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Review article

Challenges for developing pathogen-based biopesticides against *Varroa destructor* (Mesostigmata: Varroidae)

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Abstract – Control of the major pest of apiculture, the ectoparasitic mite *Varroa destructor* using pathogen-based biopesticides would resolve many of the problems experienced with other forms of control, such as chemical control, hive manipulation, or selection of resistant honeybee strains. Several research groups have developed and tested fungus-based biopesticides in laboratory and field experiments, with varying results. While biopesticides have many desirable qualities, including much lower risk of honey contamination and of pest resistance, future research needs to focus on: (1) identification of the sub-population(s) of *V. destructor* affected by biopesticides and the duration and impact of the application on mite population dynamics; (2) development of an effective, easily applied formulation, and (3) evaluation of possible side or sublethal effects on bees themselves. Biopesticides need to be evaluated on the field (colony) level in addition to the laboratory level. Researchers should consider providing data that can be easily used to evaluate effect, such as mite drop counts onto sticky boards coupled with assessments of phoretic mite density or brood cell mite density. Exploration for naturally occurring pathogens should be conducted in the native range of *V. destructor* in Asia.

***Apis mellifera* / *Varroa destructor* / biological control / entomopathogenic fungi**

1. INTRODUCTION

Varroa destructor (Anderson and Trueman 2000) is the most serious arthropod pest of honey bees. Most feral honeybee (*Apis mellifera*) colonies in Europe and the U.S.A. vanished after the appearance of *V. destructor* (Kraus and Page 1995; Moritz et al. 2007). At present, most managed colonies are infested with the mite (Ellis and Munn 2005) and those colonies of European origin will usually die within 1 to 3 years in the absence of adequate treatment (Korpela et al. 1992; Fries et al. 2006). *V. destructor* are vectors for many viral diseases of bees (Chen et al. 2006; Evans and Spivak 2010), and there is growing evidence that they have played a large role in recent heavy losses of managed colonies in the Northern Hemisphere (Guzmán-Novoa et al. 2010; Neumann and Carreck 2010; Le Conte et al. 2010; Rosenkranz et al. 2010; Schäfer et al. 2010).

V. destructor spread from Southeast Asia in the 1960s to Europe, Africa, and North and South America by the end of the 1990s. Within the past 10 years, they have been found in Hawaii and New Zealand, formerly mite-free

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areas with thriving apiculture. At present, Australia is the only continent with honeybees and free of *V. destructor* (Rosenkranz et al. 2010; Sammataro and Arlinghaus 2011). Once established in an area, *V. destructor* are difficult to eliminate. Bee researchers and beekeepers have developed and tested numerous biological or biotechnical methods to control this mite, including drone brood removal (Wantuch and Tarpy 2009), which is labor intensive, screens on bottom boards (Harbo and Harris 2004; Delaplane et al. 2005), and dusting with a powder such as flour or powdered sugar (Fakhimzadeh 2001), methods which have not always been found effective (Ellis et al. 2009; Rosenkranz et al. 2010). The most common approach (and until recently the most effective) has been chemical control, which includes synthetic miticides and organic acids as well as plant essential oils (e.g., Ruffinengo et al. 2002; Eguaras et al. 2005; Damiani et al. 2010). Given the damage that *V. destructor* can do to individual bees and entire colonies, beekeepers seek a control strategy that does not require either intensive hive manipulation or multiple applications of chemicals to beehives, which has inherent risks of honey and wax contamination, bee toxicity, and pest resistance. One option to explore is biological control.

1.1. Biological control

Biological control, defined as pest control using natural enemies (Perkins and Garcia 1999), has been used in many agricultural systems with great success (Gutierrez et al. 1999). There are two major kinds of biological control: (1) classical, in which a new organism, such as a predator, parasite, or pathogen, is released into an area where it did not previously occur in order to control an introduced pest; and (2) augmentative, in which additional numbers of a natural enemy are released in an environment where too few are present to control a pest effectively (Perkins and Garcia 1999). Classical biological control of *V. destructor* is unknown, and none of the natural enemies of *V. destructor* listed by Chandler et al. (2001) was specific for the mite. Here, we address augmentative biological control, which includes inoculative and inundative controls. Successful biological control would offer several advantages over chemical control for bees, beekeepers, and consumers, by reducing: chemical contamination of hive products; negative impacts on adult bee and brood health associated with some miticides; and the prospects for pesticide resistance by the mites (Rosenkranz et al. 2010).

