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Studies on Resistance to Vegetative (Vip3A) and Crystal (Cry1A) Insecticidal Toxins of *Bacillus thuringiensis* in *Heliothis virescens* (Fabricius)

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This thesis is dedicated to Gemma and all the members of my family, especially my Mom, for all their love and support throughout this epic journey.

And in loving memory of my Dad.

Declaration:

The work presented in this thesis is my work and mine alone.

Signed…….. Brian Pickett

Certified…….. Denis Wright

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Abstract

Bacillus thuringiensis (*Bt*) toxins expressed in commercial transgenic crop varieties are all δ-endotoxins (Cry toxins) but the identification of novel vegetative insecticidal proteins (Vip toxins) has extended the range of insecticidal proteins derived from *Bt*. One such Vip toxin, Vip3A, primarily targets the midgut epithelium cells of susceptible insects as Cry toxins do, although they appear to have different binding sites. The present study investigated the comparative toxicity of Vip3A, Cry1Ab and Cry1Ac against *Heliothis virescens* (tobacco budworm) and the impact of antibiotics on *Bt* insecticidal activity. The selection of a resistant Vip3A population led to the determination of cross-resistance, the genetics of resistance and fitness effects. There was very little variability in the natural susceptibility to Vip3A, Cry1Ab and Cry1Ac in the populations tested, although the toxicity of Vip3A was much lower compared to the Cry1A toxins. A Vip3A resistant population was successfully established within 13 selected generations, with little or no cross-resistance to Cry1Ab or Cry1Ac. The inheritance of resistance ranged from almost completely recessive to incompletely dominant with a possible paternal influence, was polygenic and relatively stable. Vip3A resistance showed a fitness benefit, reduced larval development time, and fitness costs, including survival to adult eclosion, reduced egg viability and reduced male mating success. The effects of antibiotics on *H. virescens* larval susceptibility to *Bt* toxins varied depending on antibiotic treatment, the *Bt* toxin used and the larval instar tested. *Bt* cotton expressing both Vip3A and Cry1Ab to provide activity against a wide range of pest Lepidoptera, including *H. virescens*, a major cotton pest in the USA is in the process of commercialisation. The present work will help to support a suitable insecticide resistance management strategy for continued use of *Bt* toxin in transgenic crops.

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Chapter 1

Introduction

1.1 Background to the project

Transgenic crops expressing *Bacillus thuringiensis* Berliner insecticidal crystal (Cry) toxins (*Bt* crops) were commercialised in 1996 to aid efficient control of pests and reduce reliance on chemical insecticides (James, 1997; Tabashnik, 2008). In 2008 *Bt* crops were grown on approximately 46 million hectares worldwide, a 46-fold increase since their introduction and generating one of the largest potential selections for insect resistance ever known (James, 2008; Tabashnik *et al.*, 2008a).

Field resistance has largely been absent in *Bt* crops to date, with proactive insect resistant management strategies, involving a high dose plus refuge strategy proving effective in delaying the evolution of resistance (Bates *et al.*, 2005; Tabashnik, 2008). *Bt* Cry toxins are individually active against a relatively narrow range of insect species. In addition to the introduction of double constructs (pyramided) expressing two Cry toxins, the discovery of the novel vegetative insecticidal proteins (Vips) has provided the opportunity to extend the efficacy of *Bt* crops (Estruch *et al.*, 1996). *Bt* cotton expressing Vip3A and Cry1Ab is currently in development for commercial production (Kurtz *et al.*, 2007).

1.2 Aims and objectives

The overall aim of this project is to provide an understanding of the biology and genetics of resistance to Vip toxins in insects. Knowledge of the potential risk and nature of Vip3A resistance will help to support a suitable insecticide resistance management strategy for continued use of *Bt* toxin in transgenic crops.

The present study involved field-derived populations of the tobacco budworm, *Heliothis virescens* Fabricius (Lepidoptera: Noctuidae), a major target pest of cotton in the USA.

The specific objectives of the work were to:

1) determine the base-line susceptibility of field and laboratory populations of *H. virescens* to Vip3A, Cry1Ab and Cry1Ac;

2) select a field-derived population of *H. virescens* for resistance to Vip3A;

3) test the hypothesis that there is no significant cross-resistance between Vip3A and Cry1A toxins;

4) determine the mode of inheritance and degree of dominance of the Vip3A resistant *H. virescens* population;

5) test the hypothesis that insects resistant to Vip3A will have associated fitness costs that influence the stability of resistance in the absence of selection pressure;

6) test the hypothesis that co-application of antibiotics with *Bt* toxins reduces insecticidal activity.

Chapter 2

Literature Review

2.1 *Bacillus thuringiensis* (Berliner)

Bacillus thuringiensis (*Bt*) is a ubiquitous, gram positive spore forming bacterium that is found in many habitats including soil, plant surfaces and insects (Hofte and Whiteley, 1989; Bernhard *et al.*, 1997; Schnepf *et al.*, 1998; Bravo *et al.*, 2007). *Bacillus thuringiensis* has an insecticidal toxicity that is largely attributed to its ability to form parasporal crystals that release crystal proteins (δ-endotoxins) that are toxic to insects upon ingestion, during the stationary phase (sporulation phase) of its growth (Hofte and Whiteley, 1989; Schnepf *et al.*, 1998; Bravo *et al.*, 2007; Crickmore *et al.*, 2009). Thousands of different strains have been identified worldwide, all having a limited host range, but together are active against a wide range of insect orders, predominantly Lepidoptera, Coleoptera and Diptera, along with Hymenoptera, Homoptera, Orthoptera and Mallophaga, and against other organisms such as nematodes, mites and protozoa (Feitelson *et al.*, 1992; Schnepf *et al.*, 1998; Marroquin *et al.*, 2000; de Maagd *et al.*, 2003).

Bacillus thuringiensis belongs to the *Bacillus cereus* (Frankland and Frankland) group, with classical biochemical and morphological methods and modern molecular methods failing to distinguish *Bt* from *B. cereus*. The production of parasporal crystals is the defining quality of *Bt* that distinguishes it from the remaining *B. cereus* group species, and although this is considered too narrow a criterion for taxonomic purposes, *Bt* has its own nomenclature (Crickmore *et al.*, 1998; Schnepf *et al.*, 1998; Jensen *et al.*, 2003; Vilas-Bôas *et al.*, 2007; Crickmore *et al.*, 2009).

2.1.1 Introduction

Bacillus thuringiensis was first discovered by Ishiwata (1901) who isolated the bacterium from diseased larvae of silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae) and named it ‘sotto disease’ (sudden-collapse bacillus). Berliner (1911)

formerly described *Bt*, after isolating the bacterium from a diseased granary population of the Mediterranean flour moth, *Anagasta kuehniella* Zeller (Lepidoptera: Pyralidae), naming it after the province Thuringia in Germany where it was found (Beegle and Yamamoto, 1992; van Frankenhuyzen, 1993; Glare and O'Callaghan, 2000).

The first attempts to use *Bt* for insect field control were carried out in the late 1920s to early 1930s against the European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae) in South Eastern Europe. Field testing continued against lepidopteran larvae and in 1938 the first commercial product called Sporeine® became available in France, primarily for the control of Indian mealmoth, *Plodia interpunctella* Hüb. (Lepidoptera: Pyralidae) (Beegle and Yamamoto, 1992; van Frankenhuyzen, 1993; Glare and O'Callaghan, 2000).

A resurgence in interest during the 1950s lead to the *Bt* var. *thuringiensis* commercial production of Thuricide® in 1957 for the control of lepidopteran larvae (now based on *Bt* var. *kurstaki*, *Btk*) and, in 1962, the discovery of *Bt* var. *aizawa* (*Bta*), also active against lepidopteran larvae (Bonnefoi and de Barjac, 1963; Beegle and Yamamoto, 1992; van Frankenhuyzen, 1993; Glare and O'Callaghan, 2000). An important finding by Dulmage (1970) was the discovery of *Btk* isolate HD-1. It was 20 – 200 times more active than the *Bt* isolates used in commercial production at the time, and consequently *Btk* HD-1 was adopted for commercial use and formed the basis for the majority of *Bt* commercial formulations. The HD-1 strain was also accepted as the standard to be used in the establishment of an international system for standardising and comparing the potency of *Bt* isolates and commercial products (International Units (IU) per unit product) (Beegle and Yamamoto, 1992; van Frankenhuyzen, 1993; Glare and O'Callaghan, 2000). *Bacillus thuringiensis* var. *israelensis* (*Bti*) was discovered (Goldberg and Margalit, 1977) to have activity against dipteran larvae and has proved to be an effective and potent biological pesticide for the control of mosquitoes and blackflies (Schnepf *et al.*, 1998; Glare and O'Callaghan, 2000) This was followed by the discovery of *Bt* var. *tenebrionis* (*Btt*) (Krieg *et al.*, 1983) with activity against a range of coleopterans, including the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) (Beegle and Yamamoto, 1992; van Frankenhuyzen, 1993; Glare and O'Callaghan, 2000).

Bacillus thuringiensis crystal proteins have a narrow spectrum of insecticidal activity and have demonstrated a benign environmental profile, causing little or no harm to non-target beneficial insects, animals or humans (Sjoblad *et al.*, 1992; Glare and O'Callaghan, 2000; Ferré *et al.*, 2008). Since the 1950s *Bt* formulated products have become the most successful biopesticide used in agricultural, forestry and public health, even though only a few *Bt* strains have found success commercially through a variety of formulation mixes, namely *Bt* var. *kurstaki* (*Btk*), *Bt* var. *thuringiensis* and *Bt* var. *aizawai* (*Bta*) for lepidopteran control, *Bt* var. *israelensis* (*Bti*) for dipteran control and *Bt* var. *tenebrionis* (*Btt*) for coleopteran control (Schnepf *et al.*, 1998; Glare and O'Callaghan, 2000; Ferré *et al.*, 2008).

The success of *Bt* formulated products, however, has been limited by their high specificity and reduced persistence, in comparison to synthetic chemical insecticides that have wider insect spectrums and longer persistence times. Biopesticides also incur higher production costs and carry the image of being harder to use in comparison to synthetic insecticides. These factors are reflected in the economic sales, with the market for biopesticides being less than 5 % of the total global insecticide market of roughly $8 billion (Ferré *et al.*, 2008). However, in the last two decades the development and utilisation of expressing *Bt cry* genes in the crops themselves through genetic modification has dramatically increased the use of *Bt* in many parts of the world (section 2.1.4). Importantly this has been achieved without jeopardising its excellent safety profile, while removing the issues of short environmental persistence (Bates *et al.*, 2005; Ferré *et al.*, 2008).

Bacillus thuringiensis produces various virulence factors including α-exotoxins, β-exotoxins, hemolysins, enterotoxins, chitinases and phospholipases. Some of these factors are known to be insecticidal, and may all have a role in insect pathogenesis (Hansen and Salamitou, 2000; de Maagd *et al.*, 2001). However, the most heavily researched and commercially utilised virulence factors of *Bt* are the insecticidal δ-endotoxins (Crystal proteins) (section 2.1.2) through use in *Bt* formulated products, as already briefly mentioned, and more recently in *Bt* transgenic crops (section 2.1.4); and since their discovery in the mid 1990s, the vegetative insecticidal proteins (Vips) (section 2.1.3).

2.1.2 Crystal proteins (δ-endotoxins)

In the natural environment, *Bt* produces parasporal crystals that contain crystal proteins (Cry protein/toxin) during sporulation. Cry proteins are defined as 'a parasporal inclusion protein from *Bt* that exhibits toxic effects to a target organism, or any protein that has obvious sequence similarity to a known Cry protein' (Crickmore *et al.*, 1998; Bravo *et al.*, 2007). A single *Bt* strain will normally synthesise between one and five Cry toxins in either a single parasporal crystal or multiple parasporal crystals (de Maagd *et al.*, 2001). Thousands of *Bt* strains have been discovered since the 1950s and in total over 190 different Cry protein holotypes have been identified (Crickmore *et al.*, 2009), with insecticidal activity to specific species within the orders Lepidoptera, Diptera, Coleoptera and Hymenoptera and also nematodes (de Maagd *et al.*, 2001; de Maagd *et al.*, 2003; Bravo *et al.*, 2007; van Frankenhuyzen, 2009).

2.1.2.1 Cry protein classification

The current system (Crickmore *et al.*, 1998; Crickmore *et al.*, 2009) for crystal protein classification is based on the amino acid sequence homology where each protoxin is assigned a name consisting of the mnemonic Cry (or Cyt) and four hierarchical ranks consisting of numbers, capital letters, lower case letters and numbers (e.g. Cry1Ab5) depending on its place in a phylogenetic tree (de Maagd *et al.*, 2001; Crickmore *et al.*, 2009). Proteins with the same primary rank often affect the same order of insect; those with the same secondary and tertiary rank may have altered potency and targeting within an order. The quaternary rank was established to group known toxins that differ only slightly, either because of a few mutational changes or an imprecision in sequencing (Crickmore *et al.*, 1998). The ranking system splits the proteins based on similarities in their sequence identity. Those ranked at the primary level have up to 45 % sequence identity (Cry1, Cry2 etc.), up to 78 % for secondary rank (Cry1A, Cry1C etc.) and up to 95 % for tertiary rank (Cry1Aa, Cry1Ab, Cry1Ac etc.) (Crickmore *et al.*, 1998; de Maagd *et al.*, 2001; Bravo *et al.*, 2007; Crickmore *et al.*, 2009; van Frankenhuyzen, 2009).

2.1.2.2 Cry protein structure

The three-dimensional structures of six different Cry proteins, Cry1Aa, Cry 2Aa, Cry3Aa, Cry3Bb, Cry4Aa and Cry4Ba, have been determined by X-ray crystallography with all displaying a similar structure consisting of three domains. Domain I (N-terminal) is involved in membrane insertion and pore formation, Domains II and III are involved in receptor recognition and binding, with Domain III also thought to have a role in pore function (Schnepf *et al.*, 1998; de Maagd *et al.*, 2003; Bravo *et al.*, 2007).

2.1.2.3 Cry protein mode of action

The mode of action of Cry toxins has been reviewed and described in numerous studies (e.g. Gill *et al.*, 1992; Schnepf *et al.*, 1998; de Maagd *et al.*, 2001; Bravo *et al.*, 2007; Heckel *et al.*, 2007; Hernández-Rodríguez *et al.*, 2008), and its complexity is ever increasing as new modes and activities are discovered. An overview of the mode of action is presented below.

Ingestion by the susceptible insect larva is required for the Cry protein to have an insecticidal effect. The parasporal crystals ingested by susceptible larvae dissolve in the alkaline environment of the insect gut releasing solubilised inactive protoxins (Bravo *et al,*, 2007). The solubilised inactive protoxins are cleaved by midgut proteases in stages from the carboxy- (C-terminal) and amino- (N-terminal) termini yielding a 60-70 kilodalton (kDa) activated protease-resistant protein toxin (Bravo *et al,*, 2007). In *H. virescens*, trypsins and chymotrypsins are the two major classes of proteinases (Johnston *et al.*, 1995).

The activated toxin then binds to specific receptors on the apical brush border membrane of the midgut epithelium columnar cells. Binding involves various interactions including both reversible (Hofmann and Luthy, 1986; Hofmann *et al.*, 1988; Schnepf *et al.*, 1998) and crucially for toxicity, irreversible (Van Rie *et al.*, 1989; Rajamohan *et al.*, 1995; Schnepf *et al.*, 1998) binding. For Cry1A toxins several binding protein receptors have been described in different Lepidoptera; a cadherin-like protein, a glycosylphosphatidyl-inositol (GPI)-anchored

aminopeptidase-N (APN), a GPI-anchored alkaline phosphatase (ALP) and glycolipids (Aimanova *et al.*, 2006; Bravo *et al.*, 2007). In *H. virescens* APN, ALP and cadherin-like proteins have been identified as Cry1A binding protein receptors (Jurat-Fuentes *et al.*, 2004; Xie *et al.*, 2005; Aimanova *et al.*, 2006). Cry2A toxins have also been found to bind to specific receptors, and although not identified, studies have determined that they are different to those of Cry1A toxins; an important difference with regards to resistance management (Hernández-Rodríguez *et al.*, 2008).

After the activated toxin binds to the membrane, the toxin forms a soluble oligomeric structure before insertion into the membrane leading to the formation of lytic pores in microvilli of apical membranes with subsequent cell lysis. In one model proposed for Cry1A toxins, cadherin and APN receptors have an important role (Bravo *et al.*, 2004; Bravo *et al.*, 2007). Cry1A toxins bind to the cadherin receptor, which induces proteolytical processing and oligomerization of the bound toxin. The oligomeric structure binds APN, followed by insertion into the membrane causing pore formation (Bravo *et al.*, 2004; Bravo *et al.*, 2007). A contrasting model has challenged the pore-forming model, proposing that only binding to the cadherin receptor is required, provoking cell lysis by activating a signalling pathway involving stimulation of G protein, adenylyl cyclise, increased cyclic adenosine monophosphate levels and activation of protein kinase A (Zhang *et al.*, 2006; Rodrigo-Simón *et al.*, 2008).

The events after binding are not yet clear, but the result ends in cell lysis and disruption of the midgut epithelium leading to larval death. The mechanism of death is also open to debate, with earlier proposals suggesting that disruption of the midgut epithelium leads to cessation of feeding and death by starvation or that extensive cell lysis provides access for *Bt* spores to more favourable conditions in the hemocoel for germination and reproduction leading to septicemia and death (Schnepf *et al.*, 1998; Broderick *et al.*, 2006; Broderick *et al.*, 2009). Another proposal suggests that in the larvae of some species, indigenous gut bacteria contribute to mortality, as the disruption of the midgut through consumption of *Bt* allows access for the gut bacteria into the hemocoel and consequently exerting pathogenic effects leading to death by septicemia (Broderick *et al.*, 2006; Broderick *et al.*, 2009).

2.1.3 Vegetative insecticidal protein (Vip)

Vegetative insecticidal proteins (Vips) were discovered in the mid 1990s and are a family of insecticidal proteins predominantly expressed during the vegetative phase (log-phase) of *Bt* growth (Estruch *et al.*, 1996; Warren, 1997), as well as during sporulation (Jensen *et al.*, 2003). This expression during the vegetative growth phase contrasts with that of *Bt* Cry proteins which form parasporal crystals primarily during the sporulation phase (there are a few exceptions including Cry3Aa) (Schnepf *et al.*, 1998). Vegetative insecticidal proteins have shown broad insecticidal activity against a wide variety of lepidopteran and also coleopteran pests and although still at the early stages of discovery and utilisation they may become as valuable as Cry proteins for insect control in transgenic crops (Estruch *et al.*, 1996; Warren, 1997; Yu *et al.*, 1997; Lee *et al.*, 2006; Fang *et al.*, 2007; Liu *et al.*, 2007; Beard *et al.*, 2008; Chankhamhaengdecha *et al.*, 2008). The first *Bt* transgenic cotton crop expressing Vip3A (and Cry1Ab) is being developed for commercial release in the near future (Kurtz *et al.*, 2007).

Bt has been found in the vegetative form on the phylloplane, expressing Vips, so may well have some importance in supporting natural *Bt* infections through the gut epithelium (Bizzarri *et al.*, 2007). However, Vips are most commonly expressed during the vegetative stage of growth in infected larvae, and this is ordinarily considered to occur following ingestion of *Bt* spores(Milne *et al.*, 2008). The action of Vips may therefore not be specific to gut epithelial cells and may have evolved to target other cells after initial infection and lysing of midgut cells through the action of Cry toxins (section 2.1.2). The use of Vips in pest control thus places them in a location that they are may not commonly be found through natural *Bt* infections.

2.1.3.1 Vip classification and structure

The current system for crystal protein classification (section 2.1.2.1) has been used as a basis for classification of the Vip proteins with over 20 different holotypes identified and separated into three classes: Vip1, Vip2 and Vip3 (Crickmore *et al.*, 2009). The structure of Vip2 has been assessed and reveals structurally homologous N- and C-terminal domains which are believed to more than likely represent the entire

class of binary toxins (Vip1 and Vip2) (Han *et al.*, 1999). Structural information of Vip3 is limited, however, based on the lack of sequence homology and predicted secondary structure there is no similar domain organisation in Vip3A as exists for Cry type proteins (Estruch *et al.*, 1996; Lee *et al.*, 2003).

2.1.3.2 Vip mode of action

With the discovery of Vip3Aa1 (referred hitherto as Vip3A) by Estruch *et al.* (1996) the mode of action in the insect gut has been investigated using Vip3A, and appears similar to the Cry proteins (section 2.1.2.3), although important differences are observed. The symptomatology developed upon ingestion of Vip3A resembles that caused by Cry proteins, with cessation of feeding, loss of gut peristalsis, insect paralysis and consequent death, although its timing appears to be delayed (Yu *et al.*, 1997). The symptoms caused by Vip3A ingestion develop over a period of 48 to 72 h whereas the symptoms and death caused by Cry proteins can occur within 24 h (Yu *et al.*, 1997).

Yu *et al.* (1997) demonstrated that the process of solubilisation, proteolytic processing in the midgut and binding to the gut cells of susceptible insects for Vip3A correlates with its toxicity to insects, resembling that of Cry proteins. Lee *et al.* (2003) confirmed that Vip3A is soluble from pH 5.0 to 10.0, allowing solubilisation in the alkaline lepidopteran insect midgut. The 88-kDa Vip3A full length toxin was proteolytically activated to a core toxin of approximately 62-kDa with either trypsin or lepidopteran gut juice extracts, with the Vip3 C-terminus critical to its insecticidal activity (Li *et al.*, 2007). Importantly, Lee *et al.* (2003) demonstrated through ligand blotting experiments with brush border membrane vesicles (BBMV) from the tobacco hornworm, *Manduca sexta* L. (Lepidoptera: Sphingidae), that activated Vip3A bound to 80-kDa and 100-kDa molecules which are distinct from the known Cry1Ab receptors and did not bind to the activated Cry1Ab receptors, 120-kDa amino-peptidase N (APN)-like and 250-kDa cadherin-like molecules. Lee *et al.* (2006) also demonstrated that Vip3A does not share binding sites with either Cry1Ac or Cry2Ab2 in *H. virescens* and the cotton bollworm, *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae). Support was demonstrated for the existence of a pore forming step following binding to the midgut receptors, however, through analysis of voltage

clamp assays, Vip3A and Cry1Ab differed in their principal conductance state and cation specificity (Lee *et al.*, 2003).

These findings show that while there are similarities in the symptoms and processes of insecticidal activity of Vip3A and Cry toxins, the unique protein sequence, distinct receptor binding properties and pore forming/ion channel properties of Vip3A support its use as a novel insecticidal agent (Estruch *et al.*, 1996; Yu *et al.*, 1997; Lee *et al.*, 2003; Lee *et al.*, 2006).

2.1.3.3 Vip1 and Vip2

Vip1 and Vip2 have been isolated from *B. cereus*, a strain designated AB78 (Warren, 1997) and *Bt* (Warren, 1997; Shi *et al.*, 2007), with insecticidal activity against coleopteran species. However, the Vip1 and Vip2 toxins are collectively termed a binary toxin as they only have an insecticidal effect when used in combination. Vip1 binds to the gut receptor providing a pathway for Vip2 to enter the cells and cause cell lyses (Han *et al.*, 1999; Jucovic *et al.*, 2008). It has been shown that Vip1Aa and Vip2Aa have high insecticidal activity against the western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) and the northern corn rootworm, *Diabrotica longicornis barberi* Smith and Lawrence (Warren, 1997).

2.1.3.4 Vip3

Estruch *et al.* (1996) isolated Vip3A from *Bt* strains AB88 and AB424. The spectrum of activity of Vip3A includes the black cutworm, *Agrotis ipsilon* Hufnagel, the fall armyworm, *Spodoptera frugiperda* J.E. Smith, the beet armyworm, *S. exigua* Hübner, *H. virescens*, *H. zea*, the old world bollworm, *Helicoverpa armigera* Hübner, the native budworm, *Helicoverpa punctigera* Wallengren, the soybean looper, *Pseudoplusia includens* Walker (all Lepidoptera: Noctuidae), the potato tuber moth, *Phthorimea operculella* Zeller (Lepidoptera: Gelechiidae), the diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae), the spotted stalk borer, *Chilo partellus* Swinhoe (Lepidoptera: Pyralidae) and *M. sexta* (Estruch *et al.*, 1996; Yu *et al.*, 1997; Selvapandiyan *et al.*, 2001; Liao *et al.*, 2002; Lee *et al.*, 2003; Mascarenhas *et al.*, 2003). *Agrotis ipsilon* is an agronomically important pest that is quite resistant

to δ-endotoxins, however, Vip3A provided 100% mortality at a concentration that was at least 260-fold lower than the concentrations needed to achieve only 50% mortality with Cry1Ab and Cry1Ac (MacIntosh *et al.*, 1990; Estruch *et al.*, 1996). No insecticidal activity has been observed against *O. nubilalis* (Estruch *et al.*, 1996; Yu *et al.*, 1997; Lee *et al.*, 2003) or the non-target beneficial insect the monarch butterfly, *Danaus plexippus* L. (Lepidoptera: Nymphalidae) (Lee *et al.*, 2003).

Other research on Vip3 proteins found that Vip3Ba1 led to significant growth delays of *O. nubilalis* and *P. xylostella* but no larvicidal effect (Rang *et al.*, 2005). Fang *et al.* (2007) demonstrated that Vip3Ac1 had insecticidal activity against *S. frugiperda* and *H. zea*, but as with Vip3Aa1, had no insecticidal activity against *O. nubilalis*. However, a chimeric protein Vip3AcAa combining them both became insecticidal to *O. nubilalis*, as well as having increased insecticidal activity against *S. frugiperda*. Furthermore both Vip3Ac1 and Vip3AcAa were highly insecticidal to a population of the cabbage looper, *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae) that was highly resistant to Cry1Ac, demonstrating the lack of cross-resistance between Cry1A and Vip3A toxins (Fang *et al.*, 2007). Zhu *et al.* (2006) demonstrated that *Btk* strain YBT1520 that would otherwise have no insecticidal activity against *S. exigua*, a major crop pest in China, attained high insecticidal activity against *S. exigua* after YBT1520 was transformed to include the gene that expresses Vip3Aa7. Donovan *et al.* (2001) demonstrated that Vip3A is an important component of the *Bt* strain HD1 by deleting the *vip3A* gene resulting in one-fourth toxicity to *A. ipsilon* and less than one-tenth toxicity to *S. exigua* in comparison to the full HD1 strain, although Milne *et al.* (2008) demonstrated no effect on toxicity to *L. dispar* and spruce budworm, *Choristoneura fumiferana* Clemens (Lepidoptera: Tortricidae).

2.1.4 *Bt* crops

Crops that have been genetically modified to express *Bt* genes coding for insecticidal proteins (e.g. Cry1Ac) are commonly known as *Bt* crops. In some of the first experiments involving *Bt cry* gene expression, tobacco plants were genetically modified to express modified genes derived from a toxin gene *bt2*. The genetically modified tobacco plants synthesized insecticidal proteins that protected them from feeding damage by the larvae of *M. sexta* (Vaeck *et al.*, 1987). Further research has

led to the production and commercialisation of agronomically relevant crops that confer *Bt* genes coding for insecticidal proteins to protect them from their important pest species. *Agrobacterium*-mediated transformation is the preferred protocol for transferring *Bt* genes into the plant (Peferoen, 1997; Guo *et al.*, 2007; Gatehouse, 2008). The *Bt* gene is designed to express the full length or a truncated form of the *Bt* protoxin in the plant. However, research has shown that the protoxin may be partially or completely activated by plant proteases immediately upon plant cell disruption through, for example, mechanical damage by the insect during feeding. Therefore, the potential for the first two steps of the mode of action; solubilisation and proteolytic activation, may occur in the plant prior to ingestion by susceptible insects (Andow and Hilbeck, 2004; Gao *et al.*, 2006; Li *et al.*, 2007; Anilkumar *et al.*, 2008).

Bt crops were first grown commercially in 1996, with approximately 1.1 million hectares grown in three countries (USA, Mexico and Australia). The *Bt* crops grown were cotton producing Cry1Ac, maize producing Cry1Ab and potatoes producing Cry3Aa (James, 1997; Naranjo *et al.*, 2008). Since their first commercial introduction, the global hectarage of *Bt* crops has continued to increase substantially, with second generation *Bt* crops expressing multiple *Bt* toxins becoming increasingly utilised. *Bt* crops are the most extensively planted genetically modified crops after those transformed for herbicide tolerance and in 2008 the global hectarage for genetically modified (GM) crops was 125 million hectares, of which 46 million hectares expressed *Bt* toxins, either alone (19.1 million hectares), or in combination with herbicide tolerance (26.9 million hectares) (Ferré *et al.*, 2008; James, 2008).

Other *Bt* crops that are in development include *Bt* rice, *Bt* cabbage and *Bt* eggplant (Cohen *et al.*, 2008; Ferré *et al.*, 2008; Shelton *et al.*, 2008). *Bt* rice expressing Cry1Ab was in commercial production in Iran in 2005 but production has ceased because it is no longer permitted (James, 2005; Cohen *et al.*, 2008). *Bt* rice varieties in development express either Cry1Aa, Cry1Ab, Cry1Ac, Cry1B, Cry1C or Cry2A, with a further *Bt* rice variety that contains a fusion gene expressing Cry1Ac and Cry1Ab mainly for the control of rice stem borers including the yellow stem borer, *Scirpophaga incertulas* Walker and the striped stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae) (Cohen *et al.*, 2008; Ferré *et al.*, 2008).

Bt potato protected against *L. decemlineata*, however, commercialisation was stopped in 2001 due to a number of issues including marketing difficulties (Ferré *et al.*, 2008; Grafius and Douches, 2008).

Bt maize was commercially grown in 15 countries (including USA, Argentina, Brazil, Canada, South Africa, Uruguay, Philippines and Spain) in 2008 (James, 2008). The majority of *Bt* maize expresses Cry1Ab for protection against *O. nubilalis* and the Mediterranean corn borer, *Sesamia nonagriodes* Lefebvre (Lepidoptera: Noctuidae), with alternate varieties expressing Cry1F for protection against *S. frugiperda* or Cry3Bb or Cry34Ab and Cry35Ab (binary toxin) for protection against *Diabrotica* spp. Second generation (see below) *Bt* maize varieties are at the field trial stage or close to commercialisation (Ferré *et al.*, 2008; Hellmich *et al.*, 2008).

In 2008 *Bt* cotton was commercially grown in 10 countries (USA, Australia, Mexico, Argentina, Brazil, China, Colombia, India, South Africa and Burkina Faso) occupying 15.5 million hectares (James, 2008). In the USA, Australia and South Africa more than 90 % of the cotton grown is *Bt* cotton (James, 2008). Insecticidal toxins produced in commercially available *Bt* cotton are Cry1Ac, Cry2Ab and Cry1F (Adamczyk *et al.*, 2001a; Chitkowski *et al.*, 2003; Adamczyk and Gore, 2004; Naranjo *et al.*, 2008). The first generation commercialised *Bt* cotton varieties expressed Cry1Ac only. The primary targets for Cry1Ac are the lepidopteran pests *H. virescens*, *Helicoverpa* spp., the pink bollworm, *Pectinophora gossypiella* Saunders (Lepidoptera: Gelechiidae) and a number of spiny and spotted bollworms, *Earias* spp. (Lepidoptera: Nolidae). However, Cry1Ac has limited activity against other lepidopteran pests such as *Spodoptera* spp., *T. ni* and *P. includens*. Second generation *Bt* cotton varieties that express two *Bt* genes (pyramiding) have become available, producing Cry1Ac and Cry2Ab or Cry1Ac and Cry1F and with these varieties available a wider spectrum of activity against lepidopteran pests has been accomplished. Cry1F increases the spectrum with activity against *Spodoptera* spp. and Cry2Ab having activity against *P. includens* and increasing efficacy against *H. zea* and *H. armigera* (Adamczyk *et al.*, 2001a; Chitkowski *et al.*, 2003; Adamczyk and Gore, 2004; Naranjo *et al.*, 2008). A further *Bt* cotton variety that should be commercialised in the USA will express Vip3A along with Cry1Ab to further

compliment and extend the use of currently used insecticidal toxins against lepidopteran pests, primarily *H. virescens* and *H. zea* (Kurtz *et al.*, 2007).

Bt crops are grown on the basis that environmental and human health risks would be lower than with current or alternative technologies and the benefits would be greater. It does not appear that commercial cultivation of *Bt* crops has caused environmental impacts beyond those that are caused by conventional agricultural management practices (Naranjo *et al.*, 2005; Sanvido *et al.*, 2007; Shelton, 2007). *Bt* crops have a number of benefits including specificity for key target pests, a lack of negative impact on beneficial insects, reduced insecticide use, increased yield, and season long expression. These benefits make *Bt* crops a valuable addition to sustainable integrated pest management (IPM) systems alongside, for example, biological control (maintaining natural enemy populations), cultural (modified planting dates, tillage, crop rotation) and chemical control (use of selective insecticides). However, elimination of chemical sprays is seldom achieved when non-lepidopteran pests also affect yields, e.g. aphids, mites and shield bugs (Fitt, 2000; Romeis *et al.*, 2006; Sanvido *et al.*, 2007; Fitt, 2008; Hellmich *et al.*, 2008; Naranjo *et al.*, 2008).

