Chapter 13

Physiology and Ecology of Host Defense Against Microbial Invaders

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Chapter Outline

13.1. Introduction **461**
13.2. Behavioral and Physical Barriers to Infection **462**
13.2.1. Behavioral Avoidance of Pathogens 462
Avoidance Behavior 462
Antiseptic Behavior 463
13.2.2. Morphological Barriers to Infections 463
Cuticle 464
Digestive and Tracheal Systems 464
13.3. Physiological Response to Infections **465**
13.3.1. Distinguishing Self from Non-self from Altered Self 465
13.3.2. Humoral Response System 466
Antimicrobial Peptides 466
Regulation of Humoral Defenses 467
13.3.3. Cellular Response System 467
Hemocytes 467
Phagocytosis 468
Nodulation and Encapsulation 468
13.3.4. Melanization 469
13.3.5. Intracellular Defenses 469
Involvement of the Extracellular Immune Systems Against Intracellular Pathogens 469
RNA Interference 470
Xenophagy 470
Resistance Against Intracellular Host Defenses 470
13.4. Managing Resistance to Entomopathogens **470**
13.4.1. Resistance Mechanisms to *Bacillus thuringiensis* 471
13.4.2. Managing Resistance to *Bacillus thuringiensis* 472
Delaying the Evolution of Resistance and Slowing its Spread 473
Fitness Costs Associated with Resistance 473
13.5. Future Research Directions **474**
References **475**

Summary

Insects mount a complex hierarchy of defenses that pathogens must overcome before successful infection is achieved. Behavioral avoidance and antiseptic behaviors by host insects reduce the degree of encounters between the insect and pathogens. Any pathogen that contacts or establishes on a potential host faces a series of barriers that restrict entrance into the hemocoel. Pathogens that enter the hemocoel are faced with a multipronged innate immune system. Humoral defenses primarily produce toxic molecules; hemocytes (granulocytes, plasmatocytes, oenocytoids) of the cellular defense have the capacity to phagocytose or encapsulate the target pathogen; and melanization involves both humoral and cellular components to produce several responses that are lethal to the pathogen. Intracellular pathogens must overcome cellular xenophagy and RNA interference defenses. Understanding how host resistance evolves and spreads throughout a population becomes important to preserve entomopathogens as a pest management tool. This phenomenon has been well studied in *Bacillus thuringiensis*, and the development of host resistance to this pathogen, as well as insect resistance management strategies, is discussed.

13.1. INTRODUCTION

This book focuses on the wide variety of entomopathogens and their complex ecology and physiology that

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make them such a formidable threat to insects and such a useful ally to humans. Given their abundance and the variety of virulence factors that these pathogens produce, it is startling that healthy insects can be found in nature. The insects do not idly wait to be devoured by pathogens; they detect and avoid pathogens, practice hygiene, and present multilayered barriers that block pathogens from establishing an infection. Those pathogens that do breach the behavioral and external defenses of the insect are subjected to the noxious chemicals, deadly cellular responses, and restrictive and toxic melanization responses that are presented by the innate immune systems of the host. The effectiveness of these defenses and how they evolve and spread throughout a population form the basis of resistance management plans that seek to preserve entomopathogen-based control methods for as long as possible. Insect defenses, and how we can predict and delay their effectiveness, are the focus of this chapter.

Pathogens and hosts are in an arms race. The pathogen tries to exploit the host resource to the maximum, but the condition of the host population obviously affects the capacity for a pathogen population to persist. With this in mind, pathogens fall along a continuum of virulence that ranges from causing mere sublethal effects on the host to killing the host quickly. Those pathogens with relatively mild effects on the host result in a more stable host population (i.e., the host population is not rapidly driven to local extinction). Lethal pathogens often have greater persistence in the environment so that they are present when the host population resurges; these virulent pathogens also tend to be more mobile (i.e., they have inherent dispersal means or symbioses with other sources of dispersal), which allows them to locate new host populations (Centofanti, 1995; Ewald, 1995). Vertically (mother to offspring) transmitted pathogens tend to be less virulent than those that are primarily transmitted horizontally (between individuals) (Herre, 1993). Of course, the whole concept of virulence becomes more complicated when one considers that hosts respond against infection. Indeed, virulence of a pathogen is not a consistent characteristic when considering the relative defenses possessed by different potential hosts, or even a single host under different circumstances.

Insect immunity has received substantial attention in several recent reviews, for example Beckage (2007) and Rolff and Reynolds (2009). The intent of this chapter is not to supplant these other sources. Rather, the purpose of this chapter is to introduce the hierarchy of defenses encountered by pathogens and discuss how these defenses help us to understand the similarities and differences among entomopathogens. It is hoped that understanding these defensive systems will clarify the biology and ecology of the pathogens.

13.2. BEHAVIORAL AND PHYSICAL BARRIERS TO INFECTION

Before encountering the immune responses of the host, the pathogen must breach several levels of defenses that reduce both the encounters between the host and pathogen and the likelihood that the pathogen will reach the hemocoel. Reducing pathogen encounters and establishment involves insect behavior in the case of avoidance and antiseptic practices, and it comprises the use of physical barriers that restrict the entrance of the pathogen. It is important to note that these physical barriers may be fortified by antimicrobial compounds, regulated by either the innate immune response or other sources.

13.2.1. Behavioral Avoidance of Pathogens

Avoidance Behavior

The most effective defense against disease is avoidance of contact with the disease-causing agent, which has been observed for some insect species (Alma *et al.*, 2010; Ormond *et al.*, 2011). The omnivorous pirate bug, *Anthocoris nemorum*, avoids foraging on nettle leaves containing spores of *Beauveria bassiana*. Even more striking is that this bug avoids ovipositing on tissue when it contains the fungus, thereby reducing the risk to its offspring (Meyling and Pell, 2006). The gypsy moth, *Lymantria dispar*, detects cadavers and foliage that contain nucleopolyhedrovirus (NPV), and avoids these potential sources of occlusion bodies (Parker *et al.*, 2010). In addition to being able to detect pathogenic organisms, some insects can detect and avoid some of the toxins produced by entomopathogens. For example, larvae of the beet armyworm, *Spodoptera exigua*, avoid consuming diet that contains Cry1C toxin, one of the lethal agents expressed by *Bacillus thuringiensis* (Berdegué *et al.*, 1996). Most insects must come in contact with the pathogen or a cadaver containing the pathogen in order to recognize it as something to be avoided (Meyling and Pell, 2006; Thompson *et al.*, 2007; Alma *et al.*, 2010), but this is not always the case. The termite *Macrotermes michaelseni* not only can detect and avoid sources of *B. bassiana* and *Metarhizium anisopliae*, but also assesses their virulence from a distance and is more strongly repelled by the more virulent strains (Mburu *et al.*, 2009).

Other insects are clearly unable to detect certain pathogens (Boots, 1998; Klinger *et al.*, 2006; Meyling and Pell, 2006). For instance, *Cephalonomia tarsalis*, a parasitoid of the small toothed grain beetle, *Oryxaephilus surinamensis*, is susceptible to *B. bassiana* infection but does not avoid contaminated grain (Lord, 2001). Moreover, this parasitoid oviposits equally in infected and

uninfected hosts, thereby dooming the offspring (Lord, 2001). Similarly, of two mole crickets evaluated (*Scapteriscus borellii* and *S. vicinus*), only *S. borellii* could distinguish substrates infested with *B. bassiana* and it adjusted its residence time to reduce exposure (Thompson *et al.*, 2007). Many of these studies that find instances of non-avoidance suggest that the selection pressure on the insect is simply too weak to produce positive selection of the avoidance behavior trait in the specific interactions tested. The majority of studies on this topic have focused on insects' ability to detect potential fungal infections, and it would be interesting to compare how easily some pathogen groups are recognized by insect hosts relative to other groups.

Antiseptic Behavior

Simple grooming behavior is effective in removing many pathogens from the exterior surface of the insect's body before they can cause disease. Although nearly all insects groom themselves in one way or another (many have specialized combs and brushes for just such behavior), grooming and hygienic behaviors of social insects are particularly well studied in this regard. Social insects have limited genetic variability within a colony, live in close quarters with their nestmates, and frequently store food in their nest. These characteristics intuitively should make social insects particularly prone to diseases, but in reality these insects are fairly resistant to non-specialized pathogens. This is in spite of social insects (at least honey bees, *Apis mellifera*) having only one-third of the genes responsible for innate immunity that some other insects possess (Evans *et al.*, 2006). In addition to innate immunity, social insects also practice social immunity, or those behaviors that reduce the exposure of nestmates to potential pathogens (Cremer *et al.*, 2007; Stow and Beattie, 2008). One current argument is that innate immunity incurs a cost to the host (discussed below), but that social insects have been able to replace this physiological immunity with behavioral immunity. Moreover, because behaviors are often pleiotropic and code for other useful behaviors, using these behavioral processes in immune functions may actually be more efficient than having separate genetic machinery for innate immunity (Le Conte *et al.*, 2011).

There is a wide range of examples of this social immunity in insects. Grooming in social insects is divided into autogrooming and allogrooming, depending on whether the insect is grooming itself or its nestmate (Evans and Spivak, 2010). Hygienic behavior is another form of social immunity that is well studied in honey bees. Here, the workers detect larvae that are infected with the fungus *Ascosphaera apis* (chalkbrood) or the bacterium *Paenibacillus larvae* (American foulbrood). They uncap the larval cell, remove the infected larva, and deposit it outside the nest (Evans and Spivak, 2010), and each step in this process is controlled by a suite of genes (Oxley *et al.*, 2010; Le Conte *et al.*, 2011). The chemical cue that bees use to identify infected brood is phenethyl acetate (Swanson *et al.*, 2009). Indeed, even healthy larvae that are marked with this chemical are removed and tossed from the nest by hygienic bees within 24 h. In another social insect example, ants practice necrophagy, which is similar to hygienic behavior, except that the ants sometimes deposit their dead within isolated chambers of the nest (Evans and Spivak, 2010). Both hygienic behavior and necrophagy are carried out by older members of the nest that typically do not care for brood, thereby limiting the exposure to their progeny (Evans and Spivak, 2010). If a nestmate becomes infected with a pathogen, the results are not necessarily catastrophic. Thus, when an ant colony of *Lasius neglectus* was infected with *M. anisopliae*, the workers instantly changed their behaviors to avoid contact with the brood. Even more surprising is that the uninfected ants were more likely to develop resistance to the pathogen after exposure to an infected nestmate (Ugelvig and Cremer, 2007). Hence, there may be a form of transference of immunity among nestmates. Another form of social immunity is when the workers coat the nest (and themselves or each other) in antibiotic substances. A great example of this is propolis, which is a resinous substance that honey bees encase their hives in that is simultaneously waterproof and antimicrobial (Evans and Spivak, 2010). Honey bees will even coat foreign objects that are too large to carry from their nest in propolis to seal them from the colony (Evans and Spivak, 2010). Finally, diseased social insects are frequently relegated to more risk-prone castes that operate outside the nests (e.g., foraging). It is not uncommon for these diseased insects to become disoriented outside the nest or be too weak to return to the nest, thereby limiting exposure to nestmates. It also appears that some social insects commit suicide when they are sick by quickly leaving the nest and not returning (Ruepell *et al.*, 2010). Antiseptic behaviors consisting of suicidal or heat-seeking behaviors have also been described for crickets and grasshoppers to inhibit parasite or pathogen development (Adamo, 1998). Clearly, antiseptic behaviors can have an important effect on insect–pathogen interactions.

13.2.2. Morphological Barriers to Infections

In addition to avoidance and behavioral removal of pathogens, insects possess a variety of morphological characteristics that present barriers to the establishment of pathogens. The precise barriers encountered depend on a pathogen's mode of transmission. The cuticle, tracheal

system, and midgut are the major sites of invasion, and each of these fronts has its own set of physical defenses.

Cuticle

The physical and inherent chemical characteristics of the cuticle have a great influence on which pathogens are able to establish on the external surface of a host. The cuticle covers all structures that come in contact with the external environment, including the exterior of the insect, the foregut, hindgut, and tracheal system (Chapman, 1998). The integument offers physical protection from predators, functions as an exoskeleton for muscle attachment, and reduces water loss, which allowed insects to permeate terrestrial ecosystems (Moussain, 2010). Another function of the integument is that it resists pathogen establishment.

Fungi are the main entomopathogens using the cuticular integument as a point of entry and start of infection. The entire external covering of insects is coated in a waxy layer, and the fatty acids that comprise this layer dictate which fungal entomopathogens are able to establish. Some fatty acids enhance the ability of certain fungi to adhere and germinate on the cuticle, while others restrict these processes (Boguś *et al.*, 2010). So far, it appears that fungi respond differently to the various fatty acids encountered on insect cuticles, so the insect needs to possess a wide variety of these fatty acids to have the plasticity necessary to repel the diversity of fungi they encounter. The exact mechanisms for how these fatty acids affect fungal adhesion and germination remain to be fully resolved.

