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PERSPECTIVE

Evolution and the microbial control of insects

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Abstract

Insect pathogens can be utilized in a variety of pest management approaches, from inundative release to augmentation and classical biological control, and microevolution and the consideration of evolutionary principles can potentially influence the success of all these strategies. Considerable diversity exists in natural entomopathogen populations and this diversity can be either beneficial or detrimental for pest suppression, depending on the pathogen and its mode of competition, and this should be considered in the selection of isolates for biological control. Target hosts can exhibit considerable variation in their susceptibility to entomopathogens, and cases of field-evolved resistance have been documented for *Bacillus thuringiensis* and baculoviruses. Strong selection, limited pathogen diversity, reduced gene flow, and host plant chemistry are linked to cases of resistance and should be considered when developing resistance management strategies. Pre- and post-release monitoring of microbial control programs have received little attention; however, to date there have been no reports of host-range evolution or long-term negative effects on nontarget hosts. Comparative analyses of pathogen population structure, virulence, and host resistance over time are required to elucidate the evolutionary dynamics of microbial control systems.

Introduction

Biological control of pests is primarily an exercise in applied ecology, requiring an understanding of population dynamics, predator–prey interactions, and intra- and interspecific competition. However, the relevance and application of evolutionary principles to biological control have received rather less attention (but see Roderick and Navajas 2003; Hufbauer and Roderick 2005). Evolution and biological control are most commonly discussed in the context of classical biological control, in which long-term pest suppression results from the introduction and establishment of a natural enemy collected from the native range of an exotic pest (Hufbauer and Roderick 2005). However, microevolution can also influence other forms of biological pest control.

Insect pathogens have a long history of use in biological control: in particular, bacteria [mainly various strains of *Bacillus thuringiensis* (*Bt*)], entomopathogenic fungi (e.g., *Beauveria bassiana* and *Metarhizium* species), numerous species of baculovirus, and also entomopathogenic nematodes (e.g., *Steinernema* and *Heterorhabiditis* species) (Lacey and Kaya 2007). Entomopathogens are distinguished from other means of insect biological pest control primarily by their methods of application. For the most part, insect pathogens are applied using an inundative release strategy. That is, they are applied in large numbers onto intermediate to high densities of pest populations, with the expectation of 'immediate' pest control. Immediate means that pest suppression does not rely on the long-term reproduction or establishment of the pathogen, although in reality it can take several days for the pathogens to replicate and kill their host. It is also likely that with some pathogens and with some target groups, effective control relies on secondary cycling of the pathogen [e.g., locusts (Thomas et al. 1995) and forest Lepidoptera (Woods and Elkinton 1987)], although mechanistic studies of the role of secondary transmission are rarely carried out in the pest control context. Insect pathogens have also been used successfully as classical biological control agents, with a limited number of introductions resulting in long-term pest suppression, although this approach has tended to be

more restricted to pests of forest or plantation crops (Hajek et al. 2007).

What are the evolutionary questions?

The relevance of evolution to microbial control agents primarily relates to two broad areas: efficacy and risk (e.g., Van Klinken and Edwards 2002; Thrall et al. 2011). The impact of microevolution on entomopathogen efficacy has focused heavily on the likelihood and management of the evolution of resistance to particular pathogens, although much of this research has been driven by the incorporation of entomopathogenic toxins from *Bt* into a range of crops (e.g., Tabashnik et al. 2008; Carrière et al. 2010). Directional selection favors genotypes with the highest relative fitness (Barton et al. 2007). Resistant alleles are frequently rare prior to exposure to entomopathogens (Tabashnik et al. 2009), with experimental estimates in the order of 10^{-3}; however, strong directional selection imposed by repeated use of a microbial control agent and subsequent population bottlenecks can rapidly increase the frequency of these alleles in the population. Relevant issues are determining the conditions under which resistance to a microbial control agent will occur and developing management strategies that can avoid or slow the rate of resistance development (Bates et al. 2005). With longer term classical biological control strategies, co-evolutionary responses may occur between a pathogen and a host, such that the pathogen could counter any resistance mechanisms developed by the host (Thrall et al. 2011). However, with inundative release the opportunity for co-evolution does not usually occur. Thus, the focus should be on identifying the natural variation in resistance of the insect population, the potential mechanisms of resistance, and avoidance of the conditions that select for resistance.

Several key questions should ideally be addressed to maximize the effectiveness of microbial control. The first is, how can we utilize the natural variation of entomopathogen populations to select for more effective control agents? Following on from this, how important is retaining genetic diversity of the pathogen in biological control? When addressing questions related to pathogen diversity, one must consider the evolutionary processes that produce diversity such as recombination and mutation, as well as those that maintain it, such as gene flow and balancing selection (Barton et al. 2007). Reducing the size of pathogen populations, which often occurs during production, can lead to strong genetic drift and results in reduced diversity and the fixation of alleles, which can be unfavorable. We also need to know how pathogen population structure is influenced by genotype × environment interactions and local adaptation, and whether this is important for pathogen efficacy. Selection is the sole basis for adaptation (Barton et al. 2007). Adaptation can be depicted on a fitness landscape consisting of a number of peaks and valleys, on which selection can act step by step to assemble gene combinations that represent a local optimum for the particular environment.

In terms of the potential risk of releasing microbial control agents, the focus is on possible host-range extension via evolution; can this occur and what are its likely environmental impacts? However, one key difference with many pest control programs with entomopathogens, compared with classical biological control, is that they are not solely focused on releasing an exotic pathogen onto an alien pest. Many pest control programs involve the release of a commercially available pathogen (usually non-native) onto a native pest, and increasingly, local pathogen isolates are being developed for pest control.