How a biological control agent behaves in a given system depends to some extent on the kind of agent involved. Predators and pathogens, for example, have fundamentally different ways of reproducing and of killing target pests. Van der Geest et al. (2000) reviewed the major pathogen groups that cause disease in mites, albeit with a focus on eriophyid and tetranychid mite diseases. For *V. destructor*, the most likely candidates for biological control are entomopathogenic fungi (EPF) (Chandler et al. 2001). While "entomopathogenic" refers in a strict sense to insect pathogens, many EPF also attack mites and ticks. Most EPF fall into one of two orders: Entomophthorales and Hypocreales. In a review of biological control using entomopathogens, Hayek and Delalibera (2010) point out that most entomophthoralean fungi are difficult or impossible to grow in vitro (when used in biological control, the pests are usually treated with infected cadavers, either whole or ground up); these fungi have not been isolated from or used against *V. destructor* and will not be considered further.

Chandler et al. (2001) reviewed a wide range of predators and pathogens as potential biological control agents of *V. destructor*. Some recent work has focused on viruses (Kleespies et al. 2000), bacteria and bacterial products (Tsagou et al. 2004; Tu et al. 2010), and pseudoscorpions (Donovan and Paul 2005). However, to date, only hypocrealean EPF have been evaluated in field trials as biological control agents, and most work has been conducted using two species of EPF: *Metarhizium anisopliae* (Metschnikoff) (Hypocreales: Clavicipitaceae) and *Beauveria bassiana* (Balsamo) Viullemin (Hypocreales: Cordycipitaceae). Both species have been used against other bee pests (Muerrle et al. 2006) and against pests in many agricultural systems

(Jaronski 2010) and while neither fungus is specific for *V. destructor* or even mites in general, some isolates are highly virulent against particular arthropod species. Strains of *M. anisopliae* have been used in the biological control of many arthropods, including locusts (Cherry et al. 1999) and termites (Rath 2000; Su et al. 2003). *B. bassiana* is known to have a wide host range (Tanada and Kaya 1993) and has been used against, for example, whiteflies (Islam et al. 2010) and mosquitoes (Farenhorst et al. 2009). Both fungal species have been evaluated against acarines, including ixodid ticks (e.g., Stafford and Allan 2011) and tetranychid mites (e.g., Bugeme et al. 2010).

Strains of several EPF have been evaluated in the laboratory as potential biological control agents of *V. destructor* and been found virulent (e.g., Davidson et al. 2003; James 2009; Kanga et al. 2002; Meikle et al. 2006; Rodríguez et al. 2009a; Shaw et al. 2002), but only *B. bassiana* and the recently described *B. varroae* (Rehner et al. 2011) have been found naturally occurring on the mites. *Beauveria* isolates have been reported from *V. destructor* collected in Russia (Chernov 1981, cited in Chandler et al. 2000), France (Meikle et al. 2006), Spain (García-Fernández et al. 2008), and Denmark (Steenberg et al. 2010). Calderón et al. (2004) reported *B. bassiana* from bee hives in Costa Rica.

Our purpose here is to review the published literature on pathogen-based biopesticides against *V. destructor*, with an emphasis on studies that include field applications, because this field is still emerging, the amount published modest, and there remain many possible new research directions. We would also like to draw attention to issues that we feel need more research, and to the kinds of data needed to help develop biological control as a management strategy. The issues we address here are: (1) what are the effects of EPF on adult bees and brood; (2) which sub-population of mites is most affected by EPF; (3) what is the fate of EPF in the hive; (4) which formulations and application methods have been tested; and (5) what are the contamination risks of pathogen-based biopesticides.