Taking cotton as an example, it is traditionally one of the most intensively sprayed field crops, however, the insecticide use in *Bt* cotton fields has on average reduced by 50 % across the world, with decreases of 39 % and 60 % in India and China respectively, as target pests are more effectively controlled (Fitt, 2008; James, 2008). Yields in developing countries have seen substantial increases where insect pest management has been less effective in conventional systems as compared to countries with more established IPM systems, such as the USA and Australia, where yields have changed very little (Cattaneo *et al.*, 2006; Bravo *et al.*, 2007; Fitt, 2008; James, 2008). Survival of predators and parasitoids in *Bt* cotton is demonstrably higher than in conventional cotton, and these increased beneficial populations can help to provide control against secondary pests, alongside the use of more selective insecticides (Cattaneo *et al.*, 2006; Fitt, 2008; Naranjo *et al.*, 2008). Secondary pests such as plant bugs have become more problematic in *Bt* cotton in various countries (e.g. USA, *Lygus* spp; Australia and China, Miridae) due in part to reduced insecticide use, as primary pests are controlled by *Bt*, and also due to reduced competition from target

species allowing the resurgence of non-target pest populations (Fitt, 2008; Naranjo *et al.*, 2008).

The diversity of *Bt* toxins is desirable for selectively controlling different target insect pests within crop systems and commercial varieties are currently restricted to Cry toxins. The continued commercialisation of pyramided *Bt* toxins, with the added development of *Bt* crops containing more than two *Bt* toxins, and production of chimeric Cry toxin expressing *Bt* varieties aims to increase control of multiple species and help reduce the evolution of resistance (Bravo and Soberón, 2008; Malone *et al.*, 2008). *Bt* cotton and maize expressing Vip3A and Cry1Ab are expected to be commercialised in the near future, with experimental field trials taking place over the past few years (Dively, 2005; Llewellyn *et al.*, 2007; Whitehouse *et al.*, 2007; Adamczyk and Mahaffey, 2008; Bommireddy and Leonard, 2008). The development of resistance to Cry proteins (section 2.2) is of major importance with the wide use of *Bt* crops around the world. The use of Vips alongside Cry proteins will help to better preserve and extend the usefulness of these important insect control agents using appropriate insect resistance management (IRM; section 2.2.5) measures for long term effectiveness within an IPM system (Fang *et al.*, 2007; Kurtz *et al.*, 2007).

2.2 Resistance to *Bacillus thuringiensis*

Resistance has been defined by the World Health Organisation (WHO) as 'the development of an ability in a strain of an organism to tolerate doses of a toxicant which would prove lethal to the majority of individuals in a normal (susceptible) population of the species'. Whereas Whalon *et al.* (2008) describes resistance as 'the microevolutionary process whereby genetic adaptation through pesticide selection results in populations of arthropods which present unique and often more difficult management practices'. A more simple description is 'resistance is a genetically based decrease in susceptibility of a population to an insecticide' (Tabashnik, 1994; Bravo and Soberón, 2008). Moar *et al.* (2008) emphasises that pest resistance to *Bt* crops can be defined in two different ways, based on either laboratory conditions or field conditions. Laboratory resistance is 'a statistically significant, genetically mediated reduction in sensitivity of the target organism to the controlling agent,

relative to a susceptible laboratory strain'. Field resistance is 'a genetically mediated increase in the ability of a target pest to feed and complete development on a *Bt* crop under field conditions' (Moar *et al.*, 2008).

There are many reports of insect resistance to *Bt* Cry toxins, the majority through selection in the laboratory (e.g. Gould *et al.*, 1995; Ferré and Van Rie, 2002; Gahan *et al.*, 2005; Sayyed *et al.*, 2005; Alves *et al.*, 2006; Tabashnik *et al.*, 2006; Mahon *et al.*, 2007), although there have been several cases of field-evolved resistance with *Bt* formulations and *Bt* crops (e.g. Ferré and Van Rie, 2002; Kain *et al.*, 2004; Baxter *et al.*, 2005; van Rensburg, 2007; Tabashnik, 2008). The first thoroughly studied case of resistance to *Bt* Cry toxins was with a *P. interpunctella* population from grain bins that showed a 100-fold decrease in susceptibility to *Btk* (Dipel®) following 15 generations of laboratory selection with Dipel (McGaughey, 1985).

2.2.1 Field resistance

Field-evolved resistance was first confirmed in *P. xylostella* populations in Hawaii (USA) that had been exposed to prolonged use of *Btk* (Dipel) with up to 30-fold resistance (Tabashnik *et al.*, 1990). Since then, further field resistance to *Bt* formulations in *P. xylostella* has been reported in continental USA (e.g. Florida) and Asia (e.g. Phillipines, Malaysia and Thailand) (Ferré *et al.*, 1991; Shelton *et al.*, 1993; Wright *et al.*, 1997; Imai and Mori, 1999; Sayyed *et al.*, 2000b; Sayyed *et al.*, 2004). Janmaat and Myers (2003) reported resistance to *Btk* (Dipel) in populations of *T. ni* in commercial vegetable greenhouses in British Columbia (Canada) with up to 160-fold resistance.

Reports of field-evolved resistance to *Bt* crops were reported with the African stem borer, *Busseola fusca* Fuller (Lepidoptera: Noctuidae), *S. frugiperda* and *H. zea*. Van Rensburg (2007) reported that a level of resistance was attained allowing some larvae of *B. fusca* to survive on *Bt* maize in South Africa, that expresses Cry1Ab, although there was a detrimental effect on larval growth. *Spodoptera frugiperda* developed resistance to *Bt* corn expressing Cry1F in Puerto Rico, resulting in field failure and the consequent discontinuation of commercial cultivation of the *Bt* crop variety (Matten *et al.*, 2008; Moar *et al.*, 2008; Tabashnik, 2008). Tabashnik *et al.* (2008a)

reported that laboratory bioassays of some field populations of *H. zea* from the USA showed resistance ratios of over 500-fold to Cry1Ac. Cry1Ac is expressed in *Bt* cotton, and although this level of resistance was found, resistance has not caused widespread control failures, due in part to the insecticide resistance management strategy in place (Tabashnik *et al.*, 2008a) (section 2.2.5).

2.2.2 Laboratory selection and resistance

There have been several *H. virescens* populations selected for resistance in the laboratory (Ferré and Van Rie, 2002; Gahan *et al.*, 2005; Jurat-Fuentes and Adang, 2006; Ferré *et al.*, 2008). Stone *et al.* (1989) selected a population (SEL) with Cry1Ab that produced 20-fold resistance to Cry1Ab. Selection with Cry1Ac of a population (CP73-3) by Gould *et al.* (1992) showed a 50-fold increase in resistance to Cry1Ac, 13-fold resistance to Cry1Ab and 53-fold resistance to Cry2Aa. Further selection with Cry2Aa resulted in a population (CXC) with over 250-fold resistance to Cry2Aa and increased resistance to Cry1Ac (290-fold), and further cross-resistance to Cry1Aa, Cry1Ab and Cry1Fa (Jurat-Fuentes *et al.*, 2003). In another population (YHD2) selected with Cry1Ac, over 10,000-fold resistance to Cry1Ac was developed, with cross-resistance to Cry1Aa, Cry1Ab and Cry1Fa, and more moderate cross-resistance to Cry2Aa (less than 25-fold) and very low potential cross-resistance (< 3-fold) to Cry1Ba and Cry1Ca (Gould *et al.*, 1995). A 400-fold Cry1Ac resistant population (KCB) was developed with cross-resistance to Cry1Ab and Cry2Aa (Forcada *et al.*, 1999). Further selection with Cry2Aa resulted in a population (KCBhyb) with continued resistance to Cry1Ac, over 250-fold resistance to Cry2Aa and further cross-resistance to Cry1Aa, Cry1Ab and Cry1Fa (Jurat-Fuentes *et al.*, 2003).

Selection of a *H. armigera* population (BX) with Cry1Ac resulted in up to 300-fold resistance in Cry1Ac, with cross-resistance to Cry1Ab, but not Cry2Aa or Cry2Ab (Akhurst *et al.*, 2003). A population of Cry1Ac resistance screening survivors (silver strain 2001/2) from Australian cotton areas showed 14-fold resistance to Cry1Ac and after a single selection with Cry1Ac increased to 150-fold (Gunning *et al.*, 2005). Another population (SP15) was selected with Cry2Ab, with over 6000-fold resistance

to Cry2Ab and cross-resistance to Cry2Aa, but not Cry1Ac or Dipel (Mahon *et al.*, 2007).

Two populations (AZP-R and APHIS-98R) of *P. gossypiella* selected in the laboratory with Cry1Ac had substantial cross-resistance to Cry1Aa and Cry1Ab but not to Cry1Bb, Cry1Ca, Cry1Da, Cry1Ea, Cry1Ja, Cry2Aa or Cry9Ca (Tabashnik *et al.*, 2000). Further selection with Cry1Ac increased resistance 3100-fold in AZP-R (Tabashnik *et al.*, 2002).

Laboratory selection of the field resistant population of *P. xylostella* from Hawaii (Tabashnik *et al.*, 1990) with Dipel increased resistance to over 1000-fold (NO-QA population) with high levels of resistance to Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa and Cry1Ja, but no significant resistance to Cry1Ba, Cry1Bb, Cry1C, Cry1D, Cry1I and Cry2A (Tabashnik *et al.*, 1996). Other laboratory selections of *P. xylostella* with *Btk* or specific Cry toxins showed similar patterns of cross-resistance between Cry1A toxins but a lack of cross-resistance between Cry1A and Cry1B or Cry1C (Ferré *et al.*, 1991; Tang *et al.*, 1996; Tabashnik *et al.*, 1997b; Ferré and Van Rie, 2002). A population (NO-95) that had been exposed to both *Btk* and *Bta* (which contains Cry1C) in the field displayed 22-fold resistance to Cry1Ca and was 50-130-fold less susceptible to *Btk* formulations, and with further selection with Cry1Ca developed 62-fold resistance to Cry1Ca (Liu *et al.*, 1996; Liu and Tabashnik, 1997). Another field resistant population (BCS) was shown to have 31-fold resistance to Cry1Ca, and after further selection with Cry1Ca the population (BCS-Cry1C-1) developed 1090-fold resistance to Cry1Ca and high levels of cross-resistance to Cry1Aa, Cry1Ab, Cry1Ac, Cry1F and Cry1J (more than 390-fold) (Zhao *et al.*, 2001).

2.2.3 Resistance mechanisms

It is important to know whether resistance to various Cry toxins is through the same mechanism either within or between species and also determine whether cross-resistance to other toxins is due to the same resistance mechanism in the insect or that multiple mechanisms of resistance have evolved. In principal, insects could become resistant to *Bt* toxins at any stage of the mode of action (Heckel *et al.*, 2007) leading to resistance mechanisms such as failure of protoxin solubilisation, proteolytic

processing (Forcada *et al.*, 1996; Forcada *et al.*, 1999; Oppert, 1999), toxin degradation (Forcada *et al.*, 1996), receptor binding site modification (Van Rie *et al.*, 1990; Ferré *et al.*, 1991) and replacement of dead midgut cells (Loeb *et al.*, 2001). Although the most common reported mechanisms are firstly receptor binding site modification followed by proteolytic processing (Ferré and Van Rie, 2002; Bravo and Soberón, 2008).

2.2.3.1 Binding site modifications

Receptor binding site modification is where binding of the toxin to the receptor is prevented or reduced, resulting in the absence or reduction of toxicity. Although the majority of cases of resistance have been correlated with binding site modification, determining which receptor molecules are defective has been difficult. Several Cry toxin-binding molecules have been identified and characterised by biochemical methods and include aminopeptidase N (APN), alkaline phosphatase, cadherins, and glycolipids (Schnepf *et al.*, 1998; Griffitts and Aroian, 2005).

In *H. virescens*, three Cry1 binding sites have been detected: the A binding site is recognized by Cry1Aa, Cry1Ab, Cry1Ac, Cry1F and Cry1J; the B binding site is recognized by Cry1Ab and Cry1Ac, and the C binding site is recognized by Cry1Ac only (Van Rie *et al.*, 1989; Jurat-Fuentes and Adang, 2001; Jurat-Fuentes and Adang, 2006). Jurat-Fuentes and Adang (2001) determined that Cry2Aa does not bind to any of these binding sites. Proteins that have been proposed as components of binding site A include the *H. virescens* cadherin-like protein HevCaLP (Jurat-Fuentes *et al.*, 2004) and the 170- and 130-kDa APNs (Luo *et al.*, 1997; Oltean *et al.*, 1999). A 130-kDa protein has been associated with binding site B, and proteins of less than 100-kDa have been predicted to form part of binding site C, although neither binding site B or C have been extensively studied (Jurat-Fuentes and Adang, 2001).

Binding studies with the YHD2 population, that was highly resistant to Cry1Ac and cross-resistant to Cry1Aa, Cry1Ab and Cry1Fa, determined that only Cry1Aa binding (associated with binding site A) was reduced, but binding of Cry1Ab (associated with binding sites A and B) and Cry1Ac (associated with binding sites A, B and C) was relatively unchanged. It was suggested that toxicity of Cry1 toxins in *H. virescens*

was due to the interaction with binding site A, rather than B or C which may not be involved in toxicity and that the cadherin-like protein (HevCaLP) should be considered as a component of the receptor at binding site A (Lee *et al.*, 1995; Jurat-Fuentes *et al.*, 2004). Further selection of the YHD2 population with Cry1Ac resulted in YHD2-B that had 73-fold higher resistance than YHD2 and reduced Cry1Ab and Cry1Ac binding. These changes in Cry1Ab and Cry1Ac binding implied that Cry1Ac binding sites other than HevCaLP were also involved in toxicity to *H. virescens* and a membrane bound form of alkaline phosphatase (HvALP) was identified as a Cry1 receptor, with reduced amounts contributing to resistance (Jurat-Fuentes and Adang, 2006). Altered glycosylation is also thought to be have some responsibility to increased resistance and cross-resistance to Cry1A toxins (Jurat-Fuentes *et al.*, 2002).

For KCBhyb, resistant to Cry1A and Cry2Aa toxins (Jurat-Fuentes *et al.*, 2003), Cry1A resistance is thought to be a similar mechanism of resistance to the YHD2 population due to the similar pattern of toxin binding with the loss of HevCaLP correlated with high levels of resistance to Cry1Ac (Jurat-Fuentes *et al.*, 2004; Jurat-Fuentes and Adang, 2006). Cry1A and Cry2Aa do not share binding sites (Jurat-Fuentes and Adang, 2001), and binding of Cry2Aa in KCBhyb was unaltered, and so a second mechanism of resistance is believed to exist, although putative inhibition of proteolytic processing has not been found (section 2.2.3.2) (Jurat-Fuentes *et al.*, 2003).

Proteins homologous to HevCaLP have also been proposed as mechanisms of resistance to Cry1Ac in *H. armigera* (Xu *et al.*, 2005) and *P. gossypiella* (Morin *et al.*, 2003; Fabrick and Tabashnik, 2007).

In a *S. exigua* population that is resistant to Cry1Ca, the lack of APN 1 expression is at least partly responsible for resistance to Cry1Ca (Herrero *et al.*, 2005; Bravo and Soberón, 2008).

Plutella xylostella binding site resistance has also been modelled, indicating the presence of at least four binding sites: site one is only recognised by Cry1Aa; site two is recognised by Cry1Aa, Cry1Ab, Cry1Ac, Cry1F and Cry1J; site three is recognised Cry1Ba and site four is recognised by Cry1Ca (Ferré and Van Rie, 2002; Ferré *et al.*,

2008). Reduced binding is a common mechanism seen in many of the resistant populations (Ferré *et al.*, 1991; Ferré and Van Rie, 2002; Sayyed *et al.*, 2004; Sayyed *et al.*, 2005), but the binding mechanism has not been identified, with studies rejecting possible mechanisms including the cadherin, APNs, and alkaline phosphatase (Baxter *et al.*, 2005; Heckel *et al.*, 2007; Bravo and Soberón, 2008).

2.2.3.2 Proteolytic processing

Another reported mechanism of resistance is through inhibition of proteolytic processing, where the protoxin is not successfully reduced to its active form, resulting in the loss of toxicity (Ferré and Van Rie, 2002). For example, the *H. virescens* population CXC is resistant to Cry1Ac and Cry2Aa toxins, but toxin binding is unaltered suggesting the resistance mechanism must involve modification of a step in toxin action shared by both Cry1A and Cry2A toxins. Studies have shown that CXC larvae had reduced amounts of a 35 kDa chemotrypsin-like enzyme, a reduced capacity to process Cry1Ac and Cry2Aa protoxin and an increased capacity to degrade Cry2Aa toxin. The KCBhyb population, resistant to Cry1Ac and Cry2Aa, was also hypothesised to have a similar reduced capacity to process Cry2Aa and possibly Cry1Ac due to reduced proteinase activity (Jurat-Fuentes *et al.*, 2003; Gahan *et al.*, 2005; Jurat-Fuentes and Adang, 2006), based on one of the parental populations, KCB, which had shown reduced levels of proteinases in gut extracts (Forcada *et al.*, 1999). However, no differences in protoxin activation or toxin degradation were observed in KCBhyb when compared to a susceptible population, YDK (Karumbaiah *et al.*, 2007).

Studies on the *P. interpunctella* 198-r population, resistant to *Bt* var. *entomocidus* (*Bte*), and the 133-r population, resistant to *Bta*, showed lack of a major gut trypsin-like proteinase causing a reduction in protoxin-activation abilities contributing to Cry1A toxin resistance (Oppert *et al.*, 1996; Oppert *et al.*, 1997).

2.2.4 Genetics of *Bacillus thuringiensis* resistance

2.2.4.1 Natural variability

Comparing the intraspecific variation in susceptibility to *Bt* toxins between insect field populations is one approach to estimate the potential of insect field populations to evolve resistance to *Bt* toxins and giving an indication of baseline susceptibilities. It is common practice to compare the LC_{50} (concentration that kills 50 % of the population) values. Low variability in susceptibility among populations, however, doesn't necessarily mean a low potential for selection pressure to act on, as variability within populations can still be high (Ferré and Van Rie, 2002).

Studies measuring variation in susceptibility have reported both high and low natural variations among populations. Populations of *O. nubilalis* in Germany showed no differences in susceptibility to Cry1Ab (Saeglitz *et al.*, 2006). There were small differences in susceptibilities to Cry1Ab between populations of *Diatraea saccharalis* F., sugar cane borer, in Louisiana and Texas, although all were as susceptible as a laboratory population (Huang *et al.*, 2008). Susceptibility to Cry1Ac had a 50-fold range and Cry 1Ab had a 30-fold range in populations of *Cnaphalocrocis medinalis* Guenée (Lepidoptera: Pyralidae) an important lepidopteran rice pest, in China (Han *et al.*, 2008). *Heliothis virescens* susceptibilities to Cry2Ab2 in the USA varied up to 48-fold for nine laboratory, seven laboratory-cross and 28 field populations, although the means of these three groups varied only 2-fold (Ali and Luttrell, 2007). In the same study *H. zea* susceptibilities varied up to 37-fold but the means of laboratory, laboratory-cross and field populations varied only 3-fold (Ali and Luttrell, 2007). Populations of *H. virescens* in the USA varied 12-fold to Cry1Ac, while *H. zea* varied 130-fold (Ali *et al.*, 2006).

Another method for estimating the variability of resistance genes is by measuring heritability (h^2) in laboratory experiments (Tabashnik, 1994; Ferré and Van Rie, 2002). Tabashnik (1994) estimated heritability of resistance to *Bt* products and Cry1A toxins for 27 selection experiments and showed that compared with eight other insect species, *P. interpunctella* had a relatively high h^2 value. Relatively high h^2 values were also determined in two populations from Malaysia (Wright *et al.*, 1997;

Sayyed *et al.*, 2000b). These high h^2 values indicate high additive genetic variation for susceptibility to Cry proteins in those populations (Ferré and Van Rie, 2002).

2.2.4.2 Estimation of resistance allele frequency

The initial frequency of resistance alleles is important and influences the rate at which resistance may evolve (Gould *et al.*, 1997). Therefore, it is a key element for predicting the rate of evolution in a population subjected to insecticide treatments (Ferré and Van Rie, 2002). Genetic models have assumed that initial allelic frequencies range from 10^{-2} to 10^{-6} based on theoretical assumptions regarding the balance between mutation and selection (Roush and McKenzie, 1987; Gould *et al.*, 1997). A direct estimate with *H. virescens*, using homozygous recessive resistant females (YHD2) individually mated with field captured males, calculated that the frequency of resistance alleles that confer resistance to Cry1Ac in the field population was 1.5×10^{-3} (Gould *et al.*, 1997). Tabashnik *et al.* (1997a) estimated the frequency of resistance alleles in a susceptible *P. xylostella* population to be 1.2×10^{-1}. Andow and Alstad (1998) used an F_2 screening procedure to estimate the frequency of Cry1Ab resistant alleles in *O. nubilalis* to be less than 1.3×10^{-2} for a Minnesota population (Andow *et al.*, 1998) and less than 3.9×10^{-3} for an Iowa population (Andow *et al.*, 2000). An F_2 screen estimated that resistance allele frequency for field derived *H. virescens* to Cry1Ac was 3.6×10^{-3} to 2.6×10^{-2} (Blanco *et al.*, 2009). These direct estimates have high initial frequencies, as compared to the genetic model estimates, and if these estimates are typical, resistance may evolve more quickly than expected if precautionary measures are not in place.

2.2.4.3 Mode of inheritance of resistance

The dominance level of resistance is important as it influences the rate at which resistance may evolve. It is generally assumed that alleles for *Bt* resistance are rare initially (Gould *et al.*, 1997) and individuals homozygous for resistance to *Bt* (RR) extremely rare initially. Therefore, the response of heterozygotes (RS) to *Bt* determines the initial course of evolution of resistance. If heterozygotes are killed by *Bt* then the resistance is termed recessive, and if the heterozygotes survive exposure to *Bt*, the resistance is termed dominant (Tabashnik *et al.*, 1998).

Bourguet *et al.* (2000) reported that dominance has been assessed in different ways in insecticide resistance studies. Values ranging from 0 for complete recessivity to 1 for complete dominance, have been obtained from single-dose mortality tests (D_{ML}, dominance of survival at a given insecticide dose), from LC_{50} values of dose-mortality curves (D_{LC}, dominance of insecticide resistance) and from the fitness of the 3 geneotypes in insecticide-treated areas (D_{WT}, dominance of relative fitness in the treated area). The D_{WT}, calculation is the most relevant to resistance management, but at the same time is also the most difficult to estimate. The D_{LC} value was modified from the widely used Stone's (1968) formula, for which the degree of dominance ranged from -1 to 1 (Ferré and Van Rie, 2002). It has been suggested that partial or completely recessive modes of inheritance are more closely associated with modification of binding sites, and dominant alleles seem to be associated with other mechanisms conferring broad spectrum resistance, for example, modification of midgut proteolytic activity (Gould *et al.*, 1997; Liu and Tabashnik, 1997; Tabashnik *et al.*, 1998; Bourguet *et al.*, 2000).

Recessive resistance to *Bt* was first reported in *P. interpunctella* (McGaughey, 1985). For *H. virescens*, resistance in the SEL and CP73-3 populations both showed incompletely dominant inheritance to Cry1Ac (Sims and Stone, 1991; Gould *et al.*, 1992). Gould *et al.* (1995) reported that inheritance of resistance in the YHD2 population to Cry1Ac and Cry1Ab was partially recessive, but Cry2Aa was more dominant.

For *P. xylostella* populations from Hawaii (NO-QA) and Pennsylvania (PEN), resistance to Cry1Aa, Cry1Ab, Cry1Ac and Cry1F was partly to completely recessive, however a population from the Phillipines (PHI) showed recessive inheritance of resistance to Cry1Ab, but resistance to Cry1Aa and Cry1Ac was associated with partially dominant inheritance (Tabashnik *et al.*, 1997b; Tabashnik *et al.*, 1998). Another Hawaiin population (NO-95C) resistance to Cry1C was partially dominant (Liu and Tabashnik, 1997). A *P. xylostella* population (SERD4) from Malaysia showed incompletely dominant inheritance to Cry1Ac in the Cry1Ac-selected subpopulation and to Cry1Ab in the Cry1Ab-selected subpopulation (Sayyed *et al.*, 2000b; Sayyed *et al.*, 2005).

2.2.4.4 Number of resistance genes

The number of resistance genes involved may depend on the toxins that the population is resistant to and how many mechanisms of resistance are present, since having more than one resistance mechanism would suggest the presence of more than one resistance gene (polygenic resistance). Many of the studies, using backcross data, determining the number of loci involved in resistance have fitted the single locus model or monogenic resistance (Ferré and Van Rie, 2002).

For a *H. virescens* population (SEL) resistance to Cry1Ab was polygenic (Sims and Stone, 1991), whereas for a second population (YHD2) resistance to Cry1Ab was monogenic (Gould *et al.*, 1995). Field-evolved resistance in *P. xylostella* populations NO-QA and Karak both exhibited monogenic resistance (Tabashnik *et al.*, 1997b; Sayyed *et al.*, 2004), whereas the field derived SERD4 population selected with Cry1Ac or Cry1Ab exhibited polygenic resistance with some parental sex influence (Sayyed *et al.*, 2005). Two populations of *O. nubilalis* selected with Cry1Ab have shown polygenic resistance to Cry1Ab (Alves *et al.*, 2006).

2.2.4.5 Fitness costs and stability of resistance

The evolution of resistance in a population may compromise the normal functions of individuals, such as survival, development time and fecundity, thereby causing a fitness cost associated with resistance. The nature of potential fitness costs are revealed in environments that lack the selecting agent. In the case of *Bt* crops, resistant individuals moving into refuges may exhibit fitness costs in comparison to susceptible individuals that have not been exposed to *Bt* toxin. Thus, a trade-off occurs with *Bt* resistance increasing fitness in the presence of *Bt* toxin, but causing a fitness cost when *Bt* toxin is absent (Tabashnik, 1994; Ferré and Van Rie, 2002; Gassmann *et al.*, 2009). The stability of *Bt* resistance is linked to fitness costs associated with resistance. In many studies on resistance to *Bt* Cry toxins, resistance reverted when selection was stopped (Ferré and Van Rie, 2002; Gassmann *et al.*, 2009).

2.2.5 Insect Resistance Management (IRM)

The recommended and most favoured strategy for managing resistance to *Bt* crops is the high dose plus refuge strategy, involving the combination of two separate concepts, high expression of the *Bt* toxin and the provision of refuges (non-toxin bearing plants) (Gould, 1998; Bates *et al.*, 2005; Ferré *et al.*, 2008; Tabashnik and Carrière, 2009). The expression of the *Bt* toxin is at a high enough concentration (25 times the amount needed to kill all susceptible individuals) to kill all heterozygous resistant (RS) and susceptible insects, resulting in only rare functionally recessive resistant homozygous insects surviving (RR) (Gould, 1998; Bourguet *et al.*, 2000; Bates *et al.*, 2005; Ferré *et al.*, 2008). The refuge maintains a susceptible population (SS) as it doesn't encounter *Bt* toxin. The susceptible insects mate with the surviving homozygous resistant insects and produce susceptible, heterozygous offspring, thereby successfully delaying the evolution of resistance (Bates *et al.*, 2005; Ferré *et al.*, 2008; Tabashnik and Carrière, 2009). For this strategy to be effective, several assumptions are made. The inheritance of resistance has to be recessive, initial allele frequency for resistance needs to be low, the number of susceptible insects has to outnumber the resistant survivors, the refuge has to be a close enough distance to the *Bt* plants, random mating must occur between resistant and susceptible insects, and the toxin concentration in the plant must be high enough to kill all heterozygous resistant insects (Gould, 1998; Bates *et al.*, 2005; Ferré *et al.*, 2008).

Studies have shown that one or more of these assumptions may be or have the potential to be violated by some pest species. The high dose standard has not been met with *H. zea* in *Bt* cotton expressing Cry1Ac, based on survival of susceptible larvae on *Bt* cotton and dominant inheritance of resistant (Tabashnik, 2008; Tabashnik *et al.*, 2008a) and also with *D.* vergifera vergifera in *Bt* maize expressing Cry3Bb (Ferré *et al.*, 2008; Meihls *et al.*, 2008). Non-random mating may occur due to differences in development time (Gould, 1998) between susceptible and resistant insects as shown with a resistant laboratory population of *P. gossypiella* reared on *Bt* cotton. Resistant larvae took nearly six days longer to develop on *Bt* cotton compared to susceptible larvae on non *Bt* cotton (Liu *et al.*, 1999; Ferré *et al.*, 2008). Dispersal behaviour may affect random mating, as migration from the refuge to the *Bt* crop and *vice versa* is a necessary requirement (Gould, 1998). *Ostrinia nubilalis* has been

found to mate on a more restricted spatial scale, with predispersal mating, thereby increasing the frequency of resistant homozygotes (Dively, 2005; Ferré *et al.*, 2008).

In the USA, the current refuge requirements for control of Lepidoptera in *Bt* maize (Cry1Ab) is at least 20 % non-*Bt* maize, and 50 % in cotton growing areas due to the potentially increased selection pressure on *H. zea* from *Bt* cotton expressing Cry1Ac, as it is a pest of both crops (Ferré *et al.*, 2008). The refuge must be placed within 0.5 miles either near the maize field as a separate field or as part of it. For *Bt* cotton there are three options for singularly expressed *Bt* toxin: a 5 % external unsprayed refuge, 20 % external sprayed refuge or 5 % embedded refuge. For pyramided *Bt* toxins in Texas and areas east of Texas the USEPA (US Environmental Protection Agency) has approved the use of natural refuge, no longer requiring the planting of non-*Bt* cotton refuge (Ferré *et al.*, 2008). Other countries, for example, China and India have no refuge requirements, instead relying on mixed planting systems of *Bt* and non-*Bt* crops and this mix potentially acting as natural refuges alongside wild hosts (Ferré *et al.*, 2008).

The availability of pyramided *Bt* crops (section 2.1.4) should allow better control of pest complexes, through the assumption that resistance to two or more toxins simultaneously will be very rare, provided inheritance of resistance is recessive and there is no cross-resistance between the toxins (Roush, 1997; Gould, 1998; Roush, 1998; Bates *et al.*, 2005). Models indicated that pyramided *Bt* crops require smaller refuge areas, and as already mentioned, natural refuges are sufficient in some areas of the USA. It has been shown, however, that the effectiveness of delaying resistance can be compromised if pyramided varieties are grown in close proximity with single expression varieties if the toxin expressed is the same or known to have cross-resistance, as studies have shown that selection for resistance can occur more rapidly than growing the pyramided variety alone (Bates *et al.*, 2005; Zhao *et al.*, 2005; Bravo and Soberón, 2008; Ferré *et al.*, 2008). In Australia, *Bt* cotton now only expresses the pyramided variety (Cry1Ac and Cry2Ab) having phased out the single expressing variety (Cry1Ac) (Naranjo *et al.*, 2008).

Annual monitoring of insect resistance to *Bt* crops is also an important part of the IRM strategy, as first hand reports from growers confirming unsatisfactory field

control may often be too late to implement pro-active management strategies. Monitoring methods include the dose-response bioassay, discriminating dose bioassay and F_2 screen (Bates *et al.*, 2005; Huang, 2006). Detecting increases in resistance may provide sufficient time for adjusting the IRM strategy remedial action to contain the spread of resistant individuals and adjust the strategy (Bates *et al.*, 2005; Bourguet *et al.*, 2005; Ferré *et al.*, 2008).

2.3 Lepidopteran pests of cotton

There are several major lepidopteran pests of cotton (*Gossypium* spp.) throughout the world causing severe economic damage to cotton yields without effective control. The primary pests in North and South America are *H. virescens*, *H. zea* and *P. gossypiella* (Anilkumar *et al.*, 2008). *Helicoverpa armigera* is considered the primary pest in Australia, China, India, South Africa and Indonesia (Fitt, 2008) with other important pests including *Earias* spp. found in most of Africa and the Mediterranean region and eastwards to India, China and Southeast Asia, for example, the spiny bollworm, *Earias insulana* Boisduval (Lepidoptera: Noctuidae) (Ibargutxi *et al.*, 2006) and *H. punctigera* found in Australia (Downes *et al.*, 2009).

2.3.1 *Heliothis virescens*, tobacco budworm (Lepidoptera: Noctuidae)

2.3.1.1 Distribution and economic importance

Heliothis virescens has a confined geographic range with its distribution extending through North (as far as Ontario, Canada) and South America (as far south as Argentina) (the New World) with permanent populations in most areas between latitudes 40° N and 40° S (Neunzig, 1969; Fitt, 1989; King, 1994; McCaffery, 1998). In the USA, distribution is principally in the Eastern and South Western states, with annual northward dispersal (Neunzig, 1969; Capinera, 2001) with up to five generations per year with pupal overwintering (King, 1994).