Variation and the improvement of microbial insecticides through artificial selection

Evolutionary change requires genetically based variation in phenotype. Therefore, predicting the potential for evolution requires information on pathogen variation and its means of generation. Detailed bioassays, more recently combined with molecular tools, have clearly shown variation in both pathogen virulence and host susceptibility, although the focus has primarily been on the pathogens (e.g., Wraight et al. 2010). Directional selection has a long history of use in agriculture, and the naturally high variation in entomopathogen populations could be harnessed to select for isolates with an enhanced level of a particular trait. But what are desirable traits in microbial insecticides? Initial screening of isolates generally focuses on pathogenicity (i.e., ability to cause death) and speed of kill. However, within specific pathogen groups, other traits are also seen as restricting the development and adoption of microbial insecticides, in particular their ability to cope with environmental stresses and factors that relate to their production, storage, and dissemination. The identification of new strains of *B. thuringiensis* has mainly relied on screening environmental samples, usually soils, rather than directional selection. Artificial selection has not been attempted very widely with baculoviruses either. This is perhaps surprising given the very high level of variation seen in many systems and the potential for the generation of novel isolates through recombination in mixed infections. A few attempts have been made to select for heightened pathogenicity for a specific host in the wider host-range nucleopolyhedroviruses (NPVs). For example, Kolodny-Hirsch and Van Beek (1997) serially passaged the broad host-range virus *Autographa californica* MNPV (multiply enveloped nucleopolyhedrovirus) through the diamondback moth, *Plutella xylostella*, with the aim of selecting a more virulent strain. Twenty generations of selection produced a 15-fold

increase in LC_{50} for *P. xylostella*, without any loss of pathogenicity for other species. The mechanism behind this was a change in the variant structure of the virus population, plus changes in virus morphology. More recently, selection has been used to try and overcome resistance to a granulovirus (GV) in the codling moth, *Cydia pomonella* (see below). Multiple passage of one isolate resulted in increased pathogenicity to a resistant codling moth colony and was accompanied by a change in the variant structure of the virus population (Berling et al. 2009).

Entomopathogenic fungi have received more attention, with efforts directed at selection of strains that are thermotolerant and resistant to fungicides. Fungi are relatively intolerant of high temperatures (>35°C) and a common feature of many insects is the capacity to induce a behavioral fever, which slows fungal development and can increase insect survival (e.g., Elliot et al. 2002). Many targets for entomopathogenic fungi live in environments where basking to increase body temperature above 35°C is not a problem, e.g., locusts; thus, the production of strains with thermostability at high temperatures could have significant impacts on pest suppression. Directed evolution of *Metarhizium anisopliae* to select isolates with higher thermostability, however, coincided with reduced pathogenicity but not speed of kill (de Crecy et al. 2009). Thus although this approach may have potential, trade-offs are likely to occur in this type of directed selection. A rather different approach has been the selection for strains of pathogen that will work alongside other pest management tools. Fungal plant pathogens are often key crop pests in damper climates and thus selection for fungicide resistance has been investigated in *B. bassiana* and *Metarhizium brunneum*. Although several studies have shown that improved survival against a variety of fungicides can be achieved, the impact on other fungal traits appears to be unpredictable. For example, selection for fungicide resistance in *B. bassiana* had no impact on fungal pathogenicity in one study, but resulted in a significant reduction in another (Shapiro-Ilan et al. 2002, 2011).

Entomopathogenic nematodes have received the greatest attention for trait selection often within a standard genetic framework, in which heritability has been estimated and expected selection responses have been used to monitor observed trajectories. The target traits have also been broad, ranging from enhanced dessication tolerance and nematicide resistance, to increased responsiveness to herbivore-induced plant signals. Promising results have been obtained for selecting for increased nematicide resistance and higher temperature tolerance, without a decrease in other key fitness traits (Glazer et al. 1997; Ehlers et al. 2005), but results have been more variable for cold temperature tolerance (e.g., Ehlers et al. 2005). An interesting recent example is the selection for nematodes that respond to the volatiles released by plants in response to insect attack. Maize roots damaged by the western corn rootworm *Diabrotica virgifera virgifera* emit volatiles that attract entomopathogenic nematodes (Rasmann et al. 2005). However, the most effective nematode against western corn rootworm is *Heterorhabditis bacteriphora*, which is not particularly sensitive to the main attractant (E)-β-caryophyllene (Hiltpold et al. 2009). It was possible to select a more responsive nematode strain after six generations, which, although slightly less infective compared to the unselected strain, was able to reduce western corn rootworm populations in the field (Hiltpold et al. 2010).

Is pathogen diversity beneficial or detrimental for effective control?

Although it is clear that genetic and phenotypic diversity in entomopathogens is common, its importance to biological control has largely been ignored. Various aspects of pathogen isolation and large-scale production are likely to influence diversity and population structure. It is therefore important to understand what the consequence of these changes might be for effective pest management, as there is clear evidence that parasite diversity can influence virulence (e.g., May and Nowak 1995; Van Baalen and Sabelis 1995; Frank 1996; Read and Taylor 2001; Brown et al. 2002). Some pathogens, primarily viruses, can be cloned in cell culture to isolate a specific genotype, which can be important in the characterization, registration, and commercial development of a specific product. However, production usually takes place in insects as a result of the fact that baculoviruses often adapt to cell culture, and lose genes that are crucial to insect infection (Pijlman et al. 2001). Other entomopathogens, mainly bacteria, fungi, and nematodes, are often commercially produced in artificial media, which is likely to reduce diversity and promote adaptation to conditions associated with growing outside the insect host. For example, control of the invasive wood wasp *Sirex noctillio*, a significant pest of pine (Mlonyeni et al. 2011), by the nematode *Deladenus siricidicola* declined in Australia in the late 1980s and parasitism rates went down to 20%. It turned out that this was a result of selection in the nematode cultures used for the releases. The nematode has an interesting biology in that it is has two forms: one that feeds on the wasp and another that feeds on the fungus that is introduced into the trees by the wasp. Culturing techniques for *D. siricidicola* involved repeated cycling on fungus; however, the parasitic phase was absent. Repeated selection on fungus led to enhanced *D. siricidicola* growth on fungus, but reduced the nematode's ability to parasitize *S. noctillio* (Collett and Elms 2009).