Effect of biopesticide on adult bees and bee brood Bee health is a paramount concern in any treatment against *V. destructor*. The size and vigor of adult and brood bee populations have to be assessed during any field trials, bearing in mind that an effect of a treatment on bees measured in the laboratory may be quite different from that observed in a bee hive. As Evans and Spivak (2010) state, honeybee colonies have many levels of resistance, from the individual level in the form of immunological and physiological responses, to the group level in the form of auto- and allo-grooming, to the colony level, in the form of specialized hygienic behaviors such as comb cleaning and cadaver removal. Stow et al. (2007) found a strong relationship between the strength of the antimicrobial compounds excreted by bees with their degree of sociality. Compounds emitted by chalkbrood, *Ascosphaera apis* (Maasen ex Claussen) L.S. Olive & Spiltoir (Onygenales: Ascosphaeraceae) are known to induce hygienic behavior in honeybees (Swanson et al. 2009). Among social hymenoptera in general, specialized grooming behavior against fungal pathogens has been observed in ants (Reber et al. 2011).

A primary concern is whether biopesticide applications represent a threat to bee brood, since EPF could, in theory, attack brood. Davidson et al. (2003), Fargues et al. (1992), and Rodríguez et al. (2009a) each surveyed temperature tolerances of >30 isolates of many EPF species and observed growth in several isolates of both *M. anisopliae* and *B. bassiana* at 35°C, which is about the temperature the bees maintain in the brood area (Human et al. 2006). Meikle et al. (2006) observed that 10–63% of the bee pupae exposed to mites treated with *Beauveria* isolates in bioassays subsequently sporulated, and Steenberg et al. (2010) actually collected an isolate of *B. bassiana* from a mite found in a capped brood cell. However, all field studies of biopesticide application cited here measured brood populations before and after treatment application (Table I) and, at least with the isolates in those studies, no elevated mortality due to biopesticides was observed. These results also illustrate the limits of extrap-

Table I. Summary of analyses conducted on the impact of biopesticide treatment against *V. destructor* in honey bee hives (*EPF* entomopathogenic fungus; *cfu* colony-forming units)

EPF species	Bees monitored	Mites monitored	Additional analyses	Reference
Metarhizium anisopliae	Adults, brood	Sticky boards, phoretic mites, brood mites	Infection rates of dead bees and mites; fungal cfu per bee	Kanga et al. 2003
	Adults, brood	Sticky boards, phoretic mites, brood mites	None	Kanga et al. 2010
		Sticky boards, phoretic mites	Infection rates of dead bees and mites	Rodríguez et al. 2009b
	Adults, brood	Phoretic mites, brood mites	Conidia viability over time	James et al. 2006
	Adults	Phoretic mites	Conidia viability over time	James and Hayes 2007
Beauveria bassiana	Adults, brood	Sticky boards, phoretic mites	Hive weight and food stores; infection rates of dead mites; fungal cfu per bee	Meikle et al. 2007
	Adults, brood	Sticky boards	Hive growth rate; fungal cfu per bee	Meikle et al. 2008a
	Adults, brood	Sticky boards, phoretic mites	Hive growth rate	Meikle et al. 2008b
	Brood	Sticky boards, phoretic mites	Infection rates of dead mites; fungal cfu per bee	Meikle et al. 2009

olating laboratory results to the field and the need to conduct such studies on bee colonies in order to properly evaluate efficacy and side effects.

Another main concern is the impact on the adult worker populations, since the adults are treated directly with biopesticide and become laden with infective material. By washing adult bees collected from treated colonies with a dilute detergent solution and then plating solution aliquots to determine EPF colony-forming unit (cfu) densities, average EPF cfu have been estimated to be $>10^4$ per adult worker after application (Kanga et al. 2003; Meikle et al. 2007, 2008a) so exposure is high. Two methods have been used to measure the effect of biopesticide on adult bees: (1) assessing live adult bee populations before and after biopesticide application and (2) counting dead bees around the hive and monitoring cadavers for fungal infection. Estimating the adult bee populations before and after application is recommended in order to control for any negative impact of the pathogen on adult bees. Kanga et al. (2003) and James et al. (2006) visually estimated the proportion of each frame occupied by adult bees and brood area, and Meikle et al. (2007, 2008b) subtracted the weight of hive components from the weight of the entire hive to estimate adult bee mass. Meikle et al. (2007, 2008a, b) compared hive growth rates, total adult bee masses, and the area of sealed brood for treated and control hive groups from pre-application until at least 18 days after the last application. No effects of biopesticide treatment on hive growth rates, adult bees, or brood area were found in those studies. Monitoring numbers of dead bees is useful, but to avoid a bias requires an effective dead bee trap including knowledge about the trap efficacy (e.g., Illies et al. 2002). Bees die every day for a number of reasons, and a hive with a stable population of about 10,000 adult bees with an average life span of 40 days (Winston 1992)