Heliothis virescens is a major crop pest of cotton, tobacco, tomato, sunflower and soybean (King, 1994; McCaffery, 1998; Abney *et al.*, 2007; Blanco *et al.*, 2009). Damage is caused through the feeding preference of larvae for reproductive structures (e.g. flower buds and bolls) and terminal shoots of the host plant resulting in yield losses (Fitt, 1989; King, 1994; Bommireddy and Leonard, 2008). Damage caused to the plant by the larvae can also lead to secondary infections by plant pathogens, leading to further yield losses (Fitt, 1989; King, 1994).

The multivoltine and polyphagous nature, high mobility, high fecundity, and facultative diapause of *H. virescens* along with its developed resistance to many insecticides that have been have been used against it have contributed to its success as a major pest (Sparks, 1981; Fitt, 1989; King, 1994; Kanga *et al.*, 1995; McCaffery, 1998; Ottea *et al.*, 2000; Blanco *et al.*, 2009).

2.3.1.2 Host range

Heliothis virescens has an extensive host range that spans more than 14 taxonomically diverse families of dicots including Solanaceae, Malvaceaem, Asteraceae, Fabaceae and Geraniaceae, infesting more than 19 crops, including the principal crops previously mentioned (section 2.3.1.1), and observed feeding on at least 80 wild host plants (Neunzig, 1969; Waldvogel and Gould, 1990; King, 1994; Capinera, 2001; Blanco *et al.*, 2007). Host selection is thought to be determined through the females strong preference for flowering plants, responding to a number of host plant features during selection, for example, volatiles, leaf surface chemicals, nectar, surface texture and plant height (Fitt, 1990).

2.3.1.3 Biology and ecology

Egg development ranges between 2 - 5 days, and at 25 °C is usually 3 - 4 days. Infertile eggs remain white, before becoming increasingly yellow and cone shaped as they desiccate (Neunzig, 1969; King, 1994).

The number of larval instars can vary from five to seven, although five or six instars are most common. Studies on larval development time calculated that at 25 °C larvae

that develop through six instars required 3.1, 2.0, 1.9, 2.1, 5.7 and 2.5 days for 1st to 6th instar respectively, equating to approximately 17 days for completion of larval development (Fye and McAda, 1972; King, 1994).

On emergence, the 1st instar larvae usually consume some or all of the empty egg shell before feeding on the preferred plant tissues. In cotton, larvae cause damage by feeding on the flower buds and bolls, with later instar larvae hollowing them out, if abscission has not already occurred. In the absence of buds and bolls, larvae will feed on flowers and leaves (King, 1994; Bommireddy and Leonard, 2008). Later instar larvae base colour is variable (e.g. pale green, pinkish, dark red) and determined by various factors including temperature and food substrate (King, 1994). When larval development is complete, they drop or crawl to the ground and enter the soil to pupate (Neunzig, 1969; King, 1994).

The pupal period is approximately 22 days at 20 °C, 13 days at 25 °C and 11 days at 30 °C (Fye and McAda, 1972). Facultative diapause is induced by environmental factors affecting earlier life stages, such as low temperatures and short day length (Henneberry, 1994; King, 1994).

Adults are stout-bodied, light brownish olive in colour with a wingspan of 28 – 35 mm. The fore wings have three oblique darker bands, with adjacent whitish borders, while the hind wings are pearly white with a dark band along the outer margin (less distinct in males) (King, 1994). Adults are generally active after dark (King, 1994). Females have been found to lay over 1500 eggs in the laboratory, with a mean of 963 eggs laid at 25 °C (Fye and McAda, 1972). Females lay eggs singly on many parts of the plant, but mostly near flowering and fruiting parts or growing points of the host (King, 1994).

2.3.1.4 Control of *Heliothis virescens*

The status of *H. virescens* as a major cotton pest in the USA has led to intense selection with a range of chemical insecticides resulting in the development of resistance in the field to many of those chemical insecticides (Sparks, 1981; Fitt, 1989; Sparks *et al.*, 1993; King, 1994; Kanga *et al.*, 1995; McCaffery, 1998; Ottea *et*

al., 2000; Blanco *et al.*, 2009). Mechanisms of resistance include reduced target site sensitivity, and metabolic detoxification systems (McCaffery, 1998; Huang and Ottea, 2004).

Heliothis virescens control problems first appeared in the late 1950s with reduced efficacy to the organochlorines, DDT and toxaphene, with confirmed resistance in the 1960s (Sparks, 1981; Sparks *et al.*, 1993). The switch to organophosphorous and carbamate insecticides in the 1960s initially provided adequate control of *H. virescens*, however, resistance appeared across the cotton belt in the 1970s and 1980s to these insecticide classes, including methyl parathion, monocrotophos, EPN and methomyl (Sparks, 1981; Sparks *et al.*, 1993). Synthetic pyrethroids were introduced in 1978 to replace the resistant prone and environmentally unsuitable organochlorines, organophosphates and cyclodienes. Significant resistance appeared from the mid 1980s onwards (Sparks *et al.*, 1993). Resistance often occurred through the complex interrelationship of genetic, environmental and management factors. This prompted the development of insect IRM strategies with the aim of either preventing the development of resistance or to contain it. Since the mid 1990s commercialised insecticides effective against *H. virescens* include indoxacarb, (Wing *et al.*, 2000), spinosad, (Salgado, 1998; Scott, 2008), pyridayl (Sakamoto *et al.*, 2002; Cook *et al.*, 2005) and diamides (Temple *et al.*, 2009).

Bt cotton gives effective control of *H. virescens* and chemical insecticides are generally not required for control of this species (Blanco *et al.*, 2007; Catchot *et al.*, 2008; Temple *et al.*, 2009). However, control of *H. virescens* in non-*Bt* cotton, for example in the sprayable refuge areas, and for other crop systems (e.g. tobacco), may still be needed using pyrethroids, organophosphates or carbamates that are still effective, along with spinosad, indoxacarb and diamides (Catchot *et al.*, 2008; Bacheler and Van Duyn, 2009; Burrack, 2009).

Cultural control can affect *H. virescens* populations through post- and pre-season tillage, reducing successful overwintering of pupae either by destroying them or disrupting the adult exit holes. Use of short season cultivars or ensuring early maturity to avoid late season infestations and management of border vegetation

through mowing to reduce available host plants are further practices carried out to avoid pest population build up (King, 1994; Catchot *et al.*, 2008).

Heliothis virescens has numerous natural enemies belonging to the orders Hymenoptera, Coleoptera, Hemiptera, Neuroptera, and Araneida (King and Coleman, 1989). Intensive production systems for biological control have had limited use, for example, hymenopteran parasitoid species (King and Coleman, 1989; King, 1994) and the use of nuclear polyhedrosis virus (NPV) (Bell and Romine, 1980; Bell and Hayes, 1994; King, 1994) due to inconsistent results and proving to be less cost effective in controlling *H. virescens* as chemical insecticides. However, through IRM and IPM practices naturally occurring biological control is encouraged as an additional method to control or prevent outbreaks (Catchot *et al.*, 2008; Fitt, 2008).

Chapter 3

General Materials and Methods

3.1 Artificial diet

Heliothis virescens larvae were reared on a standard artificial diet mix (Syngenta, Jealott's Hill, U.K.; Table 3.1).

Table 3.1: Artificial diet ingredients, and quantities for 400 larvae to develop through to pupae.

Ingredients	Source	Quantity
Agar	Sigma-Aldrich[1]: A7002	30 g
Distilled water		1360 ml
Pinto beans	Bio-Serv[2]: G1430	166 g
Wheat germ	Bio-Serv: G1659	133 g
Yeast	Sunshine Health, Glos., UK	85 g
Soybean flour type 1	Sigma-Aldrich: S9633	67 g
Casein from bovine milk	Sigma-Aldrich: C7078	49 g
Vitamin mixture	Bio-Serv: F8095	13.3 g
Ascorbic acid	Sigma-Aldrich: A7506	8 g
Methyl Paraben	Sigma-Aldrich: H5501	6.6 g
Sorbic acid	Sigma-Aldrich: S1626	4 g
Ampicillin sodium salt	Sigma-Aldrich: A9518	0.28 g
Distilled water		900 ml

[1] Sigma-Aldrich Ltd (Gillingham, UK); [2] Bio-Serv (New Jersey, USA)

The agar was added to 1360 ml distilled water, stirred and heated in a microwave until the agar had dissolved and the solution became clear and started to boil. In a separate container the remaining ingredients, excluding the ampicillin sodium salt, were added together and mixed thoroughly with 900 ml distilled water. The agar solution was

added to the diet mixture and stirred until a smooth even consistency was achieved. The ampicillin was mixed with 500 µl distilled water to dissolve it and added to the diet mixture. After stirring the mixture further, the diet was poured into 32-cell rearing trays (2mil PET 27HT-1 32 cells 6" x 11", Oliver Products Company, Michigan, USA), approximately 5 ml per cell, and left to cool and solidify ready for addition of larvae. The amount of diet in each cell was sufficient for completion of larval development to pupae.

3.2 *Heliothis virescens*

3.2.1 Populations

In December 2004, a field population designated GS04, was obtained from Professor J. R. Bradley (North Carolina State University) and Dr Ryan Jackson (USDA) that had been collected from flue-cured tobacco in Wilson and Pitt Counties, North Carolina, USA in June 2004.

In October 2005, three field populations were obtained from Prof Randall Luttrell and Dr Ibrahim Ali (University of Arkansas) that had been collected in June 2005. These had been designated F114 (Louisiana State), F123 (Appling County, Georgia) and F128 (Teautler County, Georgia).

Four field populations were obtained in September 2006 from the USA. Three hundred 3rd to 5th instar larvae were collected from chick pea, *Cicer arietinum*, at the University of Arkansas Research Station, Washington County, Arkansas and designated FV06. Approximately 900 eggs were collected from cutback tobacco, *Nicotiana tabacum*, in the vicinity of Clayton, Johnston County, North Carolina and designated JN06. Two hundred and ten 3rd to 5th instar larvae were collected from *C. arietinum* in the vicinity of the USDA, near Leland, Washington County, Mississippi and designated LEL06. Three hundred 3rd to 5th instar larvae were collected from velvetleaf, *Abutilon theophrasti*, on Wildy Farms, Leachville, Mississippi County, Arkansas and designated WF06.

In October 2007, a field population, designated 9607VR, was obtained from Prof Randall Luttrell and Dr Ibrahim Ali (University of Arkansas) that had been collected from *A. theophrasti* on Wildy Farms, Leachville, Mississippi County, Arkansas in September 2007.

A susceptible laboratory population was obtained from Syngenta (Jealott's Hill, Bracknell, U.K.) designated NCSU (originated from North Carolina State University laboratory population). NCSU had been maintained in the laboratory for over 140 generations without exposure to insecticides.

The populations were maintained in controlled environment (CE) rooms at 25 ± 2 °C, 65 ± 10 % relative humidity (RH) with a 16:8 (light:dark) cycle. Populations were on occasion placed at 20 ± 2 °C, 65 ± 10 % RH with a 16:8 (light:dark) cycle to slow down the population in order to synchronise development times or stages.

3.2.2 Culture maintenance

Adults were kept in large open plastic containers (L34 cm x W27 cm x D22 cm) each with a net cover held tightly in place across the top of the container with an elastic band creating a cage environment. Adults were fed sufficiently with 10 % (v/v) honey solution soaked into cotton wool pads that were placed on top of the netting. The placement of honey soaked cotton pads on top of the netting helped encourage mated females to lay eggs on the netting as opposed to the base and sides of the plastic container. The egg laden netting was removed and replaced every 24 or 48 h, and repeated until egg production decreased or no further eggs were required. The collected egg laden netting was examined for presence of hatched larvae and any found were removed as they may have dispersed from other populations in the same CE room. The netting was cut into smaller squares and placed in sealed (with a plastic lid) 250 ml round plastic containers and left in the CE room until larval emergence. With the artificial diet prepared (section 3.1), a single larva was placed in each cell of the rearing trays using a fine brush (size: 00). Larvae were separated to avoid competition and cannibalism. The trays were sealed with a breathable polyester film (10mp/2mil PET) using a self actuating tray lidder (Model #1708), which were

both products from Oliver Products Company (Michigan, USA). The larvae were left undisturbed to complete their development and pupae were collected after 21 to 24 days, allowing enough time for the majority of larva to have developed to pupae. Any remaining larvae were either allowed to continue to pupation or destroyed depending on whether population number requirements had been met. Between 240 and 360 pupae was considered adequate to continue the population to avoid potential low egg production, poor adult eclosion and genetic bottlenecks, although for some generations the pupae number collected was unavoidably lower. For each population the pupae were split into groups of approximately 120 pupae with each group being placed into a separate cage (as described previously for adult setup) to avoid overcrowding and improve population hygiene. Emerging healthy adults from these groups were transferred to a new cage within their group allocation ready for mating and egg laying.

Hygiene protocols were observed to maintain healthy cultures, including removal of waste material promptly and freezing before disposal, cleaning equipment with 1 % (w/v) Virkon solution and rinsing with distilled water. Laboratory and CE room surfaces were cleaned with 10 % (v/v) bleach solution.

3.3 *Bacillus thuringiensis* toxins

Vip3A was obtained from Syngenta (Research Triangle Park, NC, USA) and stored at -80 °C. The Vip3A protoxin had been overexpressed in *Escherichia coli* and purified as described by Yu *et al.* (1997).

Cry1Ab and Cry1Ac were obtained from Dr Neil Crickmore and Dr Ali Sayyed (University of Sussex, UK) and stored at -80 ºC. They had been expressed as crystalline inclusions in *E. coli*, with the protoxins purified by sonication and successive washes with 0.5 M NaCl and water as described by Sayyed *et al.* (2000b).

DiPel® WP (*Btk*) containing Cry1Ab, Cry1Ac, Cry1Aa, Cry2Aa and Cry2Ab (16000 IU mg^{-1} of wettable powder) was obtained from Abbott Laboratories (Chicago, USA) and stored at room temperature.

Toxins were freshly prepared in distilled water in readiness for diet incorporation.

3.4 Bioassays

Bioassays were conducted by the diet incorporation method where the toxin preparation is mixed into the molten diet as described by Dulmage *et al.* (1971), Beegle (1990) and Liao *et al.* (2002). The advantages to this method include the even distribution of the toxin throughout the diet, and thus closely reflecting expression of the toxin in *Bt* crops, and that it is a simple process to carry out. Although disadvantages are that it is labour and equipment intensive and diet can not be setup in advance and stored. Another common method is the surface contamination assay (diet overlay/surface application), where the toxin is applied to the surface of already solidified diet preparations (Beegle, 1990; Blanco *et al.*, 2008). This has the advantage of being quick and easy to setup and diet preparations can be stored for future use. However, the disadvantages make the surface contamination method less effective, with relatively uneven toxin distribution on the surface, and the potential for larvae to avoid toxin exposure depending on their feeding behaviour. Larvae that feed on the surface of the diet will be exposed to more toxin than those that burrow beneath the surface of the toxin (Beegle, 1990).

Heliothis virescens in the field, from 2nd to 3rd instar stages onwards, will tend to burrow into buds, if available, and this behaviour is noted on artificial diet in the laboratory. Therefore, the diet incorporation method is most suitable as toxin is evenly distributed throughout the diet, thereby maximising exposure of the toxin to the feeding behaviour of *H. virescens* larvae.

3.4.1 1st and 2nd instar larvae

Bioassays were conducted with 5 - 9 toxin concentrations (prepared through serial dilutions with distilled water), plus a control of distilled water only, with 48 larvae per concentration (split into 2 - 4 replicates). Each test concentration used 100 ml of artificial diet solution that included the toxin solution. The artificial diet preparation (section 3.1) was similar to that used in culture maintenance except for the exclusion of ampicillin and a 10 % reduction in distilled water content. The water content was reduced to allow the addition of the toxin solution to the diet preparation at a ratio of 1:9 (toxin:diet). The artificial diet was also allowed to cool to a temperature range of 40 - 45 °C before addition of the toxin solution to avoid high temperatures degrading the toxin.

For each toxin concentration, a 10 ml toxin solution (10 ml distilled water for control setup) was prepared at a strength equivalent to that necessary for a 100 ml solution. The 10 ml toxin solution was added to a 250 ml glass beaker, along with 90 ml of artificial diet solution and mixed thoroughly with a glass stirring rod. With Vip3A bioassay serial dilution concentrations starting above 1000 µg ml^{-1} the distilled water content was reduced by 20 % instead of 10 %, leaving the toxin:diet ratio at 2:8 to ensure enough distilled water was mixed with the Vip3A toxin for complete solubilisation. The diet incorporated toxin solution was poured evenly into 48 wells (approximately 1.5 - 2 ml per well) of 24-well bioassay plates (BD Falcon™ culture plate, Becton Dickinson Labware) and allowed to solidify and cool to room temperature. The volume of diet in each well was sufficient for survival of control larvae until the bioassay was stopped. One 1st instar larva that had hatched less than 24 h before setup was transferred using a fine brush to each well. For 2nd instar larvae setup, larvae had been reared from emergence to 2nd instar, usually taking 4 days, on artificial diet prepared without ampicillin and were transferred to the wells using a fine brush. Second instar larvae were identified by their head capsule width, that was between 0.36 and 0.53 mm (Neunzig, 1969). Breathable polyester film was cut to size and used to cover the wells and heat sealed using an iron. The bioassay plates were placed in a CE room at 25 ± 2 °C, 65 ± 10 % RH and a 16:8 (light:dark) cycle. Mortality and moult inhibition were determined after seven days, with mortality recorded as larvae that failed to respond to gentle contact with a fine brush,

and moult inhibition recorded as larvae that failed to moult to the next larval instar including dead larvae.

3.4.2 3rd and 4th instar larvae

This method was similar to that for the 1st and 2nd instar larvae with the following few exceptions. The artificial diet incorporated toxin solution for each concentration was increased from 100 ml to 200 ml (20 ml toxin solution + 180 ml diet solution), with approximately 3 - 4 ml per well. The bioassay plates were replaced by 32-well rearing trays (24 wells used) heat sealed with breathable polyester film. Larvae had been reared from emergence to 3rd or 4th instar, usually taking six and eight days respectively, on artificial diet prepared without ampicillin and were transferred to the wells using soft forceps. Third and 4th instar larvae were identified by their head capsule width, which was between 0.72 and 0.85 mm for 3rd instars, and between 1.12 and 1.25 mm for 4th instars (Neunzig, 1969). Some of these changes were made to accommodate larger larvae size and increased diet consumption of later instar larvae. Mortality and moult inhibition were determined after seven days.

3.5 Statistical analysis

Statistical package R version 2.8.1 (R Development Core Team, 2009) was used for all data analysis, such as analysis of variance (ANOVA) and generalised linear models with poisson or binomial errors.

Bioassay data used to estimate lethal concentration (LC) estimates (mortality) and moult inhibiting concentration (MIC) estimates (mortality plus 1st instar larvae failing to moult to 2nd instars) was corrected for control mortality by removing the number of dead or moult inhibited larvae in the control data from the corresponding number at each concentration and from the sample number at each concentration. Abbott's (1925) correction for control mortality was not used as it is incompatible with analysis of generalised linear models with binomial errors used in R.

The LC and MIC bioassay data was analysed by specifying a generalised linear model with binomial errors (or quasibinomial if data was overdispersed) to estimate the slope and its standard error, with significance tested at the 5 % level. A function called "dose.p" from the MASS library that used logit regession analysis calculated estimated LC_{50} (concentration that kills 50 % of the population) and MIC_{50} (concentration that moult inhibits 50 % of the population) values and their standard errors (se). Using these standard error values the 95 % Confidence Intervals (CI; $LC_{50} \pm [1.96 \times se]$; $MIC_{50} \pm [1.96 \times se]$) were calculated. Pairwise comparisons of LC_{50} values were significant at the 1 % level if their respective 95 % CI's did not overlap (Crawley, 2007).

Details of further statistics are given in relevant chapters.

Chapter 4

Susceptibility of *Heliothis virescens* field and laboratory populations to the *Bt* toxins Vip3A, Cry1Ab and Cry1Ac

4.1 Introduction

Variation in susceptibility to *Bt* toxins to different populations both within and between species may be caused by genetic differences and/or differences in bioassay methodology, such as type of bioassay (e.g. leaf dip, diet incorporation bioassay), time length of exposure, temperature of bioassay and the source of toxin (e.g. purified protoxin, activated toxin, formulations) (González-Cabrera *et al.*, 2001; Ali *et al.*, 2006; Saeglitz *et al.*, 2006).

It is important to be able to compare the susceptibilities of field-derived populations using the same methodology, so any variation in susceptibility can be attributed to genetic differences, such as through natural geographical variability or through selection for resistance. Establishing a baseline response to pesticides and reference laboratory populations is important for resistance management, allowing the re-evaluation of susceptibility at later time periods to indicate any shift in susceptibility (Luttrell *et al.*, 1999; Liao *et al.*, 2002; Blanco *et al.*, 2005; Ali *et al.*, 2006; Saeglitz *et al.*, 2006; Ali and Luttrell, 2007; Blanco *et al.*, 2008).

The objective of the present work was to establish the susceptibility of *H. virescens* field and laboratory populations to Vip3A, Cry1Ab and Cry1Ac; toxins which are already, or are planned to be, expressed in commercial *Bt* cotton varieties. Establishing a baseline for Vip3A susceptibility was also required for laboratory selection studies with Vip3A using field-derived populations of *H. virescens* (see Chapter 6). The relative toxicity of Vip3A and Cry1Ab to different larval instars of *H. virescens* was also determined. Such information may be useful in estimating

survival rates when expression levels of *Bt* toxins are sub-optimal for controlling neonate larvae.

4.2 Materials and methods

4.2.1 Populations

See section 3.2.1

4.2.2 Toxicity bioassays for *Bt* toxins against 1st instar larvae of laboratory and field populations

Diet incorporated bioassays (section 3.4.1) with Vip3A, Cry1Ab and Cry1Ac were conducted with 1st instar larvae after population establishment in the laboratory. Bioassays for populations GS04, FV06, JN06, LEL06, WF06 and 9607VR had all been carried out by the 5th generation since collection from the field. Bioassays for populations F114, F123 and F128 were not carried out until 11 generations had passed since field collection and classified as laboratory populations.

Not all bioassays produced enough data points to calculate MIC_{50} as higher concentrations resulted in 100 % moult inhibition and some lower concentrations had the same moult inhibition as the control.

4.2.3 Toxicity of Vip3A and Cry1Ab against different larval instars of NCSU and WF06 populations

Diet incorporated bioassays (sections 3.4.1 and 3.4.2) with Vip3A and Cry1Ab were conducted with 1st, 2nd, 3rd and 4th instar larvae with the established laboratory population, NCSU, and with Vip3A for the field-derived WF06 population. Mortality and moult inhibition was recorded after seven days.

4.2.4 Statistical analysis

Bioassay data was analysed to estimate LC_{50} and MIC_{50} values as described in section 3.5.

4.3 Results

4.3.1 Comparative toxicity of *Bt* toxins against 1st instar larvae of laboratory and field populations

Vip3A LC_{50} values (Table 4.1) for laboratory populations ranged from 0.58 to 2.75 µg ml^{-1} compared with 1.22 to 2.95 µg ml^{-1} for the six field populations representing up to 5-fold variation in susceptibility across all populations. While there were some significant differences in the LC_{50} values ($P<0.01$), all the LC_{50} values except F123 at 0.58 µg ml^{-1}, were within a range representing less than 3-fold variation in susceptibility. The mean of all the LC_{50} values was 2.03 µg ml^{-1}. There was no significant difference ($P>0.05$, 59 d.f., n=72) between the regression slopes for any populations.

Vip3A MIC_{50} (moult inhibition concentration) values (Table 4.2) ranged from NCSU at 0.18µg ml^{-1} to LEL06 at 1.21 µg ml^{-1}, representing up to a 7-fold variation in susceptibility across all populations. The LC_{50} for 9607VR was significantly different and lower than the remaining populations except for NCSU ($P<0.01$). There were no other significant differences in LC_{50} values ($P>0.01$). The mean of all the MIC_{50} values was 0.84 µg ml^{-1}. The regression slopes for NCSU and JN06 were significantly lower than the remaining populations ($P<0.05$, 18 d.f., n=30).

Table 4.1: LC_{50} values for laboratory and field colonies of 1st instar *Heliothis virescens* to Vip3A in a diet incorporation assay for 7 days at 25 ºC.

Population[1]	Gen[2]	LC_{50} (µg ml^{-1})	95 % CI[3]	Slope (± se)	n[4]
F123	11	0.58	0.13 – 2.58 abc	0.64 (± 0.16)	384
NCSU	LS	1.69	0.98 – 2.92 abc	0.52 (± 0.10)	336
F114	11	2.15	1.09 – 4.21 abc	0.78 (± 0.14)	384
GS04	19	2.75	1.82 – 4.16 bc	1.40 (± 0.30)	240
F128	11	2.75	1.47 – 5.16 abc	0.80 (± 0.14)	336
GS04	5	1.22	0.83 – 1.81 a	0.81 (± 0.11)	336
9607VR	3	1.39	1.04 – 1.85 ab	1.21 (± 0.13)	336
LEL06	1	2.06	1.46 – 2.91 abc	1.13 (± 0.17)	288
WF06	1	2.06	1.46 – 2.90 abc	1.00 (± 0.11)	384
FV06	1	2.69	1.93 – 3.75 c	0.94 (± 0.10)	384
JN06	2	2.95	2.03 – 4.30 c	0.81 (± 0.10)	336

[1] NCSU – North Carolina State University laboratory population; GS04 – Wilson and Pitt Counties, North Carolina, 2004; F114 – Louisiana, 2005; F123 – Appling County, Georgia, 2005; F128 – Teautler County, Georgia, 2005; FV06 – Washington County, Arkansas, 2006; LEL06 – Washington County, Mississippi, 2006; WF06– Mississippi County, Arkansas, 2006; JN06 – Johnston County, North Carolina, 2006; 9607VR – Mississippi County, Arkansas, 2007.

[2] Generation number since collection from the field; LS – Lab. population > 140 generations.

[3] Values followed by same letter are not significantly different from each other - overlapping 95 % CI (P>0.01).

[4] Number of larvae used in bioassay, including control.

Table 4.2: MIC_{50} (moult inhibition concentration) values for laboratory and field colonies of 1st instar *Heliothis virescens* to Vip3A in a diet incorporation assay for 7 days at 25 ºC.

Population[1]	Gen[2]	MIC_{50} (µg ml^{-1})	95 % CI[3]	Slope (± se)	n[4]
NCSU	LS	0.18	0.04 – 0.96 ab	0.38 (± 0.12)	336
9607VR	3	0.48	0.37 – 0.63 b	1.44 (± 0.18)	288
JN06	2	1.02	0.67 – 1.56 a	0.84 (± 0.11)	336
FV06	1	1.07	0.84 – 1.37 a	1.65 (± 0.23)	240
WF06	1	1.07	0.84 – 1.35 a	1.72 (± 0.21)	288
LEL06	1	1.21	0.91 – 1.60 a	1.61 (± 0.25)	240

[1] See Table 4.1.

[2] Generation number since collection from the field; LS – Lab. population > 140 generations.

[3] Values followed by same letter are not significantly different from each other (P>0.01).

[4] Number of larvae used in bioassay, including control.

Cry1Ab LC_{50} values (Table 4.3) ranged from 0.032 to 0.080 µg ml^{-1} for laboratory populations compared with 0.024 to 0.116 µg ml^{-1} for field populations. While there were some significant differences in the LC_{50} values (P<0.01), they were all within a range representing less than 5-fold variation in susceptibility. The mean of all the LC_{50} values was 0.060 µg ml^{-1}. There was no significant difference (P>0.05, 63 d.f., n=77) in the regression slopes between populations.

Cry1Ab MIC_{50} values (Table 4.4) for laboratory populations ranged from 0.008 to 0.039 µg ml^{-1} compared with 0.004 to 0.050 µg ml^{-1} for the field populations representing up to 13-fold variation in susceptibility between all populations. While there were some significant differences in LC_{50} values (P<0.01), all the LC_{50} values except NCSU at 0.039 µg ml^{-1} and 9607VR at 0.050 µg ml^{-1}, were within a range representing less than 4-fold variation in susceptibility. The mean of all the MIC_{50}

values was 0.017 µg ml^{-1} and excluding NCSU and 9607VR was 0.009 µg ml^{-1}. There was no significant difference in the regression slopes (P>0.05, 23 d.f., n=43).

Table 4.3: LC_{50} values for laboratory and field colonies of 1st instar *Heliothis virescens* to Cry1Ab in a diet incorporation assay for 7 days at 25 ºC.

Population[1]	Gen[2]	LC_{50} (µg ml^{-1})	95 % CI[3]	Slope (± se)	n[4]
F114	11	0.032	0.020 – 0.054 ab	0.70 (± 0.10)	336
GS04	19	0.033	0.020 – 0.053 ab	0.75 (± 0.08)	336
F123	11	0.045	0.021 – 0.100 abc	0.51 (± 0.11)	192
F128	11	0.076	0.049 – 0.117 bc	0.65 (± 0.07)	384
NCSU	LS	0.080	0.058 – 0.110 c	1.04 (± 0.13)	288
WF06	1	0.024	0.017 – 0.037 a	0.76 (± 0.08)	432
LEL06	1	0.032	0.023 – 0.046 a	0.86 (± 0.08)	432
FV06	2	0.034	0.022 – 0.054 ab	0.65 (± 0.08)	336
JN06	2	0.086	0.057 – 0.131 c	0.73 (± 0.08)	336
GS04	6	0.103	0.072 – 0.148 c	1.04 (± 0.14)	288
9607VR	2	0.116	0.078 – 0.172 c	0.73 (± 0.09)	336

[1] See Table 4.1.

[2] Generation number since collection from the field; LS – Lab. population > 140 generations.

[3] Values followed by same letter are not significantly different from each other (P>0.01).

[4] Number of larvae used in bioassay, including control.

Table 4.4: MIC_{50} values for laboratory and field colonies of 1st instar *Heliothis virescens* to Cry1Ab in a diet incorporation assay for 7 days at 25 ºC.

Population[1]	Gen[2]	MIC_{50} (µg ml^{-1})	95 % CI[3]	Slope (± se)	n[4]
GS04	19	0.008	0.005 – 0.012 ab	0.81 (± 0.12)	240
F114	11	0.010	0.007 – 0.014 ab	1.33 (± 0.18)	240
F128	11	0.014	0.010 – 0.021 bc	1.15 (± 0.17)	240
NCSU	LS	0.039	0.030 – 0.050 d	1.57 (± 0.19)	288
FV06	2	0.004	0.002 – 0.009 a	0.79 (± 0.17)	240
WF06	1	0.009	0.007 – 0.013 ab	1.18 (± 0.18)	240
LEL06	1	0.010	0.008 – 0.013 ab	1.64 (± 0.24)	240
JN06	2	0.011	0.007 – 0.018 abc	0.77 (± 0.14)	240
9607VR	2	0.050	0.035 – 0.072 d	0.87 (± 0.10)	336

[1] See Table 4.1.

[2] Generation number since collection from the field; LS – Lab. population > 140 generations.

[3] Values followed by same letter are not significantly different from each other (P>0.01).

[4] Number of larvae used in bioassay, including control.

Cry1Ac LC_{50} values (Table 4.5) for the laboratory populations ranged from 0.022 to 0.143 µg ml^{-1}, and the field populations ranged from 0.024 to 0.142 µg ml^{-1}, representing up to a 7-fold variation in susceptibility across all populations. The LC_{50} values of GS04, NCSU, LEL06, JN06 and WF06 were significantly lower than F114, F123, F128 and 9607VR (P<0.01). There were no other significant differences between the populations (P>0.01). The mean of all the LC_{50} values was 0.078 µg ml^{-1}. The regression slope for NCSU was significantly higher than the remaining populations (P<0.05, 40 d.f., n=66).

Table 4.5: LC_{50} values for laboratory and field colonies of 1st instar *Heliothis virescens* to Cry1Ac in a diet incorporation assay for 7 days at 25 ºC.

Population[1]	Gen[2]	LC_{50} (µg ml^{-1})	95 % CI[3]	Slope (± se)	n[4]
GS04	20	0.022	0.016 – 0.030 a	1.07 (± 0.13)	288
NCSU	LS	0.050	0.038 – 0.066 a	1.34 (± 0.17)	336
F114	11	0.114	0.077 – 0.170 b	0.81 (± 0.10)	336
F123	11	0.132	0.074 – 0.235 b	0.43 (± 0.06)	384
F128	11	0.143	0.079 – 0.260 b	0.46 (± 0.06)	384
LEL06	1	0.024	0.014 – 0.044 a	0.50 (± 0.06)	432
JN06	2	0.035	0.021 – 0.059 a	0.56 (± 0.08)	336
WF06	3	0.047	0.033 – 0.068 a	0.94 (± 0.12)	336
GS04	6	0.048	0.035 – 0.067 a	0.83 (± 0.09)	192
FV06	2	0.069	0.041 – 0.117 ab	0.54 (± 0.07)	336
9607VR	2	0.142	0.094 – 0.214 b	0.75 (± 0.10)	336

[1] See Table 4.1.