The importance of biodiversity in biological control, and in relation to ecosystem services, is a rapidly expanding

area. There have been numerous clear demonstrations that emergent effects on mortality or virulence can result from the combination of different species or different functional groups (e.g., Cardinale et al. 2003; Tylianakis and Romo 2010), including studies on entomopathogens (Jabbour et al. 2011). Research on the impact of intra-specific variation on mortality and effective biological control has received less attention, although studies in this area on entomopathogens are increasing.

Entomopathogen isolates are primarily chosen on their ability to kill a pest quickly. This is often described as virulence; however, the term virulence can mean different things to insect pathologists, evolutionary biologists, and mathematical modelers, and can describe both mortality and speed of kill (or mortality rate) (see Thomas and Elkinton 2004; Shapiro-Ilan et al. 2005). In addition, different definitions can alter predictions about virulence (Day 2002). Infection only refers to the pathogen gaining access to the host, whereas the amount of damage caused to the host after infection is usually referred to as virulence. There is a rich theoretical literature pertaining to the evolution of virulence. One of the most popular theories revolves around the trade-off hypothesis, which suggests that virulence is an unavoidable consequence of parasite transmission, thus implying that host mortality is detrimental to the pathogen. This results in a compromise between pathogen reproduction and virulence to optimize transmission success (Frank 1996; Alizon et al. 2009). However, this model has its critics (Ebert and Bull 2003) and there are alternative views (Brown et al. 2002, 2009). Many insect pathogens used for pest control are obligate killers and thus the host must die before pathogen transmission can occur; therefore, the trade-off between virulence and exploitation rate breaks down. The optimal level of virulence and the timing of transmission in this type of system depend on the background mortality of the host (Ebert and Weisser 1997) and are likely to be influenced by within host replication rate and host ecology. Studies on the waterflea *Daphnia magna* and a bacterium *Pasteuria ramosa*, an obligate-killing parasite that castrates its host, have shown that spore production (as a surrogate for transmission) peaks at intermediate levels of virulence (time to death) (Jensen et al. 2006). In another obligate killer, the microsporidian *Nosema whitei* attacking the beetle *Trilobium castaneum*, spore production was highest at intermediate levels of mortality (Bérénos et al. 2009). Thus even in obligate killers, there appear to be trade-offs between virulence and transmission potential.

More detailed analysis of infections has clearly shown that mixed infections are common at both the intra- and interspecies level, and the within-host dynamics of these infections are predicted to have an impact on virulence (Frank 1996; Read and Taylor 2001). Of particular relevance to entomopathogens is that the nature of the competition between competing strains is likely to have very different outcomes in terms of virulence and mortality (Mideo 2009). For example, a recent study compared intraspecific competition between strains of the entomopathenic fungus *M. anisopliae* with interspecific competition between the fungus and the nematode *Steinernema feltiae* in the greater wax moth *Galleria mellonella*. Faster speeds of kill equated with better competitive ability for different strains of fungi, whereas slower mortality rates were related to higher competitive ability against the nematode (Staves and Knell 2010). The authors suggested that this might be related to different modes of competition: straight resource competition for the different fungal strains but possibly antagonistic interactions with the nematodes.

More detailed studies on baculoviruses have started to probe the diversity and mortality (virulence) issue with some interesting results. Baculoviruses have a novel morphology as one of the main groups, the NPVs, contain multiple genomes (virus particles) in each of their transmission stages (occlusion bodies, OBs). If more than one genome coinfects a cell, recombination between different variants can take place and this is thought to be the main means whereby diversity is generated in baculoviruses (Cory 2010). Infections with more than one baculovirus variant are more pathogenic than those with either strain individually (Hodgson et al. 2004; Simón et al. 2005). In addition, natural baculovirus populations sometimes contain defective genotypes that lack certain genes necessary for infection and are incapable of transmission on their own. They survive by being occluded with viruses with intact genomes (Simón et al. 2004). The outcome of mixed infections is strain specific as the addition of defective genotypes can be either positive (López-Ferber et al. 2003) or negative (Muñoz and Caballero 2000). These experiments indicate that some type of facilitation goes on among the variants, although it remains unclear at this point as to how this might happen. Similar experiments with macro-parasites in vertebrates suggest that increased virulence could be the result of the costs of strain-specific immunity and an increased call on resources (Read and Taylor 2001). There is good evidence for genotype × genotype interactions against some groups of pathogens in a range of invertebrate species (e.g., Lively and Dybdahl 2000; Carius et al. 2001; Lambrechts et al. 2006; Luijckx et al. 2011). However, the link to specific immune responses is mainly limited to bacteria in the insects (Kurtz 2005) and there are few data that relate to groups of pest insects and their pathogens (but see Roth et al. 2009).

The situation with bacteria is equally complex. Studies on the toxins alone have shown that synergism can occur between multiple toxins for both *Bti* and *Btk* (Wu and Chang 1985; Crickmore et al. 1995; Lee et al. 1996; Xue

et al. 2005; Sharma et al. 2010), but this is not always the case (see Lee et al. 1996). Synergistic effects do not appear to be predictable and depend on both the species and toxin combination tested. For example, Lee et al. (1996) found a synergistic effect of Cry1Aa and Cry1Ac but found an antagonistic effect of Cry1Aa and Cry1Ab against the gypsy moth *Lymantria dispar*, whereas no synergistic effect was observed for either toxin combination for the silkworm *Bombyx mori*. The lack of generality of these relationships makes it difficult to predict which toxin combinations would prove fruitful. However, it is clear that native toxin combinations found in *Bt* crystalline proteins remain the most potent control method (Crickmore et al. 1995). Interactions with live bacteria are particularly interesting as interference competition is common as many bacteria secrete antagonistic compounds (bacteriocins and antibiotics) to suppress competitors. The production of these compounds is energetically costly and therefore this type of competition is predicted to result in decreased virulence, depending on relatedness (Gardner et al. 2004). To test this hypothesis, Garbutt et al. (2011) passaged two *Bt* strains in mixed infections through a lepidopteran host and found that, as predicted, antagonism (suppression of competitors) increased and virulence decreased.