Other factors, namely gland secretions and the microbial community, add antibiotic properties to the cuticle, making it an even more formidable barrier to infection. Some insects coat themselves in gland secretions that have antimicrobial properties. For instance, ants cover themselves in an antibiotic milieu produced by thoracic metapleural glands, which are unique to Formicidae (Poulsen *et al.*, 2003; Stow and Beattie, 2008). In a recent survey, all 26 species of ants examined wiped metapleural gland secretions on their bodies (Fernández-Marín *et al.*, 2006). Ants increase their use of this fungicidal secretion when challenged by a fungal entomopathogen, and this response is activated in as little as 1 h postinfection (Fernández-Marín *et al.*, 2006). When these glands are covered, ants infected with fungus die within a few days. In addition, the beneficial microbial community that resides on insect cuticles is an important source of antibiotics to resist some infections. Two hemipteran herbivores, *Dalbulus maidis* and *Delphacodes kuscheli*, are well known for their ability to resist infections by *B. bassiana*. Examining the bacterial community found on the cuticle revealed that 91 of the 155 bacterial isolates inhibited growth of the fungus, with the most effective isolates belonging to *Bacillus* (Toledo *et al.*, 2011). Only one bacterium, *Bacillus pumilis* Dm-B23, inhibited germination of the fungus. Clearly, the contributions of the symbiotic microbial community of the cuticle warrant further attention.

Digestive and Tracheal Systems

Many pathogens cannot penetrate the external defenses of the insect host, and circumvent this barrier by attempting to enter the hemocoel through the mouth (*per os*) or tracheal system. These pathogens (many viruses, bacteria, microsporidia, and protists are noteworthy examples) are confronted by a series of formidable defenses that necessitate specialized adaptations by the pathogen before successful infection is achieved. Much of the battle is waged in or near the midgut, where the cuticle is absent.

Microbes that enter the midgut must be adapted to survive and infect under the environmental conditions present in the particular insect, and these conditions vary widely among hosts. The pH, chemical milieu, and resident microbial community all play a role in which microbes are able to penetrate into the hemocoel. For example, *B. thuringiensis* protoxins are only activated under specific pHs and with specific proteases, which in part narrows the host ranges of the various strains and toxins of this pathogen (Haider *et al.*, 1986). Also, the salivary glands of some insects may produce antimicrobial compounds that permeate throughout the gut (Chouvenc *et al.*, 2010). Finally, the resident microbial community within insect guts resists some invaders in trying to reach a homeostatic condition, and even ordinarily pathogenic microbes can subsist as benign or even beneficial components of a healthy gut microbial community (Dillon and Dillon, 2004). The conditions fluctuate widely depending on the physiological status of the host and the environment in which this host lives.

If a microbe finds the environment of the gut suitable, the next barrier to infection is the peritrophic matrix. The peritrophic matrix is a semi-permeable, non-cellular envelope that encases the lumen of the midgut, and offers a barrier against physical abrasion by food and microbial invaders such as bacteria and viruses. This membrane is primarily composed of microfibrils, but also proteoglycans, proteins, and glycoproteins, and acts as a filter for microorganisms seeking access to the epithelial cells of the midgut lining (Lehane, 1997). The thickness and permeability of the peritrophic matrix can vary within an individual as well as among species (Chapman, 1998; Plymale *et al.*, 2008). Most successful pathogens that invade via the midgut (1) are small enough to pass through the peritrophic matrix (e.g., some viruses), (2) send lethal chemicals through the membrane that kill the cells that produce the envelope (e.g., δ-endotoxins of *B. thuringiensis*), or (3) degrade the membrane chemically (Chen *et al.*, 2008). This last approach is well explored in baculoviruses, which

produce various enhancins, e.g., virus enhancing protein (vep) and synergistic factor (sf) (Hoover *et al.*, 2010) (see Chapter 4). Another example of this approach comes from entomopoxviruses, which produce spindles (proteinaceous crystalline bodies) containing fusolin that disrupt the peritrophic matrix (Mitsuhashi *et al.*, 2007).

Once the peritrophic matrix is overcome, the midgut epithelial cells and basal lamina are the next barriers that must be breached. The midgut epithelial cells produce various antimicrobial proteins that are effective against Gram-positive and negative bacteria and fungal pathogens, and this process is governed by the immune deficiency (Imd) signaling pathway (discussed in Section 13.3.2) (Brennan and Anderson, 2004; Govind, 2008). In the case of intracellular pathogens, if the midgut cells are infiltrated, then the insect can simply slough them off in order to stop the infection process. At the base of the midgut epithelium (on the hemocoel side of the epithelium) lies a final acellular barrier, called the basal lamina, that must be circumvented before the hemocoel is reached (Passarelli, 2011). The basal lamina is particularly restrictive to viral infections, although viruses may pass through the basal lamina as it is recycled. In this case, the viruses express a gene that forces the cell to recycle layers of the lamina, and as this is happening, viral particles are encased in the layers of the lamina and invade the hemocoel as the lamina degrades (Passarelli, 2011). Viruses also can produce molecules that degrade the basal lamina, such as caspase and viral fibroblast growth factor (Passarelli, 2011). Also, there are occasionally small holes in the basal lamina that can be used for viral escape. Finally, some pathogens bypass the basal lamina of the midgut by infecting the tracheal system (Passarelli, 2011) (see Chapter 4).

The tracheal system is coated in cuticle and is composed of tracheae and much finer tracheoles (or tracheoblasts) (Chapman, 1998). The cuticle of the tracheoles is retained during molts, and these cells sometimes permeate other cells to oxygenate them. Tracheoles oxygenate the midgut epithelium and may be an additional conduit by which viruses infiltrate the hemocoel. But the tracheal system also possesses a basal lamina that represents a similar barrier as in the midgut epithelium (Passarelli, 2011).

13.3. PHYSIOLOGICAL RESPONSE TO INFECTIONS

If a pathogen successfully navigates the external defenses meant to keep it from becoming established, it could be faced with the defensive responses found within the insect hemocoel. Traditionally, the physiological responses of insects have been categorized as humoral or cellular in nature, although in reality the two systems are intertwined in many aspects. This notwithstanding, these categories have some function, as will be discussed below. It is noteworthy that much of the recent work in this area has focused on *Drosophila melanogaster* and *Anopheles gambiae* as models. This polarized interest is due to the availability of genomic tools and the sequenced genomes for both insects, which have provided unprecedented opportunities to explore the genetic basis of immunity. However, it is likely that important differences exist in the innate immunity expressed among the approximately 10 million species of insects (a conservative estimate) and care should be taken not to overgeneralize the important findings from these two model organisms.

Innate physiological immunity follows a series of pathways to kill and eliminate pathogens from the insect's hemocoel. The first step in the process is that the insect must recognize potentially dangerous pathogens and alert the innate immunity systems. Once this recognition is achieved, one or more of several signaling pathways are initiated. The humoral response involves the production of antibiotics that kill the pathogen. Simultaneously, a cellular response could be initiated, whereby hemocytes aggregate around the pathogen, immobilizing and killing it. Finally, these two defense systems often work together to produce melanin, a third deadly weapon targeted against the extracellular pathogens in the hemocoel. Defenses against intracellular pathogens are less well studied, although it appears that similar signaling systems are involved in at least triggering the intracellular responses (which involve antimicrobial molecules and RNA interference).

13.3.1. Distinguishing Self from Non-self from Altered Self

The first step in the innate physiological responses to pathogens is distinguishing the pathogen or pathogen-affected cells from the rest of milieu in the hemocoel. Essentially, the immune system needs to distinguish self from non-self (e.g., the pathogen or abiotic material) and altered self (e.g., dead or infected cells) (Kanost and Nardi, 2010). To do this, the immune system takes advantage of unique molecules either on or associated with a specific invader. These pathogen-associated molecular pattern (PAMP) molecules include lipopolysaccharides (LPS), lipoteichoic acid (LTA), peptidoglycans (PGN), and β-1,3-glucan (Hoffmann, 1995; Lavine and Strand, 2002; Royet, 2004a; Strand, 2008; Kanost and Nardi, 2010). While LPS, LTA, and PGN are bacterial elicitors, β-1,3-glucan triggers an antifungal response (Royet, 2004b). Although there is little research on the topic of antiviral PAMPs, viral glycoprotein VSV-G is one example that indicates that these molecules play a role in viral immunity (Sabin *et al.*, 2010).

Occasionally, endogenous signals associated with the pathogen are not needed to initiate the immune response. Wounding or stress can elicit such a response without foreign invaders (Brennan and Anderson, 2004; Kanost and Nardi, 2010). Extracellular nucleic acids can also trigger an immune response, presumably because these are a sign of cell damage that sometimes indicates infection (Kanost and Nardi, 2010; Vilcinskas, 2010). In fact, oenocytoids (a type of hemocyte) of some insects rupture in response to pathogen invasions, and their contents thereby become the signal that triggers downstream immune reactions (Vilcinskas, 2010).

Abiotic materials also can elicit immune responses, although the underlying mechanisms that drive this recognition are poorly understood. For example, Lavine and Strand (2001) injected 19 different types of chromatography beads into the lepidopteran *Pseudoplusia includens*, and while the immune system frequently encapsulated the foreign materials, there was little consensus in what signaling pathway or even hemocytes were involved in this process. Clearly, there are many more classes of recognition site than simply the PAMPs often discussed in the literature.

The molecules produced by the insect that recognize, bind to, and mark these PAMPs are called pathogen recognition receptors (PRRs) or pathogen recognition proteins (PRPs). Dozens of these receptors have been identified to date, and they are categorized by Strand (2008) as often being extracellular, cell surface, or transmembrane in nature. Gram-negative binding protein (GNBP), LPS-binding protein C-type lectins, β-1,3-glucan recognizing proteins, immunolectins, tep-proteins, Down's syndrome cell adhesion molecule (Dscam), eicosanoids, and hemolin are examples of PRRs that act against various pathogens (Christensen *et al.*, 2005; Govind, 2008; Jiang, 2008; Strand, 2008; Kanost and Nardi, 2010). Some of these PRRs are inducible, only becoming active when the host is immunochallenged. Other PRRs are produced constitutively, and thus are always probing for potential invaders.

The specificity of these PRRs allows the insect host to mount targeted defenses against the diverse army of invaders it experiences. For example, a commonly studied group of PRRs that displays specificity is the peptidoglycan receptor protein (PGRP) group (Kanost and Nardi, 2010). This group shares a 160 unit peptidoglycan recognition domain (Brennan and Anderson, 2004). Within this group of constitutively produced recognition proteins (Takehana *et al.*, 2004), the PRRs are categorized as PGRP-L and PGRP-S, based on size (Royet, 2004a). PGRP-S have fewer than 200 amino acids, and PGRP-L are proteins that are larger than 200 amino acids (Royet, 2004a). In *Drosophila*, the protein PGRP-LC protein is anchored to the hemocyte membrane, and functions against Gram-negative bacteria (Brennan and Anderson, 2004). In contrast, PGRP-S is a secreted protein in *Drosophila* acting against Gram-positive bacteria (Brennan and Anderson, 2004). Other PGRP-S proteins can act against Gram-negative infections as well (Royet, 2004b). At least two forms of PGRP-L (-LE and -LC) are present on the insect's epithelium, thus representing a first line of defense against infections. Takehana *et al.* (2004) studied these interactions on the tracheal epithelium. C-type lectins do not recognize proteins; rather, they bind to microbial-based polysaccharides (Jiang, 2008). Not all PRRs display specificity for certain pathogens. Hemolin, a hemolymph protein induced by bacteria and which is only present in Lepidoptera, is fairly non-specific to particular pathogen groups and even binds to hemocytes (Eleftherianos *et al.*, 2006; Jiang, 2008). Finally, some genes that encode for PRRs have been identified so far; *semmelweis* and *osiris* are two examples that encode specific PGRP and GNBP PRRs (Hoffmann, 2003).

Even hemocytes can function as PRRs when they adhere to a foreign target (Lavine and Strand, 2002; Carton *et al.*, 2008). In some cases, hemocytes bind to PRRs just like the humoral system does. Some hemocytes, for example, bind to immunolectins that have attached to foreign invaders (Brennan and Anderson, 2004; Strand, 2008), whereas other hemocytes possess transmembrane PRRs in their cell membranes that recognize conserved foreign molecules associated with pathogens. Within Lepidoptera, these poorly understood cell adhesion molecules include integrins, tetraspanins, and neuroglian (Lavine and Strand, 2002; Kanost and Nardi, 2010). Finally, granulocytes can sometimes produce PRRs; lacunin and hemocytin are two examples of these (Kanost and Nardi, 2010).

13.3.2. Humoral Response System

Antimicrobial Peptides

Soluble peptides constitute the main weapon that insects use during humoral responses to pathogens. Dozens of antimicrobial peptides (AMPs) (at least 50) have been identified to date, and these peptides can be very specific for specific pathogens, or classes thereof (Hoffmann, 2003; Govind, 2008; Kanost and Nardi, 2010). Lysozyme was the first AMP identified, and it was isolated in *Galleria mellonella* (Vilcinskas, 2010). Many of the AMPs fall within one of three categories, based on their structure (Bulet *et al.*, 1999). The first group of these is peptides with intramolecular disulfide bonds that form hairpin β-sheets and α-helical−β-sheet mixed structures. In comparison, the second group of AMPs has peptides that form amphipathic α-helices. Finally, the third group has a disproportionate amount of proline and/or glycine residues.

Various classes of Amp residue within these groups. For example, cecropins and defensins are two major classes of AMPs specific for Gram-positive or Gram-negative

bacteria, respectively (Hoffmann, 1995). Proline- and glycine-rich polypeptides are another class of AMP, and these are specific for Gram-negative bacteria. Acute phase glycoproteins dTEPs are another noteworthy groups of AMP that disrupt bacterial cell walls (Govind, 2008; Strand, 2008). There are exceptions to the specificities noted above; defensins sometimes act against Gram-positive bacteria as well as fungi (Bulet *et al.*, 1999), and some proline-rich polypeptides also affect Gram-positive bacteria as well (Hoffmann, 1995). Some AMPs are very specific in where they reside and function (Hoffmann, 1995). Cecropins are membrane-active proteins residing on cell membranes, while lysozyme is ubiquitous throughout insect tissues. Other AMPs are restricted to the genital tracts of insects. Finally, PGRPs that are involved in identifying PAMPs also sometimes degrade the peptidoglycan present in bacterial cell walls, and so act as antimicrobial agents as well as signaling agents (Strand, 2008). In short, new antimicrobial peptides are frequently revealed, and our understanding of this rapidly expanding source of innate immunity would benefit from some synthesis in the future.