[2] Generation number since collection from the field; LS – Lab. population > 140 generations.

[3] Values followed by same letter are not significantly different from each other (P>0.01).

[4] Number of larvae used in bioassay, including control.

Cry1Ac MIC_{50} (Table 4.6) values for the laboratory populations ranged from 0.004 to 0.029 µg ml^{-1}, and the field populations ranged from 0.004 to 0.069 µg ml^{-1}, representing up to 17-fold variation in susceptibility between all populations. While there were some significant differences in the LC_{50} values (P<0.01), all the LC_{50} values, except for NCSU and 9607VR, were within a range representing less than 5-fold variation in susceptibility. The mean of all the MIC_{50} values was 0.017 µg ml^{-1}

and, excluding NCSU and 9607VR was 0.009 µg ml^{-1}. The regression slope for NCSU was significantly higher than the remaining populations excluding GS04 ($P<0.05$, 22 d.f., n=41). There were no other significant differences in the regression slopes between populations ($P>0.05$).

Table 4.6: MIC_{50} values for laboratory and field colonies of 1st instar *Heliothis virescens* to Cry1Ac in a diet incorporation assay for 7 days at 25 ºC.

Population[1]	Gen[2]	MIC_{50} (µg ml^{-1})	95 % CI[3]	Slope (± se)	n[4]
F128	11	0.004	0.003 – 0.008 a	0.79 (± 0.12)	240
F123	11	0.007	0.005 – 0.011 a	0.96 (± 0.14)	288
GS04	20	0.009	0.007 – 0.011 a	1.75 (± 0.32)	192
F114	11	0.011	0.006 – 0.019 ab	0.79 (± 0.12)	192
NCSU	LS	0.029	0.022 – 0.037 c	1.84 (± 0.27)	192
LEL06	1	0.004	0.002 – 0.008 a	0.88 (± 0.19)	240
JN06	2	0.008	0.004 – 0.014 ab	0.66 (± 0.12)	288
FV06	2	0.009	0.005 – 0.014 ab	0.80 (± 0.13)	288
WF06	3	0.019	0.013 – 0.027 bc	0.97 (± 0.15)	240
9607VR	2	0.069	0.043 – 0.109 d	0.71 (± 0.11)	288

[1] See Table 4.1.

[2] Generation number since collection from the field; LS – Lab. population > 140 generations.

[3] Values followed by same letter are not significantly different from each other ($P>0.01$).

[4] Number of larvae used in bioassay, including control.

The toxicity of *Bt* toxins Cry1Ab and Cry1Ac to 1st instar larvae were very similar, but they both had greater toxicity to 1st instar larvae when compared to Vip3A based

on the LC_{50} values. Cry1Ab toxicity ranged from 5- to 123-fold greater and Cry1Ac toxicity ranged from 4- to 134-fold greater than Vip3A toxicity. A similar pattern occurred when comparing the MIC_{50} values. Cry1Ab moult inhibition ranged from 4- to 303-fold greater, and Cry1Ac moult inhibition ranged from 3- to 303-fold greater than Vip3A moult inhibition.

4.3.2 Comparative toxicity of Vip3A and Cry1Ab against different larval instars of the NCSU population

Vip3A LC_{50} values for 1st, 2nd and 3rd instar larvae (1.69, 1.77 and 1.5 µg ml^{-1} respectively) of the NCSU population were not significantly different from each other ($P>0.01$; Figure 4.1). The LC_{50} value for 4th instar larvae (5.11 µg ml^{-1}) was significantly greater compared with 1st, 2nd and 3rd instar larvae ($P<0.01$).

Vip3A MIC_{50} value for 1st instar larvae (0.18 µg ml^{-1}) was significantly lower than 2nd, 3rd and 4th instar larvae (1.77, 1.44 and 3.41 µg ml^{-1} respectively) of the NCSU population ($P<0.01$; Figure 4.1). The MIC_{50} values for 2nd and 3rd instar larvae were not significantly different from each other ($P>0.01$), but were significantly lower than 4th instar larvae ($P<0.01$). The 1st instar MIC_{50} value was significantly lower than the LC_{50} value ($P<0.01$). The MIC_{50} value for 2nd instar larvae was exactly the same as the LC_{50} as no surviving larvae were moult inhibited. The MIC_{50} values for 3rd and 4th instar larvae were lower than their respective LC_{50} values but were not significantly different ($P>0.01$).

Cry1Ab LC_{50} values for 1st, 2nd, 3rd and 4th instar larvae (0.085, 0.071, 0.059 and 0.092 µg ml^{-1} respectively) of the NCSU population were not significantly different ($P>0.01$; Figure 4.2).

Cry1Ab MIC_{50} values for 1st, 2nd, 3rd and 4th instar larvae (0.039, 0.029, 0.032 and 0.030 µg ml^{-1} respectively) of the NCSU population were not significantly different from each other ($P>0.01$; Figure 4.2). MIC_{50} values for 1st and 4th instar larvae were significantly lower than their respective LC_{50} values ($P<0.01$). There were no significant differences between the respective MIC_{50} and LC_{50} values for 2nd and 3rd instar larvae ($P>0.01$).

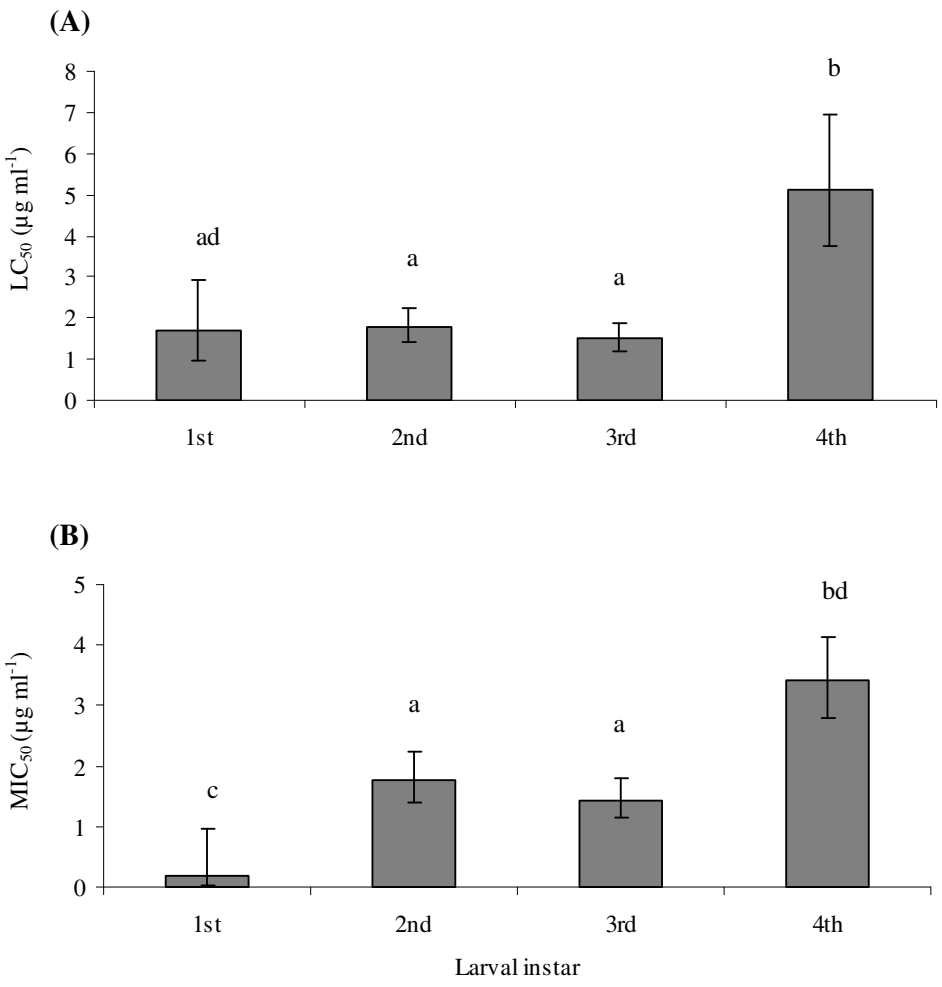

Figure 4.1: Toxicity of Vip3A to larval stages of the susceptible North Carolina State University (NCSU) *H. virescens* laboratory population: (A) LC_{50} (± 95 % CI); (B) MIC_{50} (± 95 % CI). Values with same lower case letter are not significantly different from each other (P>0.01), (see also Appendix 1, Tables A.1.1 and A.1.2).

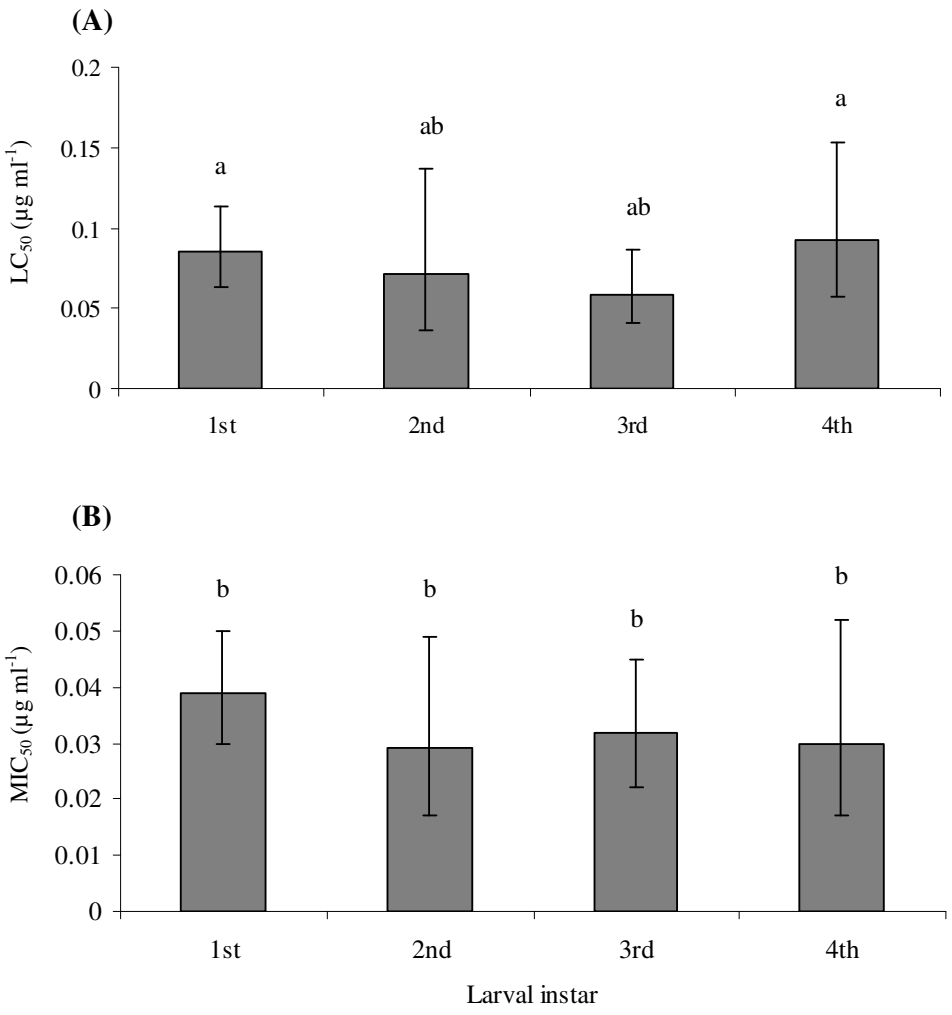

Figure 4.2: Toxicity of Cry1Ab to larval stages of the susceptible North Carolina State University (NCSU) *H. virescens* laboratory population: (A) LC_{50} (± 95 % CI); (B) MIC_{50} (± 95 % CI). Values with same lower case letter are not significantly different from each other (P>0.01), (see also Appendix 1, Tables A.1.3 and A.1.4).

4.3.3 Comparative toxicity of Vip3A against different larval instars of the WF06 population

The LC_{50} values for 1st, 2nd and 3rd instar larvae of WF06 UNSEL were not significantly different, 2.63, 2.91 and 3.76 µg ml^{-1} respectively (P>0.01) (Figure 4.3). However, for 4th instar larvae the LC_{50} was not determined as at the highest bioassay concentration of 100 µg ml^{-1}, the mortality was only 13 %.

The MIC_{50} value for 1st instar larvae of WF06 UNSEL, 0.90 µg ml^{-1}, was significantly lower than the respective LC_{50} value and significantly lower than the MIC_{50} values of 2nd (2.07 µg ml^{-1}), 3rd (1.88 µg ml^{-1}) and 4th (9.21 µg ml^{-1}) instar larvae of WF06 UNSEL ($P<0.01$). There was no significant difference between the respective MIC_{50} and LC_{50} values for 2nd and 3rd instar larvae ($P>0.01$). The MIC_{50} value for 4th instar larvae was significantly higher than 1st, 2nd and 3rd instar larvae ($P<0.01$).

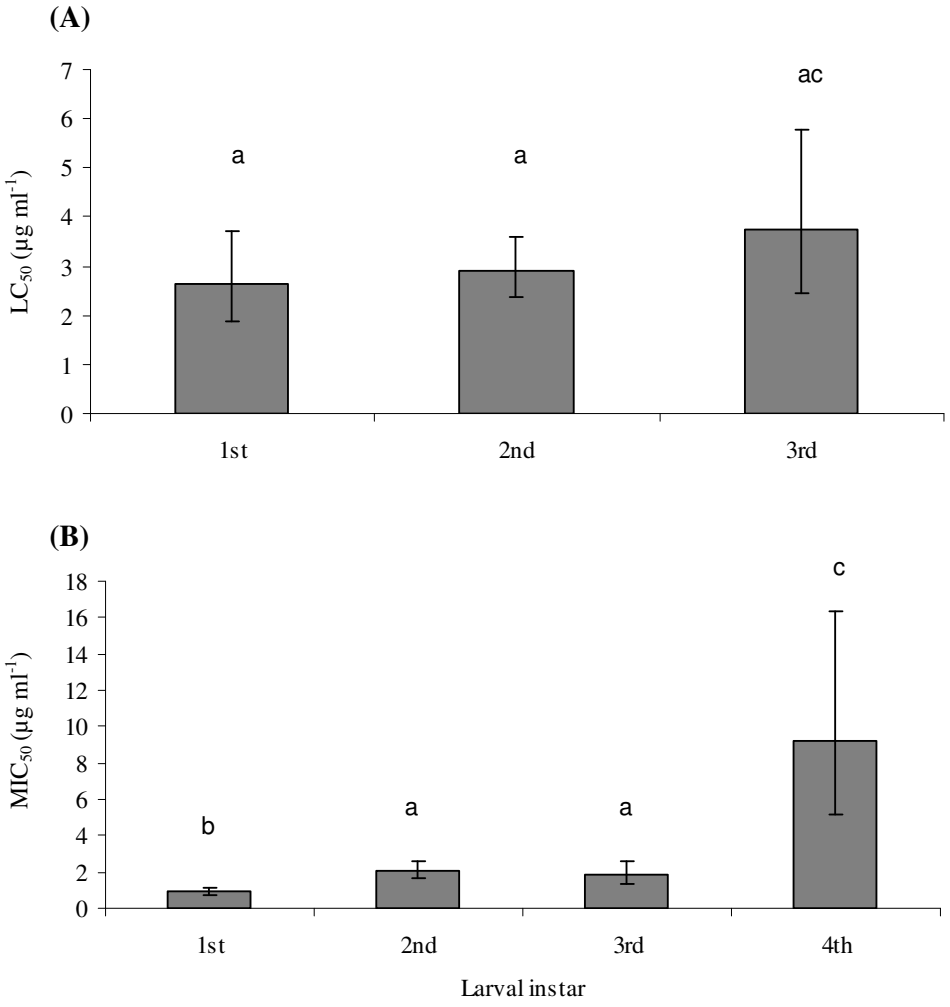

Figure 4.3: Toxicity of Vip3A to larval stages of the WF06 UNSEL *H. virescens* population: (A) LC_{50} (± 95 % CI); (B) MIC_{50} (± 95 % CI). Values with same lower case letter are not significantly different from each other ($P>0.01$), (see also Appendix 1, Tables A.1.5 and A.1.6.)

4.4 Discussion

Previous laboratory studies with Vip3A or Vip3-related toxins have not compared variation in susceptibility, only presenting data on the toxicity range to various lepidopteran larvae (Estruch *et al.*, 1996; Yu *et al.*, 1997; Selvapandiyan *et al.*, 2001; Lee *et al.*, 2003; Fang *et al.*, 2007). In the present study, there was generally little variability in the susceptibility to Vip3A, Cry1Ab and Cry1Ac in the field-derived and laboratory populations of *H. virescens* examined. Variation within each toxin for both LC_{50} and MIC_{50} values was less than 7-fold. The exceptions were the laboratory population NCSU and field-derived population 9607VR that had higher MIC_{50} values for Cry1Ab and Cry1Ac resulting in up to 13- and 17-fold susceptibility compared to the remaining populations. MIC_{50} values were on average 2- to 5-fold lower than the LC_{50} values for Vip3A, Cry1Ab and Cry1Ac demonstrating that not all surviving larvae successfully developed on the diet. None of the data suggested that resistance to Vip3A or Cry1Ab / Cry1Ac was present in the populations examined. The apparent low variability in susceptibility may also have been due in part as a result of these populations becoming established as laboratory cultures. With limited individuals taken from the field, the populations may have gone through genetic bottlenecks, even with a single generation (Table 4.1), possibly eliminating rare genes or genes that may have imposed fitness costs in the field that have no impact in the laboratory, and *vice versa*. Population bottlenecks and small populations are expected to decrease genetic variance and reduced evolutionary potential (Willi *et al.*, 2006). The behaviour of the populations may have altered in the laboratory environment, for example, mating preferences or the use of artificial diet as opposed to natural food (e.g. cotton plants). These laboratory effects may have altered the response of individuals to the toxins.

Using moult inhibition data may present a more realistic view of the toxicity of Vip3A, Cry1Ab and Cry1Ac, as the vast majority of surviving 1st instar larvae fail to successfully develop on artificial diet (personal observation) and such data can help to highlight antifeedant effects and/or other sub lethal effects of the toxin. Gore *et al.* (2005) found that *H. virescens* showed some avoidance of Cry1Ac and Cry2Ab in bioassays in choice experiments, selectively feeding on non-toxin treated diet

compared with Cry1Ac treated diet, although avoidance was less prominent with Cry2Ab.

Moult inhibition data is useful provided identification of the instar stage is determined accurately, and not just based on larval size. Head capsule width is the most accurate way of determining instar stage, although it has been argued that this can take too much time for use in bioassays (Blanco *et al.*, 2005). However, with practice, consistent identification of larval instars can be achieved through observation of head capsule size by eye rather than the need to measure all individuals (personal experience). Head capsule size is fairly distinct when looking at reference larvae for all stages, particularly for 1st to 4th larval instars.

The variation in susceptibility to *Bt* toxins determined for the populations in the present study appears to be typical for *H. virescens* when compared to other work. Jackson *et al.*, (2007) found that susceptibility to Vip3A in a laboratory insecticide susceptible population of *H. virescens* (YDK) and three Cry1Ac resistant populations (YHD2, KCBhyb and CXC) were similar with LC_{50} values ranging from 179 µg ml^{-1} to 210 µg ml^{-1}. This indicated that these Cry1Ac resistant populations did not show any signs of cross-resistance to Vip3A. The Vip3A LC_{50} values reported in the above work are approximately 100-fold greater than those calculated in the present study (based on a mean Vip3A LC_{50} of 2 µg ml^{-1}). This difference may, in part, be because the Vip3A was prepared in distilled water in the present study rather than in an ammonium carbonate buffer as in Jackson *et al.* (2007). This illustrates the potential dangers of direct comparisons made on absolute values gathered from differing bioassay methodology as opposed to relative comparisons to respective reference populations (González-Cabrera *et al.*, 2001; Liao *et al.*, 2002; Ali *et al.*, 2006).

Using a diet incorporation assay, Ali *et al.* (2006) found that *H. virescens* varied 12-fold in susceptibility to Cry1Ac (MPV II formulation) (LC_{50} 0.36 µg ml^{-1} to 4.54 µg ml^{-1}) in five laboratory, seven laboratory-cross and 10 field populations collected from 2002 to 2004. The difference was only 4-fold across all laboratory and field populations when LC_{50} values were pooled, demonstrating little change in relative differences compared with populations collected in 1992 and 1993 (LC_{50} 0.02 µg ml^{-1} to 0.13 µg ml^{-1}) (Luttrell *et al.*, 1999; Ali *et al.*, 2006). Similarly low (6-fold)

variability in toxicity of Cry1Ab to laboratory and field populations of *H. virescens* was reported by Luttrell *et al.* (1999).

Susceptibility of *H. virescens* populations to other *Bt* toxins has also demonstrated a similar pattern of low natural variability. Blanco *et al.* (2008) found a 3-fold variation in baseline LC_{50} values for Cry1F using a diet overlay method, while Ali and Luttrell (2007) found only a 2-fold variation in mean LC_{50} values for Cry2Ab2 across tested laboratory and field populations collected from 2002 to 2005 using a diet incorporation method.

In the related heliothine species *H. zea* the pattern of susceptibility has been reported to be more varied. Ali and Luttrell (2007) found *H. zea* LC_{50} values for Cry2Ab2 varied up to 37-fold in eight laboratory, 10 laboratory-cross and 64 field populations collected from 2002 to 2005, and only 3-fold across all laboratory and field populations based on mean LC_{50} values. *Helicoverpa zea* susceptibility to Cry1Ac (MPV II formulation) varied over 500-fold in five laboratory, nine laboratory-cross and 57 field populations collected from 2002 to 2004 and relative susceptibility appeared to have decreased in comparison with earlier collections in 1992 and 1993 (Luttrell *et al.*, 1999; Ali *et al.*, 2006). This reduced susceptibility has led to the suggestion that *H. zea* has evolved field resistance to Cry1Ac (*Bt* cotton), although there have been no reports of widespread control failures or changes in efficacy of *Bt* cotton (Tabashnik *et al.*, 2008a). There has also been some discussion among researchers over whether this conclusion meets the classification of field-evolved resistance (Moar *et al.*, 2008; Tabashnik *et al.*, 2008b).

Analysis of published monitoring data for five other major lepidopteran pests targeted by *Bt* crops concluded that field-evolved resistance had not occurred to Cry1Ac (*Bt* cotton) in *H. armigera*, *H. virescens* and *P. gossypiella* and not to Cry1Ab (*Bt* corn) in *O. nubilalis*, and *S. nonagrioides* (Tabashnik *et al.*, 2008a).

In the present study, the LC_{50} and MIC_{50} values for Cry1Ab and Cry1Ac were similar. Vip3A toxicity was up to 134- and 300-fold lower compared with Cry1A toxins based on LC_{50} and MIC_{50} values respectively. The lower toxicity for Vip3A could be due to a lower saturation of functional toxin-binding sites, or to the assembly of pores and/or

the flux through pores may differ from that of Cry1A toxins (Lee *et al.*, 2003; Jackson *et al.*, 2007). Jackson *et al.* (2007) reported that *H. virescens* Vip3A toxicity was on average 110-fold lower than that of Cry1Ac in a laboratory susceptible population (YDK) using the diet incorporation method. The lower toxicity of Vip3A may also be a result of their lack of specificity to gut cells, due to the prevalent nature of their production during vegetative growth (section 2.1.3). In contrast, the *H. armigera* ANGR laboratory population using a surface application bioassay method found that Vip3A and Cry1Ac toxicities were not significantly different and Cry1Ab was less toxic (Liao *et al.*, 2002).

Cry1Ac and Vip3A expression levels in *Bt* cotton have been shown to decrease in various plant parts throughout the growing season, potentially reducing efficacy to target pests (Greenplate, 1999; Adamczyk *et al.*, 2001b; Olsen *et al.*, 2005; Llewellyn *et al.*, 2007), and Cry1Ac expression was found to be greater in terminal foliage than in fruiting structures (Greenplate, 1999). Observed differences in *H. virescens* larval survival among *Bt* cotton expressing Vip3A could be due to protein expression variation in different plant structures and although they would not likely survive to pupation, larval feeding may cause economic damage (Bommireddy and Leonard, 2008). Information on the effect of larval stage of *H. virescens* on susceptibility to Vip3A and Cry1Ab may thus help to predict the possible impact of declining expression of *Bt* toxins in the plant with age and/or variation in expression of *Bt* toxins in the plant on the survival of *H. virescens* larvae.

In the present study, the LC_{50} values for Vip3A were found to be similar against 1st, 2nd and 3rd instar larvae of the NCSU and the field-derived population WF06 of *H. virescens*. Fourth instar larvae of the NCSU population had approximately 3-fold reduced susceptibility while for WF06, the tolerance of the 4th instar larvae to Vip3A was at least 27-fold greater than the earlier instars. MIC_{50} estimates showed that there was greater inhibition of 1st instar development compared with later instars in both populations. Thus, larval age effects on susceptibility to Vip3A indicated that higher concentrations are needed against 4th instar larvae, and that there is greater inhibition of 1st instar development compared with later instars.

First, 2nd, 3rd and 4th instar larvae of the NCSU population all had similar susceptibilities to Cry1Ab based on LC_{50} and MIC_{50} values. Only 1st and 4th instar larvae showed significant effects due to moult inhibition. Thus, larval age had little effect on susceptibility to Cry1Ab indicating that the dose of toxin doesn't have to be greater to kill or inhibit development of later instar larvae.

As larval age increases so does diet consumption, and although the toxicity of Vip3A and Cry1Ab were similar between some larval instars, differences in tolerance were present as later instar larvae consumed more diet than earlier instars (personal observation). This increased tolerance with 4^{th} instar larvae may be a result of increased effectiveness of cell-mending or a more effective immune response in later instar larvae (Loeb *et al.*, 2001; Heckel *et al.*, 2007). Difference in tolerance demonstrates that later instar larvae would potentially cause more damage in a field crop situation (Liao *et al.*, 2002).

Chapter 5

Selection of a Vip3A resistant population of *Heliothis virescens* with studies on cross-resistance and the genetics of resistance

5.1 Introduction

Selecting for resistance in field-derived populations in the laboratory has produced many resistant populations (section 2.2.2). The production of laboratory selected resistant populations enables the study of the genetics of resistance (section 2.2.4) gaining knowledge of what potentially may occur in field populations and how to delay potential field resistance with suitable IRM strategies (section 2.2.5).

In this chapter the selection of resistance to Vip3A in a field-derived population of *H. virescens* is described, together with subsequent work on cross-resistance to Cry1Ab and Cry1Ac and crossing studies to investigate the inheritance of Vip3A resistance and whether resistance is monogenic or polygenic.

5.2 Materials and Methods

5.2.1 *Heliothis virescens* populations

Populations LEL06, FV06 and WF06 (section 3.2.1) were initially selected with Vip3A. After three generations this was reduced to the continuation of selection with WF06 only. The LEL06 and FV06 populations were stopped as their cultures were not as healthy, and limitations on resources including equipment, CE room space, and labour meant the reduction to continuation of one selected population.

For experiments investigating the genetics of resistance (section 5.2.7), the resistant WF06-Vip3ASEL population at generation 15, which had undergone 12 selections with Vip3A, and the unselected WF06-UNSEL population were used.

5.2.2 *Bacillus thuringiensis* toxin

Vip3A, Cry1Ab and Cry1Ac toxins were used (section 3.3).

5.2.3 Selection for resistance with Vip3A

The WF06 population was divided into two sub-populations at the larval stage of the 2nd generation of laboratory culture. One sub-population was left unselected (WF06-UNSEL) and the other selected with Vip3A (WF06-Vip3ASEL) at the 1st instar larval stage from the 2nd generation onwards.

The selection method incorporated the Vip3A toxin into the diet in a similar way to the diet incorporation bioassay method (section 3.4). The artificial diet preparation (section 3.1) was similar to that used in culture maintenance except for the exclusion of ampicillin and a reduction in distilled water content of 10 % (226 ml). The appropriate Vip3A concentration for 2260 ml of artificial diet was made up in the 226 ml of distilled water. Once the artificial diet solution had cooled to between 40 and 45 °C the Vip3A solution was added and mixed vigorously into the diet. After mixing, the diet was poured into 32-cell rearing trays with approximately 3 ml per cell and left to cool ready for addition of larvae. Larvae were setup on the diet as described in section 3.2.2. The number of larvae selected per generation ranged from approximately 600 to 1200; with the exception of the initial selection when the number of larvae (330) available was low. After seven days surviving larvae were removed from the diet and those that had moulted to 2nd instar or higher were used to continue the population to the next generation. Toxin free artificial diet (section 3.1) was setup and poured into 1 oz. plastic cups (9051, Bio-Serv, NJ, USA) with 5 ml diet per cup. A single larva was placed in each diet cup and sealed with a snap on lid (9053, Bio-Serv, NJ, USA) that had a single slit for aeration. Larvae were left in the cups for the remainder of their larval development. Surviving 1st instar larvae were also used if the number of successfully moulted larvae was low (less than 250) in an

attempt to maintain a large population size. Pupae collection, adult setup and egg collection methods were as described in section 3.2.2.

5.2.4 Evaluation of resistance in Vip3A selected (WF06-Vip3ASEL) and unselected (WF06-UNSEL) populations

First instar bioassays (section 3.4.1) with Vip3A were carried out throughout the selection process to determine changes in LC_{50} and MIC_{50} values and determine resistance ratios by comparing LC_{50} and MIC_{50} values to those of the WF06-UNSEL population.

5.2.5 Cross-resistance studies in the Vip3A selected (WF06-Vip3ASEL) population

First instar bioassays (section 3.4.1) were carried out on WF06-Vip3ASEL with Cry1Ab after 11, 13 and 14 selections with Vip3A and with Cry1Ac after 13 and 14 selections, to evaluate cross-resistance. LC_{50} and MIC_{50} values were determined and compared to WF06-UNSEL.

5.2.6 Stability of resistance

A sub-population of WF06-Vip3ASEL was setup at generation 13 that had undergone 11 selection episodes with Vip3A. The sub-population, WF06-Vip3AREV, was maintained continuously without selection and at generation 18, after five generations without exposure to Vip3A, 1st instar larvae bioassays (section 3.4.1) were carried out to establish LC_{50} and MIC_{50} values for Vip3A, Cry1Ab and Cry1Ac for comparison to WF06-UNSEL.

5.2.7 Evaluation of maternal/paternal effects, genetic variation, dominance of resistance and mode of inheritance in the Vip3A selected (WF06-Vip3ASEL) population

The response of F_1 and F_2 progeny to Vip3A was evaluated at concentrations of 100 µg ml^{-1} and 500 µg ml^{-1} of Vip3A using diet incorporated bioassays based on the

method described in section 3.4.1. The pupae from the WF06-UNSEL and WF06-Vip3ASEL populations were used in conjunction with those setup for fitness studies (Chapter 6). The pupal sex was determined with males and females placed separately in sealed 250 ml round plastic containers.

Pupal sex was determined by examining the genetalia and abdominal segment pattern at the posterior end under a microscope. Males were confirmed by observation of their genetalia; two joined circular shapes (gonads), at the partition of the 9th and 10th abdominal segments on the ventral surface of the pupae. Females were confirmed by the lack of gonads and the 'V' shaped pattern of the partitions between the 8th and 9th abdominal segments and the 9th and 10th abdominal segments, in the direction of the anterior end (personal communication, Syngenta, and personal observation).

Upon adult emergence, single-pair crosses were setup for WF06-Vip3ASEL (two pairs successful), WF06-UNSEL (five pairs successful) and the reciprocal crosses WF06-Vip3ASEL female x WF06-UNSEL male (seven pairs successful) and WF06-UNSEL female x WF06-Vip3ASEL male (five pairs successful). Each single-pair was setup in a 250 ml round plastic container covered with netting held in place by an elastic band. They were fed *ad lib* with 10 % (v/v) honey solution soaked into cotton wool pads that were placed on top of the netting allowing the adults to feed but preventing egg lay on the cotton pad. Once the female had started laying eggs, the adult pair was transferred to a new 250 ml container setup every two days, as eggs were laid on the container itself as well as the netting. The netting (if eggs were present) was placed inside the container and sealed with a plastic lid. Hatching usually occurred 3 - 4 days after being laid. F_1 progeny from each of the single-pair reciprocal crosses were split between use for bioassays and use for continuation in culture of the specific family line for the F_2 progeny evaluation. F_1 progeny of the WF06-Vip3ASEL and WF06-UNSEL populations were required for bioassays only. For bioassay setup, the F_1 larvae from each family were reared on artificial diet containing Vip3A concentrations of 100 µg ml^{-1} and 500 µg ml^{-1}, or with no Vip3A as a control setup. Four replicates of 12 1st instar larvae (48 in total) for each single-pair cross at each concentration were carried out. The bioassays were kept in a CE room at 25 °C ± 2 °C, 65 ± 10 % RH and 16:8 (light:dark) cycle. Mortality and moult inhibition was determined after seven days and used to determine the genetic variation

within the populations, maternal or paternal influence and the dominance of resistance.