Entomopathogenic nematodes increase the complexity of different interactions even further. They exist in a symbiotic relationship with a bacterium; the bacterium helps to overcome host resistance and protects the body from other potential scavengers, in addition to being a source of nutrition for the nematode (Ciche et al. 2006). *Xenorhabdus nematophila*, a bacterium found in the nematode *Steinernema carpocapsae*, produces a bacteriocin (xenorhabdicin) that has been shown to kill unrelated bacteria and other isolates of *X. nematophila*, but only when it is found in a different nematode species (Morales-Soto and Forst 2011). Studies on the interactions among different strains of one *bacterium, Xenorhabdus bovienii,* in isolation (without the nematode) showed that where inhibition of other strains did occur, infection took longer (decreased virulence) (Bashey et al. 2012). However, the result of competition of both partners at the intraspecific level is less clear. When the same bacterium is present in two different nematode species, it appears that it is the nematode that determines virulence (Bashey et al. 2011), whereas it is clear that presence of the bacteria *per se* has a large impact on the result of interactions, at least at the interspecific level (Sicard et al. 2006).

These data indicate that the impact of pathogen diversity on effective pest control is likely to be dependent on the particular group of pathogens, their mode of competition, and probably their relatedness or genetic similarity. For baculoviruses, the data indicate that mixed genotype infections are generally beneficial, resulting in enhanced mortality, although the results with defective viruses imply that this is likely to be genotype dependent. However, intraspecific interactions among bacteria and nematodes (plus bacteria) could result in negative impacts of mixed infections, but it remains unclear how much relatedness alters this outcome.

The evolution of resistance to microbial insecticides

The prime area where an evolutionary focus has impacted the use of insect pathogens as biological control agents involves the evolution of resistance. As inundative release strategies are only intended to be short term, it is likely that in some situations effective pest control will require multiple, high-density pathogen applications that potentially create the environment for selection for resistance to occur. However, less attention has been paid to the investigation of the natural variation in resistance in field populations of host insects, despite its importance for assessing the likelihood of the evolution of resistance in target pests (for exceptions, see Table 1). Here, we use the term resistance to mean a significant decrease in the susceptibility of a tar-

Table 1. Natural variation in resistance to insect pathogens recorded in field populations.

Type	Pathogen	Insect order	Species	Variation in resistance
Bacterium	*Bt subsp. kurstaki*	Lepidoptera	*Plodia interpunctella*	X 42*
			Trichoplusia ni	X 4 and X 2†‡
	Bt subsp. israelensis	Diptera	*Culex pipiens*	X 4§
Baculovirus	*Phthorimaea operculella* granulovirus	Lepidoptera	*P. operculella*	X 11¶
	Malacosoma californicum pluviale nucleopolyhedrovirus		*M. californicum pluviale*	X 50**

*Kinsinger and McGaughey (1979).
†Janmaat and Myers (2003).
‡Franklin and Myers (2008).
§Vasquez et al. (2009).
¶Briese and Mende (1981).
**Cory and Myers (2009).

get insect to a specific pathogen isolate or formulation: the development of total resistance to an entomopathogen is less common.

Bacillus thuringiensis has become the mainstay in microbial control and currently comprises 75% of the biopesticide market in the USA, Canada, and Mexico (Bailey et al. 2010). *Bt*'s complex mode of action and multiple toxin formulations were expected to impede the evolution of resistance; however, this has not been the case (Table 2; Tabashnik 1994; Janmaat and Myers 2003; Van Rensburg 2007; Tabashnik et al. 2008; Bagla 2010; Storer et al. 2010; Gao et al. 2011; Gassmann et al. 2011). During the early stages of *Bt* use, researchers were unsuccessful at evolving resistance through laboratory selection experiments. This ended in 1985, when McGaughey (1985) reported resistance to *Bt kurstaki* (*Btk*) in *Plodia interpunctella* after several generations of selection in the laboratory and the occurrence of low levels of resistance in *Bt*-treated grain bins. Following this, numerous selection experiments performed in the laboratory have demonstrated the ability of many insects to evolve resistance, including those belonging to the Orders Lepidoptera, Coleoptera, and Diptera (reviewed in Tabashnik 1994; Ferré and Van Rie 2002). Despite this, for over 20 years no cases of field-evolved resistance had been documented. The first case of field-evolved resistance was reported by Kirsch and Schmutterer (1988) in *P. xylostella* populations from the Philippines, with subsequent cases reported worldwide for *P. xylostella* populations (reviewed in Tabashnik 1994). Following this, in 2000, resistance to *Btk* sprays was reported for *T. ni* in commercial vegetable greenhouses in British Columbia, Canada, although not in nearby fields (Janmaat and Myers 2003). In addition, in 2003, one population of the mosquito *Culex pipiens* in New York was found to be resistant to *Bt israelensis* (*Bti*)(Paul et al. 2005). However, this remains the only reported case of *Bti* resistance. In 1996, the first genetically modified (GM) crops expressing *Bt* toxins were used in the field and at least five lepidopteran and one coleopteran pest have evolved resistance to *Bt* crops expressing single Cry toxins (Van Rensburg 2007; Tabashnik et al. 2008; Bagla 2010; Storer et al. 2010; Gao et al. 2011; Gassmann et al. 2011; Wan et al. 2012).

Table 2. Reported cases of resistance development to bacterial and viral pathogens in field populations. No cases of field-evolved resistance have been reported for entomopathogenic fungi or nematodes.