Regulation of Humoral Defenses

Antimicrobial peptides are produced in the fat body, and to a lesser extent in other somatic cells and by circulating granulocytes and plasmatocytes (Hoffmann, 1995; Lavine and Strand, 2002; Govind, 2008; Strand, 2008). Upon encountering a foreign target, usually one of two signal transduction pathways (Toll and Imd, although there are others) that resist different pathogen classes is triggered (Govind, 2008). The Toll pathway is mobilized in response to Gram-positive and fungal infections, while Imd is mobilized by Gram-negative bacteria and infections of multiple classes associated with barrier membranes. The specificity of the pathways is truly remarkable given the genetic divergence observed in pathogen groups that trigger the same response pathway. For instance, fungi and Gram-positive bacteria share only 30% genetic similarity, but somehow both activate the Toll pathway (Hoffmann, 2003). Both the Toll and Imd pathways are triggered upon exposure to certain viruses, although the mechanisms involved are poorly understood (Sabin *et al.*, 2010). Both of these pathways belong to the intracellular nuclear factor-κB (NF-κB) related signal transduction pathway, which ultimately produces the AMPs. The Toll pathway is not mobilized by direct interactions with microbes themselves, but rather by interacting with the cytokine intermediate Spätzle, which is activated through cleavage by a serine protease (Hoffmann, 2003; Govind, 2008). Serine proteases are responsible for cleaving the proSpätzle into its active form (Royet, 2004a). During fungal infections, the gene *persephone* is involved in the production of these serine proteases (Brennan and Anderson, 2004). The Imd pathway is regulated by *Relish* (Royet, 2004a). It appears that hemocytes are particularly important in the Imd-mediated response of *Drosophila* (Brennan and Anderson, 2004). When hemocytes function as elicitors of the Imd pathway, PGRP-LC is a transmembrane protein on the hemocytes. When the hemocytes bind to a foreign target, nitric oxide (NO) produced by the hemocytes triggers the fat body to produce the necessary AMPs (e.g., diptericin). Other signaling pathways, such as the Jak-STAT and JNK pathways, also initiate the humoral defense system in immune-challenged insects (Brennan and Anderson, 2004). The immunity provided by AMPs generally lasts for two to three days (Kanost and Nardi, 2010). Although it is known that they block the growth of microbes in the hemolymph (Hoffmann, 2003), the mechanisms by which the AMPs kill foreign targets remain poorly understood.

13.3.3. Cellular Response System

Inherently tied to humoral-based immunity is the cellular defense system of insects which, as the name suggests, involves various types of hemocytes. These hemocytes kill and remove pathogenic targets through phagocytosis of small or unicellular targets, or through nodulation/encapsulation of larger elements, groups of pathogens, or multicellular organisms. Aspects of the cellular defense system are also coupled with the humoral defense network in melanization of pathogens.

Hemocytes

Several hemocytes have been characterized by their morphology, the presence of specific antigenic markers, and their functional responses to entomopathogens (Strand, 2008). All types of hemocyte are derived from prohemocytes, which are a type of stem cell (Lavine and Strand, 2002; Kanost and Nardi, 2010). Hemocytes are produced during two life stages of the insect, during embryogenesis and during late larval development (Strand, 2008). The latter prohemocytes produced by larvae are of mesodermal origin and are derived from hematopoietic organs (Carton *et al.*, 2008). Hematopoietic organs are lymph glands (Carton *et al.*, 2008; Strand, 2008); in *Drosophila*, pairs of these glands form along the anterior area of the dorsal vessel during embryogenesis. There are three groups of these organs in *Drosophila*: the posterior signaling center, the medullary zone, and the cortical zone. The first two of these groups contain only quiescent prohemocytes, whereas the cortical zone has active production of both plasmatocytes and crystal cells (Strand, 2008). Some lepidopteran larvae have four hematopoietic organs located in the thorax near the wing imaginal disks (Strand, 2008). Some insects also have secondary hematopoietic organs and free-living prohemocytes that are involved in the synthesis of

plasmatocytes (Strand, 2008). Knowledge on the generation of hemocytes comes entirely from studies on *Drosophila.* In this insect, some genes have been identified that regulate the initial production and proliferation of prohemocytes (*Srp* and *Pvf2*). Once the cells are produced, they differentiate from prohemocytes into their specific forms, and several genes that regulate this process have also been identified. In *Drosophila*, *gcm* and *gcm2* regulate the differentiation of plasmatocytes, and *Notch* and *Lz* genes regulate the differentiation of crystal cells (Carton *et al.*, 2008; Strand, 2008). Several signaling pathways have also been implicated in hemocyte proliferation and differentiation, including Toll, Jak/Stat, Jun kinase, and Ras-mitogen pathways (Carton *et al.*, 2008; Strand, 2008). Once the hemocytes have been created and have differentiated, they are able to proliferate in response to pathogens, although the mechanisms deserve further study.

Various hemocyte types comprise the hemolymph. The most abundant hemocytes in many insects are granulocytes, followed in abundance by plasmatocytes, oenocytoids, and spherulocytes (Lavine and Strand, 2002). Sometimes, granulocytes differentiate after foreign invasion. In *Manduca sexta* larvae, one group of enlarged granulocytes, called phagocytes, behaves differently from other granulocytes in response to invasion, spreading out asymmetrically during encapsulation and being capable of forming capsules around foreign targets (Strand, 2008). To confound the literature on cellular responses, the hemocytes of *Drosophila* follow a different nomenclature altogether. In this insect, plasmatocytes (90% of hemocytes) dominate in number, and lamellocytes and crystal cells are less abundant (Lavine and Strand, 2002). There are clear analogies to the hemocytes of the other insects (granulocytes and plasmatocytes, oenocytoids and crystal cells, plasmatocytes and lamellocytes), although the two nomenclature systems are not perfectly interchangeable (Strand, 2008). Not all hemocytes are found in all insects, and even congeners can vary in the composition of hemocyte types in their hemolymph (Carton *et al.*, 2008). Also, foreign invasion can induce differentiation of prohemocytes; healthy *Drosophila* do not have lamellocytes and are produced only following infection (Strand, 2008).

Each of these hemocytes has a fairly distinct function within the immune system of insects. Plasmatocytes and granulocytes are the only hemocytes that adhere to foreign molecules and pathogens, and thus are the primary agents involved in phagocytosis and encapsulation of pathogens (Lavine and Strand, 2002). Oenocytoids produce phenoloxidase components that are involved in melanization, which will be discussed below (Lavine and Strand, 2002). The function of spherulocytes is less well understood, but they appear to transport cuticular materials and have an unknown function in cellular immunity (Lavine and Strand, 2002). In *Drosophila*, lamellocytes are adhesive and play a role in encapsulation. Crystal cells are not adhesive, but they produce phenoloxidase components (Brennan and Anderson, 2004). Similar to other insects, plasmatocytes of *Drosophila* adhere to foreign targets and play a role in phagocytosis (Brennan and Anderson, 2004). In sum, these hemocytes work together in mounting the various cellular responses to invasion by pathogenic agents, which are fairly easily categorized as involving either phagocytosis or nodulation/encapsulation, depending on the size and type of pathogen involved.

Phagocytosis

The word "phagocytosis" has a Greek origin and when translated to English it means "cell eating", which clearly describes this process. In phagocytosis, granulocytes adhere to a foreign molecule or cell and envelop the invader, thereby killing it. During phagocytosis, the pathogen is encased intracellularly in a phagosome. The hemocyte releases reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs), which are toxic to the pathogen (Lavine and Strand, 2002). These ROIs and RNIs may also trigger the humoral signaling pathway that leads to the production of antimicrobial peptides (Lavine and Strand, 2002). For example, NO activates the NF-κB pathway discussed above. Regardless of the mechanism, the result is death for the pathogen. This process is constrained by the size of the hemocyte and pathogen involved, and the cellular immune system must overcome larger invaders or groups of invaders using other means.

Nodulation and Encapsulation

Insects rely on nodulation and encapsulation to ensnare larger invaders that cannot be killed with a single granulocyte (Strand, 2008; Vilcinskas, 2010); these two processes differ in their degree rather than in approach. When a pathogen is located in the hemocoel, granulocytes (and sometimes plasmatocytes), or lamellocytes in *Drosophila*, adhere to a PAMP of the invading organism. It is common for the hemocytes to spread out and apoptose when they encounter a foreign target (Brennan and Anderson, 2004; Strand, 2008; Kanost and Nardi, 2010). This process is initiated by one or several activator molecules; one such molecule is plasmatocyte spreading peptide (PSP), which belongs to the ENF-peptide family (Kanost and Nardi, 2010). ProPSP is cleaved to generate the active form which elicits spreading of the hemocyte. Another protein class known to elicit spreading is Rac GTPases (Carton *et al.*, 2008), which function in cytoskeletal organization, regulation of cellular adhesion, and related transcriptional activation (Carton *et al.*, 2008).

Spreading hemocytes overlap each other to encapsulate the target (Lavine and Strand, 2002). Different insects produce different patterns in how the hemocytes aggregate

to infections. Sometimes, granulocytes form the initial sheath around the target, and plasmatocytes and granulocytes build upon this. In other species, plasmatocytes and granulocytes arrange randomly, and granulocytes are not ubiquitously necessary for encapsulation to occur. Encapsulation is almost always reinforced by melanization, a process discussed further below. Encapsulation almost always kills the target, either through asphyxiation or through the production of toxic chemicals during the melanization process.

13.3.4. Melanization

The humoral and cellular components of innate immunity often both contribute to melanization of a foreign target. Foremost, encapsulation is often accompanied by melanization, but not in all insect species (Lavine and Strand, 2002; Christensen *et al.*, 2005; Carton *et al.*, 2008; Strand, 2008; Kanost and Nardi, 2010). Also, when hemocytes are scarce, melanization can still occur in some insects (Christensen *et al.*, 2005). Melanization is a series of chemical reactions that results in the production of darkened pigment around a wound or pathogen, and there are three general steps in this chemical process (Carton *et al.*, 2008).

The first step is the activation of the phenoloxidase cascade, which occurs within minutes of infection (Royet, 2004b). Phenoloxidases are a key chemical in the cascade of reactions that ultimately leads to melanization. The few insects in which this process has been studied to date have been found to contain at least six active phenoloxidases (Christensen *et al.*, 2005). The process begins when serine proteases, which are produced with the involvement of immunolectin-2 in *M. sexta* (Eleftherianos *et al.*, 2006), trigger the hydroxylation of tyrosine (derived from phenylalanine) into dihydroxyphenylalanine (DOPA), and ends with the production of eumelanins and indolequinones (Christensen *et al.*, 2005; Carton *et al.*, 2008; Jiang, 2008). To protect themselves from random melanization events, phenoloxidases circulate in the hemolymph as prophenoloxidase (Kanost and Nardi, 2010). As mentioned above, this prophenoloxidase is produced by oenocytoids (or crystal cells in *Drosophila*), a type of hemocyte (Strand, 2008; Kanost and Nardi, 2010). The cleavage of prophenoloxidase into phenoloxidase is blocked by the protein Spn27A, which inhibits a prophenoloxidase activating enzyme (PPAE) (Brennan and Anderson, 2004). When cleavage is required, the insect produces an inhibitor to Spn27A, or it overproduces PPAE (Brennan and Anderson, 2004). Genes that regulate the creation of prophenoloxidase have been identified and different genes are expressed in response to various pathogens. For example, in *Heliothis virescens* larvae, only one of two genes (the one producing HvPPO-1) is upregulated when the host is challenged by a bacterial invader (*Micrococcus lysodeikticus*); the other gene product is produced constitutively. The prophenoloxidase is activated by one of several prophenoloxidase-activating proteases.

The next step in melanization is the proliferation and physical alterations of hemocytes at the site of the wound or invader. Much of this process is discussed above in the section on encapsulation. In brief, hemocytes adhere to receptors on the foreign target, spread, and aggregate to form a multicellular sheath that asphyxiates the foreign body.

The final step in melanization is the production of toxic chemicals that kill the offending agent. The melanization process produces several chemicals that are known to be toxic to microbes. Specifically, ROIs (O_2^-, H_2O_2), RNIs (NO), quinones, hydroquinones, and most importantly, L-DOPA are produced (Lavine and Strand, 2002; Christensen *et al.*, 2005; Carton *et al.*, 2008). Antimicrobial peptides such as defensins also aggregate to sites of melanization, adding another lethal agent to the milieu (Hillyer and Christensen, 2005).

13.3.5. Intracellular Defenses

Defending against intracellular pathogens requires additional tools on the part of the host than those previously discussed. Indeed, innate immunity has received much more attention from the perspective of extracellular pathogens, and the mechanisms that cells use to defend themselves against intracellular bacteria and viruses remain to be fully resolved. Nevertheless, a growing body of research is beginning to shed light on how xenophagy and RNA interference (RNAi) combine with antimicrobial molecules to defend against this major class of pathogens.