The progeny used for family line continuation from the reciprocal crosses were reared through to pupae and split into males and females that were kept in separate cages for each individual family. A group of pupae from the WF06-Vip3ASEL population was also split into males and females. Emerging adults were setup in single-pair crosses with F_1 adult males backcrossed to WF06-Vip3ASEL females and *vice versa*, along with within family F_2 crosses as shown in Table 5.1. Some families and single-pair crosses within families were unsuccessful through lack of viable eggs, as observed in the variation in the number of successful families and single-pair crosses setup in Table 5.1. The single-pair setup was the same as mentioned previously, as were the Vip3A treatments (0 (control), 100 and 500 µg ml^{-1}). Mortality and moult inhibition was determined after seven days and used to determine the genetic variation within the populations and the mode of inheritance (monogenic or polygenic).

Table 5.1: Backcrosses and F_2 crosses setup for evaluation of response to Vip3A.

Backcross and F_2 crosses	No.[1]	pairs[2]
F_1 (WF06-Vip3ASEL ♀ x WF06-UNSEL ♂) ♀ x WF06-Vip3ASEL ♂	4	14
F_1 (WF06-Vip3ASEL ♀ x WF06-UNSEL ♂) ♂ x WF06-Vip3ASEL ♀	4	16
F_1 (WF06-Vip3ASEL ♀ x WF06-UNSEL ♂) ♂ x ♀	4	16
F_1 (WF06-UNSEL ♀ x WF06-Vip3ASEL ♂) ♀ x WF06-Vip3ASEL ♂	3	11
F_1 (WF06-UNSEL ♀ x WF06-Vip3ASEL ♂) ♂ x WF06-Vip3ASEL ♀	2	8
F_1 (WF06-UNSEL ♀ x WF06-Vip3ASEL ♂) ♂ x ♀	2	8

[1] Number of families setup.

[2] Number of single-pairs in total for each cross type (2 – 5 single-pairs per family).

5.2.8 Statistical analysis

Bioassay data with multiple concentrations was analysed to estimate the LC_{50} and MIC_{50} as described in section 3.5.

The degree of dominance (h) was estimated using the single concentration method, based on Hartl's (1992) definition of dominance and on survival at any single concentration (Liu and Tabashnik, 1997). The calculation is as follows:

$$h = (w_{12} - w_{22}) / (w_{11} - w_{22})$$

where w_{11}, w_{12} and w_{22} are the fitness values at a particular concentration for resistant homozygotes, heterozygotes and susceptible homozygotes, respectively. The fitness of treated resistant homozygotes (WF06-Vip3ASEL) is defined as 1. The fitness for treated susceptible homozygotes (WF06-UNSEL) was determined as the survival rate of treated WF06-UNSEL larvae divided by the survival rate of treated WF06-Vip3ASEL larvae. For treated heterozygotes (WF06-Vip3ASEL x WF06-UNSEL), the fitness was determined as the survival rate of treated F_1 larvae divided by the survival rate of treated WF06-Vip3ASEL larvae. Mortality and moult inhibition was corrected for control mortality using Abbott's (1925) method. The survival rate was estimated as 100 % - mortality %. Values of h range from 0 (completely recessive) to 1 (completely dominant) (Liu and Tabashnik, 1997).

The genetic variation within WF06-UNSEL, WF06-Vip3ASEL, F_1 reciprocal crosses, backcrosses and F_2 crosses was determined using analysis of variance (ANOVA) to test for significant variation in mortality and moult inhibition among families produced by the single-pair crosses. Percentage mortality and moult inhibited data were arcsine transformed prior to ANOVA.

The backcross data was used as a direct test of a monogenic model of resistance (Tabashnik, 1991). The null hypothesis is that resistance is controlled by one locus with two alleles (monogenic resistance), S (susceptible) and R (resistant), with the parental resistant population RR, and the F_1 offspring RS. If so, then a backcross of F_1 (WF06-Vip3ASEL x WF06-UNSEL) RS x WF06-Vip3ASEL RR will produce progeny that are 50 % RR and 50 % RS. This hypothesis is tested through calculation of the expected mortality, followed by a χ^2 test for goodness of fit between the expected and observed mortality and moult inhibition of the backcross data at each concentration. The expected mortality or moult inhibition, $Y_{(x)}$, for the backcross progeny at concentration (x) is calculated as:

$Y_{(x)} = 0.50(W_{RS} + W_{RR})$,

where W_{RS} and W_{RR} are the mortality or moult inhibition values of the presumed RS (F_1) and RR (resistant parental line: WF06-Vip3ASEL) geneotypes at concentration (x), respectively (Tabashnik, 1991; Wyss *et al.*, 2003). The χ^2 test for goodness of fit between the backcross and expected mortality or moult inhibition is calculated, as described by Sokal and Rohlf (1995), as:

$\chi^2 = (F_1 - pn)^2 / pqn$,

where F_1 is the observed number dead in the backcross generation at concentration (x), p is the expected proportion dead calculated as $Y_{(x)}$, n is the number of backcross progeny exposed to concentration (x) and $q = 1 - p$. The χ^2 value is compared with the χ^2 distribution with one degree of freedom, and if $P<0.05$ the null hypothesis of monogenic resistance is rejected (Tabashnik, 1991; Wyss *et al.*, 2003).

5.3 Results

5.3.1 Selection with Vip3A

The average survival rate to pupation of Vip3A selected larvae was 42 % (Table 5.2). The Vip3A concentrations remained constant at 2 µg ml^{-1} from the 3rd generation to the 9th generation, but were increased from the 10th generation (selection nine) onwards as resistance increased dramatically. No selection took place at generation 15 and 16, as bioassay results indicated that LC_{50} values were unattainable as even high concentrations failed to kill sufficient larvae. A relaxation in selection aimed to reduce the level of resistance to allow the calculation of the LC_{50}.

5.3.2 Response to selection with Vip3A in the WF06 population

The LC_{50} values (Table 5.3) for the WF06 unselected population (WF06-UNSEL) remained relatively unchanged. The only exception being at generation seven where

there was around a 2- to 3-fold decrease in the LC_{50}, however, there was also a decrease in the LC_{50} for the WF06 selected population (WF06-Vip3ASEL) at the same generation (time). The LC_{50} values for WF06-Vip3ASEL indicated a large increase in the resistance ratio after 13 selection episodes. There were reversing fluctuations in the LC_{50} estimates with the LC_{50} at selection nine (516 µg ml^{-1}) approximately 2-fold greater than at selection 10 (246 µg ml^{-1}). After 12 selections the LC_{50} was undetermined as the mortality at the highest bioassay concentration, 4000 µg ml^{-1}, was only 21 %. Therefore, the LC_{50} could only be assumed to be greater than 4000 µg ml^{-1}. No Vip3A selection occurred at generations 15 and 16 in an attempt to lower the LC_{50} so that it could be determined. This proved successful as at generation 17, after 13 selection episodes, the LC_{50} was 2300 µg ml^{-1} with a resistance ratio of 2040.

The MIC_{50} values for WF06-UNSEL ranged from 0.1 to 1.07 µg ml^{-1}, with CI's overlapping for the majority of values (Table 5.4). The MIC_{50} values for WF06-Vip3ASEL determined a 6-fold increase in resistance after 13 selection episodes. There were fluctuations in the MIC_{50} values during the selection process. The resistance ratio increased 7-fold after nine selection episodes before rising to 809 fold after 12 selections. The resistance ratio then decreased to 6-fold after 13 selection episodes, as Vip3A selection was relaxed to allow determination of the LC_{50} as previously mentioned (Table 5.4).

Table 5.2: Summary of selection experiment for Vip3A against WF06 population of *Heliothis virescens*.

Generation	Selection	No. of larvae selected	Vip3A concentration (µg ml^{-1})	No. of larvae transferred to normal diet[1]	No. of healthy pupae	Survival (%)[3]
2	1	330	1.5	140[2]	95	29
3	2	768	2	362	276	36
4	3	768	2	244	214	28
5	4	864	2	414	345	40
6	5	1120	2	430[2]	241	22
7	6	1184	2	368[2]	252	21
8	7	821	2	440	350	43
9	8	1161	2	624	532	46
10	9	1232	2.5	600	482	39
11	10	1152	3	745	542	47
12	11	663	4	534	422	64
13	12	800	20	395	305	38
14	13	768	20	594	478	54
15	-	-	-	403	307	76
16	-	-	-	576	386	67
17	14	715	20	480	380	53

[1] Only larvae that had developed to 2nd Instar or higher were transferred.
[2] Number of larvae included 1st instars as there was poor larval development.
[3] Survival rate of larvae setup to pupation.

Table 5.3: Toxicity of Vip3A against a field population of *Heliothis virescens* unselected (WF06-UNSEL) and selected (WF06-Vip3ASEL) in the laboratory.

Population	Gen [1]	Sel [2]	LC_{50} (µg ml^{-1})	95% CI	Slope (± se)	n [3]	RR [4]
WF06-	1	-	2.06	1.46 - 2.90	1.00 (±0.11)	384	-
UNSEL	7	-	0.73	0.43 - 1.24	0.80 (±0.17)	288	-
	11	-	2.47	1.65 - 3.69	0.81 (±0.16)	288	-
	12	-	2.63	1.87 - 3.70	0.82 (±0.10)	384	-
	15	-	1.76	1.14 – 2.72	0.56 (±0.07)	384	-
	18	-	1.13	0.72 - 1.75	0.66 (±0.08)	336	-
WF06-Vip3A	5	3	2.44	0.92 - 6.48	0.26 (±0.08)	336	1
SEL	7	5	2.02	1.19 - 3.43	0.47 (±0.11)	336	3
	9	7	38.1	7.39 - 197	0.48 (±0.14)	336	19
	11	9	516	111 - 2400	0.33 (±0.07)	384	209
	12	10	246	102 - 595	0.29 (±0.05)	480	94
	14	12	>4000 [5]				>2000
	17	13	2300	1010 - 5260	0.24 (±0.05)	432	2040

[1] Number of laboratory generations. WF06-UNSEL generations 15 and 18 were synchronised with WF06-Vip3ASEL generations 14 and 17.
[2] Number of selections with Vip3A.
[3] Number of larvae tested, including control.
[4] Resistance ratio (WF06-Vip3ASEL / WF06-UNSEL). RR for selections 3 and 7 were compared to WF06-UNSEL generation 1.
[5] LC_{50} undetermined as mortality at highest concentration of 4000 µg ml^{-1} was only 21 %.

Table 5.4: Moult inhibiting toxicity of Vip3A against a field population of *Heliothis virescens* unselected (WF06-UNSEL) and selected (WF06-Vip3ASEL) in the laboratory.

Population	Gen [1]	Sel [2]	MIC_{50} (µg ml^{-1})	95% CI	Slope (± se)	n [3]	RR [4]
WF06	1	-	1.07	0.84 - 1.35	1.72 (±0.21)	384	-
UNSEL	7	-	0.10	0.01 - 0.90	0.76 (±0.32)	240	-
	11	-	0.63	0.48 - 0.82	1.09 (±0.14)	336	-
	12	-	0.90	0.69 - 1.18	0.82 (±0.10)	336	-
	15	-	0.46	0.33 - 0.65	0.96 (±0.11)	384	-
	18	-	0.36	0.25 - 0.50	1.08 (±0.15)	336	-
WF06-Vip3A	5	3	0.67	0.39 - 1.16	0.76 (±0.14)	336	0.6
SEL	7	5	0.20	0.07 - 0.64	0.46 (±0.12)	336	2.0
	9	7	4.59	2.51 - 8.41	0.52 (±0.11)	336	4.3
	11	9	4.63	2.52 - 8.49	0.43 (±0.06)	384	7.4
	12	10	2.41	1.20 - 4.85	0.38 (±0.04)	480	2.7
	14	12	372	137 - 1012	0.22 (±0.04)	432	810
	17	13	2.02	0.39 - 10.6	0.21 (±0.05)	432	5.6

[1] Number of laboratory generations. WF06-UNSEL generations 15 and 18 were synchronised with WF06-Vip3ASEL generations 14 and 17.
[2] Number of selections with Vip3A.
[3] Number of larvae tested, including control.
[4] Resistance ratio (WF06-Vip3ASEL / WF06-UNSEL). RR for selections 3 and 7 were compared to WF06-UNSEL generation 1.

5.3.3 Stability of resistance in the Vip3A selected (WF06-Vip3ASEL) population

After five generations without exposure to Vip3A, the Vip3A LC_{50} for WF06-Vip3AREV, 709 µg ml^{-1}, was approximately 630-fold greater than and significantly different from WF06-UNSEL, 1.13 µg ml^{-1} ($P<0.01$) (Table 5.5). The MIC_{50} for WF06-Vip3AREV, 39.8 µg ml^{-1}, was also significantly greater (110-fold) than that for WF06-UNSEL, 0.36 µg ml^{-1} ($P<0.01$).

The Cry1Ab LC_{50} for WF06-Vip3AREV was not significantly different to that of WF06-UNSEL ($P>0.01$), but the MIC_{50} was significantly lower than WF06-UNSEL ($P<0.01$) (Tables 5.5 and 5.6).

There were no significant differences in either the Cry1Ac LC_{50} or MIC_{50} values when comparing WF06-Vip3AREV with WF06-UNSEL ($P>0.01$).

5.3.4 Cross-resistance to Cry1Ab and Cry1Ac in the Vip3A selected (WF06-Vip3ASEL) population

Cry1Ab LC_{50} values for WF06-Vip3ASEL after both 11 and 13 Vip3A selections, although respectively 2- and 3-fold greater, were not significantly different from the LC_{50} values of WF06-UNSEL ($P>0.01$). However, after 14 selections the Cry1Ab LC_{50} value for WF06-Vip3ASEL was 7-fold greater than that of WF06-UNSEL, a significant increase ($P<0.01$) (Table 5.5). The Cry1Ab MIC_{50} values for WF06-Vip3ASEL after 11, 13 and 14 Vip3A selections were all significantly lower than the respective WF06-UNSEL values ($P<0.01$) (Table 5.6).

There was a significant increase in the Cry1Ac LC_{50} value for WF06-Vip3ASEL after 13 selections with Vip3A, approximately 7-fold greater than that of WF06-UNSEL ($P<0.01$). However, the respective MIC_{50} values were similar with CIs determining no significant difference ($P>0.01$). After 14 selections there were no significant differences in either the LC_{50} or MIC_{50} values ($P>0.01$) (Tables 5.5 and 5.6).

Table 5.5: LC_{50} values of *Heliothis virescens* 1st instar larvae determining resistance and cross-resistance of Vip3A and Cry1Ab / Ac in the WF06-Vip3ASEL and WF06-Vip3A-REV.

Population	No. of generations [1]	Vip3A selections	Toxin	LC_{50} (µg ml^{-1})	95% CI	Slope (± se)	n [3]	RR [4]
WF06-UNSEL	14	-	Cry1Ab	1.20	0.74 - 1.94	0.68 (± 0.08)	384	-
WF06-Vip3ASEL	13	11	Cry1Ab	2.89	1.50 - 5.62	0.62 (± 0.10)	336	2.4
WF06-UNSEL	16	-	Cry1Ab	1.46	0.92 - 2.31	0.76 (± 0.10)	288	-
WF06-Vip3ASEL	15	13	Cry1Ab	4.63	1.89 - 11.3	0.40 (± 0.22)	288	3.2
WF06-UNSEL	19	-	Cry1Ab	2.49	1.84 - 3.38	1.17 (± 0.15)	288	-
WF06-Vip3ASEL	18	14	Cry1Ab	16.7	4.06 - 68.5	0.37 (± 0.09)	288	6.7
WF06-Vip3AREV	18 [2]	11	Cry1Ab	2.03	1.36 - 3.02	0.79 (± 0.10)	384	0.8
WF06-UNSEL	16	-	Cry1Ac	0.41	0.27 - 0.61	1.16 (± 0.16)	240	-
WF06-Vip3ASEL	15	13	Cry1Ac	2.95	0.91 - 9.58	0.59 (± 0.17)	336	7.1
WF06-UNSEL	19	-	Cry1Ac	1.67	1.06 - 2.63	0.87 (± 0.14)	288	-
WF06-Vip3ASEL	18	14	Cry1Ac	1.67	1.14 - 2.43	0.85 (± 0.10)	384	1
WF06-Vip3AREV	18 [2]	11	Cry1Ac	0.93	0.68 - 1.29	1.00 (± 0.11)	336	0.6
WF06-UNSEL	19	-	Vip3A	1.13	0.72 - 1.75	0.66 (± 0.08)	336	-
WF06-Vip3AREV	18 [2]	11	Vip3A	709	246 - 2040	0.27 (± 0.04)	480	627

[1] WF06-UNSEL generations 14, 16, 19 were respectively synchronised with WF06-Vip3ASEL generations 13, 15 and 18 and WF06-Vip3AREV gen 18.
[2] Absence of selection with Vip3A from generation 13 to 17 (5 continuous generations).
[3] Number of larvae tested, including control.
[4] Resistance ratio: WF06-Vip3ASEL LC_{50} / WF06-UNSEL LC_{50}; or WF06-Vip3AREV LC_{50} / WF06-UNSEL LC_{50}.

Table 5.6: MIC_{50} values of *Heliothis virescens* 1st instar larvae determining resistance and cross-resistance of Vip3A and Cry1Ab / Ac in the WF06-Vip3ASEL and WF06-Vip3A-REV.

Population	No. of generations [1]	Vip3A selections	Toxin	MIC_{50} (µg ml^{-1})	95% CI	Slope (± se)	n [3]	RR [4]
WF06-UNSEL	14	-	Cry1Ab	0.35	0.25 - 0.48	1.54 (± 0.33)	384	-
WF06-Vip3ASEL	13	11	Cry1Ab	0.15	0.11 - 0.20	1.35 (± 0.16)	288	0.4
WF06-UNSEL	16	-	Cry1Ab	0.49	0.36 - 0.66	1.19 (± 0.16)	288	-
WF06-Vip3ASEL	15	13	Cry1Ab	0.19	0.14 - 0.26	1.66 (± 0.36)	192	0.4
WF06-UNSEL	19	-	Cry1Ab	0.86	0.66 - 1.12	1.44 (± 0.20)	240	-
WF06-Vip3ASEL	18	14	Cry1Ab	0.36	0.25 - 0.45	1.13 (± 0.13)	336	0.4
WF06-Vip3AREV	18 [2]	11	Cry1Ab	0.20	0.15 - 0.25	1.44 (± 0.18)	288	0.2
WF06-UNSEL	16	-	Cry1Ac	0.25	0.17 - 0.35	1.29 (± 0.17)	240	-
WF06-Vip3ASEL	15	13	Cry1Ac	0.31	0.22 - 0.44	1.05 (± 0.25)	336	1.2
WF06-UNSEL	19	-	Cry1Ac	0.50	0.37 - 0.67	1.18 (± 0.15)	288	-
WF06-Vip3ASEL	18	14	Cry1Ac	0.43	0.32 - 0.59	1.07 (± 0.12)	336	0.9
WF06-Vip3AREV	18 [2]	11	Cry1Ac	0.30	0.23 - 0.38	1.71 (± 0.24)	240	0.6
WF06-UNSEL	19	-	Vip3A	0.36	0.25 - 0.50	1.08 (± 0.15)	336	-
WF06-Vip3AREV	18 [2]	11	Vip3A	39.8	19.0 - 83.4	0.28 (± 0.04)	480	110

[1] WF06-UNSEL generations 14, 16, 19 were respectively synchronised with WF06-Vip3ASEL generations 13, 15 and 18 and WF06-Vip3AREV gen 18.
[2] Absence of selection with Vip3A from generation 13 to 17 (5 continuous generations).
[3] Number of larvae tested, including control.
[4] Resistance ratio: WF06-Vip3ASEL LC_{50} / WF06-UNSEL LC_{50}; or WF06-Vip3AREV LC_{50} / WF06-UNSEL LC_{50}.

5.3.5 Degree of dominance

Bioassays of F_1 progeny from single-pair crosses with two concentrations of Vip3A showed that dominance of resistance depended upon the F_1 reciprocal cross, the analysis of mortality or moult inhibition data and the concentration of Vip3A (Tables 5.7 and 5.8). The mean dominance values of F_1 progeny from WF06-Vip3ASEL males x WF06-UNSEL females showed that the degree of dominance increased with an increase in Vip3A concentration. Resistance was incompletely recessive (mean h = 0.47) and incompletely dominant (mean h = 0.58) at 100 and 500 µg ml^{-1} respectively, based on larval mortality and resistance based on larval moult inhibition at 100 and 500 µg ml^{-1} was incompletely recessive (h = 0.22 and 0.37, respectively). In comparison, the mean dominance values of F_1 progeny from WF06-Vip3ASEL females x WF06-UNSEL males showed that resistance was incompletely recessive at 100 and 500 µg ml^{-1} for both mortality and moult inhibition data (Tables 5.7 and 5.8).

5.3.6 Evaluation of genetic variation within the populations by single-pair crosses

The mortality and moult inhibition with Vip3A of the progeny of F_1 families from crosses between WF06-Vip3ASEL and WF06-UNSEL (Tables 5.7 and 5.8) indicated that there were significant differences at three levels.

There were significant differences within the seven single-pair families at 100 µg ml^{-1} (mortality: $F_{6,17}$ = 44.51, P<0.001; moult inhibition: $F_{6, 17}$ = 9.11, P<0.001) and within the 11 single-pair families at 500 µg ml^{-1} (mortality: $F_{11, 32}$ = 5.98, P<0.001; moult inhibition: $F_{11, 32}$ = 9.43, P<0.001). There was a significant difference between the reciprocal crosses (WF06-Vip3ASEL female x WF06-UNSEL male and WF06-Vip3ASEL male x WF06-UNSEL female) at 100 µg ml^{-1} (mortality: $F_{1, 22}$ = 13.55, P<0.01; moult inhibition: $F_{1, 22}$ = 4.63, P<0.05) and 500 µg ml^{-1} (mortality: $F_{1, 42}$ = 24.67, P<0.001; moult inhibition: $F_{1, 42}$ = 25.44, P<0.001). There were significant differences within the WF06-Vip3ASEL female x WF06-UNSEL male cross at 100 µg ml^{-1} for the four single-pair crosses (mortality: $F_{3, 10}$ = 52.67, P<0.001; moult inhibition: $F_{3, 10}$ = 8.23, P<0.01) and at 500 µg ml^{-1} for the seven single-pair crosses (mortality: $F_{6, 19}$ = 4.58, P<0.05; moult inhibition: $F_{6, 19}$ = 12.70, P<0.001). Likewise, there were significant differences within the WF06-Vip3ASEL male x WF06-UNSEL

female cross at 100 µg ml^{-1} for the three single-pair crosses (mortality: $F_{2, 7} = 8.19$, $P<0.05$; moult inhibition: $F_{2, 7} = 8.90$, $P<0.05$). However, at 500 µg ml^{-1} there was no significant difference in the mortality or moult inhibition within the five single-pair crosses (mortality: $F_{4, 13} = 1.85$, $P>0.05$; moult inhibition: $F_{4, 13} = 2.53$, $P>0.05$).

There were significant differences in the mortality and moult inhibition with Vip3A of the progeny of F_2 families of F_1 (WF06-Vip3ASEL x WF06-UNSEL) x WF06-Vip3ASEL backcrosses and F_1 (WF06-Vip3ASEL x WF06-UNSEL) female x male crosses (Tables 5.9 and 5.10). There were significant differences within the 19 single-pair families at 100 µg ml^{-1} (mortality: $F_{18, 54} = 4.99$, $P<0.001$; moult inhibition: $F_{18, 54} = 3.76$, $P<0.001$) and at 500 µg ml^{-1} (mortality: $F_{18, 54} = 4.35$, $P<0.001$; moult inhibition: $F_{18, 54} = 2.4$, $P<0.01$). There were significant differences between the F_2 cross types, with the mean mortality at 100 µg ml^{-1} of F_1 (WF06-UNSEL female x WF06-Vip3ASEL male) female x F_1 (WF06-UNSEL female x WF06-Vip3ASEL male) male, and the WF06-Vip3ASEL female x F_1 (WF06-UNSEL female x WF06-Vip3ASEL male) male, 51 and 64 % respectively, significantly lower than the remaining F_2 cross types ($F_{5, 67} = 5.2$, $P<0.001$). The same trend was present with the mean mortality at 500 µg ml^{-1} for F_1 (WF06-UNSEL female x WF06-Vip3ASEL male) female x F_1 (WF06-UNSEL female x WF06-Vip3ASEL male) male, and the WF06-Vip3ASEL female x F_1 (WF06-UNSEL female x WF06-Vip3ASEL male) male, 49 and 60 % respectively, significantly lower than the remaining F_2 cross types ($F_{5, 67} = 6.97$, $P<0.001$). There were no significant differences in mean moult inhibition between the F_2 cross types at 100 µg ml^{-1} ($F_{5, 67} = 0.83$, $P>0.05$) and at 500 µg ml^{-1} ($F_{5, 67} = 1.52$, $P>0.05$).

5.3.7 Mode of inheritance in the Vip3A selected (WF06-Vip3ASEL) population

The direct test for a monogenic mode (single gene) of inheritance of Vip3A resistance showed significantly greater ($P<0.001$) observed mortality and moult inhibition than expected values at 100 µg ml^{-1} and 500 µg ml^{-1} of Vip3A for the backcross progeny F_1 (WF06-Vip3ASEL x WF06-UNSEL) and the WF06-Vip3ASEL (Tables 5.11, 5.12, 5.13 and 5.14). This indicated that the null hypothesis was rejected, thus a monogenic model was not an acceptable fit of the data at the concentrations tested.

Table 5.7: Dominance (*h*) of resistance to Vip3A in the WF06-Vip3ASEL *Heliothis virescens* population using mortality values as a function of the concentration of Vip3A for single-pair hybrid F_1families.

Population/families	Characteristics of larvae at Vip3A concentration					
	100 µg ml^{-1}			500 µg ml^{-1}		
	Mortality (%) [1]	Fitness [2]	*h* [3]	Mortality (%)	Fitness	*h*
Vip3ASEL	38	1.00		27	1.00	
UNSEL	90	0.16		89	0.16	
Single-pair F_1 families						
(Vip3ASEL ♀ x UNSEL ♂) A	100	0.00	0.00	81	0.26	0.12
(Vip3ASEL ♀ x UNSEL ♂) B	65	0.56	0.48	65	0.48	0.38
(Vip3ASEL ♀ x UNSEL ♂) C	-	-	-	94	0.08	0.00
(Vip3ASEL ♀ x UNSEL ♂) D	-	-	-	91	0.13	0.00
(Vip3ASEL ♀ x UNSEL ♂) E	100	0.00	0.00	96	0.06	0.00
(Vip3ASEL ♀ x UNSEL ♂) F	-	-	-	98	0.20	0.05
(Vip3ASEL ♀ x UNSEL ♂) G	77	0.37	0.25	67	0.46	0.36
(Vip3ASEL ♀ x UNSEL ♂) mean	**88**	**0.19**	**0.03**	**86**	**0.20**	**0.05**
(UNSEL ♀ x Vip3ASEL ♂) H	-		-	68	0.45	0.34
(UNSEL ♀ x Vip3ASEL ♂) I	65	0.56	0.47	28	0.99	0.98
(UNSEL ♀ x Vip3ASEL ♂) J	60	0.63	0.56	47	0.73	0.69
(UNSEL ♀ x Vip3ASEL ♂) K	-	-	-	67	0.46	0.36
(UNSEL ♀ x Vip3ASEL ♂) L	87	0.21	0.06	65	0.48	0.38
(UNSEL ♀ x Vip3ASEL ♂) mean	**66**	**0.55**	**0.47**	**53**	**0.65**	**0.58**

[1] Adjusted for control mortality by Abbott's (1925) method.

[2] Fitness is the survival rate of the larvae divided by the survival rate of the Vip3ASEL larvae (survival rate is estimated as 100 - % mortality) (section 5.2.8).

[3] Estimates of dominance range from 0 (completely recessive resistance) to 1 (completely dominant) (section 5.2.8).

Table 5.8: Dominance (*h*) of resistance to Vip3A in the WF06-Vip3ASEL *Heliothis virescens* population using moult inhibition (MI) values as a function of the concentration of Vip3A for single-pair hybrid F_1families with WF06-UNSEL.

Population/families	Characteristics of larvae at Vip3A concentration					
	100 µg ml^{-1}			500 µg ml^{-1}		
	MI (%) [1]	Fitness [2]	*h* [3]	MI (%)	Fitness	*h*
WF06-Vip3ASEL	42	1.00		31	1.00	
WF06-UNSEL	97	0.06		100	0.00	
Single-pair F_1 families						
(Vip3ASEL ♀ x UNSEL ♂) A	100	0.00	0.00	98	0.03	0.03
(Vip3ASEL ♀ x UNSEL ♂) B	79	0.36	0.32	79	0.30	0.30
(Vip3ASEL ♀ x UNSEL ♂) C	-	-	-	100	0.00	0.00
(Vip3ASEL ♀ x UNSEL ♂) D	-	-	-	98	0.03	0.03
(Vip3ASEL ♀ x UNSEL ♂) E	100	0.00	0.00	100	0.00	0.00
(Vip3ASEL ♀ x UNSEL ♂) F	-	-	-	100	0.00	0.00
(Vip3ASEL ♀ x UNSEL ♂) G	83	0.29	0.24	79	0.30	0.30
(Vip3ASEL ♀ x UNSEL ♂) mean	**92**	**0.14**	**0.08**	**94**	**0.08**	**0.08**
(UNSEL ♀ x Vip3ASEL ♂) H	-	-	-	78	0.32	0.32
(UNSEL ♀ x Vip3ASEL ♂) I	78	0.38	0.34	52	0.69	0.69
(UNSEL ♀ x Vip3ASEL ♂) J	88	0.20	0.15	88	0.17	0.16
(UNSEL ♀ x Vip3ASEL ♂) K	-	-	-	81	0.28	0.28
(UNSEL ♀ x Vip3ASEL ♂) L	96	0.08	0.02	78	0.31	0.31
(UNSEL ♀ x Vip3ASEL ♂) mean	**85**	**0.27**	**0.22**	**74**	**0.37**	**0.37**

[1] Adjusted for control moult inhibition by Abbott's (1925) method.

[2] Fitness is the survival rate of the larvae divided by the survival rate of the Vip3ASEL larvae (survival rate is estimated as 100 - % moult inhibition) (section 5.2.8).

[3] Estimates of dominance range from 0 (completely recessive resistance) to 1 (completely dominant) (section 5.2.8).

Table 5.9: Mortality and moult inhibition (MI) in WF06 population of *Heliothis virescens* larvae from single-pair F_2 families to Vip3A.

Backcross and F_2 family[2]	Vip3A concentration			
	100 µg ml^{-1}		500 µg ml^{-1}	
	Mortality (%)[1]	MI (%)[1]	Mortality (%)	MI (%)
F_1 progeny (Vip3ASEL ♀ x UNSEL ♂) backcrossed with Vip3ASEL population				
F_1 progeny ♀ (G) (n = 4)	89	94	90	92
F_1 progeny ♀ (E) (n = 2)	93	99	83	94
F_1 progeny ♀ (A) (n = 4)	75	91	73	90
F_1 progeny ♀ (F) (n = 4)	60	60	63	63
F_1 progeny ♀ mean	**79**	**85**	**78**	**84**
F_1 progeny ♂ (G) (n = 3)	79	97	80	97
F_1 progeny ♂ (E) (n = 4)	85	94	85	93
F_1 progeny ♂ (A) (n = 5)	75	84	77	88
F_1 progeny ♂ (F) (n = 4)	70	72	66	69
F_1 progeny ♂ mean	**78**	**90**	**78**	**90**
F_1 progeny (Vip3ASEL ♀ x UNSEL ♂) ♀ x ♂				
(G) (n = 4)	92	98	89	97
(E) (n = 4)	85	97	92	97
(A) (n = 4)	75	92	79	93
(F) (n = 4)	76	78	76	76
mean	**82**	**90**	**83**	**90**

[1] Adjusted for control mortality and moult inhibition by Abbott's (1925) method.

[2] Capital letters in brackets refer to the family connection in the F_1 crosses in Tables 5.7 and 5.8; n is the number of successful pairs setup for that family.

Table 5.10: Mortality and moult inhibition (MI) in WF06 population of *Heliothis virescens* larvae from single-pair F_2 families to Vip3A.