Type	Pathogen	Insect order	Species
Bacterium	*Bt subsp. kurstaki*	Lepidoptera	*Plutella xylostella**†
			Trichoplusia ni‡§
			Plodia interpunctella¶
Bacterium	*Bt subsp. israelensis*	Diptera	*Culex pipiens***
Baculovirus	*Cydia pomonella* granulovirus	Lepidoptera	*Cydia pomonella*††

*Kirsch and Schmutterer (1988).
†Tabashnik et al. (1990).
‡Janmaat and Myers (2003).
§Franklin and Myers (2008).
¶McGaughey (1985).
**Paul et al. (2005).
††Eberle and Jehle (2006).
‡‡Asser-Kaiser et al. (2007).

Why did resistance to *Bt* develop in *T. ni* populations inhabiting greenhouses, but not in the field? The first contributing factor is the greater frequency with which *Bt* is sprayed in greenhouses. As temperatures are warm in greenhouses, *T. ni* population cycles occur at a faster rate than in the field. This results in greater insect pressures and more frequent use of *Bt* sprays. In addition, the growing season is extended in greenhouses when compared to that in the field in British Columbia, and this can result in 4–5 more generations of *T. ni* annually in the greenhouse. Also, when greenhouse growers do not perform an adequate clean-up in the winter, resistant *T. ni* populations can persist into the following growing season (Cervantes et al. 2011). However, the extended generation time can only partially explain the difference in susceptibility between field and greenhouse populations, as in areas of California where field populations persist year-round, *T. ni* remain susceptible to *Bt* (Franklin and Myers 2008). Gene flow can also act to delay or enhance the spread of resistance to pathogens, depending on the pattern of movement of susceptible and resistant individuals between treated and untreated areas (Caprio and Tabashnik 1992; Croft and Dunley 1993). Movement of moths among fields is not constrained and thus there are no barriers to gene flow. However, in greenhouses, resistant genes reach a high frequency early in the growing season and can spread to populations in other greenhouses in close proximity (Franklin and Myers 2008; Franklin et al. 2010, 2011). This is in agreement with a model developed for the high-dose refuge strategy that found that resistance spreads rapidly if a susceptible population does not persist (Ives and Andow 2002). *Trichoplusia ni* is a migratory pest, and susceptible moths travel on wind currents from as far as southern California to British Columbia each spring; however, the limited gene flow that occurs between greenhouse and field populations during the summer is not sufficient to reduce resistance (Franklin et al. 2010). These results are in line with the model predictions of Caprio and Tabashnik (1992) who found that moderate levels of gene flow were required to spread resistance, but high levels were necessary to impede resistance. Furthermore, the fitness costs that are present at high levels of resistance are likely minimal or absent and may explain why resistance genes were maintained in greenhouse populations that were not selected with *Bt* (Franklin et al. 2010).

Whereas resistance to *Bt* products was not anticipated, the development of resistance to other insect pathogens, such as viruses and fungi, in the field was considered even less likely. Infection by baculoviruses, fungi, and other pathogens is complex and likely to involve more diverse polygenic resistance mechanisms. In addition, they are not used frequently enough or widely enough to select for resistance. However, this expectation has turned out not to be true. Several laboratory-based studies have assessed the potential for the development of resistance in baculoviruses. For example, a modest increase of ×3 was seen after several generations of selection in the fall armyworm *Spodoptera frugiperda* and its NPV, and this was lost when selective pressure was removed (Fuxa and Richter 1989). The largest scale use of a baculovirus (NPV) (in terms of crop area) has been to control a pest of soybean, the velvetbean caterpillar *Anticarsia gemmatalis*, in Brazil (Moscardi 1999). The potential for *A. gemmatalis* to develop resistance was assessed as part of this huge and very successful program. Significant resistance to NPV could be selected for within 3–4 generations; however, interestingly, whereas insects from Brazil eventually developed resistance of up to 1000-fold, insects collected in the USA reached a plateau at approximately fivefold resistance (Abot et al. 1996). The authors suggest that this is because of the fact that the NPV is apparently not naturally present in populations in the USA and the NPV is not used there for biological control. Further selection experiments on *A. gemmatalis* in the USA showed that the insects could revert to their earlier levels of susceptibility within three generations (Fuxa and Richter 1998). Both these examples indicate that there are costs of resistance to baculoviruses, usually in the form of longer life spans, lower pupal weights, and reduced fecundity and offspring viability. In contrast, detailed selection experiments in which both the cabbage looper, *T. ni*, and its singly-enveloped NPV (TnSNPV) were co-selected for many generations demonstrated that *T. ni* could develop resistance to the virus of up to ×22-fold. However, the resistance was stable and no costs of resistance could be found (Milks and Myers 2000; Milks et al. 2002).

One baculovirus which has been commercialized very successfully is codling moth, *Cydia pomonella*, granulovirus (CpGV). Codling moth is a major global pest of apples, pears, and related pome fruits; the young larvae burrow into the apple, rapidly causing sufficient damage to reduce the marketability of the fruit. With such a low damage threshold, any control agent has to act rapidly to be considered effective. CpGV is highly pathogenic and rapid acting; however, one of its limitations is that it needs to be applied relatively frequently during the crucial period of egg hatch, which can mean every 7–14 days where *C. pomonella* is multivoltine (Lacey et al. 2008). In the early 2000s, codling moth populations with reduced susceptibility to GV were reported in Germany and France. Preliminary bioassays indicated that some field populations were ×1000 more resistant to the commercial CpGV isolate than the standard laboratory strains, and resistance appeared to be autosomal, incompletely dominant, and possibly polygenic or non-additive (Eberle and Jehle 2006). However, patterns of resistance in this study were determined using mass crosses. Further, more detailed individual crosses showed a more complex pattern, where the resistance was sex-linked and monogenic, and dominance varied with the concentration of the virus applied (Asser-Kaiser et al. 2007). Thus, resistance could spread rapidly in the population, as females only required a single gene to become resistant and at lower virus concentrations, heterozygotes survived (the resistance gene was dominant), but at higher concentrations, it was recessive (Asser-Kaiser et al. 2007). When the gene is fixed, resistance levels can approach ×100 000 (Asser-Kaiser et al. 2007). However, it appears that despite variation in resistance in the field, resistance, in Germany at least, is based on this one gene (Asser-Kaiser et al. 2010). The exact mechanism of resistance has not yet been determined, but it appears to be related to a block in early virus replication, rather than a change to the peritrophic membrane, gut receptors, or immunity (Asser-Kaiser et al. 2011). Other CpGV isolates that can overcome the resistance have already been identified (Eberle et al. 2008; Berling et al. 2009).