Involvement of the Extracellular Immune Systems Against Intracellular Pathogens

Outside the host cell, intracellular bacteria often do not elicit the innate immune responses of the hosts, although the humoral and cellular responses are often effective against these pathogens. Phenoloxidase is antiviral, although injection with baculovirus does not necessarily elicit the production of phenoloxidase (Shelby and Popham, 2008). Likewise, antimicrobial genes are not upregulated when *Drosophila* is infected with the intracellular bacterium *Spiroplasma poulsonii* (Hurst *et al.*, 2003). This pathogen resembles the Gram-positive bacteria, but they lack a cell wall and presumably the PAMP necessary to instigate the humoral and cellular responses. However, *Drosophila* mutants that produce seven different antimicrobial peptides constitutively are able to repel *S. poulsonii* infections, as are *Drosophila* that have their immune systems "primed" by injection with dead bacteria

(Hurst *et al.*, 2003). Similarly, *Wolbachia*, one of the most well known of the intracellular bacteria (see Chapter 9), does not trigger the Gram-negative specific immune response in *Anopheles* or *Aedes* mosquitoes, even though this microbe resides within this guild of pathogens (Bourtzis *et al.*, 2000; Brennan *et al.*, 2008). In *Drosophila*, *Wolbachia pipientis* infection also does not instigate the production of two antimicrobial peptides (diptericin and cecropin, which are both specific to the Gram-negative bacteria) (Siozos *et al.*, 2008). An exception is a virulent strain of *W. pipientis* named "popcorn", which ruptures the host cells and presumably leaves the pathogen exposed to the extracellular innate immune response (Siozos *et al.*, 2008). In any case, it seems clear that prior exposure to other pathogens influences the success of these intracellular disease agents.

RNA Interference

Insect cells respond to viral infection by initiating an antiviral program involving an RNA silencing pathway; this RNAi is one of the most important components of the antiviral defenses of insects (Sabin *et al.*, 2010). Some of the key genes involved in the biogenesis of RNAi that functions against viruses are *Dcr-2*, *r2d2*, and *AGO2*, and it is likely that additional contributing genes that govern this process will be discovered (Sabin *et al.*, 2010). The protein Dcr-2 attaches to viral dsRNA, and it functions as a PAMP recognizable by other antiviral molecules (Sabin *et al.*, 2010). Vago is one such PRR that recognizes Dcr-2, and Vago also kills the virus (Sabin *et al.*, 2010). So far, the Jak-STAT signaling pathway appears to be the most important for antiviral defenses of cells. Research on antiviral defenses involving RNAi of insects is still in its infancy, and it is also unknown whether RNAi pathways function in defending against other pathogenic agents apart from viruses.

Xenophagy

Under specific adverse conditions, cells can undergo autophagy, a catabolic process by which cells digest old or damaged organelles (Amano *et al.*, 2006). The process whereby the cell directs its autophagy machinery toward killing pathogens is termed xenophagy (Levine, 2005). Xenophagy is thought to play a role in antiviral defenses (Sabin *et al.*, 2010), although there are few data on these interactions. While the specific molecules controlling this process are poorly understood, they are expected to share similarities with general steps described for autophagy processes. During autophagy, old organelles are engulfed by autophagosomes, which are essentially multimembrane structures involving cisternae and isolation membranes (Amano *et al.*, 2006). Lysosomes then fuse with the autophagosome and degrade the contents (Amano *et al.*, 2006). The role of autophagy in maintaining healthy cells has been known for some time, although its role in destroying pathogens was discovered only recently.

Resistance Against Intracellular Host Defenses

To avoid or circumvent detection, intracellular pathogens either hide from the defensive system or co-opt it entirely at a genetic level. To hide from the immune response, intracellular bacteria will sometimes encase themselves in membranes of the host cell, called endosomes or phagosomes (Amano *et al.*, 2006). *Wolbachia* is a good example of this (McGraw and O'Neill, 2004), but the lysosomes can recognize these endosomes and degrade them through acidification (Amano *et al.*, 2006). In turn, the pathogen resists this process. Obligate endosymbionts usually have reduced genomes and rely on their host's genes for key functions, including defense. In the case of *Wolbachia*, the bacterium actually co-opts the production of antioxidants to defend against the host's reactive oxygen species, a major aspect of the humoral defense system of insects (Brennan *et al.*, 2008). Indeed, *Wolbachia* infection of *An. gambiae* upregulates more than 700 host genes, and many (but not all) of the immune, stress, and detoxification-related transcripts are downregulated upon *Wolbachia* infections (Hughes *et al.*, 2011). Viruses that are targeted by RNAi can evolve the ability to suppress these nucleic acids, and the resulting arms race between viruses and their host cells has made RNAi genes some of the fastest evolving within the *Drosophila* genome (Sabin *et al.*, 2010).

13.4. MANAGING RESISTANCE TO ENTOMOPATHOGENS

Understanding and predicting how insect hosts become resistant to pathogens or their toxins is important as land managers (especially farmers) have come to rely on entomopathogens or their toxins more heavily for controlling pests. Declaring an insect resistant can be controversial, since commercial products that rely on entomopathogens as their active ingredient could then lose applicability and market share. Thus, having a clear definition of resistance becomes imperative. As mentioned throughout this chapter, most insect populations have a range of defensive capabilities against particular pathogens. For the purposes of this discussion, the general definition of Fuxa (2004) is adopted. Fuxa (2004) describes resistance as "the development of an ability in a strain of insects to tolerate doses of a [pathogen] that would cause disease or prove lethal in the majority of individuals in a normal population of the same species." Heritability is also a key aspect to resistance; the trait must be passed on to the offspring in order for a strain to be considered resistant (Gassmann *et al.*, 2009a). Thus, we recognize that there is a continuum in resistance, and

use the background or average resistance in a population as a benchmark against which to compare comparing selected strains.

Given the diversity of defensive mechanisms that pathogens must overcome for successful infection, there are remarkably few well-studied examples of host resistance to pathogens when using the definition provided above. The few instances where the evolution of resistance has been well studied are in ecosystems where tremendous selective pressure has been placed upon particular target pests. For example, the velvetbean caterpillar, *Anticarsia gemmatalis*, a key pest of soybeans in Brazil, is managed using the nucleopolyhedrosis virus AgNPV. Although field resistance in the pest has not been discovered, laboratory strains of resistant pests have been selected for in the laboratory and resistance mechanisms and stability are being revealed (Fuxa and Richter, 1998; Piubelli *et al.*, 2006; Levy *et al.*, 2007). Another system where resistance has been well studied is the case of *Bacillus sphaericus*, which is applied to control mosquitoes of medical importance. High levels of field resistance have been documented in *Culex* spp. in Asia and Europe (Charles *et al.*, 1996; Park *et al.*, 2010), which is probably the result of only a single insecticidal toxin being produced by this species of *Bacillus* (rather than the multiple toxins produced by *B. thuringiensis*). The mechanisms underlying these cases of resistance to *B. sphaericus* have been fairly well studied, and usually involve the inability of the Bin toxin (a potent protein toxin with two components, BinA and BinB) to adhere to the binding sites on the midgut epithelium of resistant individuals (Darboux *et al.*, 2002, 2007). This is certainly not always the case, and behavioral avoidance of the toxin (Rodcharoen and Mulla, 1995) and other, unidentified, mechanisms (Nielsen-Leroux *et al.*, 1997, 2002) may also be at play.

With regard to host resistance and its implications for pathogen−insect dynamics, no species has received more attention than *B. thuringiensis*. Various strains of *B. thuringiensis* have been widely applied to entire landscapes for controlling dipteran and lepidopteran pests, and now various genetically modified crops express the Cry and/or Vip toxins. Cry toxins are the crystal proteins produced by *B. thuringiensis* that destroy the midgut epithelial cells; Vip toxins are vegetative insecticidal proteins produced by *B. thuringiensis* that also target the midgut epithelium, but adhere to different receptors on the midgut wall than the Cry toxins. The wide use of these toxins increases the selective pressure towards evolving resistance in the target pests. For these reasons, this organism will be used as a model for discussing more in depth how resistance evolves within host insects, and how information on this process is crucial to preserve *B. thuringiensis* as a management option.

13.4.1. Resistance Mechanisms to *Bacillus thuringiensis*

Insecticidal products containing *B. thuringiensis* as the active ingredient have been used extensively in pest control, mostly against dipteran pests of medical concern and lepidopteran pests of agriculture. For years it had been assumed that because *B. thuringiensis* was a biological agent, capable of evolving alongside its host, host resistance was of minimal concern (Shelton *et al.*, 2007). As will be discussed below, this was an incorrect assumption. In 1996, the first transgenic crops genetically engineered to constitutively produce δ-endotoxins of *B. thuringiensis* (Bt crops) were commercialized and have quickly come to dominate cotton and corn acreages throughout North America and other countries. In the USA, this level of selection pressure − 4.2 million ha of cotton and 24.3 million ha of corn expressed one or more Bt toxins in 2011 (NASS, 2011) − has resulted in tremendous selection pressure against not only the Cry toxins expressed in the plants, but possibly the pathogen itself. Efforts to mitigate this resistance have advanced our understanding of the ecology and dynamics of how hosts evolve defenses against pathogens.

Resistance to *B. thuringiensis* was first reported in grain storage bins (McGaughey, 1985) and was subsequently found under greenhouse (Janmaat *et al.*, 2004; Meihls *et al.*, 2008) and field conditions (Tabashnik *et al.*, 1990; Tang *et al.*, 1997); these instances include resistance to transgenic Bt crops (van Rensburg, 2007; Storer *et al.*, 2010). However, resistance to *B. thuringiensis* toxins may be seen as a rare event when considering the level of adoption of these technologies (Tabashnik *et al.*, 2009). A diverse group of insect species has been selected to be resistant to *B. thuringiensis* toxins in the laboratory (Schnepf *et al.*, 1998; Ferré and Van Rie, 2002), and these strains have facilitated the characterization of resistance mechanisms that may potentially evolve in field populations. Alterations of host defenses in resistant strains are limited to Cry toxins; there are no data yet available on resistance against Vip toxins.

The most well-studied mechanism of resistance is the alteration of receptors on the midgut brush border membranes and consequent disruption of toxin binding (Ferré and Van Rie, 2002). This type of resistance mechanism was described for strains of the diamondback moth, *Plutella xylostella* (Ferré *et al.*, 1991; Wright *et al.*, 1997), and the Indian meal moth, *Plodia interpunctella* (van Rie *et al.*, 1990), which had developed resistance to commercial *B. thuringiensis* formulations, although the specific altered receptor was not reported. Since diverse Cry toxins may recognize the same midgut receptors, cross-resistance to toxins that were not present in the environment of selection is possible. For instance, selection of lepidopteran larvae with Cry1Ac toxin usually results in cross-resistance

to all Cry1A toxins. One specific resistance phenotype commonly described in diverse lepidopteran species and designated as "Mode 1 resistance" (Tabashnik *et al.*, 1998) is characterized by reduced binding by the toxin and more than 500-fold resistance to at least one Cry1A toxin, recessive inheritance, and little or no cross-resistance to Cry1C. The first gene linked to this Mode 1 resistance was a cadherin from *H. virescens* (Gahan *et al.*, 2001). This cadherin gene appeared disrupted by a retrotransposon insertion, resulting in lack of a full-length cadherin protein on the surface of midgut cells in Cry1Ac-resistant larvae (Jurat-Fuentes *et al.*, 2004). Alterations in cadherin genes linked to resistance against Cry toxins have also been confirmed in the pink bollworm, *Pectinophora gossypiella* (Morin *et al.*, 2003), and the cotton bollworm, *Helicoverpa armigera* (Xu *et al.*, 2005; Yang *et al.*, 2007). Cadherin resistance alleles are not detected in field populations of *P. gossypiella* (Tabashnik *et al.*, 2006) and *H. virescens* (Gahan *et al.*, 2007), probably owing to the low frequency of resistance alleles. This observation suggests that other mechanisms may be involved in resistance, a conclusion supported by the finding that field resistance in *P. xylostella* is not linked to cadherin (Baxter *et al.*, 2005). Recently, a mutation in an ABC transporter gene has been reported to be genetically linked to resistance and lack of Cry1Ac binding in strains of *H. virescens* (Gahan *et al.*, 2010). However, further work would be necessary to characterize the role of this transporter protein in the Cry1A intoxication process.

Although alteration of the midgut receptors sites has received the most attention from researchers, this is not the only mechanism by which insects may evolve resistance to *B. thuringiensis*. Because of the importance of toxin activation during the intoxication process, alterations in the midgut protease composition of the host can result in resistance. For example, resistance to *B. thuringiensis* subsp. *entomocidus* or subsp. *aizawai* in two strains of *P. interpunctella* was associated with the loss of a major trypsin-like protease (Oppert *et al.*, 1997). Although alterations in protease gene expression in other insect-resistant strains have been reported (Karumbaiah *et al.*, 2007; Khajuria *et al.*, 2009; Rajagopal *et al.*, 2009), their genetic linkage to resistance has not been established. Since activation is a common step in the mode of action of diverse Cry toxins, cross-resistance to other Cry toxins would be expected from alteration of this process. However, not all cases of cross-resistance to diverse toxins correlate with alterations in proteases. For example, a strain of *S. exigua* selected for resistance with Cry1Ab displayed cross-resistance to toxins not expected to share receptors with Cry1A toxins, such as Cry1D and Cry1Ca, but no protease alterations were detected compared to susceptible larvae (Hernández-Martínez *et al.*, 2009).