Backcross and F_2 family	Vip3A concentration			
	100 µg ml^{-1}		500 µg ml^{-1}	
	Mortality (%)[1]	MI (%)[1]	Mortality (%)	MI (%)
F_1 progeny (UNSEL ♀ x Vip3ASEL ♂) backcrossed with Vip3ASEL population				
F_1 progeny ♀ (J) (n = 5)	84	94	78	95
F_1 progeny ♀ (I) (n = 4)	90	98	81	96
F_1 progeny ♀ (H) (n = 2)	68	78	79	84
F_1 progeny ♀ mean	**83**	**93**	**79**	**93**
F_1 progeny ♂ (J) (n = 4)	78	93	67	93
F_1 progeny ♂ (I) (n = 4)	40	76	46	86
F_1 progeny ♂ mean	**64**	**86**	**60**	**91**
F_1 progeny (UNSEL ♀ x Vip3ASEL ♂) ♀ x ♂				
(J) (n = 4)	64	94	61	89
(I) (n = 4)	38	71	36	71
mean	**51**	**83**	**49**	**80**

[1] Adjusted for control mortality and moult inhibition by Abbott's (1925) method.

[2] Capital letters in brackets refer to the family connection in the F_1 crosses in Tables 5.7 and 5.8; n is the number of successful pairs setup for that family.

Table 5.11: Direct test of monogenic inheritance for resistance to Vip3A by comparing expected and observed mortality of the backcross of F_1 (Vip3ASEL x UNSEL) and Vip3ASEL population of *Heliothis virescens* at a Vip3A concentration of 100 µg ml^{-1}.

Single-pair matings	n[1]	Observed mortality (%)	Expected mortality (%)[2]	χ^2 (df=1)[3]	P
Vip3ASEL ♀ x ♂	96	44			
UNSEL ♀ x ♂	240	90			
Vip3ASEL ♀ x UNSEL ♂	168	89			
UNSEL ♀ x Vip3ASEL ♂	120	70			
F_1 (Vip3ASEL ♀ x UNSEL ♂) ♀ x Vip3ASEL ♂	733	80	67	64.41	<0.001
F_1 (UNSEL ♀ x Vip3ASEL ♂) ♀ x Vip3ASEL ♂	635	86	57	210.61	<0.001
F_1 (Vip3ASEL ♀ x UNSEL ♂) ♂ x Vip3ASEL ♀	834	79	67	62.07	<0.001
F_1 (UNSEL ♀ x Vip3ASEL ♂) ♂ x Vip3ASEL ♀	507	69	57	31.95	<0.001

[1] Number of larvae tested.

[2] Expected number of larvae dead at a given dose = 0.5 (observed mortality of F_1 larvae + observed mortality of Vip3ASEL) (section 5.2.8).

[3] df = degrees of freedom.

Table 5.12: Direct test of monogenic inheritance for resistance to Vip3A by comparing expected and observed moult inhibition (MI) of the backcross of F_1 (Vip3ASEL x UNSEL) and Vip3ASEL population of *Heliothis virescens* at a Vip3A concentration of 100 µg ml^{-1}.

Single-pair matings	n	Observed MI (%)	Expected MI (%)	χ^2 (df=1)	P
Vip3ASEL ♀ x ♂	96	48			
UNSEL ♀ x ♂	240	97			
Vip3ASEL ♀ x UNSEL ♂	168	93			
UNSEL ♀ x Vip3ASEL ♂	120	87			
F_1 (Vip3ASEL ♀ x UNSEL ♂) ♀ x Vip3ASEL ♂	733	86	71	88.63	<0.001
F_1 (UNSEL ♀ x Vip3ASEL ♂) ♀ x Vip3ASEL ♂	635	94	68	198.71	<0.001
F_1 (Vip3ASEL ♀ x UNSEL ♂) ♂ x Vip3ASEL ♀	834	91	71	160.85	<0.001
F_1 (UNSEL ♀ x Vip3ASEL ♂) ♂ x Vip3ASEL ♀	507	88	68	100.59	<0.001

[1] Number of larvae tested.

[2] Expected number of larvae moult inhibited at a given dose = 0.5 (observed MI of F_1 larvae + observed MI of Vip3ASEL) (section 5.2.8).

[3] df = degrees of freedom.

Table 5.13: Direct test of monogenic inheritance for resistance to Vip3A by comparing expected and observed mortality of the backcross of F_1 (Vip3ASEL x UNSEL) and Vip3ASEL population of *Heliothis virescens* at a Vip3A concentration of 500 µg ml^{-1}.

Single-pair matings	n	Observed mortality (%)	Expected mortality (%)	χ^2 (df=1)	P
Vip3ASEL ♀ x ♂	96	34			
UNSEL ♀ x ♂	336	89			
Vip3ASEL ♀ x UNSEL ♂	312	87			
UNSEL ♀ x Vip3ASEL ♂	216	59			
F_1 (Vip3ASEL ♀ x UNSEL ♂) ♀ x Vip3ASEL ♂	742	80	61	118.72	<0.001
F_1 (UNSEL ♀ x Vip3ASEL ♂) ♀ x Vip3ASEL ♂	636	82	47	317.86	<0.001
F_1 (Vip3ASEL ♀ x UNSEL ♂) ♂ x Vip3ASEL ♀	830	80	61	130.44	<0.001
F_1 (UNSEL ♀ x Vip3ASEL ♂) ♂ x Vip3ASEL ♀	502	66	47	73.14	<0.001

[1] Number of larvae tested.

[2] Expected number of larvae dead at a given dose = 0.5 (observed mortality of F_1 larvae + observed mortality of Vip3ASEL) (section 5.2.8).

[3] df = degrees of freedom.

Table 5.14: Direct test of monogenic inheritance for resistance to Vip3A by comparing expected and observed moult inhibition (MI) of the backcross of F_1 (Vip3ASEL x UNSEL) and Vip3ASEL population of *Heliothis virescens* at a Vip3A concentration of 500 µg ml^{-1}.

Single-pair matings	n	Observed MI (%)	Expected MI (%)	χ^2 (df=1)	P
Vip3ASEL ♀ x ♂	96	39			
UNSEL ♀ x ♂	336	100			
Vip3ASEL ♀ x UNSEL ♂	312	95			
UNSEL ♀ x Vip3ASEL ♂	216	78			
F_1 (Vip3ASEL ♀ x UNSEL ♂) ♀ x Vip3ASEL ♂	742	86	67	121.25	<0.001
F_1 (UNSEL ♀ x Vip3ASEL ♂) ♀ x Vip3ASEL ♂	636	94	58	341.80	<0.001
F_1 (Vip3ASEL ♀ x UNSEL ♂) ♂ x Vip3ASEL ♀	830	91	67	216.44	<0.001
F_1 (UNSEL ♀ x Vip3ASEL ♂) ♂ x Vip3ASEL ♀	502	92	58	241.47	<0.001

[1] Number of larvae tested.

[2] Expected number of larvae moult inhibited at a given dose = 0.5 (observed MI of F_1 larvae + observed MI of Vip3ASEL) (section 5.2.8).

[3] df = degrees of freedom.

5.4 Discussion

The present study reports the first known laboratory selection of an insect population for resistance to Vip3A. The development of Vip3A resistance in the *H. virescens* selected population was rapid with 200-fold resistance after nine selection episodes and over 2000-fold resistance after 13 selection episodes based on mortality data (Table 5.3). No direct comparison of Vip3A resistance in other populations can be made, but in comparison to selection with Cry toxins other *H. virescens* populations appear to have developed resistance to Cry1Ac more slowly (Gould *et al.*, 1992; Gould *et al.*, 1995). In a *H. zea* population fast development of Cry1Ac resistance was observed with 123-fold resistance after 11 selection episodes (Anilkumar *et al.*, 2008). In *P. xylostella* populations, the speed and intensity of selection for resistance appears to vary with the Cry1 toxin being selected (Liu and Tabashnik, 1997; Sayyed *et al.*, 2000b; Sayyed and Wright, 2001a) although other factors such as initial frequency of resistance alleles and the selection pressure would also be involved. In the present study, the relatively fast development of resistance may have also been helped by the procedure of only selecting larvae that had moulted to at least 2nd instar after the 7 day bioassay period (three exceptions; Table 5.2), thus removing more susceptible individuals, a procedure also carried out by Anilkumar *et al.* (2008). Moult inhibition data indicated that larval development of some individuals was inhibited by the presence of Vip3A toxin in the diet, also demonstrating resistance to Vip3A.

In the absence of exposure to Vip3A for 5 generations, resistance to Vip3A in WF06-Vip3AREV was still 627-fold greater than the WF06-UNSEL. Although no direct comparison could be made to assess the change in resistance over the five unselected generations, the high LC_{50} and MIC_{50} values relative to the WF06-Vip3ASEL after 10 selections suggests that resistance to Vip3A was relatively stable over this period. A study on the stability of Cry resistance in many other populations of insects of various species found that, on average, resistance ratios decreased by a factor of 10 in seven generations without exposure to Cry toxins (Gassmann *et al.*, 2009). Fitness costs associated with Cry resistance are thought to cause instability in resistance (Ferré and

Van Rie, 2002). In the present study, this association appears to be absent (Chapter 6).

Little or no cross-resistance was apparent between Vip3A and Cry1Ab or Cry1Ac based on mortality of the resistant Vip3A population. There was 7-fold resistance to Cry1Ab and Cry1Ac based on mortality data. However, only resistance ratios that are more than 10-fold will generally reflect heritable decreases in susceptibility (Tabashnik, 1994; Tabashnik *et al.*, 2008a), thus no significant cross-resistance can be assumed. The moult inhibition data indicated no cross-resistance between Vip3A and Cry1Ab or Cry1Ac. These findings are supported indirectly by previous work demonstrating the lack of sequence homology and differing modes of action between Vip3A and Cry toxins (Estruch *et al.*, 1996; Yu *et al.*, 1997; Lee *et al.*, 2003; Lee *et al.*, 2006) thus reducing the likelihood of cross-resistance mechanisms based on altered target site, the most commonly observed resistance mechanism (Ferré and Van Rie, 2002). The lack of observed cross-resistance is also consistent with the results of Jackson *et al.* (2007) that found no cross-resistance to Vip3A in three *H. virescens* populations that had been selected for resistance to Cry1 toxins and Cry2A. A Cry1Ac resistant *H. zea* population also demonstrated a lack of cross-resistance to Vip3A (Anilkumar *et al.*, 2008).

The significantly lower mortality and moult inhibition of larvae (Tables 5.7 and 5.8) from the WF06-Vip3ASEL male x WF06-UNSEL female cross compared to the WF06-Vip3ASEL female x WF06-UNSEL male cross, suggested a paternal influence on Vip3A resistance. While paternal influences have not been noted in other studies, maternal influences have been suggested in Cry1Ac and Cry1Ab resistant *P. xylostella* populations (Martinez-Ramirez *et al.*, 1995; Sayyed and Wright, 2001a; Sayyed *et al.*, 2005), although sex linkage was rejected in one study as no significant difference in the number of male and female survivors was found (Martinez-Ramirez *et al.*, 1995). In the present study, male and female survival was not recorded but a paternal influence would have resulted in greater female survival as males are the heterogametic sex in Lepidoptera (Hartl and Jones, 1999). It is important to highlight that paternal and maternal effects may also be caused by epigenetic effects or genomic imprinting and not by sex linkage (Reik and Walter, 2001). Therefore data in the present study cannot indicate the mechanism for a potential paternal influence

of Vip3A resistance. The majority of other studies have found no influence of sex, maternal or paternal, and have assumed an autosomal mode of inheritance, for example, with Cry1Ac resistant *H. virescens* populations (Sims and Stone, 1991; Gould *et al.*, 1992; Gould *et al.*, 1995), Cry1Ac and Cry2Ab resistant *H. armigera* populations (Akhurst *et al.*, 2003; Xu *et al.*, 2005; Mahon *et al.*, 2007), and Cry1 toxin resistant *P. xylostella* populations (Liu and Tabashnik, 1997; Tabashnik *et al.*, 1997b; Sayyed *et al.*, 2000a; Sayyed *et al.*, 2000b; Sayyed *et al.*, 2004).

The degree of dominance of Vip3A depended on the F_1 reciprocal cross, the Vip3A concentration and the use of mortality or moult inhibition data. Inheritance of resistance in WF06-Vip3ASEL female x WF06-UNSEL male cross was almost completely recessive using mortality and moult inhibition data at two toxin concentrations (100 and 500 $\mu g\ ml^{-1}$). Whereas the dominance of resistance for the WF06-Vip3ASEL male x WF06-UNSEL female cross increased from incompletely recessive to incompletely dominant with an increase in toxin concentration using the mortality data, while dominance remained incompletely recessive using moult inhibition data, although greater at 500 $\mu g\ ml^{-1}$. This apparent split mode of dominance gives further evidence of a possible paternal influence on Vip3A resistance. A similar split, but reversed, was found in a *P. xylostella* population with incomplete dominance in resistant females crossed with susceptible males, but incomplete recessivity in resistant males crossed with susceptible females (Sayyed and Wright, 2001a). Dominance of resistance in other *H. virescens* populations against Cry1Ac and Cry1Ab were either incompletely recessive or incompletely dominant (Sims and Stone, 1991; Gould *et al.*, 1992; Gould *et al.*, 1995). This variation in degree of dominance has also been found in *P. xylostella* (Sayyed *et al.*, 2000a; Sayyed *et al.*, 2000b), *H. armigera* (Xu *et al.*, 2005; Mahon *et al.*, 2007) and *P. gossypiella* populations (Tabashnik *et al.*, 2004; Carrière *et al.*, 2006). Dominance of resistance in other selected populations against Cry toxins has revealed both recessive and incompletely dominant resistance that can vary depending on the concentration of the toxin used. However, the general pattern frequently found shows that the degree of dominance decreases with increasing toxin concentration (Liu and Tabashnik, 1997; Sayyed and Wright, 2001a; Tabashnik *et al.*, 2002), the opposite trend to that found in the present study.

The estimation of dominance was based on the assumption that the resistant population and susceptible population were completely homozygous when F_1 progeny were produced. The presence of heterozygotes in a selected population would tend to lower the survival rate of F_1 progeny and underestimate the degree of dominance, with heterozygotes in the susceptible population having the opposite effect (Liu and Tabashnik, 1997; Tabashnik *et al.*, 1997b; Sayyed *et al.*, 2005). There was significant variation in mortality and moult inhibition for single-pair F_1 families, suggesting that the resistant population was not homozygous for resistance at the time of the crosses and so that the estimates of dominance may be lower than the true values. The significant variation within the single pair F_2 families suggests that differences in resistance between single-pairs was due to different combinations of resistance alleles being carried by individuals (Tabashnik *et al.*, 1997; Sayyed *et al.*, 2005), which would indicate that more than one locus was involved in Vip3A resistance. However, the potential for underestimation of dominance indicated by the heterozygous resistant line may compromise the suggestion of more than one locus involved in Vip3A resistance (Liu and Tabashnik, 1997).

In the present study, further analysis involving the backcross experiments suggested that resistance to Vip3A in WF06-Vip3ASEL was due to more than one locus (polygenic) at both concentrations tested for mortality and moult inhibition data. Other populations resistant to Cry toxins have also been shown to exhibit polygenic resistance, for example, a Cry2Aa resistant *H. virescens* population (Gahan *et al.*, 2005) and Cry1Ac resistant *P. xylostella* (Sayyed *et al.*, 2000b; Sayyed and Wright, 2001a; Sayyed *et al.*, 2005) and *P. gossypiella* populations (Tabashnik *et al.*, 2006). In contrast monogenic resistance has been found in other populations of *H. virescens* (Gould *et al.*, 1995), *H. armigera* (Xu *et al.*, 2005; Mahon *et al.*, 2007), *P. xylostella* (Tabashnik *et al.*, 1992; Sayyed *et al.*, 2000a; Sayyed *et al.*, 2004) and *O. nubilalis* (Alves *et al.*, 2006) and is generally regarded as the most common form of Cry resistance (Ferré and Van Rie, 2002).

Chapter 6

Fitness studies on a Vip3A resistant population of *Heliothis virescens*

6.1 Introduction

Evaluation of the fitness of resistant populations in comparison to susceptible populations can reveal whether resistant populations have associated fitness costs that may put them at a disadvantage for there continuing survival in the field, therefore delaying resistance. Fitness costs of Cry resistance occur in the absence of *Bt,* with the fitness of resistant insects lower than that of susceptible insects. Knowledge of fitness costs can aid the design of insect management strategies, such as improving the effectiveness of refuges through designs to increase the dominance or magnitude of fitness costs. Greater expression of fitness costs of resistance in refuges will actively select against resistance (Carrière and Tabashnik, 2001; Gould *et al.*, 2006; Raymond *et al.*, 2007b; Gassmann *et al.*, 2009). However, estimating the total fitness of a population is often impossible, with only measurements of some of its components recorded. Therefore, care must be taken when drawing conclusions about an individual's or a population's fitness based on only a few fitness components as there will be a lack of information on trade-offs amongst life history traits and how they may affect a field population (Reed and Bryant, 2004).

Fitness costs linked to Cry resistance have been reported in studies on various insect species, (e.g. *H. virescens*, *H. armigera*, *P. gossypiella*, *P. xylostella* and *T. ni*) including effects on survival, development rate, larval and pupal weight, and fecundity (Sayyed and Wright, 2001b; Janmaat and Myers, 2003; Bird and Akhurst, 2005; Carrière *et al.*, 2005; Bird and Akhurst, 2007; Gassmann *et al.*, 2009). However, not all studies have demonstrated fitness costs. Studies on a *Btk* resistant population of *H. virescens* found no difference in larval weight, larval survival and egg viability compared with a susceptible population (Gould and Anderson, 1991),

while in a Cry1Ac resistant population of the same species (BRX8.8) there was no apparent effect on growth rate (Gahan *et al.*, 2005).

The present study compares the biology of the Vip3A resistant population (WF06-Vip3ASEL) and unselected population (WF06-UNSEL) of *H. virescens* (Chapter 5) to investigate the effects of Vip3A resistance on fitness. The fitness effects studied were development time, pupal weight, survival to eclosion, adult sex ratio, mating success, fecundity and egg viability.

6.2 Materials and Methods

6.2.1 *Heliothis virescens* populations

The populations used were the resistant WF06-Vip3ASEL population at generation 15, which had undergone 12 selections with Vip3A, and the unselected WF06-UNSEL population.

6.2.2 Development time, pupal weight and sex ratio of WF06-UNSEL and WF06-Vip3ASEL

One hundred and fifty 1st instar larvae from WF06-UNSEL and WF06-Vip3ASEL, in 15 replicates of 10 larvae each, were setup on artificial diet (six WF06-UNSEL and two WF06-Vip3ASEL individuals lost through handling error). The artificial diet was prepared as described in section 3.1, except that the diet was setup in 1 oz. plastic cups (9051, Bio-Serv, NJ, USA) as opposed to the 32-cell rearing trays. A single 1st instar larva (< 24 h old) was placed in each diet cup and sealed with a snap on lid (9053, Bio-Serv, NJ, USA) that had a single slit for aeration. The development time from larva to pupa was recorded and two day old pupae were weighed. The sex of the pupae was determined as described in section 5.2.7. The pupae were placed individually in 250 ml round plastic containers covered with netting held in place by an elastic band. Development times from pupae to adult and from egg to adult were recorded. The number of larvae that survived to successful adult eclosion was

recorded, although adults with wing deformities were not classed as successful. The populations were maintained in CE rooms at 25 ± 2 °C, 65 ± 10 % relative humidity (RH) with a 16:8 (light:dark) cycle.

6.2.3 Mating success, fecundity and egg viability of WF06-UNSEL and WF06-Vip3ASEL populations and their reciprocal crosses

Newly emerged adult males and females from the development studies (section 6.2.2) were paired at random into WF06-UNSEL female and male (21 pairs), WF06-Vip3ASEL female and male (19 pairs), WF06-Vip3ASEL female x WF06-UNSEL male (17 pairs) and WF06-UNSEL female x WF06-Vip3ASEL male (20 pairs) crosses. Each pair was setup in a 250 ml round plastic container covered with netting held in place by an elastic band. They were fed *ad lib* with 10 % (v/v) honey solution soaked into cotton wool pads that were placed on top of the netting allowing the adults to feed but preventing egg lay on the cotton pad. Once the female had started laying eggs, the adult pair was transferred to a new 250 ml container setup every 2 days, as eggs were laid on the container itself as well as the netting. The eggs were counted and the netting (if eggs were present) was placed inside the container and sealed with a plastic lid. Hatching usually occurred after 3 - 4 days, but the eggs were given up to seven days to hatch before the container was discarded. Emerging larvae were counted and removed each day. Mating pair success was recorded by the number of females that laid eggs and the number of pairs that had viable progeny. The populations were maintained in CE rooms at 25 ± 2 °C, 65 ± 10 % relative humidity (RH) with a 16:8 (light:dark) cycle.

6.2.4 Statistical analysis

Statistical analysis was carried out using R version 2.8.1 (R Development Core Team, 2009). Larval, pupal and egg to adult development times were modelled using analysis of variance (ANOVA). The pupal development time data was log transformed to correct for non-normal distribution. Pupal weight was modelled using ANOVA. The sex ratio was analysed using the chi squared test for given probabilities (chisq.test), where the null hypothesis probability is equal to 0.5 (50:50 sex ratio). The proportion of larvae that survived to adult eclosion was analysed using

a generalised linear model with a binomial error structure. Mating success for both proportion of pairs laying eggs and pairs producing viable progeny was analysed using the binomial proportions test (prop.test) to compare four proportions. The mean number of eggs was modelled using a generalised linear model with a poisson error structure corrected for overdispersion. The mean egg viability was modelled using a generalised linear model with binomial errors corrected for overdispersion. For all analyses significance differences between treatments were accepted at the 5 % level ($P<0.05$).

6.3 Results

6.3.1 Development time, pupal weight and sex ratio of the WF06-UNSEL and WF06-Vip3ASEL populations

There was a significant effect on mean larval development time ($F_{3,157} = 7.97$, $P<0.01$). The WF06-Vip3ASEL population had significantly lower larval development times compared with the WF06-UNSEL population (Table 6.1). There was no significant difference between male and female larval development times within each population ($P>0.05$).

The mean pupal development time was significantly different ($F_{3,157} = 12.51$, $P<0.01$). The mean male pupal development time was significantly longer in both the WF06-UNSEL and WF06-Vip3ASEL populations compared with the mean female pupal development time (Table 6.1). There was no significant difference within either male or female pupal development times ($P>0.05$).

There was a significant effect on mean development time from egg to adult eclosion ($F_{3,157} = 5.59$, $P<0.01$), with WF06-Vip3ASEL female development significantly faster compared with WF06-Vip3ASEL males, and WF06-UNSEL female and males (Table 6.1). There were no further significant differences between WF06-Vip3ASEL males and WF06-UNSEL females and males ($P>0.05$).

The proportion of larvae that survived to adult eclosion was significantly different between WF06-Vip3ASEL (72 out of 148) and WF06-UNSEL (89 out of 144) ($z_{1, 28}$ = -2.253, P<0.05; Table 6.1).

There were significant effects on pupal weight between the populations ($F_{3, 157}$ = 15.37, P<0.01; Table 6.1). Mean female and male pupal weights were significantly greater in the WF06-UNSEL population compared with the WF06-Vip3ASEL population (P<0.05). There were no significant differences in mean pupal weight between females and males within each population (P>0.05).

There were no significant differences in the sex ratio for both populations from the expected 50:50 split, with the WF06-UNSEL population having 41 females and 48 males (χ^2=0.55, 1 d.f., P>0.05), and the WF06-Vip3ASEL population having 35 females and 37 males (χ^2 = 0.056, 1 d.f., P>0.05)

Table 6.1: Mean (± se) development parameters for WF06-UNSEL and WF06-Vip3ASEL populations of *Heliothis virescens*.

Life history trait	Sex	WF06-UNSEL[1]	WF06-Vip3ASEL[1]
Larval development time (days)	female	19.3 ± 0.3	17.1 ± 0.5
	male	18.9 ± 0.5	17.9 ± 0.5
Pupal development time (days)	female	14.0 ± 0.2	14.3 ± 0.3
	male	15.2 ± 0.3	15.3 ± 0.3
Egg to adult development time (days)	female	36.3 ± 0.5	34.4 ± 0.7
	male	37.1 ± 0.6	36.1 ± 0.7
Pupal weight (mg)	female	288 ± 4	264 ± 4
	male	290 ± 4	259 ± 5
Adult eclosion (%)	-	62 ± 3	49 ± 2

[1] Sample size: WF06-UNSEL n (females) = 41, n (males) = 48; WF06-Vip3ASEL n (females) = 35, n (males) = 37.

6.3.2 Mating success, fecundity and egg viability of WF06-UNSEL and WF06-Vip3ASEL populations and their reciprocal crosses

There was no significant difference in the proportion of pairs that produced eggs between any of the four crosses (χ^2=4.47, 3 d.f., P>0.05; Table 6.2). There were significant differences in the proportion of pairs that produced viable progeny. In the WF06-Vip3ASEL and WF06-UNSEL female x WF06-Vip3ASEL male crosses fewer pairs produced viable progeny compared with WF06-UNSEL and WF06-Vip3ASEL female x WF06-UNSEL male crosses (χ^2=8.26, 3 d.f., P<0.05; Table 6.2).

There was no significant difference in the mean number of eggs laid excluding pairs that produced no viable offspring between any of the four crosses (P>0.05, 28 d.f.,

n=31; Table 6.2). There was also no significant difference in the mean number of eggs laid that included pairs which produced no viable offspring between any of the four crosses (P>0.05, 63 d.f., n=66).

The mean egg viability of the WF06-Vip3ASEL cross was significantly lower compared with the WF06-UNSEL cross, or the WF06-UNSEL female x WF06-Vip3ASEL male and WF06-Vip3ASEL female x WF06-UNSEL male crosses (P<0.05, 63 d.f., n=66; Table 6.2). There were no other significant differences in mean egg viability (P>0.05, 63 d.f., n=66).

Table 6.2: Mean reproductive parameters for WF06-UNSEL, WF06-Vip3ASEL, WF06-Vip3ASEL female x WF06-UNSEL male and WF06-UNSEL female x WF06-Vip3ASEL male populations of *Heliothis virescens*.

Life history trait	WF06-UNSEL [1]	WF06-Vip3ASEL [1]	WF06-Vip3ASEL female x WF06-UNSEL male [1]	WF06-UNSEL female x WF06-Vip3ASEL male [1]
Mating pair success [2] - eggs laid (%)	81	79	100	90
Mating pair success [3] - viable progeny (%)	57	26	59	25
Mean egg no. per viable pair [2] (± se)	802 ± 157	856 ± 297	939 ± 209	1020 ± 338
Egg viability (%) (± se)	43 ± 4	10 ± 7	48 ± 6	43 ± 13

[1] Number of pairs: WF06-UNSEL = 21; WF06-Vip3ASEL = 19; WF06-Vip3ASEL female x WF06-UNSEL male = 17; WF06-UNSEL female x WF06-Vip3ASEL male = 20.

[2] Number of egg laying pairs: WF06-UNSEL = 17; WF06-Vip3ASEL = 15; WF06-Vip3ASEL female x WF06-UNSEL male = 17; WF06-UNSEL female x WF06-Vip3ASEL male = 18.

[3] Number of viable pairs: WF06-UNSEL = 12; WF06-Vip3ASEL = 5; WF06-Vip3ASEL female x WF06-UNSEL male = 10; WF06-UNSEL female x WF06-Vip3ASEL male = 5.

6.4 Discussion

In the present study, the Vip3A resistant population of *H. virescens* showed faster larval development and faster female development to adult eclosion compared with an unselected population. A Cry1Ac resistant population of *P. xylostella* (SERD4) has also been reported to show faster larval development time (Sayyed *et al.*, 2003) but other reports on Cry resistant populations have generally shown an increased development time or no effect (Gassmann *et al.*, 2009; Table 6.3). For example, a reduced growth rate was found in a *H. virescens* population resistant to Cry1Ac and Cry2Aa, although the authors suggested that this cost may have been a result of inbreeding (Gahan *et al.*, 2005). Differences in development time have the potential to lead to non-random mating with resistant adults mating with each other and not susceptible populations. However, the relatively small differences in faster development time for resistant insects observed in the laboratory, in the present study, would probably be mitigated by overlapping generations in the field, particularly as the season progresses (Wu *et al.*, 2002; Bird and Akhurst, 2004). The bias of only using larvae that had moulted during selection to continue the population may also have had an impact on the observed faster development rates of the Vip3A resistant population in comparison to the susceptible population.

The reduced pupal weight for the Vip3A resistant population observed in the present work was similar to findings in other studies (Table 6.3), although an increase in pupal weight was reported in the Cry1Ac resistant SERD4 population of *P. xylostella* (Sayyed and Wright, 2001b; Sayyed *et al.*, 2003). The decrease in survival to adult eclosion for the Vip3A resistant population in the present study has also been observed in other studies with *Bt* toxins (Table 6.3), and while survival in some resistant populations was not affected, there appears to have been no reported increase in survival. Larval survival was also found to be reduced in populations of *H. armigera* and *P. gossypiella* (Table 6.3).

Fitness studies that include F_1 reciprocal crosses allow the dominance of fitness costs to be further classified. Nonrecessive fitness costs, expressed in the F_1 reciprocal

Table 6.3: A summary demonstrating the variety of fitness effects correlated with Cry resistance in some lepidopteran pest species.

Species	larval development	larval survival	pupal weight	survival to adult	fecundity	egg viability	mating success
H. virescens (present study)	+ve		-ve	-ve	NE	-ve	-ve
H. virescens [1]	NE / -ve	NE				NE	
H. armigera [2]	NE / -ve	-ve	-ve		-ve	-ve	-ve
P. gossypiella [3]	NE	-ve			NE	NE	NE / -ve
P. xylostella [4]	NE / +ve		NE / +ve	NE / -ve	-ve	NE / -ve	-ve
P. interpunctella [5]	NE / -ve			NE / -ve			
T. ni [6]	-ve		-ve				

+ve means positive effect on fitness of resistant population compared to susceptible population; -ve means negative effect on fitness of resistant population compared to susceptible population; NE means no effect on fitness of resistant population.

[1] (Gould and Anderson, 1991; Gahan *et al.*, 2005)
[2] (Akhurst *et al.*, 2003; Bird and Akhurst, 2004; Bird and Akhurst, 2005; Liang *et al.*, 2008; Zhao *et al.*, 2008)
[3] (Carrière *et al.*, 2001a; Higginson *et al.*, 2005)
[4] (Groeters *et al.*, 1993; Groeters *et al.*, 1994; Sayyed and Wright, 2001b; Sayyed *et al.*, 2003)
[5] (Oppert *et al.*, 2000)
[6] (Janmaat and Myers, 2003)

cross as well as in the resistant population, would be expected to be most effective for delaying resistance (Gassmann *et al.*, 2009).

In the present study, studies on mating success, fecundity and egg viability revealed variability between the resistant WF06-Vip3ASEL and susceptible WF06-UNSEL populations and their F_1 reciprocal crosses. The number of females that successfully laid eggs was not affected by the cross, indicating that the ability of females to produce eggs was not effected, and this was further demonstrated by no effect on fecundity among the crosses. Successful mating with pairs involving WF06-Vip3ASEL males was reduced as determined by the proportion of pairs producing viable progeny demonstrating a nonrecessive fitness cost. This suggests that resistant males may have reduced virility. However, only the resistant population exhibited reduced egg viability in comparison to WF06-UNSEL and the F_1 reciprocal crosses, indicating that expression of reduced egg viability requires mating between resistant males and females, a recessive fitness cost. It may be more likely, therefore, that mating frequency was reduced or mating did not occur with some resistant males.

Fitness costs on fecundity and egg viability have been found in other studies (Table 6.3), for example, with Cry1Ac resistant *H. armigera* (Liang *et al.*, 2008), and *Btk* resistant *P. xylostella* (Groeters *et al.*, 1994). Fitness effects on resistant males have also been reported. Male mating frequency was reduced in a *Btk* resistant population of *P. xylostella* population (Groeters *et al.*, 1993). While Zhao *et al.* (2008) found that the incidence of successful mating was reduced in a Cry1Ac resistant population of *H. armigera*, where differences between F_1 crosses suggested that resistant males reduce the incidence of mating paternity (egg viability) rather than mating frequency. Reduced male fertility was also observed in another Cry1Ac resistant population of *H. armigera* and in F_1 crosses (Bird and Akhurst, 2005). Higginson *et al.* (2005) found no effect on mating frequency or egg viability in the absence of competition for several Cry1Ac resistant populations of *P. gossypiella*. However, competition studies with susceptible males resulted in reduced egg viability with resistant males that had mated first, caused perhaps by reduced sperm precedence.

Inbreeding within laboratory populations can be problematic for resistance studies (Groeters *et al.*, 1994; Bird and Akhurst, 2004; Zhao *et al.*, 2008), and care was taken

in the present study to maintain large populations of selected and unselected populations to ensure that differences in fitness of populations are a result of Vip3A resistance. However, the nature of establishing a laboratory population will no doubt result in the genetic background of such a population differing over time with that of a wild population, therefore, measuring the fitness of a population is very difficult and it is important to understand the limitations of drawn conclusions (Reed and Bryant 2004; section 6.1).