Why has resistance developed in the codling moth, whereas it has not developed in the velvetbean caterpillar? It is likely that numerous factors are involved; the first is virus variation. The CpGV used in Europe (and virtually everywhere else) originated from an isolate collected in Mexico in the 1960s and it is reported to be genetically homogenous (Asser-Kaiser et al. 2007). In contrast, the Brazilian NPV used for *A. gemmatalis* management is a field isolate that is more mixed. Tied to this is the structure of the two viruses; a GV contains a single virus particle, whereas NPVs carry multiple virus particles. Thus, as previously discussed, NPVs maintain variation as a result of their co-occluded structure and can respond rapidly to changes in the host population. Second, the viruses are produced in very different ways for application in the field. CpGV formulations all originate from the single isolate and are produced in laboratory colonies, whereas AgNPV is amplified in field populations by spraying and collecting infected insects each year (Moscardi 1999). This means that the CpGV is unlikely to change over time and may well become adapted to the insect culture that it is reared on, depending on how frequently new insects are introduced, whereas AgNPV is actually evolving with the insects in the field and can respond to changes in those insects. Third, the spraying regimes are very different in the two target crops. CpGV is sprayed frequently during a season, whereas soybean can

tolerate some damage and the virus is applied fewer times per season. Finally, the mode of resistance is important to the speed of the development of resistance. Codling moth GV resistance had spread rapidly because it was sex-linked and heterozygotes survived at low virus concentrations. A key aim in the future should therefore be to develop genetic markers for resistance genes and to investigate the basis of resistance in multiple populations and species.

The impact of genotype × environment interactions

Local adaptation

Pathogens do not occur in a homogeneous environment; interactions with hosts will vary over different spatial scales because of variation in, inter alia, resource quality, climatic factors, and other competitors. It is therefore predicted that within this spatial patchwork, host and pathogen will evolve traits that are beneficial in their particular environment, such that local host–pathogen combinations will result in higher mortality (fitness) than more distant pairings (Kawecki and Ebert 2004; Greischar and Koskella 2007). This is known as local adaptation and raises the question, 'should the source (and target) of an entomopathogen be considered as part of the selection process?' Knowing over what spatial scale local adaptation might take place is particularly important as microbial agents are often released in several areas as registering individual variants as biocontrol agents can be a lengthy and expensive process. A wealth of literature, both theoretical and empirical, on local adaptation exists (e.g., Dybdahl and Storfer 2003; Kawecki and Ebert 2004; Nuismer and Gandon 2008) and it has been discussed in relation to biological control (Hufbauer and Roderick 2005). In general, local adaptation has often been found, but not always (e.g., Kaltz et al. 1999; Greischar and Koskella 2007; Adiba et al. 2010). Genotype × environment interactions and local adaptation are also important because they will maintain polymorphism in host and pathogen populations (Byers 2005; Laine et al. 2011). Other factors will act to oppose or restrict local adaptation, in particular the level of gene flow between populations, plus genetic drift, environmental variability, and limited adaptive genetic variation (Gandon and Michalakis 2002; Laine et al. 2011). In addition, intrinsic factors such as genetic background, life span, and host range can be important (Kawecki and Ebert 2004; Hufbauer and Roderick 2005; Hoeksema and Forde 2008), and thus it is not surprising that results vary.

Very little information on local adaptation is available for entomopathogen groups used for biological control, although there is some indication that *Bt* can acquire genes to increase their fitness in the local environment (Swiecicka et al. 2011). In addition, data from clonal invertebrate-parasite systems indicate that local adaptation is possible (e.g., Ebert 1994; Lively and Dybdahl 2000; Carius et al. 2001). In general, local adaptation is predicted to occur in situations where selection is strongest (Greischar and Koskella 2007). Therefore, the highly virulent, obligate pathogens which make up the majority of microbial control agents would be expected to be strong contenders, and this situation should be exacerbated by their capacity to generate new variation rapidly (at a rate faster than the host). This would be an interesting area to focus future work.

Whereas local adaptation primarily focuses on host–pathogen interactions, there is another potential player in this relationship, the host plant on which the insect host feeds. Many of the insect pathogens used for biological control are capable of surviving outside of their hosts, either on plants or soil, and, in fact, may spend longer in the environment than they will in their host. Thus, they all interact with the host plant of the insect and this can affect the insect–pathogen interaction both directly and indirectly (Cory and Hoover 2006). Several insect pathogen groups must be ingested to initiate infection (baculoviruses, bacteria), whereas fungi infect through the insect cuticle and nematodes enter through body orifices or wounds. Pathogens that interact directly with host plant secondary chemicals in the gut are particularly affected, as some chemicals will inhibit the infection process, resulting in decreased mortality. It is therefore possible that some insect pathogens could overcome these inhibitory effects and adapt to a particular host plant. In the pine beauty moth *Panolis flammea* and its NPV system, host plant affected the performance of different NPV genotypes, with mortality depending on genotype–plant combination (Hodgson et al. 2002). This implies that there could be selection for successful NPV genotypes on the locally dominant host plant. This appears to be the case with the western tent caterpillar and its NPV; spatially distinct moth populations occur in sites where a different host plant dominates. When viruses collected at these sites were compared against a common stock of insects, viruses performed best on the host plant from which they were isolated (Cory and Myers 2004). Experiments have also indicated that entomopathogenic nematodes can respond to selection to insects fed on different host plants. *Steinernema carpocapsae* fed on corn-reared insects improved in their ability to kill corn rootworm *Diabrotica undecimptinetata howardi* compared to squash-fed rootworms, although this varied with nematode isolate (Barbercheck et al. 2003).