Another potential mechanism that would result in resistance to diverse Cry toxins is a midgut regenerative response that would prevent disruption of the midgut epithelium barrier and colonization of the hemocoel by vegetative *B. thuringiensis* or other bacterial cells. Early reports suggested that larvae of *H. virescens* infected with *B. thuringiensis* could recover from intoxication when presented with a toxin-free diet after a short exposure (Dulmage and Martinez, 1973; Dulmage *et al.*, 1978). A midgut regenerative response to intoxication with *B. thuringiensis* was described in larvae of the rice moth *Corcyra cephalonica* (Chiang *et al.*, 1986) and *M. sexta* (deLello *et al.*, 1984). This defensive midgut regenerative mechanism has also been demonstrated *in vitro* (Loeb *et al.*, 2001) and was proposed to be involved in resistance to Cry toxins in strains of *H. virescens* (Forcada *et al.*, 1999; Martínez-Ramírez *et al.*, 1999). In these insects, faster regeneration of damaged midgut epithelium correlated with survival on a diet containing Cry1Ac toxin. However, the specific genes involved in this regenerative process and their linkage with resistance have not been reported.

While feeding avoidance has been reported and could also potentially result in resistance to diverse toxins, there are no documented cases of resistance correlated with selective feeding. Cross-resistance to diverse toxins can also be a result of the existence of multiple mechanisms. For instance, strains of *H. virescens* resistant to Cry1Ac and Cry2A toxins were found to present altered toxin binding and protease patterns (Jurat-Fuentes *et al.*, 2003; Karumbaiah *et al.*, 2007). Resistance to Cry1Ab in strains of *P. interpunctella* (Herrero *et al.*, 2001), and Cry3Aa in the Colorado potato beetle, *Leptinotarsa decemlineata* (Loseva *et al.*, 2002), has also been reported to correlate with alterations in both protease activity and toxin binding. Remarkably, cross-resistance to Cry1Ac toxin and the chemical pesticide deltamethrin has been described in strains of *P. xylostella* (Sayyed *et al.*, 2008). Tests looking for complementary mechanisms in these *P. xylostella* strains suggest that a common genetic locus or loci controlled resistance to both insecticides, although the specific mechanism of cross-resistance has not been identified. If common, this type of resistance would preclude the use of some chemical pesticides to delay or control episodes of resistance to *B. thuringiensis* toxins.

13.4.2. Managing Resistance to *Bacillus thuringiensis*

Once resistance to a pathogen evolves in an insect, effective resistance control practices become crucial to preserve the future utility of the pathogen for insect control. The importance of *B. thuringiensis* as a biopesticide, especially for organic farmers who have few equivalent options for managing pests, and the high adoption rate of Bt crops,

made proactively mitigating the spread of Bt-resistant pest populations a major priority during the early regulation of Bt crops (Ferré and Van Rie, 2002; Tabashnik *et al.*, 2003; Carrière *et al.*, 2010). Transferring methods from the toxicology literature, an insect resistance management (IRM) strategy was developed to delay resistance evolution and spread (Bourguet *et al.*, 2005; Sivasupramaniam *et al.*, 2007). However, the evolution of resistance is difficult to predict, and several factors (including fitness costs associated with resistance and how stress factors influence these dynamics) complicate these interactions between the host and its pathogens.

Delaying the Evolution of Resistance and Slowing its Spread

As has been discussed in Sections 13.2 and 13.3, the hierarchy of host defenses that must be overcome by a pathogen is substantial and dynamic. Therefore, it is impossible to predict which host defense phenotypes will evolve in response to selection pressure from a pathogen. To overcome this hurdle, IRM strategies have partially avoided trying to predict what resistance will look like by focusing on the genetics that underlie the resistant phenotypes. After all, every phenotype is simply the product of one or several gene alleles, and approaching IRM from the perspective of nameless resistant and susceptible alleles is more manageable than trying to visualize what actual resistance will look like. The assumption frequently made (at least in the Bt literature) is that field resistance will be produced by a single autosomal recessive allele, producing a recessive trait (but see Gassmann *et al.*, 2009b). Based on this assumption, homozygotes are expected to be the only resistant members of the population. Thus, current IRM strategies for Bt crops are based on a high-dose/refuge strategy to target heterozygous individuals for resistance alleles in the population (Gould, 1988; Caprio, 1994). The high-dose approach involves making plants express high doses of Cry toxins so that any insects that are heterozygous for resistance are killed; this dose amounts to 25 times the LD_{99} of the pathogen against the targeted pest. This high-dose strategy ensures that 100% of the pest population is killed, but it also puts substantial pressure on the pest population to evolve resistance to the pathogen (Gould, 1998; Andow and Ives, 2002; Mendelsohn *et al.*, 2003; Bourguet *et al.*, 2005; Tabashnik *et al.*, 2009). It is notable that this high-dose strategy is sometimes not achieved owing to low susceptibility of some of the targeted insect pests and levels of toxin production being dependent on plant development and physiology (Abel and Adamczyk, 2004; Olsen *et al.*, 2005). In these cases, the use of plants producing combinations of Bt toxins with diverse mode of action (toxin pyramiding) can increase the product's effectiveness and delay the onset of resistance (Stewart *et al.*, 2001; Zhao *et al.*, 2003; Onstad and Meinke, 2010).

An essential component of the high-dose strategy for delaying resistance to pathogens is to incorporate an untreated refuge of non-Bt plants so that Bt-susceptible individuals can mate with the resistant individuals emerging from Bt plants and dilute the recessive resistance alleles in the population (Ives and Andow, 2002). This refuge component of IRM for Bt crops is difficult to implement and monitor. Depending on the crop and the country, these refuge mandates vary in size, type, and distance from the Bt field. With the introduction of pyramided Bt crops, refuge regulations have been relaxed or altered substantially. Given that host resistance to Bt has been documented at several stages of the infection process, the presumption that resistance is exclusively tied to simple alterations to key midgut receptors seems short sighted, and alterations to the refuge strategy have drawn concern from some stakeholders (Alyokhin, 2011; Onstad *et al.*, 2011).

Fitness Costs Associated with Resistance

Evolving a high level of resistance to a pathogen often comes at a cost to the insect host. Often, these costs are manifested in slower development, smaller size, lower fecundity, reduced overwintering success, and reduced survival (Fuxa and Richter, 1998; Carrière *et al.*, 2001; Gassmann *et al.*, 2009a). For example, field resistance to *B. sphaericus* was associated with slower development and reduced fecundity in the mosquito *Culex quinquefasciatus* (de Oliveira *et al.*, 2003). Thirty-four percent of published studies reveal reductions in fitness (survival, development rates, and body mass) in individuals resistant to *B. thuringiensis* relative to susceptible strains (Gassmann *et al.*, 2009a). In one study involving *B. thuringiensis* subsp. *kurstaki* sprays against *P. xylostella*, the 1,500-fold resistance diminished to only 300-fold resistance after exposure was ceased. This reduced level of resistance persisted without obvious fitness costs, and resistance quickly increased to 1,000-fold by the fourth generation of exposure (Tang *et al.*, 1997). This case illustrates that resistance level in a host is balanced against fitness costs of this resistance, and maintaining low levels of resistance to a pathogen may provide some flexibility for the host when the pathogen re-emerges. Because of fitness costs, a population that is resistant to a pathogen often reverts to susceptibility once the selection pressure is removed or relaxed. Gassmann *et al.* (2009a) showed that insects that were resistant to *B. thuringiensis* lost this resistance once selection pressure was reduced in 62% of published studies. In these studies, the average degree of resistance loss was 10-fold over seven generations of the pest. These examples aside, host resistance does not always lower the fitness of the host (Amorim *et al.*, 2010). Under these circumstances,

the resistance can become fixed in the population for many generations. For instance, larvae of the cabbage looper (*Trichoplusia ni*) that show one form of resistance to its NPV (TnSNPV) remain resistant to the virus for up to 22 generations after the selection pressure is removed in the laboratory (Milks and Myers, 2000; Milks *et al.*, 2002). A question that remains to be answered in depth is why or how these forms of resistance cause harm to the host. In any case, fitness costs associated with resistance must be considered as models are developed to predict how host resistance to pathogens spreads through a population.

Healthy insects are better at defending themselves from pathogens than are stressed insects, and there are myriad potential stressors that affect the dynamics of host resistance to pathogens. These external factors influence the performance of the pathogen or the host, and thereby disrupt the normal dynamics of the two organisms. Climatic conditions are one of the best documented forms of stressor. The optimal environmental conditions for pathogen performance have largely been discussed in the chapters on the different pathogen groups. Suffice it to say here that temperature, humidity, winter conditions, and sunlight are a few of the conditions known to influence the performances of both the pathogen and the host insect (Ignoffo, 1992; Fuxa *et al.*, 1999; Jaronski, 2010). In fact, insects seek out or produce higher temperatures in order to create suboptimal environments for a pathogen and fend off infections, a term called behavioral fever (Watson *et al.*, 1993; Evans and Spivak, 2010). Age of the insect also affects its ability to resist pathogens (Hochberg, 1991b; Armitage and Boomsma, 2010). For example, larval insects are often killed by NPVs, whereas these infections are sometimes sublethal in the adult stage (Fuxa, 2004). Older larvae can be more resistant to pathogens such as NPVs (Fuxa, 2004) or *B. thuringiensis* toxins (Beegle *et al.*, 1981; Kouassi *et al.*, 2001), although this is not always the case.

13.5. FUTURE RESEARCH DIRECTIONS

For a pathogen to mount a successful infection on an insect, it must circumvent or overcome three strategic components of the insect antipathogen defenses: avoid recognition, overcome barriers, and withstand the innate immune response. The antipathogen defenses of insects are inherently tied to the insect's ability to recognize the pathogen on several levels. First, pathogens may be entirely avoided if the insect can use its senses to proactively detect and avoid potential disease agents. Once the pathogen invades the insect, the ability to distinguish pathogens from "self" is critical to alerting the innate immune system of the host. Next, there are several physical barriers that deter entomopathogens from invading the host. The cuticle, peritrophic matrix, and basal lamina are particularly effective at filtering the microbiota that infiltrates the hemocoel. Cellular membranes are another barrier that limit intracellular pathogens from establishing infections. Entomopathogens must have adaptations that allow them to overcome these barriers if they are to survive in the host. Finally, the innate immune system possesses several weapons, including AMPs and the chemicals associated with the phenoloxidase cascade, hemocytes capable of phagocytosis and encapsulation, and xenophagy and RNAi-based defenses designed to kill intracellular pathogens. The pathogen must be able to hide from or disable the defenses, or offer counter-defenses in order to persist and reproduce in the host.

The in-depth examination of *B. thuringiensis* with regard to host-resistance evolution and management illustrates several key points and challenges. First, it is clear from this system that resistance can arise from alterations of the host at multiple points along the infection process, and predicting what form resistance will take is virtually impossible. Nevertheless, designing IRM plans that reduce the evolution of insect resistance and the spread of this resistance through the population is necessary in modern agricultural systems. Current IRM strategies are predicated on using a high dose of the pathogen alongside a non-Bt refuge that increases the likelihood that highly resistant individuals will mate with susceptible conspecifics. But these strategies are changing rapidly and the implications of these alterations and reductions on resistance spread are unknown. Clearly, numerous knowledge gaps remain in predicting resistance to entomopathogens even in the well-studied system of *B. thuringiensis*.

There is no shortage of questions that can be confronted in future research efforts. Immunochallenged insects upregulate hundreds of genes, and in most cases we know almost nothing regarding their function (Hoffmann, 2003; Baton *et al.*, 2008). Thus, although our understanding of host defenses as a branch of insect pathology is growing rapidly, our concept of immune responses may still be somewhat limited. One topic that has received a fair amount of research, but little synthesis, is that insects and pathogens live in communities, and we know very little about how community interactions affect host resistance and defensive capabilities against pathogens.

A future frontier is understanding the interaction between the biological communities and their effect on insect resistance. Insects seldom live in isolation, and their interactions within their community affect their ability to defend themselves against pathogens. On the smallest scale, microbial symbionts of insects form complex communities in the gut and on the cuticle of insects that resist the establishment of pathogenic microbes (discussed briefly in Section 13.2). In part, this is through the production of antimicrobial compounds that kill invading pathogens; but competitive interactions are also likely to play a role. Pathogens can be an important part of this

community, and only attain pathogen status when the normal milieu is disrupted. For example, *Enterococcous faecalis* is a common gut bacterium that is believed to be pathogenic under some circumstances, but may facilitate herbivory in insects under other conditions (Lundgren and Lehman, 2010). The key players in these microbial communities, how they restrict or promote pathogenicity, and what the implications are for host resistance remain compelling questions.

The host plant, as a form of either diet or habitat, has a great effect on the success of infection (Cory and Hoover, 2006). Indeed, some plants form symbioses with entomopathogens to resist herbivorous insects (Clay and Schardl, 2002; Arnold and Lewis, 2005; Lundgren, 2009). The morphology and chemistry of leaves affect the suitability of the phylloplane as a habitat for potential microorganisms (Duetting *et al.*, 2003; Jaronski, 2010). The nutritional suitability of the plant affects the rate at which herbivorous insects ingest phylloplane resident microbes (Verkerk and Wright, 1996). In addition, ingesting phytochemicals can enhance or diminish the infection, depending on the circumstances (Ekesi *et al.*, 2000; Cory and Hoover, 2006). For example, the aphid *Sitobion avenae* is more susceptible to the fungus *Erynia neoaphidis* when the infection occurs on aphid-resistant wheat plants than on susceptible plants (Fuentes-Contreras *et al.*, 1998).