Fitness studies on Vip3A resistant *H. virescens* could be extended to include other parameters investigated in Cry toxin resistant insect populations, such as emergence of overwintering populations (Carrière *et al.*, 2001b; Bird and Akhurst, 2004; Carrière *et al.*, 2007), the effect of host plants on larval development and survival (Raymond *et al.*, 2005; Bird and Akhurst, 2007) and the effect of pathogens (Raymond *et al.*, 2007a).

Fitness costs expressed in the present study revealed a variety of effects on the Vip3A resistant WF06-Vip3ASEL population. The reduced mating success observed in resistant males may help to limit an increase in the frequency of the resistant allele (Zhao *et al.*, 2008), and with the possible paternal influence on Vip3A resistance (Chapter 5), contribute to delays in the evolution of resistance in the field with the involvement of current management strategies involving the use of refuges (Gassmann *et al.*, 2009).

Chapter 7

The effect of antibiotics on *Heliothis virescens* larval susceptibility to *Bacillus thuringiensis* toxins

7.1 Introduction

The mechanisms responsible for the death of insect larvae exposed to *Bt* toxins are not fully understood (section 2.1.2.3). Broderick *et al.* (2006) used broad spectrum antibiotics (gentamicin, penicillin, rifampicin, streptomycin in a combined cocktail) to eliminate the gut microbiota from larvae of the gypsy moth, *Lymantria dispar* L. (Lepidoptera: Lymantriidae). They found that mortality caused by *Btk* (Dipel) was greatly reduced in antibiotic-treated larvae compared with untreated larvae, thus suggesting a novel mechanism of larval death involving the insect gut microbiota. Further research has suggested that this mechanism has an important role in a range of Lepidoptera taxa (Broderick *et al.*, 2009).

The present study, investigates Broderick *et al.*'s (2006) findings, using the same antibiotics, both singly and in combination, on the toxicity of Dipel, Cry1Ab, and Vip3A on 1st and 3rd instar larvae of *H. virescens*.

The use of antibiotics in insect artificial diet is standard practice to help prevent disease establishing on the diet, e.g. ampicillin sodium salt for *H. virescens* artificial diet (section 3.1). Previous research has found that antibiotics, such as aureomycin can have variable effects on insect susceptibility to *Bt* toxins in the artificial diet bioassays (Beegle *et al.*, 1981; Beegle, 1990). Antibiotics are therefore generally excluded from toxin bioassays to avoid misleading results (sections 3.4.1 and 3.4.2). The present experiments, in using single as well as mixtures of antibiotics, were also designed to investigate whether all antibiotics had similar interactions with a range of *Bt* toxins.

7.2 Materials and Methods

7.2.1 *Heliothis virescens* populations

The established WF06 unselected population (WF06-UNSEL) was used as the test population for these experiments. First instar larvae used in the experiments had hatched less than 24 h before experimental setup. Third instar larvae used in the experiments were within 24 h of having moulted to 3rd instar larvae.

7.2.2 Antibiotic preparation

Gentamicin, penicillin, rifampicin and streptomycin were all used at a concentration of 500 µg ml^{-1} (Broderick *et al.*, 2006). For each 100 ml treatment combination, 50 mg of the required antibiotic(s) were therefore needed: gentamicin sulphate salt (G3632, Sigma, UK; 600 µg ml^{-1} 83.3 mg equivalent to 50 mg antibiotic); penicillin G potassium salt (P7794, Sigma; 1596 units mg^{-1}); rifampicin (R8883, Sigma; 1010 µg ml^{-1}); streptomycin sulphate salt (S6501, Sigma; 753 units mg^{-1}).

Ampicillin sodium salt was used at a concentration of 124 µg ml^{-1}, as per the concentration used in the standard artificial diet (section 3.1). Therefore, 12.4 mg was required for each 100 ml treatment combination.

The single antibiotic treatments were then mixed with 10 ml distilled water. For the antibiotic cocktail treatment, gentamicin, penicillin, rifampicin and streptomycin were added together and mixed in 10 ml distilled water. For the treatments not containing any antibiotic, 10 ml of distilled water alone was prepared and used instead.

Treatments using 3rd instar larvae required 200 ml of artificial diet solution. Therefore, the antibiotic cocktail treatments required 166.6 mg of gentamicin and 100 mg for each of penicillin, rifampicin and streptomycin were required in 20 ml distilled water, or 20 ml distilled water alone for treatments not containing the antibiotic cocktail. Some treatment combinations also required larvae reared from

emergence to 3rd instar on artificial diet containing the antibiotic cocktail at a concentration of 500 µg ml^{-1}.

7.2.3 *Bacillus thuringiensis* toxin preparation

The toxin concentrations for Cry1Ab, Dipel and Vip3A treatments were based on their respective LC_{50} values for that particular instar. The Cry1Ab concentration for 1st instar larvae was 0.024 µg ml^{-1}. In earlier results the LC_{50} for 1st and 3rd instar larvae was similar, however, the toxicity of the Cry1Ab protoxin batch used for this experiment was reduced in comparison to earlier batches; the calculated 3rd instar larvae concentration was 0.3 µg ml^{-1}. The Vip3A concentration for 1st instar larvae was 2 µg ml^{-1}. Two concentrations were setup for 1st instar larvae with Dipel, 0.047 µg ml^{-1} and 0.0047 µg ml^{-1} as an accurate LC_{50} value was not successfully established. The Dipel concentration for 3rd instar larvae was 5 µg ml^{-1}. The required toxin volume was calculated for 100 ml treatments for 1st instar larvae, and 200 ml treatments for 3rd instar larvae and added to either 10 ml or 20 ml respectively, of distilled water.

Control treatments did not contain toxin, instead 10 ml (for 1st instar treatments) or 20 ml (for 3rd instar treatments) of distilled water alone was prepared.

7.2.4 1st instar larvae antibiotic bioassay method

Each treatment combination used 100 ml of artificial diet solution that included the toxin solution and antibiotic solution. The bioassay procedure was similar to that described in section 3.4.1 with the exception of a requirement of 20 % reduction in distilled water content in the diet preparation (section 3.1) to allow the addition of the antibiotic and toxin solutions to the diet preparation at a ratio of 1:1:8 (antibiotic:toxin:diet). For each treatment the required 10 ml antibiotic solution (or control alternative), 10 ml toxin solution (or control alternative) and 80 ml artificial diet solution were mixed together vigorously using a glass stirring rod in a 250 ml beaker and setup as previously described in section 3.4.1, with 48 larvae per treatment split into four replicates of 12 larvae. Mortality and moult inhibition were both determined after seven days.

7.2.5 3rd instar larvae antibiotic bioassay method

The method was similar to that for the 1st instar larvae antibiotic bioassay method with these following few exceptions. The artificial diet solution for each treatment was increased from 100 ml to 200 ml (20 ml antibiotic solution, 20 ml toxin solution and 160 ml diet solution), with approximately 3 – 4 ml per well. The bioassay plates were replaced by 32-well rearing trays (24 wells used) sealed with breathable polyester film. Depending on the treatment combination, larvae had been reared from emergence to 3rd instar on either artificial diet prepared without any antibiotics or with the antibiotic cocktail (500 μg ml^{-1} for each antibiotic). 3rd instar larvae were transferred to the wells using soft forceps. Some of these changes were made to accommodate larger larval size and increased food consumption of later instar larvae. Mortality and moult inhibition were both determined after seven and 14 days.

7.2.6 Treatments

1) To investigate the effect of the antibiotic cocktail and ampicillin alone on larval mortality and moult inhibition (MI) of Cry1Ab and Vip3A the following treatments were used:

Treatment	Antibiotic	*Bt* toxin
1	none	none
2	cocktail	none
3	ampicillin	none
4	none	Cry1Ab
5	cocktail	Cry1Ab
6	ampicillin	Cry1Ab
7	none	Vip3A
8	cocktail	Vip3A
9	ampicillin	Vip3A

2) To investigate the effect of individual antibiotics on larval mortality and moult inhibition (MI) of Cry1Ab and Vip3A the following treatments were used:

Treatment	Antibiotic	*Bt* toxin
1	none	none
2	gentamicin	none
3	penicillin	none
4	rifampicin	none
5	streptomycin	none
6	none	Cry1Ab
7	gentamicin	Cry1Ab
8	penicillin	Cry1Ab
9	rifampicin	Cry1Ab
10	streptomycin	Cry1Ab
11	none	Vip3A
12	gentamicin	Vip3A
13	penicillin	Vip3A
14	rifampicin	Vip3A
15	streptomycin	Vip3A

3) To investigate the effect of the antibiotic cocktail on larval mortality and moult inhibition (MI) of Dipel the following treatments were used:

Treatment	Antibiotic	*Bt* toxin
1	none	none
2	cocktail	none
3	none	Dipel (0.047 µg ml^{-1})
4	cocktail	Dipel (0.047 µg ml^{-1})
5	none	Dipel (0.0047 µg ml^{-1})
6	cocktail	Dipel (0.0047 µg ml^{-1})
7	none	Cry1Ab
8	cocktail	Cry1Ab

4) To investigate the effect of individual antibiotics on larval mortality and moult inhibition (MI) of Dipel (0.0047 µg ml^{-1}) the following treatments were used:

Treatment	Antibiotic	*Bt* toxin
1	none	none
2	cocktail	none
3	gentamicin	none
4	penicillin	none
5	rifampicin	none
6	streptomycin	none
7	none	Dipel
8	cocktail	Dipel
9	gentamicin	Dipel
10	penicillin	Dipel
11	rifampicin	Dipel
12	streptomycin	Dipel

5) To investigate the effect of an antibiotic cocktail on the larval mortality and moult inhibition (MI) of Dipel (0.0047 µg ml^{-1}) and Cry1Ab on 3rd instar larvae reared in the presence or absence of antibiotics the following treatments were used:

Treatment	Antibiotic pre treatment	Antibiotic	*Bt* toxin
1	Yes	none	none
2	Yes	cocktail	none
3	Yes	none	Dipel
4	Yes	cocktail	Dipel
5	Yes	none	Cry1Ab
6	Yes	cocktail	Cry1Ab
7	No	none	none
8	No	cocktail	none
9	No	none	Dipel
10	No	cocktail	Dipel
11	No	none	Cry1Ab
12	No	cocktail	Cry1Ab

7.2.7 Statistical analysis

Statistical analysis was carried out using R version 2.8.1 (R Development Core Team, 2009). Percentage mortality and moult inhibition data were arcsine transformed and modelled using analysis of variance (ANOVA) to determine the effects of antibiotic treatment and *Bt* toxin. Significance differences between treatments were tested at the 5 % level ($P<0.05$) through comparison of standard errors as calculated in the ANOVA.

7.3 Results

7.3.1 Effect of an antibiotic cocktail and ampicillin alone on larval mortality and moult inhibition (MI) of Cry1Ab and Vip3A

Larval mortality was unaffected ($F_{2, 9} = 0.41$, $P>0.05$; Figure 7.1A) by the antibiotics tested alone (mortality ≤ 5 %). There were significant effects on larval mortality with treatments involving Cry1Ab ($F_{5, 18} = 50.7$, $P<0.001$) and Vip3A ($F_{1, 22} = 100.2$, $P<0.001$).

A significant effect on larval mortality within Cry1Ab treatments was found ($F_{2, 9} = 79.1$, $P<0.001$). Larval mortality was significantly greater in the treatment combining Cry1Ab and the antibiotic cocktail when compared to Cry1Ab alone (89 and 35 % mortality respectively; $P<0.001$). When Cry1Ab was combined with ampicillin, larval mortality (18 %) was significantly lower compared with Cry1Ab alone ($P<0.05$).

There were no significant differences in larval mortality between Vip3A alone and Vip3A combined with the antibiotic cocktail or ampicillin (47, 57 and 60 % mortality respectively; $F_{2, 9} = 0.7$, $P>0.05$).

The moult inhibition (MI) of larvae was unaffected ($F_{2, 9} = 0.21$, $P>0.05$; Figure 7.1B) by the antibiotics tested alone (MI ≤ 8 %). There were significant effects on MI with

treatments involving Cry1Ab ($F_{5, 18}$ = 74.53, P<0.001) and Vip3A ($F_{1, 24}$ = 274.2, P<0.001).

A significant effect on MI within Cry1Ab treatments was found ($F_{2, 9}$ = 83.9, P<0.001). There was no significant difference (P>0.05) in larval MI between Cry1Ab alone and Cry1Ab combined with ampicillin (48 and 36 % MI respectively). The MI of Cry1Ab combined with the antibiotic cocktail (100 %) was significantly greater (P<0.001) than the other Cry1Ab treatments.

There were no significant differences in larval MI between Vip3A alone and Vip3A combined with the antibiotic cocktail or ampicillin (72, 76 and 77 % MI respectively; $F_{2, 9}$ = 0.27, P>0.05).

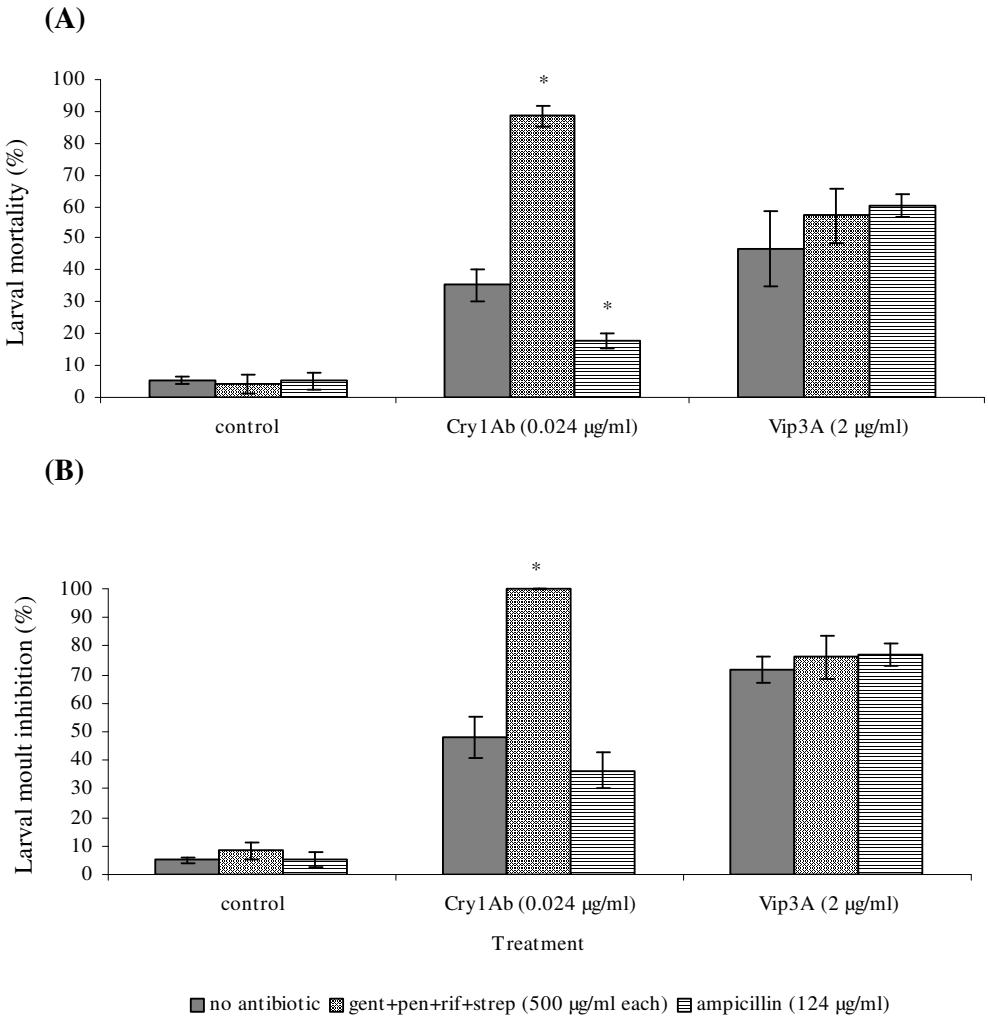

Figure 7.1: The effect of an antibiotic cocktail (gentamicin, penicillin, rifampicin and streptomycin) or ampicillin on toxicity of Cry1Ab and Vip3A to 1st instar *Heliothis virescens* in a diet incorporation assay after 7 days: (A) mean percent mortality (± se); (B) mean percent moult inhibition (± se); * indicates a within treatment significant difference ($P<0.05$) to the toxin alone.

7.3.2 Effect of individual antibiotics on larval mortality and moult inhibition (MI) of Cry1Ab and Vip3A

Larval mortality was unaffected ($F_{4, 15}$ = 1.65, P>0.05; Figure 7.2A) by the antibiotics tested alone (mortality ≤15 %). There were significant effects on larval mortality with treatments involving Cry1Ab ($F_{5, 34}$ = 8.995, P<0.001) and Vip3A ($F_{5, 34}$ = 15.04, P<0.001).

There were no significant differences in larval mortality between Cry1Ab alone and combined with either gentamicin, penicillin, rifampicin, or streptomycin (25, 42, 13 19 and 46 % mortality respectively; ($F_{4, 15}$ = 4.963, P>0.05), despite greater mortality with either gentamicin or streptomycin.

A significant effect on larval mortality within Vip3A treatments was found ($F_{4, 15}$ = 5.601, P<0.01). There were no significant differences in larval mortality between Vip3A alone and combined with rifampicin or streptomycin (54, 54 and 42 % mortality respectively; P>0.05). Vip3A combined with gentamicin or penicillin had significantly lower mortality than the other treatments (19 and 25 % mortality respectively; P<0.05), but were not significantly different from each other (P>0.05).

Larval MI was unaffected ($F_{4, 15}$ = 0.4, P>0.05; Figure 7.2B) by the antibiotics tested alone (MI ≤ 17%). There were significant effects on MI with treatments involving Cry1Ab ($F_{9, 30}$ = 18.17, P<0.001) and Vip3A ($F_{5, 34}$ = 26.38, P<0.001).

A significant effect of larval MI within Cry1Ab treatments was found ($F_{4, 15}$ = 23.51, P<0.001). There were no significant differences in larval MI between Cry1Ab alone and combined with penicillin or rifampicin (25, 21 and 38 % MI respectively; P>0.05). The MI of Cry1Ab combined with gentamicin (83 %) or streptomycin (92 %) were significantly greater than the other treatments (P<0.001), although they were not significantly different from each other (P>0.05).

A significant effect of larval MI within Vip3A treatments was found ($F_{4, 15}$ = 5.957, P<0.01). There were no significant differences in larval MI between Vip3A alone and combined with rifampicin or streptomycin (81, 81 and 65 % MI respectively; P>0.05).

Vip3A combined with gentamicin or penicillin both had significantly lower MI than Vip3A alone and combined with rifampicin ($P<0.05$), but they were not significantly different from each other or streptomycin ($P>0.05$).

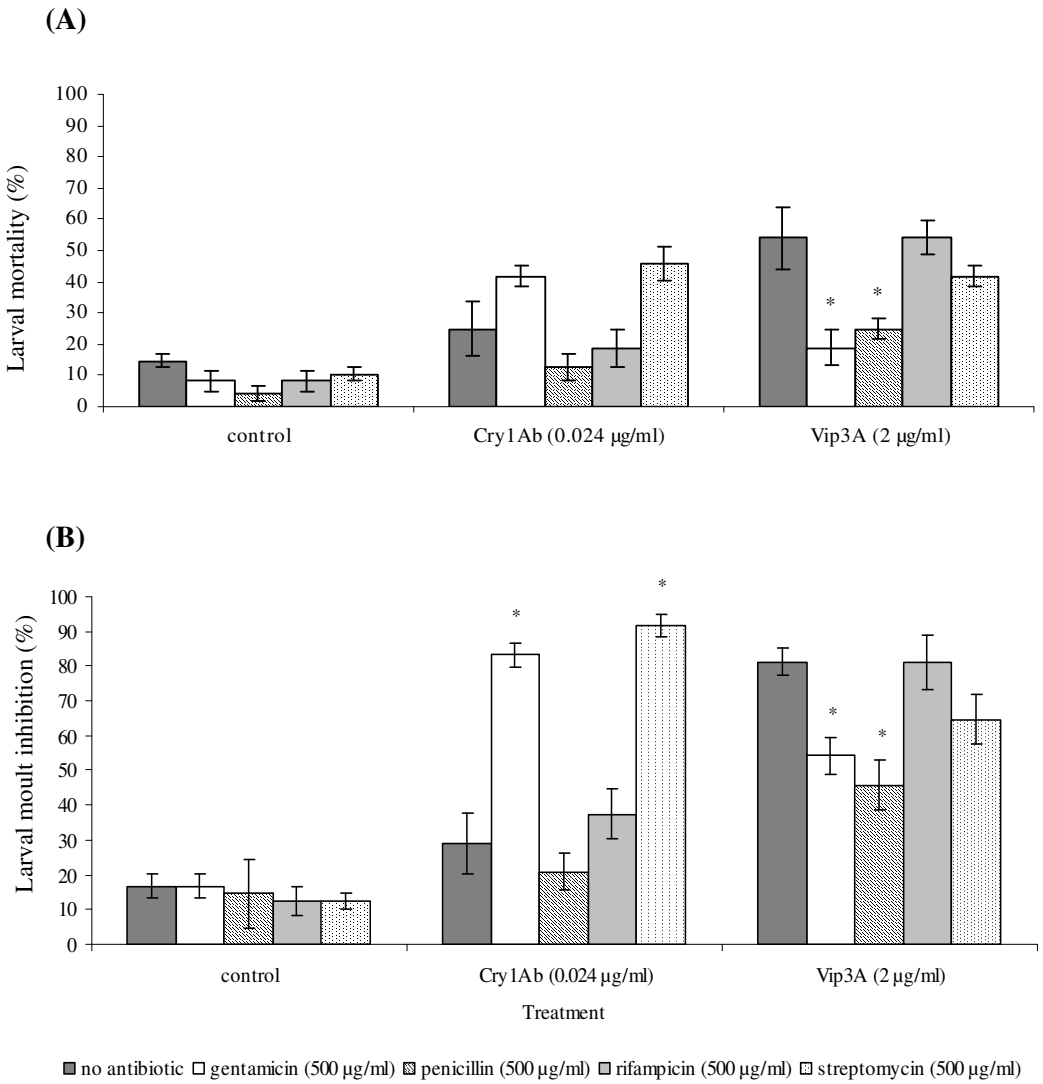

Figure 7.2: The effect of gentamicin, penicillin, rifampicin and streptomycin on toxicity of Cry1Ab and Vip3A to 1st instar *Heliothis virescens* in a diet incorporation assay after 7 days: (A) mean percent mortality (± se); (B) mean percent moult inhibition (± se); * indicates a within treatment significant difference ($P<0.05$) to the toxin alone.

7.3.3 Effect of the antibiotic cocktail on larval mortality and moult inhibition (MI) of Dipel

Larval mortality was unaffected ($F_{1, 6}$ = 0.45, P>0.05; Figure 7.3A) by the antibiotics alone (mortality ≤ 15 %). There were significant effects on larval mortality with treatments involving Dipel at 0.047 µg ml^{-1} ($F_{1, 14}$ = 88.19, P<0.001), Dipel at 0.0047 µg ml^{-1} ($F_{1, 14}$ = 45.88, P<0.001) and Cry1Ab ($F_{1, 14}$ = 9.304, P<0.01).

There was no significant difference in larval mortality between Dipel at 0.047 µg ml^{-1} with no antibiotic and the antibiotic cocktail (83 and 94 % mortality respectively; $F_{1, 6}$ = 4.15, P>0.05), between Dipel alone at 0.0047 µg ml^{-1} and combined with the antibiotic cocktail (54 and 69 % mortality respectively; $F_{1, 6}$ = 1.869, P>0.05) and between Cry1Ab alone and combined with the antibiotic cocktail (25 and 48 % mortality respectively; $F_{1, 6}$ = 4.47, P>0.05).

Larval MI was unaffected ($F_{1, 6}$ = 0.08, P>0.05; Figure 7.3B) by the antibiotics alone (MI ≤ 19 %). There were significant effects on larval MI with treatments involving Dipel at 0.047 µg ml^{-1} ($F_{1, 14}$ = 192.1, P<0.001), Dipel at 0.0047 µg ml^{-1} ($F_{3, 12}$ = 51.3, P<0.001) and Cry1Ab ($F_{3, 12}$ = 29.38, P<0.001).

Larval MI was significantly greater in the treatment combining Dipel at 0.047 µg ml^{-1} and the antibiotic cocktail compared to Dipel alone (100 and 92 % MI respectively; $F_{1, 6}$ = 7.97, P<0.05). Significant increases in MI were also confirmed with Dipel at 0.0047 µg ml^{-1} and the antibiotic cocktail compared to Dipel alone (98 and 65 % MI respectively; $F_{1, 6}$ = 22.45, P<0.01) and with Cry1Ab combined with the antibiotic cocktail compared to Cry1Ab alone and (92 and 29 % MI respectively; $F_{1, 6}$ = 31.67, P<0.01).

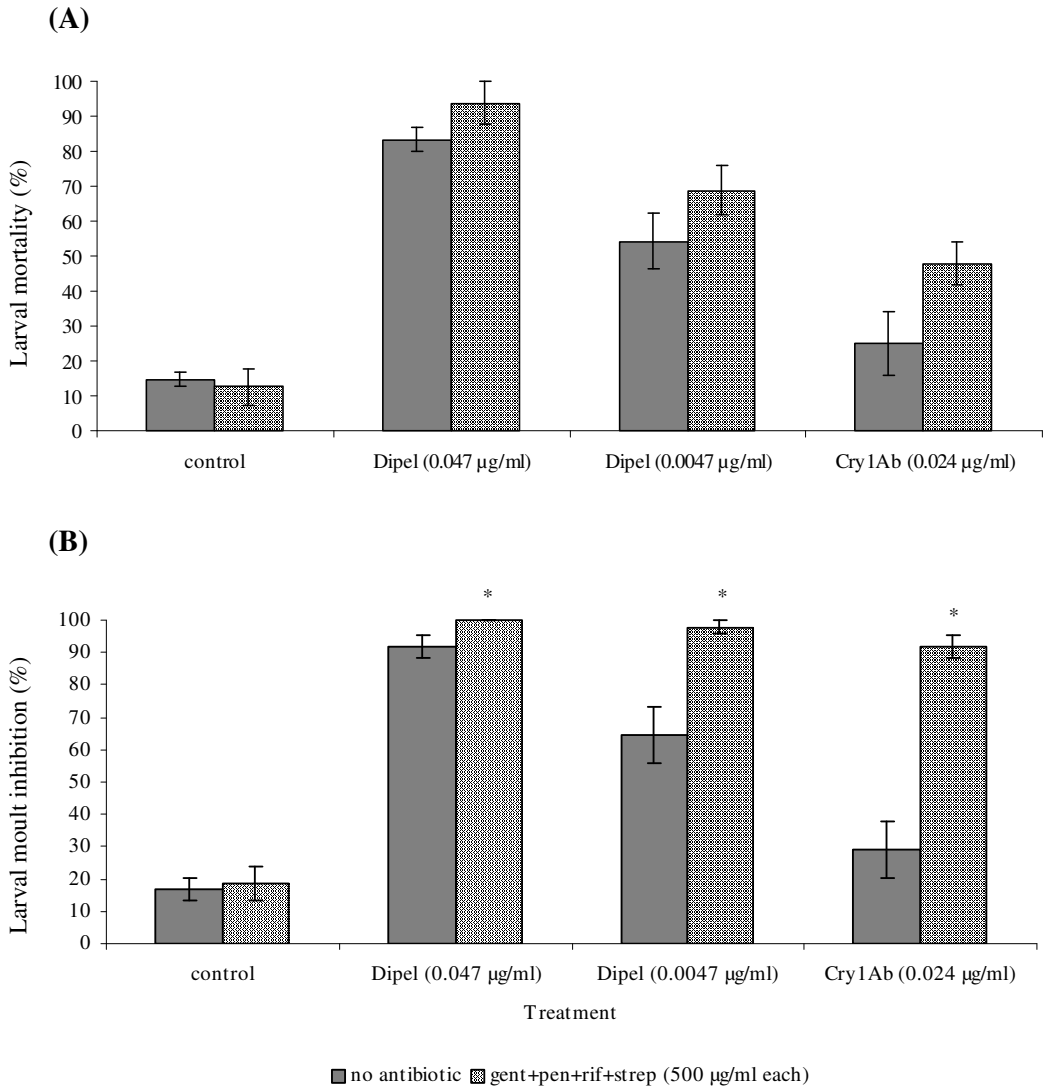

Figure 7.3: A comparison of the effect of an antibiotic cocktail (gentamicin, penicillin, rifampicin and streptomycin) on the toxicity of Dipel (at two concentrations) and Cry1Ab to 1st instar *Heliothis virescens* in a diet incorporation assay after 7 days: (A) mean percent mortality (± se); (B) mean percent moult inhibition (± se); * indicates a within treatment significant difference ($P<0.05$) to the toxin alone.

7.3.4 Effect of individual antibiotics on larval mortality and moult inhibition (MI) of Dipel (0.0047 µg ml^{-1})

Larval mortality was unaffected ($F_{5, 18}$ = 0.1795, P>0.05; Figure 7.4A) by the antibiotics alone (mortality ≤ 6 %). There were significant effects on larval mortality with treatments involving Dipel at 0.0047 µg ml^{-1} ($F_{11, 36}$ = 10.98, P<0.001).

A significant effect on larval mortality within Dipel treatments was found ($F_{5, 18}$ = 11.1, P<0.001). There were no significant differences in larval mortality between Dipel alone at 0.0047 µg ml^{-1} and combined with gentamicin, or penicillin or rifampicin (29, 42, 23 and 13 % mortality respectively; P>0.05). There was a significant increase in larval mortality in Dipel at 0.0047 µg ml^{-1} combined with the antibiotic cocktail (73 % mortality; P<0.001) and a significant decrease in larval mortality in Dipel at 0.0047 µg ml^{-1} combined with streptomycin (10 % mortality; P<0.05).

Larval MI was unaffected ($F_{5, 18}$ = 0.2041, P>0.05; Figure 7.4B) by the antibiotics alone (mortality ≤ 10 %). There were significant effects on larval MI with treatments involving Dipel at 0.0047 µg ml^{-1} ($F_{11, 36}$ = 19.33, P<0.001).

A significant effect on MI within Dipel treatments was found ($F_{5, 18}$ = 13.11, P<0.001). There were no significant differences (P>0.05) in larval MI between Dipel alone at 0.0047 µg ml^{-1} and combined with penicillin, or rifampicin (31, 58 and 23 % MI respectively; P>0.05). Larval MI was significantly greater in the treatments combining Dipel at 0.0047 µg ml^{-1} with either the antibiotic cocktail, gentamicin, or streptomycin (94, 88 and 73 % MI respectively; P<0.01).

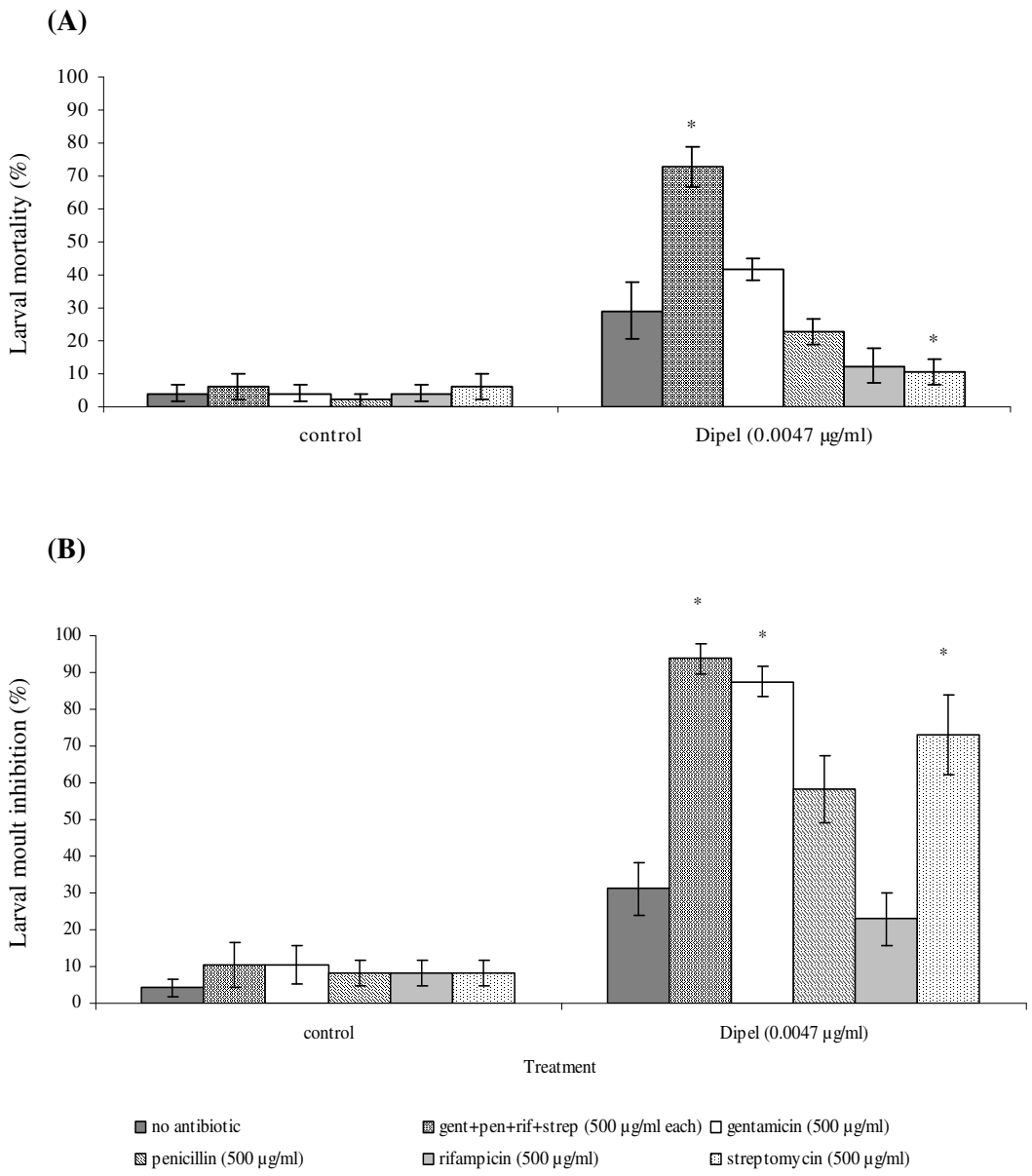

Figure 7.4: A comparison of the effect of an antibiotic cocktail (gentamicin, penicillin, rifampicin and streptomycin) and individual doses of those antibiotics on the toxicity of Dipel to 1st instar *Heliothis virescens* in a diet incorporation assay after 7 days: (A) mean percent mortality (± se); (B) mean percent moult inhibition (± se); * indicates a within treatment significant difference ($P<0.05$) to the toxin alone.