The impact of resource availability on the evolution of resistance

Resisting pathogens can be energetically costly, and there is a wealth of evidence showing both the standing costs of

resistance and the cost of evolving the resistant trait, particularly in reference to immunocompetence (Schmid-Hempel 2005). Therefore, one would predict that the evolution of resistance to entomopathogens, and any associated costs, would be strongly influenced by the availability of resources (McKean et al. 2008). In addition, the shape of any trade-offs between resistance and other life history traits is predicted to be as important as their strength, but there are little data on trade-off curves, particularly for insect pathogens (Mealor and Boots 2006). There is recent evidence from a selection experiment; however, that indicates that higher resistance to a baculovirus evolves when there are greater resources and the costs are stronger in poorer resource environments (Boots 2011).

The area where the interaction of the evolution of resistance and resources has received the most attention is the evolution of resistance to *Bt*. Documented fitness costs include increased development time, and reduced mass, fecundity, and survival (Janmaat and Myers 2003, 2005; Raymond et al. 2007a; Gassmann et al. 2009; Paris et al. 2011). It is clear that variation in host plant nutritional quality, which includes the influence of secondary chemicals, can also be a major determinant of fitness costs in *Bt*-resistant insects (Janmaat and Myers 2005; Raymond et al. 2007b, 2011). For example, the cost of *Bt* resistance for *T. ni* larvae varies with host plant, with the longest development time, lowest larval weight, and poorest survival occurring on the poorest host, pepper, followed by tomato and cucumber, the best host (in terms of growth rate) (Janmaat and Myers 2005, 2006); thus, it appears that costs of resistance are condition dependent. This implies that when insects are reared on a poor-quality host, limited resources may be available to develop an adaptive trait, such as pathogen resistance (Janmaat and Myers 2006). This reduction in fitness is also likely to be associated with secondary plant chemicals that can act as major stressors, such as phenolics found in peppers (Estiarte et al. 1994) and chlorogenic acid and rutin found in tomato (Isman and Duffey 1982). Raymond et al. (2007b) similarly found an increase in development time for the *Bt*-resistant diamondback moth, *P. xylostella*, populations reared on the *Brassica* host *B. oleracea*, a host with greater defences than the alternative host *B. pekinensis*; however larval survival did not follow this trend. In addition, fitness costs were found to vary between the populations tested and thus indicate specificity of host plant effects at the population level.

A further layer of complexity in tritrophic interactions is whether the dominance of pathogen resistance can interact with the environment. Dominance has been found to decline as the environment becomes less favorable (Bourguet et al. 1996; Janmaat and Myers 2007). The dominance of *Bt* resistance varied with host plant and not necessarily following the pattern found with costs to resistance (Janmaat and Myers 2007). On the best host for *T. ni* growth and development, cucumber, *Bt* resistance was partially recessive, whereas on the least preferred host it was recessive or possibly under-dominant. However, on the intermediate host, tomato, resistance was dominant, albeit with a lot of variation (Janmaat and Myers 2007). We would therefore predict that resistance to *Bt* would develop more rapidly or frequently on tomatoes, and this indeed appears to be the case for *T. ni* (Janmaat and Myers 2003). Given the clear evidence that crop plant–insect interaction is likely to have a significant effect on *Bt*, from expression of toxicity, to the level of costs incurred and the degree of dominance, it would seem wise to consider host plant factors in the development of any management scheme design to delay the development of resistance to *Bt* in polyphagous insect pests (Janmaat and Myers 2007). Manipulation of plant traits is possible by plant breeders and thus, one approach for growers to manage resistance may be to select varieties where resistance is recessive and fitness costs are large (Raymond et al. 2011). However, because of variation in the response at the population level, varieties may have to be designed that act to reduce the fitness of the target pest population, rather than the species as a whole.

Post-release changes and potential risks

Are post-release evolutionary changes, such as adaptation, important to the success of biological control using pathogens? As the majority of insect pathogens are applied inundatively, with no expectation of ongoing pest suppression or establishment, the answer to this question must be no. However, there are numerous examples where pathogens have been used in classical or augmentative control programs. In these situations, understanding what happens to the pathogen after release and how that impacts effective control is important. The first use of an entomopathogen as a classical biological control agent was in the late 1800s, and extends through to the present day (see Hajek et al. 2007 for a review). However, in many of the earlier studies, documentation of the release is lacking and post-release monitoring is absent. The questions that we are mainly interested in are what levels of genetic and phenotypic diversity are necessary for successful establishment and pest suppression, and what types of evolutionary changes occur post-release? The likely relevance of diversity has been discussed above, but few studies have documented genetic changes in pathogen population structure after release. For pathogens, a key question is whether changes in pathogenicity or virulence occur after release? The trade-off hypothesis predicts that pathogens should evolve to levels where virulence is optimized to maximize fitness. The classic example of this is often cited as the interaction between

rabbits and myxoma virus in Australia, where both increased resistance in the rabbits and decreased virulence in the virus were shown over a relatively short time period (Fenner 2010). However, this example has been criticized as being not representative of a natural interaction, as the rabbit was not the natural host of the virus and virus isolate released had very high virulence (Ebert and Bull 2003).