Finally, insects are often the target of multiple infections simultaneously, and the intraguild interactions among the pathogens and parasites become very important in the dynamics of hosts and pathogens (Furlong and Pell, 2005). In general, studies on intraguild interactions are interested in identifying the victor. More often than not, the rule of precedence comes into play. When two infectious agents invade the same host, the winner is the one that got there first (Hochberg, 1991a; Milks *et al.*, 2001; Fuxa, 2004). In some cases, prior infection "primes" the immune system and makes the host insect more resistant to subsequent pathogens (Vilcinskas, 2010). Fytrou *et al.* (2006) found that *Drosophila simulans* encapsulated larvae of the hymenopteran parasitoid *Leptopilina heterotoma* more efficiently when the fly was previously infected with *Wolbachia*, but not with *B. bassiana*. This beneficial effect of *Wolbachia* on host resistance has been documented in other insects as well (Hughes *et al.*, 2011). Finally, pathogens sometimes specialize on certain tissues of the host, and at least one example suggests that pathogens that are able to live in more than one tissue may be more competitive in direct competition with tissue specialists. *Wolbachia pipientis* and *Spiroplasma* both live intracellularly in *D. melanogaster*, but *Spiroplasma* can also live extracellularly. When both are present in the same host, *Spiroplasma* dominates the host more frequently than *W. pipientis* (Goto *et al.*, 2006). There is a growing understanding of the physiology and genetics that control insect–pathogen interactions, and this understanding will provide the basis for asking more complex questions about how other organisms with which insects live affect the dynamics of insect pathology.

REFERENCES

Abel, C. A., & Adamczyk, J. J., Jr. (2004). Relative concentration of Cry1A in maize leaves and cotton bolls with diverse chlorophyll content and corresponding larval development of fall armyworm (Lepidoptera: Noctuidae) and southwestern corn borer (Lepidoptera: Crambidae) on maize whorl leaf profiles. *J. Econ. Entomol., 97*, 1737–1744.

Adamo, S. A. (1998). The specificity of behavioral fever in the cricket *Acheta domesticus*. *J. Parasitol., 84*, 529–533.

Alma, C. R., Gillespie, D. R., Roitberg, B. D., & Goettel, M. S. (2010). Threat of infection and threat-avoidance behavior in the predator *Dicyphus hesperus* feeding on whitefly nymphs infected with an entomopathogen. *J. Insect Behav., 23*, 90–99.

Alyokhin, A. (2011). Scant evidence supports EPA's pyramided *Bt* corn refuge size of 5%. *Nat. Biotechnol., 29*, 577–578.

Amano, A., Nakagawa, I., & Yoshimori, T. (2006). Autophagy in innate immunity against intracellular bacteria. *J. Biochem., 140*, 161–166.

Amorim, L. B., de Barros, R. A., de Melo Chalegre, K. D., de Oliveira, C. M. F., Regis, L. N., & Lobo Silva-Filha, M. H. N. (2010). Stability of *Culex quinquefasciatus* resistance to *Bacillus sphaericus* evaluated by molecular tools. *Insect Biochem. Mol. Biol., 40*, 311–316.

Andow, D. A., & Ives, A. R. (2002). Monitoring and adaptive resistance management. *Ecol. Appl., 12*, 1378–1390.

Armitage, S. A. O., & Boomsma, J. J. (2010). The effects of age and social interactions on innate immunity in a leaf-cutting ant. *J. Insect Physiol., 56*, 780–787.

Arnold, A., & Lewis, L. C. (2005). Ecology and evolution of fungal endophytes and their roles against insects. In F. E. Vega & M. Blackwell (Eds.), *Insect–Fungal Associations: Ecology and Evolution* (pp. 74–96). Oxford: Oxford University Press.

Baton, L. A., Garver, L., Xi, Z., & Dimopoulos, G. (2008). Functional genomics studies on the innate immunity of disease vectors. *Insect Sci., 15*, 15–27.

Baxter, S. W., Zhao, J. Z., Gahan, L. J., Shelton, A. M., Tabashnik, B. E., & Heckel, D. G. (2005). Novel genetic basis of field-evolved resistance to Bt toxins in *Plutella xylostella*. *Insect Mol. Biol., 14*, 327–334.

Beckage, N. E. (2007). *Insect Immunology.* San Diego: Academic Press.

Beegle, C. C., Lewis, L. C., Lynch, R. E., & Martinez, A. J. (1981). Interaction of larval age and antibiotic on the susceptibility of 3 insect species to *Bacillus thuringiensis*. *J. Invertebr. Pathol., 37*, 143–153.

Berdegué, M., Trumble, J. T., & Moar, W. J. (1996). Effect of CryIC toxin from *Bacillus thuringiensis* on larval feeding behavior of *Spodoptera exigua*. *Entomol. Exp. Appl., 80*, 389–401.

Boguś, M. I., Czygier, M., Gołębiowski, M., Kedra, E., Kucińska, J., Mazgajska, J., Samborski, J., Wieloch, W., & Włóka, E. (2010). Effects of insect cuticular fatty acids on *in vitro* growth and pathogenicity of the entomopathogenic fungus *Conidiobolus coronatus*. *Exp. Parasitol., 125*, 400–408.

Boots, M. (1998). Cannibalism and the stage-dependent transmission of a viral pathogen of the Indian meal moth, *Plodia interpunctella*. *Ecol. Entomol., 23*, 118–122.

Bourguet, D., Desquilbet, M., & Lemarié, S. (2005). Regulating insect resistance management: the case of non-Bt corn refuges in the US. *J. Environ. Manag., 76*, 210−220.

Bourtzis, K., Pettigrew, M. M., & O'Neill, S. L. (2000). *Wolbachia* neither induces nor suppresses transcripts encoding antimicrobial peptides. *Insect Mol. Biol., 9*, 635−639.

Brennan, C. A., & Anderson, K. V. (2004). *Drosophila*: the genetics of innate immune recognition and response. *Annu. Rev. Immunol., 22*, 457−483.

Brennan, L. J., Keddie, B. A., Braig, H. R., & Harris, H. L. (2008). The endosymbiont *Wolbachia pipientis* induces the expression of host antioxidant proteins in an *Aedes albopictus* cell line. *PLoS ONE, 3*. e2083.

Bulet, P., Hetru, C., Dimarcq, J.-L., & Hoffmann, D. (1999). Antimicrobial peptides in insects; structure and function. *Dev. Comp. Immunol., 23*, 329−344.

Caprio, M. A. (1994). *Bacillus thuringiensis* gene deployment and resistance management in single- and multi-tactic environments. *Biocontrol Sci. Technol., 4*, 487−497.

Carrière, Y., Ellers, K. C., Patin, A. L., Sims, M. A., Meyer, S., Liu, Y. B., Dennehy, T. J., & Tabashnik, B. E. (2001). Overwintering cost associated with resistance to transgenic cotton in the pink bollworm (Lepidoptera: Gelechiidae). *J. Econ. Entomol., 94*, 935−941.

Carrière, Y., Crowder, D. W., & Tabashnik, B. E. (2010). Evolutionary ecology of insect adaptation to Bt crops. *Evol. Appl., 3*, 561−573.

Carton, Y., Poirié, M., & Nappi, A. J. (2008). Insect immune resistance to parasitoids. *Insect Sci., 15*, 67−87.

Centofanti, M. (1995). Playing by the rules. How and why organisms turn nasty. *Sci. News, 148*, 382−383.

Chapman, R. F. (1998). *The Insects: Structure and Function* (4th ed.). Cambridge: Cambridge University Press.

Charles, J.-F., Nielsen-LeRoux, C., & Delécluse, A. (1996). *Bacillus sphaericus* toxins: molecular biology and mode of action. *Annu. Rev. Entomol., 41*, 451−472.

Chen, K., Weng, Z.-H., & Zheng, L. (2008). Innate immunity against malaria parasites in *Anopheles gambiae*. *Insect Sci., 15*, 45−52.

Chiang, A. S., Yen, D. F., & Peng, W. K. (1986). Defense reaction of midgut epithelial cells in the rice moth larva (*Corcyra cephalonica*) infected with *Bacillus thuringiensis*. *J. Invertebr. Pathol., 47*, 333−339.

Chouvenc, T., Su, N.-Y., & Robert, A. (2010). Inhibition of the fungal pathogen *Metarhizium anisopliae* in the alimentary tracts of five termite (Isoptera) species. *Fla. Entomol., 93*, 467−469.

Christensen, B. M., Li, J., Chen, C.-C., & Nappi, A. J. (2005). Melanization immune responses in mosquito vectors. *Trends Parasitol., 21*, 192−199.

Clay, K., & Schardl, C. (2002). Evolutionary origins and ecological consequences of endophyte symbioses with grasses. *Am. Nat., 160*, S99−S127.

Cory, J. S., & Hoover, K. (2006). Plant-mediated effects in insect−pathogen interactions. *Trends Ecol. Evol., 21*, 278−286.

Cremer, S., Armitage, S. A., & Schmid-Hempel, P. (2007). Social immunity. *Curr. Biol., 17*, R693−R702.

Darboux, I., Pauchet, Y., Castella, C., Silva-Filha, M. H., Nielsen-LeRoux, C., Charles, J.-F., & Pauron, D. (2002). Loss of the membrane anchor of the target receptor is a mechanism of bioinsecticide resistance. *Proc. Natl. Acad. Sci. USA, 99*, 5830−5835.

Darboux, I., Charles, J.-F., Pauchet, Y., Warot, S., & Pauron, D. (2007). Transposon-mediated resistance to *Bacillus sphaericus* in a field-evolved population of *Culex pipiens* (Diptera: Culicidae). *Cell. Microbiol., 9*, 2022−2029.

Dillon, R. J., & Dillon, V. M. (2004). The gut bacteria of insects: nonpathogenic interactions. *Annu. Rev. Entomol., 49*, 71−92.

Duetting, P. S., Ding, H., Neufeld, J., & Eigenbrode, S. D. (2003). Plant waxy bloom on peas affects infection of pea aphids by *Pandora neoaphidis*. *J. Invertebr. Pathol., 84*, 149−158.

Dulmage, H. T., & Martinez, E. (1973). The effects of continuous exposure to low concentrations of the δ endotoxin of *Bacillus thuringiensis* on the development of the tobacco budworm, *Heliothis virescens*. *J. Invertebr. Pathol., 22*, 14−22.

Dulmage, H. T., Graham, H. M., & Martinez, E. (1978). Interactions between the tobacco budworm, *Heliothis virescens*, and the δ-endotoxin produced by the HD-1 isolate of *Bacillus thuringiensis* var. *kurstaki*: relationship between length of exposure to the toxin and survival. *J. Invertebr. Pathol., 32*, 40−50.

Ekesi, S., Maniania, N. K., & Lwande, W. (2000). Susceptibility of the legume flower thrips to *Metarhizium anisopliae* on different varieties of cowpea. *BioControl, 45*, 79−95.

Eleftherianos, I., Millichap, P. J., ffrench-Constant, R. H., & Reynolds, S. E. (2006). RNAi suppression of recognition protein mediated immune responses in the tobacco hornworm *Manduca sexta* causes increased susceptibility to the insect pathogen *Photorhabdus*. *Dev. Comp. Immunol., 30*, 1099−1107.

Evans, J. D., & Spivak, M. (2010). Socialized medicine: individual and communal disease barriers in honey bees. *J. Invertebr. Pathol., 103*, S62−S72.

Evans, J. D., Aronstein, K., Chen, Y. P., Hetru, C., Imler, J.-L., Jiang, H., Kanost, M., Thompson, G. J., Zou, Z., & Hultmark, D. (2006). Immune pathways and defence mechanisms in honey bees *Apis mellifera*. *Insect Mol. Biol., 15*, 645−656.

Ewald, P. W. (1995). The evolution of virulence: a unifying link between parasitology and ecology. *J. Parasitol., 81*, 659−669.

Fernández-Marín, H., Zimmerman, J. K., Rehner, S. A., & Wcislo, W. T. (2006). Active use of the metapleural glands by ants in controlling fungal infection. *Proc. R. Soc. B., 273*, 1689−1695.

Ferré, J., & Van Rie, J. (2002). Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. *Annu. Rev. Entomol., 47*, 501−533.

Ferré, J., Real, M. D., Van Rie, J., Jansens, S., & Peferoen, M. (1991). Resistance to the *Bacillus thuringiensis* bioinsecticide in a field population of *Plutella xylostella* is due to a change in a midgut membrane receptor. *Proc. Natl. Acad. Sci. USA, 88*, 5119−5123.

Forcada, C., Alcácer, E., Garcerá, M. D., Tato, A., & Martínez, R. (1999). Resistance to *Bacillus thuringiensis* Cry1Ac toxin in three strains of *Heliothis virescens*: proteolytic and SEM study of the larval midgut. *Arch. Insect Biochem. Physiol., 42*, 51−63.

Fuentes-Contreras, E., Pell, J. K., & Niemeyer, H. M. (1998). Influence of plant resistance at the third trophic level: interactions between parasitoids and entomopathogenic fungi of cereal aphids. *Oecologia, 117*, 426−432.

Furlong, M. J., & Pell, J. K. (2005). Interactions between entomopathogenic fungi and arthropod natural enemies. In F. E. Vega & M. Blackwell (Eds.), *Insect−Fungal Associations: Ecology and Evolution* (pp. 51−73). Oxford: Oxford University Press.

Fuxa, J. R. (2004). Ecology of insect nucleopolyhedroviruses. *Agric. Ecosyst. Environ., 103*, 27−43.

Fuxa, J. R., & Richter, A. R. (1998). Repeated reversion of resistance to nucleopolyhedrovirus by *Anticarsia gemmatalis*. *J. Invertebr. Pathol., 71*, 159−164.

Fuxa, J. R., Sun, J.-Z., Weidner, E. H., & LaMotte, L. R. (1999). Stressors and rearing diseases of *Trichoplusia ni*: evidence of vertical transmission of NPV and CPV. *J. Invertebr. Pathol., 74*, 149−155.