loses an average of 250 workers per day. If the researcher collects only a fraction of that, then it is important to have some idea which subset is being sampled since ailing bees may die far from the hive. Once collected, a dead bee is placed in a sporulation chamber with high humidity and examined for signs of infection by EPF. Even if the cadaver is surface sterilized, however, attributing the death of a particular bee to fungal infection can be difficult. Both *Metarhizium* and *Beauveria* represent groups of facultative, rather than obligatory, pathogens that can readily attack insect cadavers (Tanada and Kaya 1993). Given the cfu density per bee, how quickly fungi can attack a fresh cadaver, and the unknown length of time between a bee's death and its collection by the researcher, the probability of a false positive (a conclusion that the fungus killed the bee when it did not) may be high. Kanga et al. (2003) used dead bee traps and found higher numbers of dead bees in the hives treated with *M. anisopliae*-based biopesticide than among hives either treated chemically or not at all, indicating that the biopesticide may have affected the adult population, even if it did not significantly impact colony health.

To our knowledge, potential sublethal effects of EPF on honeybees have not been investigated. However, Rosengaus et al. (2007) evaluated the contact of a sublethal dose of *M. anisopliae* on another social insect, the dampwood termite *Zootermopsis angusticollis* Hagen (Isoptera: Termopsidae) and found that exposure to the fungus induced the production of protective proteins in nymphs, pseudergates (false workers), and soldiers. Exposing honeybees to EPF may provoke a similar immune response, although whether such a response measurably affects colony health or reduces hive activity is another issue and is not known.

Varroa sub-population affected by the biopesticide Obviously, any increase in *V. destructor* mortality caused by the treatment is appreciated by beekeepers. However, to understand and improve biological control, it is important to know which mites are being attacked. Mites living in bee colonies belong to one of three sub-populations: (1) the phoretic mite population, consisting of mites attached to adult bee hosts; (2) the brood mite population, consisting of mites in capped brood cells that are protected from direct exposure to biopesticide application; and (3) the comb mite population, consisting of mites on the comb but not in a capped brood cell; these mites may be either immature at the time of bee emergence from a cell, or male (although male mites usually die within the capped cell [Rosenkranz et al. 2010]), or females seeking a cell with a larva, or mites that had not attached to a bee host. When interpreting the number of mites fallen on to sticky boards, a common measure of mite density in hives (Ostiguy and Sammataro 2000), one must bear in mind that those mites come from one of the three populations and they fall due to a number of reasons, including but not limited to: death, losing their grip, being cleaned off a bee or comb, or the application of a powder. Common ways to measure overall treatment efficacy at the end of an experiment are either to kill the colonies and wash the mites from the adult bees and brood, or to treat hives with a highly effective miticide over several weeks and count the fallen mites, a method which assumes little or no miticide resistance in the *V. destructor* population being treated.

