implicated in affecting the proportions of single- and multi-nucleocapsid ODVs. This suggests that the rate of genome replication may have downstream effects on the enveloping of nucleocapsids singly or in groups. The assembly of nucleocapsids in association with microvesicle-rich regions of the nuclear virogenic stroma, from which ODV membranes may be derived, also appears likely to influence ODV composition but is poorly understood [92]. Spatial segregation of genomes during replication and encapsidation was recently implicated in low levels of midgut coinfection by AcMNPV recombinant viruses [97]. As this finding would appear to contradict the findings of others [137], the possibility that variant genomes are segregated among different ODVs merits closer examination.

5.2. Transmission of Diversity

It is clear that baculoviruses face genetic bottlenecks during horizontal transmission, initially to establish a primary infection in midgut cells, and during systemic infection of host cells that is restricted to a brief (~16 h) temporal window. Transovum or transovarial vertical transmission to offspring is likely to represent an additional bottleneck. We have a poor understanding of the magnitude of these bottlenecks, although attempts have been made to quantify the number of founder genomes following oral inoculation in some host-nucleopolyhedrovirus systems [66,99]. If the number of variants present in progeny OBs exceeds that of the founders, the additional diversity must have been generated

during replication and there is indeed growing evidence for the de novo generation of variants [22,41,152].

The advantages of each occlusion strategy for nucleopolyhedroviruses (multiple virions in each OB) and granuloviruses (a single virion in each OB) are unclear, as the choice of strategy dictates the likelihood of coinfection (and the transmission of genetic diversity) at the organismal level. In general, granuloviruses have a narrower host range and are more specialized in infecting a particular host species than nucleopolyhedroviruses, so that the granulovirus strategy appears to be based on maximizing the likelihood of establishing infection from a single OB. In consequence, granuloviruses prove more successful than nucleopolyhedroviruses when the amount of leaf surface consumed by a larva is small, as is the case for insects that develop inside plant structures, in which opportunities for horizontal transmission are restricted in time and space. Notable examples include the tortricids *C. pomonella*, *Cryptophlebia leucotreta*, and *Epinotia* (*Crocidosema*) *aporema*, the gelechiids *Tecia solanivora* and *Phthorimaea operculella,* and the crambid *Diatraea saccharalis*. As such, the granulovirus strategy involves producing an exceptionally large number of highly infectious progeny OBs in each infected larva.

By contrast, the occlusion strategy involving groups of ODVs seen in nucleopolyhedroviruses is becoming elucidated through new sociovirology approaches that highlight the benefits of collective transmission in establishing infection, replication, and the ability to subjugate host defenses [78,153]. Nonetheless, a number of issues related to the social interactions of viruses have been highlighted including the role of defective (cheater) genotypes, the trade-offs that arise from optimizing within- and between-host transmissions, and the optimal group size for coinfecting genotypes, all of which are faced by nucleopolyhedroviruses [154].

An additional unresolved question focuses on the conditions under which the multi-nucleocapsid strategy is favored over the single-nucleocapsid strategy of genome delivery to midgut cells. As the number and distribution of nucleocapsids among ODVs is a virus-specific trait, this would suggest that this trait is optimized for the midgut characteristics and cell sloughing behavior of the host. It would be interesting to compare the success of single- and multi-nucleocapsid ODVs in viruses that are host-specific, such as SeMNPV, with those that encounter a broader range of potential hosts, such as AcMNPV and MbMNPV, in order to examine the correlation between inoculum repackaging and pass-through events and cell sloughing rate and whether this differs for viruses that differ in host specificity. ODV enrichment studies could prove useful for this type of study [94].

5.3. Host Resistance

As resistance is of key importance in pest control, it deserves close attention. Experience with CpGV proves that insects can develop resistance to single-genotype insecticides, but to date, no resistance has been observed to genotypically diverse insecticides under field conditions [155]. Indeed, virus diversity likely hinders the development of host resistance. In a laboratory study on *T. ni*, resistance was generally higher and developed faster when exposed to single variants than mixtures of variants or a natural isolate of AcMNPV [156]. In addition, various virus fitness components (productivity, virulence) appear to be independently selected, leading to different strategies in each host, which results in an increased virus population diversity [157].

Resistance often imposes a cost to host fitness, such as slower development or reduced body weight [155]. For example, fitness costs were more severe when the inoculum was genotypically diverse compared to the costs of exposure to single variants of AcMNPV [156]. Resistance costs were also higher on poor-quality diets [158–160], so we might predict higher host resistance and greater diversity in virus populations associated with high-

quality food plants and the opposite on marginal food plants, despite the marked effects that food plants can have on the host immune response [161]. However, no cost was observed in codling moths for resistance to CpGV-M [162].

5.4. Independent Action of Virions

Finally, and importantly, the hypothesis underlying all previous studies is that of the independence of infection of each cell by ODVs and BVs. This assumption warrants scrutiny, as sequential infection of the codling moth by different genotypes of CpGV suggests the production of helper molecules that facilitate the action of other genotypes [113]. If confirmed, these findings could significantly change our understanding of the infection process and the functional importance of genotypic diversity in this family of viruses.

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