Fytrou, A., Schofield, P. G., Kraaijeveld, A. R., & Hubbard, S. F. (2006). *Wolbachia* infection suppresses both host defence and parasitoid counter-defence. *Proc. R. Soc. B., 273*, 791−796.

Gahan, L. J., Gould, F., & Heckel, D. G. (2001). Identification of a gene associated with Bt resistance in *Heliothis virescens*. *Science, 293*, 857−860.

Gahan, L. J., Gould, F., López, J. D., Jr., Micinski, S., & Heckel, D. G. (2007). A polymerase chain reaction screen of field populations of *Heliothis virescens* for a retrotransposon insertion conferring resistance to *Bacillus thuringiensis* toxin. *J. Econ. Entomol., 100*, 187−194.

Gahan, L. J., Pauchet, Y., Vogel, H., & Heckel, D. G. (2010). An ABC transporter mutation is correlated with insect resistance to *Bacillus thuringiensis* Cry1Ac toxin. *PLoS Genet, 6*. e1001248.

Gassmann, A. J., Carrière, Y., & Tabashnik, B. E. (2009a). Fitness costs of insect resistance to *Bacillus thuringiensis*. *Annu. Rev. Entomol., 54*, 147−163.

Gassmann, A. J., Onstad, D. W., & Pittendrigh, B. R. (2009b). Evolutionary analysis of herbivorous insects in natural and agricultural environments. *Pest Manag. Sci., 65*, 1174−1181.

Goto, S., Anbutsu, H., & Fukatsu, T. (2006). Asymmetrical interactions between *Wolbachia* and *Spiroplasma* endosymbionts coexisting in the same insect host. *Appl. Environ. Microbiol., 72*, 4805−4810.

Gould, F. (1988). Evolutionary biology and genetically engineered crops: consideration of evolutionary theory can aid in crop design. *Bioscience, 38*, 26−33.

Gould, F. (1998). Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. *Annu. Rev. Entomol., 43*, 701−726.

Govind, S. (2008). Innate immunity in *Drosophila*: pathogens and pathways. *Insect Sci., 15*, 29−43.

Haider, M. Z., Knowles, B. H., & Ellar, D. J. (1986). Specificity of *Bacillus thuringiensis* var. *colmeri* insecticidal δ-endotoxin is determined by differential proteolytic processing of the protoxin by larval gut proteases. *Eur. J. Biochem., 156*, 531−540.

Hernández-Martínez, P., Ferré, J., & Escriche, B. (2009). Broad-spectrum cross-resistance in *Spodoptera exigua* from selection with a marginally toxic Cry protein. *Pest Manag. Sci., 65*, 645−650.

Herre, E. A. (1993). Population structure and the evolution of virulence in nematode parasites of fig wasps. *Science, 259*, 1442−1445.

Herrero, S., Oppert, B., & Ferré, J. (2001). Different mechanisms of resistance to *Bacillus thuringiensis* toxins in the indianmeal moth. *Appl. Environ. Microbiol., 67*, 1085−1089.

Hillyer, J. F., & Christensen, B. M. (2005). Mosquito phenoloxidase and defensin colocalize in melanization innate immune responses. *J. Histochem. Cytochem., 53*, 689−698.

Hochberg, M. E. (1991a). Intra-host interactions between a braconid endoparasitoid, *Apantales glomeratus*, and *a baculovirus for larvae of Pieris brassicae*. *J. Anim. Ecol., 60*, 51−63.

Hochberg, M. E. (1991b). Extra-host interactions between a braconid endoparasitoid, *Apanteles glomeratus*, and *a baculovirus for larvae of Pieris brassicae*. *J. Anim. Ecol., 60*, 65−77.

Hoffmann, J. A. (1995). Innate immunity of insects. *Curr. Opin. Immunol., 7*, 4−10.

Hoffmann, J. A. (2003). The immune response of *Drosophila*. *Nature, 426*, 33−38.

Hoover, K., Humphries, M. A., Gendron, A. R., & Slavicek, J. M. (2010). Impact of viral *enhancin* genes on potency of *Lymantria dispar* multiple nucleopolyhedrovirus in *L. dispar* following disruption of the peritrophic matrix. *J. Invertebr. Pathol., 104*, 150−152.

Hughes, G. L., Ren, R., Ramirez, J. L., Sakamoto, J. M., Bailey, J. A., Jedlicka, A. E., & Rasgon, J. L. (2011). *Wolbachia* infections in *Anopheles gambiae* cells: transcriptomic characterization of a novel host−symbionet interaction. *PLoS ONE, 7*. e1001296.

Hurst, G. D. D., Anbutsu, H., Kutsukake, M., & Fukatsu, T. (2003). Hidden from the host: *Spiroplasma* bacteria infecting *Drosophila* do not cause an immune response, but are suppressed by ectopic immune activation. *Insect Mol. Biol., 12*, 93−97.

Ignoffo, C. M. (1992). Environmental factors affecting persistence of entomopathogens. *Fla. Entomol., 75*, 516−525.

Ives, A. R., & Andow, D. A. (2002). Evolution of resistance to Bt crops: directional selection in structured environments. *Ecol. Lett., 5*, 792−801.

Janmaat, A. F., Wang, P., Kain, W., Zhao, J.-Z., & Myers, J. (2004). Inheritance of resistance to *Bacillus thuringiensis* subsp. *kurstaki* in *Trichoplusia ni*. *Appl. Environ. Microbiol., 70*, 5859−5867.

Jaronski, S. (2010). Ecological factors in the inundative use of fungal entomopathogens. *BioControl, 55*, 159−185.

Jiang, H. (2008). The biochemical basis of antimicrobial responses in *Manduca sexta*. *Insect Sci., 15*, 53−66.

Jurat-Fuentes, J. L., Gould, F. L., & Adang, M. J. (2003). Dual resistance to *Bacillus thuringiensis* Cry1Ac and Cry2Aa toxins in *Heliothis virescens* suggests multiple mechanisms of resistance. *Appl. Environ. Microbiol., 69*, 5898−5906.

Jurat-Fuentes, J. L., Gahan, L. J., Gould, F. L., Heckel, D. G., & Adang, M. J. (2004). The HevCaLP protein mediates binding specificity of the Cry1A class of *Bacillus thuringiensis* toxins in *Heliothis virescens*. *Biochemistry, 43*, 14299−14305.

Kanost, M. R., & Nardi, J. B. (2010). Innate immune responses of *Manduca sexta*. In M. R. Goldsmith & F. Marec (Eds.), *Molecular Biology and Genetics of the Lepidoptera* (pp. 271−291). Boca Raton: CRC Press.

Karumbaiah, L., Oppert, B., Jurat-Fuentes, J. L., & Adang, M. J. (2007). Analysis of midgut proteinases from *Bacillus thuringiensis*-susceptible and -resistant *Heliothis virescens* (Lepidoptera: Noctuidae). *Comp. Biochem. Physiol. B Biochem. Mol. Biol., 146*, 139−146.

Khajuria, C., Zhu, Y. C., Chen, M. S., Buschman, L. L., Higgins, R. A., Yao, J. X., Crespo, A. L. B., Siegfried, B. D., Muthukrishnan, S., & Zhu, K. Y. (2009). Expressed sequence tags from larval gut of the European corn borer (*Ostrinia nubilalis*): exploring candidate genes potentially involved in *Bacillus thuringiensis* toxicity and resistance. *BMC Genomics, 10*, 286.

Klinger, E., Groden, E., & Drummond, F. A. (2006). Beauveria bassiana horizontal infection between cadavers and adults of the Colorado potato beetle, *Leptinotarsa decemlineata (Say)*. *Environ. Entomol., 35*, 992−1000.

Kouassi, K. C., Lorenzetti, F., Guertin, C., Cabana, J., & Mauffette, Y. (2001). Variation in the susceptibility of the forest tent caterpillar (Lepidoptera: Lasiocampidae) to *Bacillus thuringiensis* variety

kurstaki HD-1: effect of the host plant. *J. Econ. Entomol., 94*, 1135−1141.

Lavine, M. D., & Strand, M. R. (2001). Surface characteristics of foreign targets that elicit an encapsulation response by the moth *Pseudoplusia includens*. *J. Insect Physiol., 47*, 965−974.

Lavine, M. D., & Strand, M. R. (2002). Insect hemocytes and their role in immunity. *Insect Biochem. Mol. Biol., 32*, 1295−1309.

Le Conte, Y., Alaux, C., Martin, J.-F., Harbo, J. R., Harris, J. W., Dantec, C., Séverac, D., Cros-Arteil, S., & Navajas, M. (2011). Social immunity in honeybees (*Apis mellifera*): transcriptome analysis of varroa-hygienic behaviour. *Insect Mol. Biol., 20*, 399−408.

Lehane, M. J. (1997). Peritrophic matrix structure and function. *Annu. Rev. Entomol., 42*, 525−550.

deLello, E., Hanton, W. K., Bishoff, S. T., & Mish, D. W. (1984). Histopathological effects of *Bacillus thuringiensis* on the midgut of tobacco hornworm larvae (*Manduca sexta*): low doses compared with fasting. *J. Invertebr. Pathol., 43*, 169−181.

Levine, B. (2005). Eating oneself and uninvited guests: autophagy-related pathways in cellular defense. *Cell, 120*, 159−162.

Levy, S. M., Falleiros, A. M. F., Moscardi, F., & Gregório, E. A. (2007). Susceptibility/resistance of *Anticarsia gemmatalis* larvae to its nucleopolyhedrovirus (AgMNPV): structural study of the peritrophic membrane. *J. Invertebr. Pathol., 96*, 183−186.

Loeb, M. J., Martin, P. A., Hakim, R. S., Goto, S., & Takeda, M. (2001). Regeneration of cultured midgut cells after exposure to sublethal doses of toxin from two strains of *Bacillus thuringiensis*. *J. Insect Physiol., 47*, 599−606.

Lord, J. C. (2001). Response of the wasp *Cephalonomia tarsalis* (Hymenoptera: Bethylidae) to *Beauveria bassiana* (Hyphomycetes: Moniliales) as free conidia or infection in its host, the sawtoothed grain beetle, *Oryzaephilus surinamensis* (Coleoptera: Silvanidae). *Biol. Control, 21*, 300−304.

Loseva, O., Ibrahim, M., Candas, M., Koller, C. N., Bauer, L. S., & Bulla, L. A., Jr. (2002). Changes in protease activity and Cry3Aa toxin binding in the Colorado potato beetle: implications for insect resistance to *Bacillus thuringiensis* toxins. *Insect Biochem. Mol. Biol., 32*, 567−577.

Lundgren, J. G. (2009). *Relationships of Natural Enemies and Non-prey Foods*. Dordrecht: Springer International.

Lundgren, J. G., & Lehman, R. M. (2010). Bacterial gut symbionts contribute to seed digestion in an omnivorous beetle. *PLoS ONE, 5*. e10831.

Martínez-Ramírez, A. C., Gould, F., & Ferré, J. (1999). Histopathological effects and growth reduction in a susceptible and a resistant strain of *Heliothis virescens* (Lepidoptera: Noctuidae) caused by sublethal doses of pure Cry1A crystal proteins from *Bacillus thuringiensis*. *Biocontrol Sci. Technol., 9*, 239−246.

Mburu, D. M., Ochola, L., Maniania, N. K., Njagi, P. G. N., Gitonga, L. M., Ndung'u, M. W., Wanjoya, A. K., & Hassanali, A. (2009). Relationship between virulence and repellency of entomopathogenic isolates of *Metarhizium anisopliae* and *Beauveria bassiana* to the termite *Macrotermes michaelseni*. *J. Insect Physiol., 55*, 774−780.

McGaughey, W. H. (1985). Insect resistance to the biological insecticide *Bacillus thuringiensis*. *Science, 229*, 193−195.

McGraw, E. A., & O'Neill, S. L. (2004). *Wolbachia pipientis*: intracellular infection and pathogenesis in *Drosophila*. *Curr. Opin. Microbiol., 7*, 67−70.

Meihls, L. N., Higdon, M. L., Siegfried, B. D., Miller, N. J., Sappington, T. W., Ellersieck, M. R., Spencer, T. A., & Hibbard, B. E. (2008). Increased survival of western corn rootworm on transgenic corn within three generations of onplant greenhouse selection. *Proc. Natl. Acad. Sci. USA, 105*, 19177−19182.

Mendelsohn, M., Kough, J., Vaituzis, Z., & Matthews, K. (2003). Are Bt crops safe? *Nat. Biotechnol., 21*, 1003−1009.

Meyling, N. V., & Pell, J. K. (2006). Detection and avoidance of an entomopathogenic fungus by a generalist insect predator. *Ecol. Entomol., 31*, 162−171.

Milks, M. L., & Myers, J. H. (2000). The development of larval resistance to a nucleopolyhedrovirus is not accompanied by an increased virulence in the virus. *Evol. Ecol., 14*, 645−664.

Milks, M. L., Lepitch, M. K., & Theilmann, D. A. (2001). Recombinant and wild-type nucleopolyhedroviruses are equally fit in mixed infections. *Environ. Entomol., 30*, 972−981.

Milks, M. L., Myers, J. H., & Leptich, M. K. (2002). Costs and stability of cabbage looper resistance to a nucleopolyhedrovirus. *Evol. Ecol., 16*, 369−385.

Mitsuhashi, W., Kawakita, H., Murakami, R., Takemoto, Y., Saiki, T., Miyamoto, K., & Wada, S. (2007). Spindles of an entomopoxvirus facilitate its infection of the host insect by disrupting the peritrophic membrane. *J. Virol., 81*, 4235−4243.