Phoretic mite density is an important measure, both because phoretic mites are reproductive females and because phoretic mite densities are often monitored by beekeepers to judge overall infestation levels. The phoretic phase lasts between about 4 and 11 days (Fries et al. 1994) but is not obligatory, and a female can complete up to seven reproductive cycles in her lifetime (Rosenkranz et al. 2010). Kanga et al. (2003) reported significant reductions in the densities of phoretic mites, using *M. anisopliae* conidia either coated on strips placed between frames or sprinkled as a dust in the hive; phoretic mite densities in the test hives of those experiments ranged from 2.1 to 6.1 mites per 100 bees prior to application to 0.1 to 1.2 after application. However, neither James et al. (2006), James and Hayes (2007), nor Rodríguez et al. (2009b) reported reductions in phoretic

mite density compared to controls using *M. anisopliae* applied using strips, as a powder, in an auto-applicator, as a liquid or as a solid (see Table II). Meikle et al. (2009) reported lower mite fall on to sticky boards after three consecutive applications of a *B. bassiana*-based biopesticide applied as a dust but found no significant differences in phoretic mite densities between treated hives and controls. The results of Kanga et al. (2003) thus remain the exception rather than the rule.

When brood is present, brood mites are probably the most numerous, at least compared to the phoretic mites (Martin 1998). Data on brood mite density are valuable in understanding the *V. destructor* population structure, particularly combined with phoretic mite and/or sticky board data. However, estimating brood mite density is usually more complicated than counting phoretic mites or using sticky boards because it requires removing one or more frames of capped brood and either opening cells and counting mites in the field, in order to return sampled frames to the hives, or taking the frames to the lab for assessment and risking the loss of the brood in the frames. The issue of brood mites can be avoided entirely by limiting biopesticide application to broodless colonies, as is done with oxalic and lactic acid treatments (Rosenkranz et al. 2010), although this would likewise limit the use of a potential varroacide. Kanga et al. (2003) treated broodless colonies (that produced brood before the end of the experiment) which probably contributed to the success of those trials.

Duration of treatment effect "Duration" as it is used here describes the period during which the pathogen is causing new, lethal infections among mites. Duration of an application in a beehive is best measured with time trends of mite mortality, such as mite fall onto sticky boards, usually for at least 2 weeks post-application. Mite fall data can provide important information on the start and duration of the treatment impact, but the data must be properly

Table II. Different types of formulations tested in fungus-based biopesticides used against *V. destructor* in honey bee hives (*EPF* entomopathogenic fungus)

EPF species	Formulation type	Main ingredient	Application method	Reference
Metarhizium anisopliae	Powder	None	Sprinkled between frames	Kanga et al. 2003; Rodríguez et al. 2009b
	Powder	None	Strips between frames	Kanga et al. 2003; Rodríguez et al. 2009b
	Powder	None	Auto-applicator at hive entrance	Rodríguez et al. 2009b
	Powder	None	Dusted on bees between frames	James et al. 2006; James and Hayes 2007
	Powder	Rice flour or talc	Dusted on bees between frames	James and Hayes 2007
	Liquid (aqueous)	Water+0.01% Tween 20 (wetting agent)	Sprayed between frames	James et al. 2006
	Solid	Sporulating fungus culture+media	Frame placed in hive	James et al. 2006
	Solid	Protein patty	Fed to bees	Kanga et al. 2010
Beauveria bassiana	Powder	None	Blown between frames	Meikle et al. 2009
	Powder	Plant wax powder+0.5% silica (flow agent)	Blown between frames	Meikle et al. 2007, 2008a, b, 2009
	Powder	Wheat flour+0.5% silica	Blown between frames	Meikle et al. 2008b

interpreted. The start of the treatment effect due to the EPF itself does not begin the day of application. All EPF require an incubation period before killing a host. This period is usually 3 or more days: Kanga et al. (2002) reported the LT_{90} of *M. anisopliae* in laboratory bioassays was 5.9 days at 34±1°C and a concentration of 2×10^8 conidia per milliliter; and Meikle et al. (2006) found that the most virulent *M. anisopliae* isolate they examined took an average of 4.3 days to kill their host at a concentration of 10^7 conidia per milliliter. How long an EPF will take to kill mites in the hive can be expected to vary with respect to factors such as fungal strain, formulation, individual resistance, and exposure level (application method). Even mites exposed to conidia rapidly and thoroughly in laboratory bioassays have a distribution in mortality over time. Also, because application of a powder is associated with mites falling from their bee hosts (Fakhimzadeh 2001; Macedo et al. 2002), a spike in mite fall within 24 h after application of a powder formulation (e.g., Kanga et al. 2005; Meikle et al. 2007) is usually observed. This "powder" effect should either be removed or statistically controlled from the analysis of treatment effect where powders are used, either by excluding mite fall for the first day from the analysis or by applying a "blank" powder treatment as a control.