One of the most successful classical biological control programs using pathogens is the control of rhinoceros beetles *Oryctes* spp. in palm plantations in the South Pacific and countries around the Indian Ocean (e.g., Zelazny et al. 1992). The beetles are infected by a virus, not a type of baculovirus as was originally thought, which is now known as a nudivirus (Wang et al. 2011b). Virus dissemination is very efficient in these beetles as the adults excrete the virus and spread it to breeding sites (Huger 2005). This could have potentially been an excellent system for studying post-release evolution and the impact of pathogen diversity on sustained pest suppression as all of the original introductions to various island populations were made with the original isolate from Malaysia. However, the tools for rapid genetic characterization of the virus were not readily available then, and the focus was on practical pest management. However, it is interesting to note that Zelazny (1973) comments that infection levels were higher in Samoa where previously no virus occurred, compared to the Philippines where it is endemic. *Oryctes* virus is genetically diverse and in a unique study, Crawford and Zelazny (1990) released three distinct strains of *Oryctes* virus into field sites in the Maldive Islands and then monitored the outcome over 4 years. The viruses remained unchanged for the first 2 years, but after 4 years a new recombinant isolate was formed, although the impact on virus phenotype was not clear. Studies of this type need to be expanded if we are to understand the nature and relevance of diversity and post-release evolution in microbial control agents. In the *Oryctes* system, control has recently started to break down in some areas of the Pacific; this appears to not only be related in part to changing management practices, but may also be related to the evolution of the virus (Jackson et al. 2005).

Designing new releases of entomopathogens to include pre- and post-release monitoring of genetic and phenotypic diversity might be able to provide answers to questions about the importance of post-release adaptation and pre-release population structure. One option might be to use genetically marked pathogens. A recent study has taken this approach using the entomopathogenic fungus *Metarhizium robertsii* (although not an obligate insect pathogen). Wang et al. (2011a,b) carried out a 4-year study in which they monitored the changes in a fluorescent marked *M. robertsii* following transfer from tropical to temperate soils. They found adaptive changes related to cell wall components and stress factors, but not to virulence determinants. This implies that selection was habitat rather than host specific. Insights can also be obtained from experimental evolution studies where host and pathogens are left to coevolve within microcosms. Many of these studies involve clonal organisms, which provide interesting insights on the role of interacting genotypes, but are perhaps less relevant for biocontrol. An exception is a study by Bérénos et al. (2011) where the beetle *T. castaneum* was left to interact with its microsporidian parasite *N. whitei* for 13 generations. Comparison of pathogens collected from previous generations indicated a general decrease in virulence over time, either suggesting evolution toward optimal virulence or perhaps that the beetle hosts were keeping ahead in terms of coevolved defences.

Could microbial insecticides present a risk to other species through host-range evolution? Whether pathogens are released in short- or long-term control strategies, there is always the risk that they could adapt to nontarget species in the environment. Given that many entomopathogens that are used in pest control are indigenous species, rather than introduced, host-range evolution is less of an issue as it is assumed that they are already present in nature. However, unlike larger organisms, it is usually not possible to define the original distribution of a pathogen. For example, should it be assumed that it occurs naturally everywhere that the host or hosts are found? Pathogens are applied in very large numbers with the expectation that they will not establish, and as far as we know they do not, also indicating that their environmental risk is low. Transient effects are possible as nontarget insects will be swamped with a density of pathogen that might well be above endemic levels in the environment, potentially endangering local populations of vulnerable species (e.g., Boulton and Otvos 2004); but as far as we are aware, there is no evidence that they become established in these species. There are also no reports of the entomopathogens that have been released as classical biological control agents causing any negative, long-term effects; however, post-release monitoring is rare (Hajek et al. 2007). Pre-release samples are not always taken, so if an entomopathogen is applied over wide-ranging areas, it might be difficult to ascertain whether it really is endemic or is being introduced into a novel population. One study has looked at whether introducing *Bt* can impact natural populations and found that adding *Bt* and insects increased the number of *Bt* strains in the population which produced insecticidal toxins (Raymond et al. 2010). However, the dominant *Bt* strain in the soil and on leaves was the same as that which has been developed for pest control products. When a pathogen has a single host, risks are reduced considerably, as with all biological control agents. With generalist species, however, it is difficult to say whether they have expanded their host range or not. Occasionally scenarios occur where it is possible to

record host-range shifts. One example is the potato pest *Tecia solanivora*. This species recently expanded its range into South America where it now co-occurs with the potato tuber moth *Phthorimeae operculella*, and it appears to have acquired a baculovirus from *P. operculella* which has now adapted to its new host (Espinel-Correal et al. 2010). It is interesting to note that several examples of baculoviruses and fungi that produce regular epizootics are obligate pathogens with a very narrow host range, often restricted to a single host (Cory and Myers 2003; Hajek et al. 2007). This perhaps indicates that pathogens with wider host ranges do not present a large risk as they either do not compete well against more specialized organisms or do not possess the characteristics necessary to persist in populations and cause epizootics.

Conclusions

Insect pathogens, although primarily applied as short-term pest control agents, could benefit from consideration of evolutionary principles in several areas. Given the large natural variation of insect pathogens, selection for particular traits is an approach that could yield benefits if it were clear what traits were required for a particular strategy. We also need to increase our knowledge of changing patterns of virulence at both spatial and temporal scales to be able to select the most effective isolates. In addition, production methods can have major impacts on the evolution of the pathogen used for biological control; isolates cultured on artificial media can lose their pathogenicity for target hosts, and can lose natural variation, which might decrease their effectiveness in the field. It may be beneficial to enhance variation in commercially produced pathogens by periodically introducing new isolates and diversifying the culturing media, but this remains to be tested. Field-evolved resistance has been documented in insect pests targeted by the commercially produced bacterial product, *B. thuringiensis*, and GV of the codling moth. Understanding what scenarios are likely to promote its development will be crucial to prolong the use of microbial control agents; frequent applications, constrained gene flow, reduced pathogen variation, gene × environment interactions, and the genetic basis of resistance all appear to be contributing factors to the evolution of resistance. Finally, little research has focused on post-release evolutionary changes for inundative or classical biological control. With short-term use, post-release evolutionary change could be less important; however, it is difficult to assess risk as the pre-introductory range of these pathogens remains unknown. The molecular tools are now available to characterize individual isolates readily and to probe pathogen population structure, and incorporation of such studies into microbial control programs to facilitate pre- and post-release monitoring is strongly encouraged.

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