Morin, S., Biggs, R. W., Sisterson, M. S., Shriver, L., Ellers-Kirk, C., Higginson, D., Holley, D., Gahan, L. J., Heckel, D. G., Carrière, Y., Dennehy, T. J., Brown, J. K., & Tabashnik, B. E. (2003). Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. *Proc. Natl. Acad. Sci. USA, 100*, 5004−5009.

Moussain, B. (2010). Recent advances in understanding mechanisms of insect cuticle differentiation. *Insect Biochem. Mol. Biol., 40*, 363−375.

NASS. (2011). *National Agriculture Statistics Service*. USDA. http://www.nass.usda.gov/

Nielsen-Leroux, C., Pasquier, F., Charles, J. F., Sinegre, G., Gaven, B., & Pasteur, N. (1997). Resistance to *Bacillus sphaericus* involves different mechanisms in *Culex pipiens* (Diptera: Culicidae) larvae. *J. Med. Entomol., 34*, 321−327.

Nielsen-Leroux, C., Pasteur, N., Pretre, J., Charles, J. F., Sheikh, H. B., & Chevillon, C. (2002). High resistance to *Bacillus sphaericus* binary toxin in *Culex pipiens* (Diptera: Culicidae): the complex situation of west Mediterranean countries. *J. Med. Entomol., 39*, 729−735.

de Oliveira, C. M. F., Costa Filho, F., Beltràn, J. F. N., Silva-Filha, M. H., & Regis, L. (2003). Biological fitness of a *Culex quinquefasciatus* population and its resistance to *Bacillus sphaericus*. *J. Am. Mosq. Control Assoc., 19*, 125−129.

Olsen, K. M., Daly, J. C., Holt, H. E., & Finnegan, E. J. (2005). Season-long variation in expression of Cry1Ac gene and efficacy of *Bacillus thuringiensis* toxin in transgenic cotton against *Helicoverpa armigera* (Lepidoptera: Noctuidae). *J. Econ. Entomol., 98*, 1007−1017.

Onstad, D. W., & Meinke, L. J. (2010). Modeling evolution of *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) to transgenic corn with two insecticidal traits. *J. Econ. Entomol., 103*, 849−860.

Onstad, D. W., Mitchell, P. D., Hurley, T. M., Lundgren, J. G., Porter, R. P., Krupke, C. H., Spencer, J. L., DiFonzo, C. D., Baute, T. S., Hellmich, R. L., Buschmann, L. L., Hutchison, W. D., & Tooker, J. F. (2011). Seeds of change: corn seed mixtures for resistance management and IPM. *J. Econ. Entomol., 104*, 343−352.

Oppert, B., Kramer, K. J., Beeman, R. W., Johnson, D., & McGaughey, W. H. (1997). Proteinase-mediated insect resistance to *Bacillus thuringiensis* toxins. *J. Biol. Chem., 272*, 23473–23476.

Ormond, E. L., Thomas, A. P. M., Pell, J. K., Freeman, S. N., & Roy, H. E. (2011). Avoidance of a generalist entomopathogenic fungus by the ladybird, *Coccinella septempunctata*. *FEMS Microbiol. Ecol., 77*, 229–237.

Oxley, P. R., Spivak, M., & Oldroyd, B. P. (2010). Six quantitative trait loci influence task thresholds for hygienic behaviour in honeybees (*Apis mellifera*). *Mol. Ecol., 19*, 1452–1461.

Park, H. W., Bideshi, D. K., & Federici, B. A. (2010). Properties and applied use of the mosquitocidal bacterium, *Bacillus sphaericus*. *J. Asia Pac. Entomol., 13*, 159–168.

Parker, B. J., Elderd, B. D., & Dwyer, G. (2010). Host behaviour and exposure risk in an insect–pathogen interaction. *J. Anim. Ecol., 79*, 863–870.

Passarelli, A. L. (2011). Barriers to success: how baculoviruses establish efficient systemic infections. *Virology, 411*, 383–392.

Piubelli, G. C., Hoffmann-Campo, C. B., Moscardi, F., Miyakubo, S. H., & de Oliveira, M. C. N. (2006). Baculovirus-resistant *Anticarsia gemmatalis* responds differently to dietary rutin. *Entomol. Exp. Appl., 119*, 53–60.

Plymale, R., Grove, M. J., Cox-Foster, D., Ostiguy, N., & Hoover, K. (2008). Plant-mediated alteration of the peritrophic matrix and baculovirus infection in lepidopteran larvae. *J. Insect Physiol., 54*, 737–749.

Poulsen, M., Bot, A. N. M., & Boomsma, J. J. (2003). The effect of metapleural gland secretion on the growth of a mutualistic bacterium on the cuticle of leaf-cutting ants. *Naturwissenschaften, 90*, 406–409.

Rajagopal, R., Arora, N., Sivakumar, S., Rao, N. G., Nimbalkar, S. A., & Bhatnagar, R. K. (2009). Resistance of *Helicoverpa armigera* to Cry1Ac toxin from *Bacillus thuringiensis* is due to improper processing of the protoxin. *Biochem. J., 419*, 309–316.

van Rensburg, J. B. J. (2007). First report of field resistance by stem borer, *Busseola fusca* (Fuller) to Bt-transgenic maize. *S. Afr. J. Plant Soil, 24*, 147–151.

van Rie, J., McGaughey, W. H., Johnson, D. E., Barnett, B. D., & Van Mellaert, H. (1990). Mechanism of insect resistance to the microbial insecticide *Bacillus thuringiensis*. *Science, 247*, 72–74.

Rodcharoen, J., & Mulla, M. S. (1995). Comparative ingestion rates of *Culex quinquefasciatus* (Diptera, Culicidae) susceptible and resistant to *Bacillus sphaericus*. *J. Invertebr. Pathol., 66*, 242–248.

Rolff, J. & Reynolds, S. E. (Eds.) (2009). *Insect Infection and Immunity: Evolution, Ecology, and Mechanisms*. Oxford: Oxford University Press.

Royet, J. (2004a). Infectious non-self recognition in invertebrates: lessons from *Drosophila* and other insect models. *Mol. Immunol., 41*, 1063–1075.

Royet, J. (2004b). *Drosophila melanogaster* innate immunity: an emerging role for peptidoglycan recognition proteins in bacteria detection. *Cell. Mol. Life Sci., 61*, 537–546.

Ruepell, O., Hayworth, M. K., & Ross, N. P. (2010). Altruistic self-removal of health-compromised honey bee workers from their hive. *J. Evol. Biol., 23*, 1538–1546.

Sabin, L. R., Hanna, S. L., & Cherry, S. (2010). Innate antiviral immunity in *Drosophila*. *Curr. Opin. Immunol., 22*, 4–9.

Sayyed, A. H., Moores, G., Crickmore, N., & Wright, D. J. (2008). Cross-resistance between a *Bacillus thuringiensis* Cry toxin and non-Bt insecticides in the diamondback moth. *Pest Manag. Sci., 64*, 813–819.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D. R., & Dean, D. H. (1998). *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev., 62*, 775–806.

Shelby, K. S., & Popham, H. J. R. (2008). Cloning and characterization of the secreted hemocytic prophenoloxidases of *Heliothis virescens*. *Arch. Insect Biochem. Physiol., 69*, 127–142.

Shelton, A. M., Wang, P., Zhao, J.-Z., & Roush, R. T. (2007). Resistance to insect pathogens and strategies to manage resistance: an update. In L. A. Lacey & H. K. Kaya (Eds.), *Field Manual of Techniques in Invertebrate Pathology* (pp. 793–811). Dordrecht: Springer.

Siozos, S., Sapountzis, P., Loannidis, P., & Bourtzis, K. (2008). *Wolbachia* symbiosis and insect immune response. *Insect Sci., 15*, 89–100.

Sivasupramaniam, S., Head, G. P., English, L., Li, Y. J., & Vaughn, T. T. (2007). A global approach to resistance monitoring. *J. Invertebr. Pathol., 95*, 224–226.

Stewart, S. D., Adamczyk, J. J., Jr., Knighten, K. S., & Davis, F. M. (2001). Impact of Bt cottons expressing one or two insecticidal proteins of *Bacillus thuringiensis* Berliner on growth and survival of noctuid (Lepidoptera) larvae. *J. Econ. Entomol., 94*, 752–760.

Storer, N. P., Babcock, J. M., Schlenz, M., Meade, T., Thompson, G. D., Bing, J. W., & Huckaba, R. M. (2010). Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico. *J. Econ. Entomol., 103*, 1031–1038.

Stow, A., & Beattie, A. J. (2008). Chemical and genetic defenses against disease in insect societies. *Brain Behav. Immun., 22*, 1009–1013.

Strand, M. R. (2008). The insect cellular immune response. *Insect Sci., 15*, 1–14.

Swanson, J. A. I., Torto, B., Kells, S. A., Mesce, K. A., Tumlinson, J. H., & Spivak, M. (2009). Odorants that induce hygienic behavior in honeybees: identification of volatile compounds in chalkbrood-infected honeybee larvae. *J. Chem. Ecol., 35*, 1108–1116.

Tabashnik, B. E., Cushing, N. L., Finson, N., & Johnson, M. W. (1990). Field development of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). *J. Econ. Entomol., 83*, 1671–1676.

Tabashnik, B. E., Liu, Y.-B., Malvar, T., Heckel, D. G., Masson, L., & Ferré, J. (1998). Insect resistance to *Bacillus thuringiensis*: uniform or diverse? *Philos. Trans. R. Soc. Lond. B., 353*, 1751–1756.

Tabashnik, B. E., Carrière, Y., Dennehy, T. J., Morin, S., Sisterson, M. S., Roush, R. T., Shelton, A. M., & Zhao, J.-Z. (2003). Insect resistance to transgenic Bt crops: lessons from the laboratory and field. *J. Econ. Entomol., 96*, 1031–1038.

Tabashnik, B. E., Fabrick, J. A., Henderson, S., Biggs, R. W., Yafuso, C. M., Nyboer, M. E., Manhardt, N. M., Coughlin, L. A., Sollome, J., Carrière, Y., Dennehy, T. J., & Morin, S. (2006). DNA screening reveals pink bollworm resistance to Bt cotton remains rare after a decade of exposure. *J. Econ. Entomol., 99*, 1525–1530.

Tabashnik, B. E., Van Rensburg, J. B. J., & Carrière, Y. (2009). Field-evolved insect resistance to Bt crops: definition, theory, and data. *J. Econ. Entomol., 102*, 2011–2025.

Takehana, A., Yano, T., Mita, S., Kotani, A., Oshima, Y., & Kurata, S. (2004). Peptidoglycan recognition protein (PGRP)-LE and PGRP-LC act synergistically in *Drosophila* immunity. *EMBO J., 23*, 4690–4700.

Tang, J. D., Gilboa, S., Roush, R. T., & Shelton, A. M. (1997). Inheritance, stability, and lack-of-fitness costs of field-selected resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae) from Florida. *J. Econ. Entomol., 90*, 732−741.

Thompson, S. R., Brandenburg, R. L., & Roberson, G. T. (2007). Entomopathogenic fungi detection and avoidance by mole crickets (Orthoptera: Gryllotalpidae). *Environ. Entomol., 36*, 165−172.

Toledo, A. V., Alippi, A. M., & de Remes Lenicov, A. M. M. (2011). Growth inhibition of *Beauveria bassiana* by bacteria isolated from the cuticular surface of the corn leafhopper, *Dalbulus maidis* and the planthopper, *Delphacodes kuscheli*, two important vectors of maize pathogens. *J. Insect Sci., 11*, 29.

Ugelvig, L. V., & Cremer, S. (2007). Social prophylaxis: group interaction promotes collective immunity in ant colonies. *Curr. Biol., 17*, 1967−1971.

Verkerk, R. H. J., & Wright, D. J. (1996). Effects of interactions between host plants and selective insecticides on larvae of *Plutella xylostella* L. (Lepidoptera: Yponomeutidae) in the laboratory. *Pestic. Sci., 46*, 171−181.

Vilcinskas, A. (2010). Lepidopterans as model mini-hosts for human pathogens and as a resource for peptide antibiotics. In M. R. Goldsmith & F. Marec (Eds.), *Molecular Biology and Genetics of the Lepidoptera* (pp. 293−306). Boca Raton: CRC Press.

Watson, D. W., Mullens, B. A., & Petersen, J. J. (1993). Behavioral fever response of *Musca domestica* (Diptera: Muscidae) to infection by *Entomophthora muscae* (Zygomycetes: Entomophthrorales). *J. Invertebr. Pathol., 61*, 10−16.

Wright, D. J., Iqbal, M., Granero, F., & Ferré, J. (1997). A change in a single midgut receptor in the diamondback moth (*Plutella xylostella*) is only in part responsible for field resistance to *Bacillus thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *aizawai*. *Appl. Environ. Microbiol., 63*, 1814−1819.

Xu, X., Yu, L., & Wu, Y. (2005). Disruption of a cadherin gene associated with resistance to Cry1Ac δ-endotoxin of *Bacillus thuringiensis* in *Helicoverpa armigera*. *Appl. Environ. Microbiol., 71*, 948−954.

Yang, Y., Chen, H., Wu, Y., Yang, Y., & Wu, S. (2007). Mutated cadherin alleles from a field population of *Helicoverpa armigera* confer resistance to *Bacillus thuringiensis* toxin Cry1Ac. *Appl. Environ. Microbiol., 73*, 6939−6944.

Zhao, J. Z., Cao, J., Li, Y., Collins, H. L., Roush, R. T., Earle, E. D., & Shelton, A. M. (2003). Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution. *Nat. Biotechnol., 21*, 1493−1497.