Duration of an application in the hive is subject to three main factors: bee hygienic behavior, conidium longevity in the hive environment, and formulation (discussed below). Bee hygienic behavior is likely very important. Bees will clean out much of an application rapidly, even if many still have high conidia counts on their bodies. The peaks in post-application mite fall observed by Meikle et al. (2007, 2008b, 2009) would be consistent with effect duration of only a day or two per application, if the data are interpreted conservatively. While applying a biopesticide powder between frames distributes the conidia well (Meikle et al. 2007, 2008a), a single application has not been sufficient, probably because untreated brood mites continue to emerge with their bee hosts up to 2 weeks after application while the conidia density in the brood area is dropping due to bee cleaning. Slow release of EPF, such as using frames with sporulating fungi (James et al. 2006), or auto-inoculation (Rodríguez et al. 2009b) would seem to solve some problems, but nevertheless were not effective in those studies. Slow-release and auto-inoculation methods usually depend on bees independently making contact with the conidia, rather than being forcibly treated with them, and thus reduce dosage control, which may partly explain the lack of positive results.

Although conidia of some isolates of *M. anisopliae* and *B. bassiana* can germinate and grow at brood temperatures, there is a limit to their life span under those conditions. Using data on the longevity characteristics of several *B. bassiana* isolates (Hong et al. 2001) and a simulation model of conidia longevity (Meikle et al. 2003) conidia longevity for some strains was estimated to be between 4 and 13 days at 35°C and 70% relative humidity, although up to 10 times longer if the humidity was lowered to 40% (Meikle et al. 2007). James et al. (2006) measured conidia viability over time in the hives and found that conidia placed in protected packets had measurable viability for over 3 weeks, but conidia sampled from hive frames were inactive within 2 weeks after application. Several studies reported estimates of mite infection rates obtained by placing samples of fallen mites in fungal growth chambers or on non-nutritive agar in Petri dishes (see Table I). Because of the propensity of these EPF to attack cadavers, adequate precautions have to be taken (see above) to reduce the probability of false positives. Infected mite cadavers, which indicate the presence of viable conidia, have been collected up to several weeks after application (Meikle et al. 2008a).

Biopesticides are based on biological control principles, in which a living organism attacks and kills the target. In some circumstances, biopesticides are thought to generate "secondary cycling" of the biological control agent, which in the case of EPF means conidia released from sporulating cadavers produced by the original

application infect more target pests and thus increase the effect duration. Secondary cycling is difficult to measure. Thomas et al. (1995) attributed some insect mortality to secondary cycling after biopesticides were applied on migratory locusts. Secondary cycling is unlikely to occur in beehives because bees would remove most or all mite cadavers before conidia are produced and thus remove the source of conidia for more infections.

Formulation A variety of biopesticide formulations and application methods have been used in the quest for control of *V. destructor* (Table II). An ideal formulation would be nontoxic, augment EPF efficacy against *V. destructor*, and not induce the EPF to attack bees. Burges (1998) reviewed biopesticide formulations for use at high temperatures and humidites, but not all of those formulations are practical or desirable for treating beehives. James and Hayes (2007) and Meikle et al. (2008b) tested flour as a carrier, recommended by Burges (1998) for application of EPF in high-humidity situations, but neither found it effective. Because flour, whether wheat or rice, can be used as a food source for germinating conidia (Burges 1998), it can influence fungal growth and thus is not "inert". Meikle et al. (2008b) found that, compared to flour or no carrier, wax powder worked best for a *B. bassiana*-based biopesticide. The wax powders, which are inert and permitted as food additives in the U.S. (Meikle et al. 2008b), were made from carnauba wax, obtained from *Copernicia cerifera* Mart. or *Copernicia prunifera* (Mill) (both Aracales: Aracaceae), and candelilla wax, obtained from *Euphorbia antisyphilitica* Zucc. and *Pedilanthus pavonis* Boissier (both Malpighiales: Euphorbiaceae).

As noted above, Kanga et al. (2003) reported good results using powders or strips to which conidia had been affixed, but cumulative mites fall on sticky boards post-application was on average <250 mites over 42 days (<6 mites per day); whether these results can be extrapolated to hives with higher infestations, which are more likely to be treated, remains to be seen. Kanga et al. (2010) formulated conidia of the same isolate of *M. anisopliae* into a protein patty for trials in Texas and Florida, and while the Texas trial was reported to be a success, with phoretic mite densities in treated hives far lower 24 to 47 days after treatment than in the control, post-treatment densities were not lower compared to pre-treatment densities for the same hives. Also, none of the tests included a control for the effects of protein patties themselves, making it difficult to attribute observed effects solely to the presence of biopesticide. Protein patties are used by beekeepers because they improve bee nutrition, increase worker bee production, and help colonies recover from diseases (Herbert 1992), and these effects would have confounded any biopesticide effects in those trials.

Several approaches to formulation and application method have been taken. Rodríguez et al. (2009b) reported some positive results from conidia applied either between frames or on strips. Kanga et al. (2003), Meikle et al. (2007, 2008b), James et al. (2006) and James and Hayes (2007) applied conidia by blowing or sprinkling them between hive frames. While effective in distributing conidia, this method would need to be refined for use in large numbers of hives. James et al. (2006) tried a number of different application methods but observed no impact of treatment on mite densities and felt that the *M. anisopliae* conidia were possibly not coming into contact with enough *V. destructor*.

Another consideration is fungal spore type used in the biopesticide. While only aerial conidia were used in the colony-level studies reported here, Hypocrealean fungi produce other spore types: blastospores and submerged conidia (see Holder et al. 2007 for examples for *B. bassiana*) that might be better suited for certain application methods or formulations.

Contamination risks An important selling point of a biopesticide is that it reduces the risk of chemical contamination of hive products. This is the case, provided (a) there is no problem from fungus-produced compounds (mycotoxins), (b) the formulation did not involve

undesirable materials (see above), and (c) conidia were not mixed with objectionable substances from the fungal media. Regarding the production of mycotoxins, Strasser et al. (2000) reviewed most of the important compounds with potential mammalian toxicity issues, such as the destruxins (produced by *Metarhizium* sp.) and beauvericin (produced by *Beauveria* sp.) and observed that there was a great deal of variability among species and among isolates of the same species. Because these compounds are mostly host specific and secreted at very low levels, they do not pose a health risk. However, Niu et al. (2011) reported that propolis (bee-collected plant resins) helped bees break down mycotoxins found in bee bread by enhancing the activity of enzymes involved in detoxification. This finding illustrates the advantage of testing biopesticides on bee colonies as opposed to caged bees in a laboratory. As Evans and Spivak (2010) pointed out, some hive protection features occur on the level of the colony, and those features may not be present in laboratory cages.

In addition, the media used to produce these fungi may need scrutiny. Two reasons for concern are: (1) some media contain compounds such as antibiotics; and (2) if the media are contaminated with other microbes during fermentation, these microbes can end up in the final product. The importance of compounds in the media depends largely on whether particles of media are present in the final product. Contaminating microbes might be a more important issue, since some microbes can interfere with either the pathogen or with honeybee health. *Pseudomonas fluorescens* (Pseudomonadales: Pseudomonadaceae), for example, is a bacterial contaminant often found in wastewater (Sacchetti et al. 2007) and stored food (Dogan and Boor 2003); it has been found in high densities in commercially prepared *B. bassiana* conidia (Meikle et al. 2012). In addition to unknown health effects on bees, *P. fluorescens* is known to inhibit growth and activity of fungal pathogens of plants (Haas and Dèfago 2005), so such bacteria could interfere with the activity of insect pathogens. Conidia preparations should therefore be tested for such contaminants before formulation and application.

2. CONCLUSIONS

We reviewed the recent work on biological control of *V. destructor* in an attempt to help promote future research by indicating promising areas that need clarification and further study. Areas most in need of attention include: (1) standardization of test methods to ensure comparable data between laboratories; (2) identification of the sub-population(s) of *V. destructor* affected by biopesticides, and the duration and impact of the application on mite population dynamics; (3) evaluating possible side- or sublethal effects on bees themselves; and most importantly from a beekeeper's point of view, (4) development of an inexpensive and easily applied formulation.

It is clear from the variability of the results that not enough is known about interactions among *V. destructor*, honeybee colonies, and EPF to state definitively whether biopesticides have a potential role in controlling this mite. Part of the reason for these mixed results is the wide variability in the studies in terms of biopesticide dose, formulation and application, and how impact was measured. This illustrates the need to standardize methods so that results can be compared, to improve aspects of biopesticide use against *V. destructor*, and to understand which mites get infected, how they get infected, and what levels of infection are needed to significantly affect mite populations. The occurrence of hypocrealean EPF in beehives may be common—*Beauveria* strains have been isolated from *V. destructor* in Russia, France, Spain, and Denmark (see above)—but basic questions concerning how the fungi enter a hive, their ecology in the hive, and why *Beauveria* species are the only Hypocrealean fungi collected from mites, still need attention. Development of an inexpensive yet effective biopesticide will require innovative thinking. Traps combining attractants and biological

control agents have been deployed with success against pest arthropods (Reddy and Guerrero 2010; Vargas et al. 2010). Attractants for *V. destructor* have been identified (Calderone and Lin 2001; Maggi et al. 2010) but not reported for use in biological control of this mite. Likewise, EPF spore types other than aerial conidia have received little attention.

Further exploration is also needed for novel *V. destructor* natural enemies, including pathogens, in *Apis cerana* populations, particularly in the Korean peninsula from which the most widespread and damaging *V. destructor* haplotype originated (k-haplotype, cf. Rosenkranz et al. 2010). While the main reasons for the spread and impact of *V. destructor* on *Apis mellifera* are likely due to the low level of development, or absence, of specific behaviors to kill and remove mites (e.g., hygienic behavior and "entombing behavior" as found in *A. cerana* [Rath 1999]), and the ability of *V. destructor* to reproduce on worker brood of *A. mellifera* (but not *A. cerana*) (Rosenkranz et al. 2010), it is possible that in invading *A. mellifera* nests, the mites also escaped many natural enemies associated with the *A. cerana* hive ecosystem. Mitchell and Power (2003) and Torchin et al. (2003) presented the "enemy release hypothesis" and argued that in invading a new geographical area, pests escape control from many pathogens, parasites, and predators which, as a group, exert significant control on the pest in its native range.

It is clear that, while laboratory studies are needed to explore some issues such as the mechanism of infection, biopesticides against *V. destructor* need to be evaluated at the colony level. Bee larvae, for example, are highly susceptible to EPF in the laboratory but are largely protected from biopesticides in bee hives. Likewise, bees in colonies can apparently mitigate the effects of mycotoxins using propolis, and adults treated topically with biopesticides may have different experiences depending whether they are in a colony or a hoarding cage. Populations of adult bees and brood in hives should therefore be measured before and after treatment to ensure that the non-target impact on bees is low or none. Lastly, researchers should consider estimating at least two of the within-hive *V. destructor* populations (phoretic, brood, and comb) in order to best understand the impact of a treatment on mite population dynamics when evaluating EPF effects, and evaluations should include pre- and post-treatment effects. In light of the urgent need to find sustainable treatment solutions for the mite *V. destructor*, biological control in general and biopesticides in particular merit further examination.

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Défis à relever pour le développement de biopesticides à base de pathogènes contre *Varroa destructor* (Mesostigmata: Varroidae).

***Apis mellifera* / *Varroa destructor* / lutte biologique / champignons entomopathogènes**

Anforderungen für die Entwicklung von Biopestiziden auf Pathogenbasis gegen *Varroa destructor* (Mesostigmata: Varroidae).

***Apis mellifera* / *Varroa destructor* / biologische Kontrollle / entomopathogene Pilze**

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