7.3.5 Effect of an antibiotic cocktail on the larval mortality and moult inhibition (MI) of Dipel and Cry1Ab on 3rd instar larvae reared in the presence or absence of antibiotics

There was no larval mortality without the presence of *Bt* toxins, or with the antibiotic cocktail alone after either 7 days or 14 days with larvae reared in the presence of the antibiotic cocktail (Figure 7.5). There was a significant increase in mortality with treatments involving Dipel and Cry1Ab after both 7 days ($F_{3, 20} = 3.823$, $P<0.05$) and 14 days ($F_{5, 18} = 41.41$, $P<0.001$).

The mortality within Dipel treatments after 7 days, with larvae reared in the presence of the antibiotic cocktail, was significantly greater with Dipel alone than combined with the antibiotic cocktail (13 and 3 % mortality respectively; $F_{1, 6} = 9$, $P<0.05$). The same pattern was found after 14 days, with mortality greater in Dipel alone than combined with the antibiotic cocktail (34 and 3 % mortality respectively; $F_{1, 6} = 30.73$, $P<0.01$).

There were no significant differences in mortality associated with Cry1Ab treatments with larvae reared in the presence of the antibiotic cocktail. After 7 days mortality with Cry1Ab alone was 6 % and combined with antibiotic cocktail was 12 % ($F_{1, 6} = 0.74$, $P>0.05$). After 14 days, mortality with Cry1Ab alone was 34 %, and combined with antibiotic cocktail was 44 % ($F_{1, 6} = 1.11$, $P>0.05$).

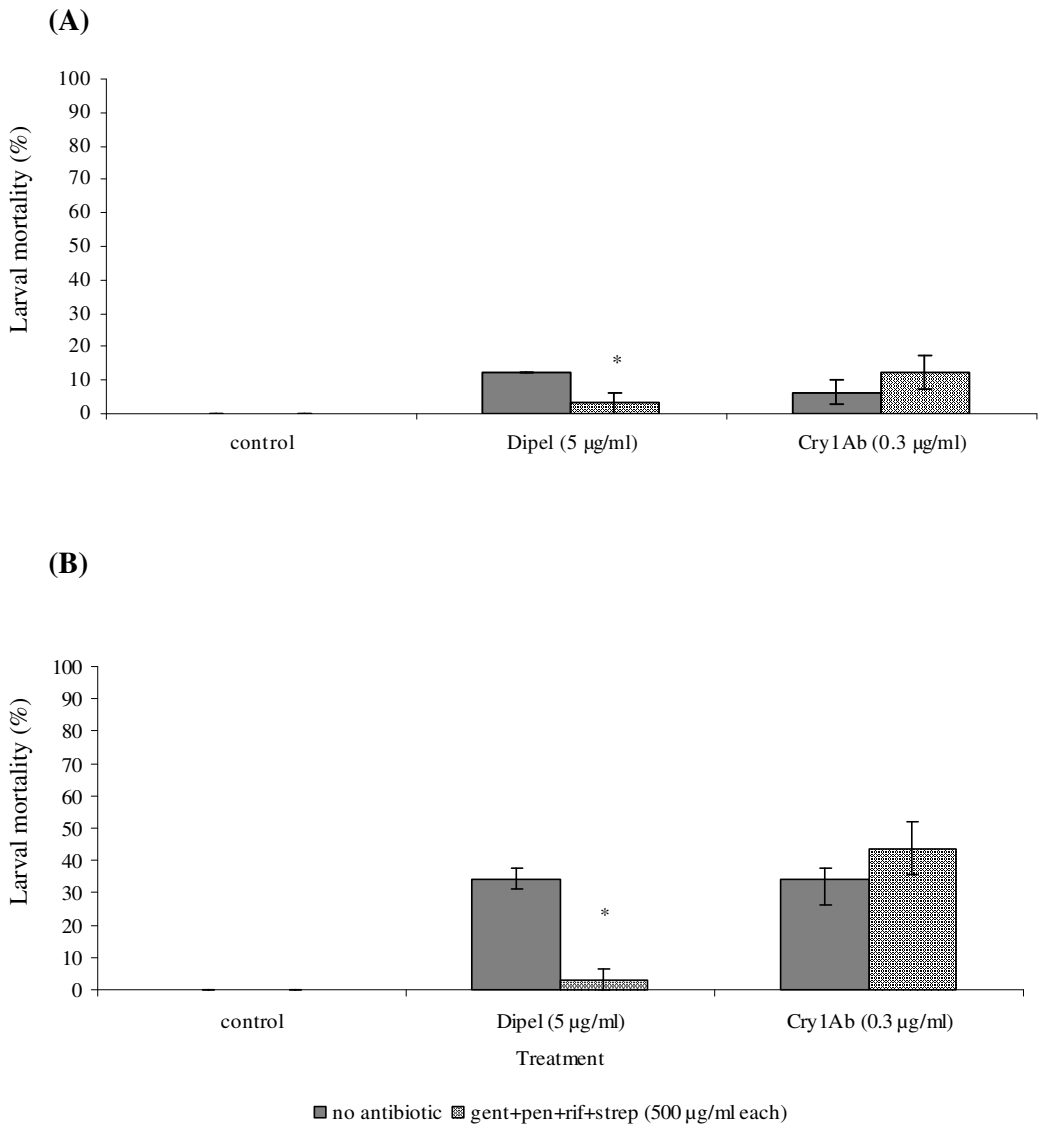

Figure 7.5: A comparison of the effect of an antibiotic cocktail (gentamicin, penicillin, rifampicin and streptomycin) on the mortality of Dipel and Cry1Ab to 3rd instar *Heliothis virescens* larvae that had been reared from 1st instar on artificial diet containing the antibiotic cocktail: (A) mean percent mortality after 7 days (± se); (B) mean percent mortality after 14 days (± se); * indicates a within treatment significant difference ($P<0.05$) to the toxin alone.

There was no MI without the presence of *Bt* toxins, or with the antibiotic cocktail alone, after either 7 days or 14 days with larvae reared in the presence of the antibiotic cocktail (Figure 7.6). There is a significant increase in MI with treatments involving Dipel and Cry1Ab after both 7 days ($F_{5, 18}$ = 57.56, P<0.001) and 14 days ($F_{5, 18}$ = 31.09, P<0.001).

The MI after 7 days, with larvae reared in the presence of the antibiotic cocktail, was significantly greater in the treatment combining Dipel and the antibiotic cocktail when compared to Dipel alone (63 and 16 % MI respectively; $F_{1, 6}$ = 26.41, P<0.01). The reverse pattern was demonstrated after 14 days, with significantly greater MI with Dipel alone than combined with the antibiotic cocktail (34 and 9 % MI respectively; $F_{1, 6}$ = 13.48, P<0.05).

There were no significant differences in larval MI between Cry1Ab alone and combined with the antibiotic cocktail after both 7 days (75 and 69 % mortality respectively; $F_{1, 6}$ = 0.0004, P>0.05) and 14 days (53 and 75 % mortality respectively; $F_{1, 6}$ = 2.84, P>0.05), with larvae reared in the presence of the antibiotic cocktail.

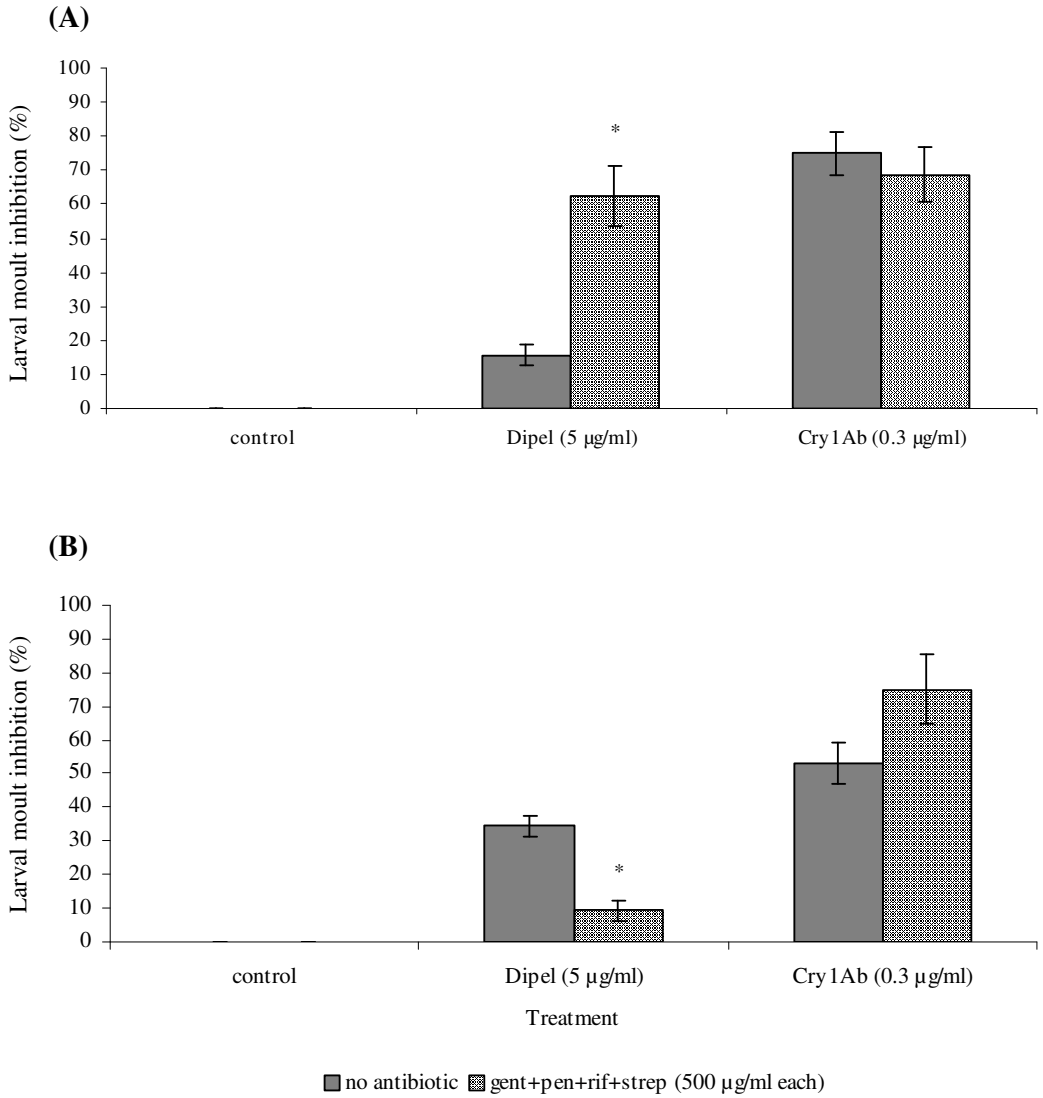

Figure 7.6: A comparison of the effect of an antibiotic cocktail (gentamicin, penicillin, rifampicin and streptomycin) on the moult inhibition of Dipel and Cry1Ab to 3rd instar *Heliothis virescens* larvae that had been reared from 1st instar on artificial diet containing the antibiotic cocktail: (A) mean percent moult inhibition after 7 days (± se); (B) mean percent moult inhibition after 14 days (± se); * indicates a within treatment significant difference ($P<0.05$) to the toxin alone.

There was no larval mortality without the presence of *Bt* toxins, or with the antibiotic alone, after both 7 days and 14 days with larvae reared in the absence of the antibiotic cocktail (Figure 7.7). There was a significant increase in mortality with treatments involving Dipel and Cry1Ab after both 7 days ($F_{5, 18} = 4.69$, $P<0.01$) and 14 days ($F_{5, 18} = 21.39$, $P<0.001$).

The mortality within Dipel treatments after 7 days, with larvae reared in the absence of the antibiotic cocktail, was significantly greater with Dipel alone than combined with the antibiotic cocktail (34 and 0 % mortality respectively; $F_{1, 6} = 6.64$, $P<0.05$). There was no significant difference in mortality after 14 days between Dipel alone combined with the antibiotic cocktail (41 and 16 % mortality respectively; $F_{1, 6} = 3.655$, $P>0.05$) despite the trend of greater mortality with Dipel alone.

There were no significant differences in mortality between Cry1Ab alone and combined with the antibiotic cocktail after both 7 days (3 and 13 % mortality respectively; $F_{1, 6} = 2.4$, $P>0.05$) and 14 days (34 and 44 % mortality respectively; $F_{1, 6} = 1.191$, $P>0.05$), with larvae reared in the absence of the antibiotic cocktail.

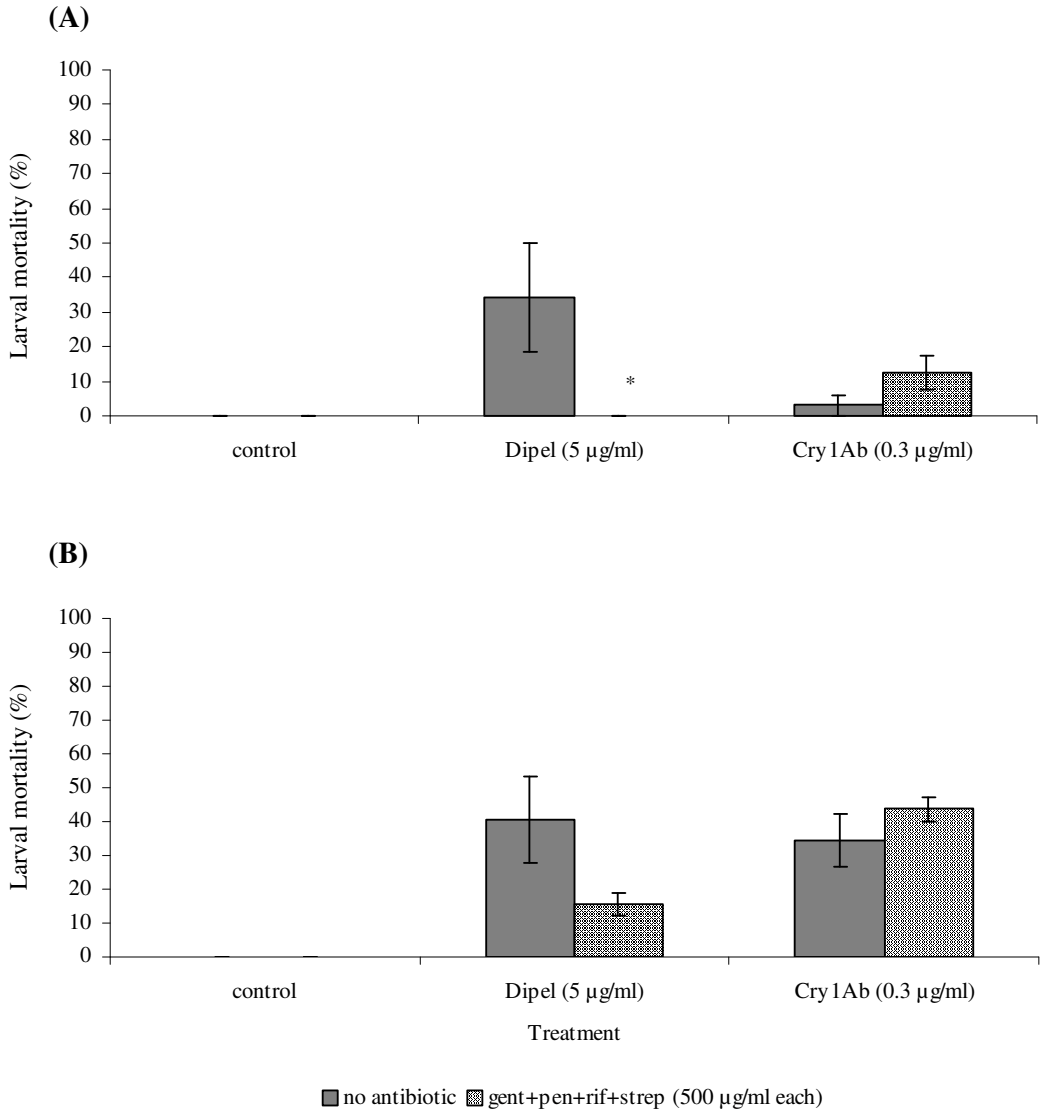

Figure 7.7: A comparison of the effect of an antibiotic cocktail (gentamicin, penicillin, rifampicin and streptomycin) on the mortality of Dipel and Cry1Ab to 3rd instar *Heliothis virescens* larvae that had been reared from 1st instar on artificial diet without containing the antibiotic cocktail: (A) mean percent mortality after 7 days (± se); (B) mean percent mortality after 14 days (± se); * indicates a within treatment significant difference ($P<0.05$) to the toxin alone.

There was no MI without the presence of *Bt* toxins, or the antibiotic cocktail alone, after both 7 days and 14 days, with larvae reared in the absence of the antibiotic cocktail (Figure 7.8). There was a significant increase in MI with treatments involving Dipel and Cry1Ab after both 7 days ($F_{5, 18} = 12.40$, $P<0.01$) and 14 days ($F_{5, 18} = 17.86$, $P<0.001$).

There were no significant differences in MI between Dipel alone and combined with the antibiotic cocktail after both 7 days (34 and 13 % MI respectively; $F_{1, 6} = 1.27$, $P>0.05$) and 14 days (41 and 16 % MI respectively; $F_{1, 6} = 3.66$, $P>0.05$), with larvae reared in the absence of the antibiotic cocktail, despite the trend of greater MI with Dipel alone.

The MI within Cry1Ab treatments after 7 days, with larvae reared in the presence of the antibiotic cocktail, was significantly greater with Cry1Ab and the antibiotic combined than with Cry1Ab alone (78 and 38 % MI respectively; $F_{1, 6} = 13.15$, $P<0.05$). After 14 days the trend was similar although there was no significant difference between Cry1Ab alone and when combined with the antibiotic cocktail (47 and 75 % MI respectively; $F_{1, 6} = 3.10$, $P>0.05$).

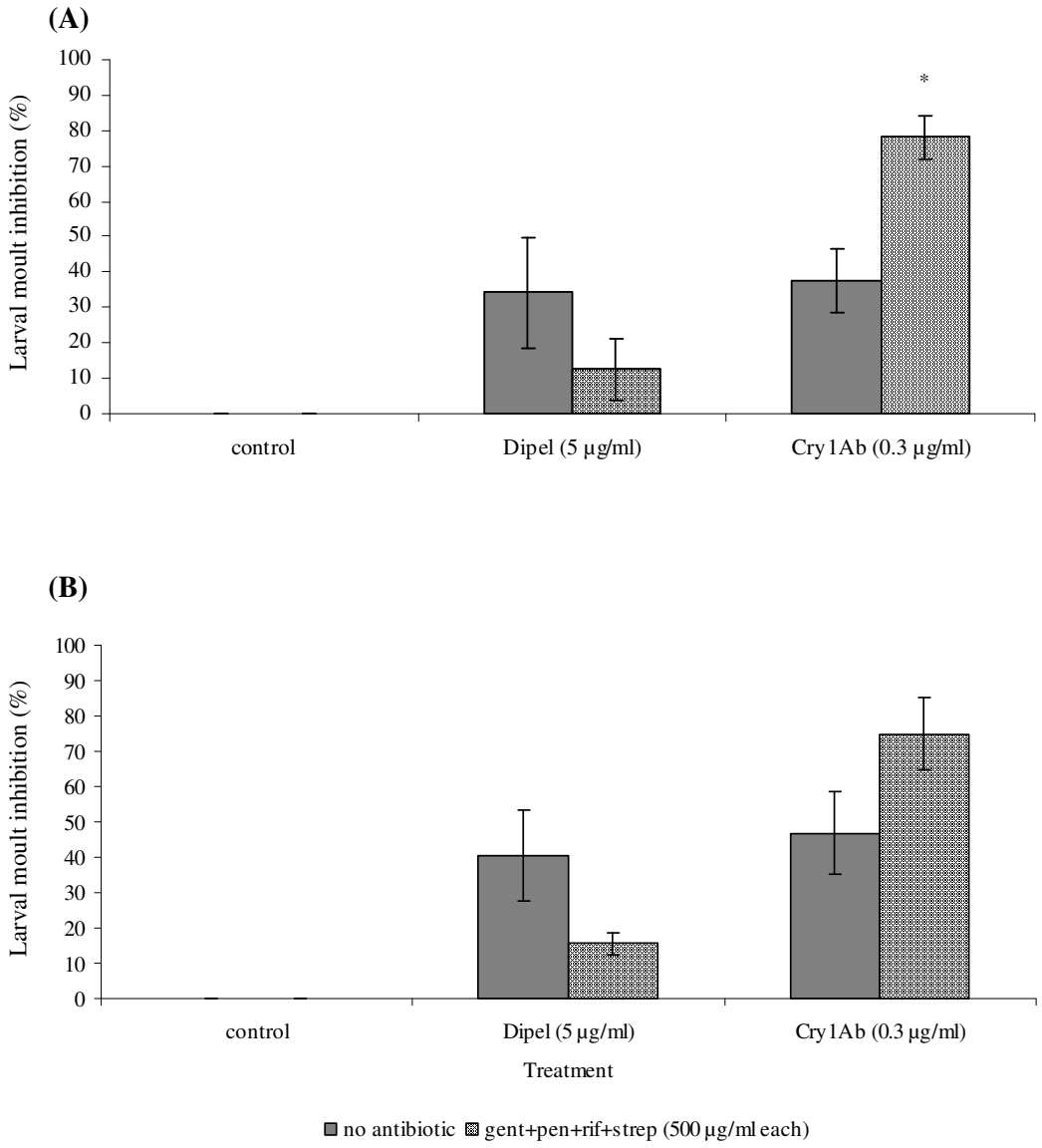

Figure 7.8: A comparison of the effect of an antibiotic cocktail (gentamicin, penicillin, rifampicin and streptomycin) on the moult inhibition of Dipel and Cry1Ab to 3rd instar *Heliothis virescens* larvae that had been reared from 1st instar on artificial diet without containing the antibiotic cocktail: (A) mean percent moult inhibition after 7 days (± se); (B) mean percent moult inhibition after 14 days (± se); * indicates a within treatment significant difference ($P<0.05$) to the toxin alone.

There was no significant difference in larval mortality of 3rd instar larvae reared in the presence or absence of the antibiotic cocktail with Dipel treatments after 7 days ($F_{3, 12}$ = 4.73, P>0.05; Figures 7.5A and 7.7A) or 14 days ($F_{3, 12}$ = 9.38, P>0.05; Figures 7.5B and 7.7B). The same pattern was found with MI after 14 days ($F_{3, 12}$ = 4.80, P>0.05; Figures 7.6B and 7.8B).

The MI of larvae reared in the presence of the antibiotic cocktail after 7 days on the Dipel and antibiotic cocktail treatment was significantly greater than the MI of larvae reared in the absence of the antibiotic cocktail (69 and 13 % MI respectively; $F_{3, 12}$ = 3.90, P<0.05; Figures 7.6A and 7.8A). There was no significant difference in MI of larvae reared in the presence or absence of the antibiotic cocktail with Dipel alone (P>0.05).

There was no significant difference in the larval mortality of 3rd instar larvae reared in the presence or absence of the antibiotic cocktail with Cry1Ab treatments after 7 days ($F_{3, 12}$ = 1.07, P>0.05; Figures 7.5A and 7.7A). The same pattern was found with larval mortality after 14 days ($F_{3, 12}$ = 0.77, P>0.05; Figures 7.5B and 7.7B) and MI after 14 days ($F_{3, 12}$ = 2.02, P>0.05; Figures 7.6B and 7.8B).

The MI of larvae reared in the presence of the antibiotic cocktail after 7 days on Cry1Ab alone was significantly greater than the MI of larvae reared in the absence of the antibiotic cocktail (75 and 38 % MI respectively; $F_{3, 12}$ = 5.21, P<0.05; Figures 7.6A and 7.8A). There was no significant difference in MI of larvae reared in the presence or absence of the antibiotic cocktail with Cry1Ab combined with the antibiotic cocktail ($F_{3, 12}$ = 5.21, P>0.05).

7.4 Discussion

In the present study the effect of antibiotics on *H. virescens* larval susceptibility to *Bt* toxins varied depending on the antibiotic treatment, the *Bt* toxin used and the larval instar tested (Tables 7.1, 7.2).

The antibiotic cocktail had a generally antagonistic effect on mortality due to Dipel against 3rd instar *H. virescens* larvae when they had been reared in the presence or absence of the antibiotic cocktail, although for some treatments the effect was not significant (Table 7.1). The trend was less clear for moult inhibition, where the antibiotic cocktail had no significant effect when insects were not reared with the antibiotic and a synergistic effect on the moult inhibition was found after 7 days with the antibiotic pre-treatment, although after 14 days this effect was reversed. This apparent anomaly may have been due to environmental variation or to variability in susceptibility within the population.

Broderick *et al.* (2006) found a similar antagonistic trend for mortality due to Dipel in 3rd instar *L. dispar* larvae reared in the presence of the same antibiotic cocktail. Further studies (Broderick *et al.*, 2009) with 3rd instar larvae of the painted lady, *Vanessa cardui* L. (Lepidoptera: Nymphalidae), the small white, *Pieris rapae* L. (Lepidoptera: Pieridae), *M. sexta* and *H. virescens* also showed that mortality caused by Dipel was reduced when the larvae were reared in the presence of antibiotics. The toxicity of Cry1Ac (MVPII), the primary Cry toxin in Dipel, was also antagonised by the antibiotic cocktail against 3rd instar larvae of *L. dispar*. However, these authors found the opposite effect for antibiotics with Cry1Ac against 3rd instar larvae of *P. gossypiella.*

The present study suggested that the antibiotic cocktail had a different effect on the toxicity of Cry1Ab compared to Dipel against 3rd instar larvae (Table 7.1). In all but one treatment antibiotics had no significant effect on mortality or moult inhibition due to Cry1Ab, however, the trend was for increased toxicity or moult inhibition.

Table 7.1: Summary of the effects on mortality and moult inhibition (MI) of Dipel or Cry1Ab combined with the antibiotic cocktail in comparison to the toxicity of toxins alone using 3rd instar larvae of *Heliothis virescens* that had been reared from 1st instars in the presence or absence of the antibiotic pre-treatment.

Antibiotic pre-treatment	Toxin	Mortality (7 days)[1]	Mortality (14 days)[1]	MI (7 days)[1]	MI (14 days)[1]
Yes	Dipel	-ve	-ve	+ve	-ve
No	Dipel	-ve	ne[2]	ne[2]	ne[2]
Yes	Cry1Ab	ne	ne	ne	ne[3]
No	Cry1Ab	ne	ne	+ve	ne[3]

[1] Significantly increased toxicity with antibiotic cocktail (+ve); significantly decreased toxicity with antibiotic cocktail (-ve); no significant effect of antibiotic cocktail (ne).

[2] No significant effect but a negative trend.

[3] No significant effect but a positive trend.

The effect of Vip3A on the mortality and moult inhibition of 1st instar larvae was unaffected by the antibiotic cocktail, whereas the cocktail had a synergistic effect with Dipel and Cry1Ab (Table 7.2). The synergistic effect with Dipel is in contrast to the antagonistic effect/trend observed with 3rd instar larvae of *H. virescens* (Table 7.1) and by Broderick *et al.* (2006, 2009) with other insect species, with the exception of *P. gossypiella*.

The effects of individual antibiotics on mortality and moult inhibition due to Vip3A for 1st instar larvae of *H. virescens* indicated that gentamicin and penicillin significantly antagonised Vip3A toxicity but that these decreases in susceptibility were masked when the antibiotics were incorporated into the cocktail mixture (Table 7.2). Individual antibiotic treatments had either no significant effect or the opposite (antagonistic) effect on mortality due to Cry1Ab or Dipel in contrast to the antibiotic cocktail. While gentamicin and, in this case, streptomycin had the same synergistic effects on moult inhibition due to Cry1Ab and Dipel as the antibiotic cocktail.

Table 7.2: Summary of the effects of antibiotics as a cocktail or individually on mortality and moult inhibition due to Vip3A, Cry1Ab and Dipel on 1st instar larvae of *Heliothis virescens.*

	Mortality[1] (7 days)			Moult inhibition[1] (7 days)		
Antibiotic	Vip3A	Cry1Ab	Dipel	Vip3A	Cry1Ab	Dipel
cocktail	ne	+ve	+ve	ne	+ve	+ve
gentamicin	-ve	ne	ne	-ve	+ve	+ve
penicillin	-ve	ne	ne	-ve	ne	ne
rifampicin	ne	ne	ne	ne	ne	ne
streptomycin	ne	ne	-ve	ne	+ve	+ve
ampicillin	ne	-ve	NT	ne	ne	NT

[1] Significantly increased toxicity with antibiotic treatment (+ve) compared with toxins alone; significantly decreased toxicity with antibiotic treatment (-ve); no significant effect of antibiotic treatment (ne); not tested (NT).

The complexities of the relationship between antibiotic effects, larval susceptibility and *Bt* toxin used are apparent from the findings of the present study and other research. Broderick *et al.* (2006, 2009) proposed that the effect of reduced *Bt* pathogenicity observed in lepidopteran larvae when reared on antibiotics was due to the elimination of important culturable gut microbiota. Re-establishment of a gut bacterium, *Enterobacter* sp. NAB3, restored the pathogenicity of *Bt* in four of the five species examined (Broderick *et al.*, 2006; Broderick *et al.*, 2009). They suggested therefore that gut microbiota contribute to the mortality associated with *Bt*, probably through initiation of septicemia after permeabilisation of the gut epithelium by *Bt toxins*, allowing access of the gut microbiota to the hemocoel. Pathogenicity was not restored in *H. virescens* which had been noted to not contain detectable gut microbiota before antibiotic administration (Broderick *et al.*, 2009). This different response with *H. virescens* suggests that the gut microbiota may have been undetectable (Broderick *et al.*, 2009) or that reduced pathogenicity of *Bt* is due to the direct effects of

antibiotic treatment (Raymond *et al.*, 2009). Raymond *et al.* (2009) demonstrated with 2nd and 3rd instar larvae of *P. xylostella*, that rifampicin reduced the pathogenicity of *Btk* in aseptic larvae. This suggested that antibiotics can persist in the tissues and haemolymph of lepidopteran larvae.

With *P. gossypiella* antibiotics increased susceptibility to Cry1Ac, which suggested a protective role of gut microbiota with their suppression resulting in the increased pathogenicity of Cry1Ac (Broderick *et al.*, 2009). This role was also suggested by the observed significant reduction in larval mortality with *Btk* HD-1 on *P. xylostella* larvae with culturable gut microbiota (Raymond *et al.*, 2009). Another study has demonstrated synergism of zwittermicin A with *Btk* in *L. dispar* and it was hypothesized that the antimicrobial properties of this antibiotic altered the gut microbiota (Broderick *et al.*, 2000). The ingestion of a high-antibiotic secreting *B. cereus* strain has been observed to synergise *Bt* mortality in *P. xylostella* larvae, with confirmed reduction in the gut microbiota (Raymond *et al.*, 2008).

The applicability of these hypotheses to results in the present study is not clear, as information on the gut microbiota for *H. virescens* was not investigated. However, the effects of the antibiotic cocktail on Dipel pathogenicity differed between 1st and 3rd instar larvae and antibiotic effects on other *Bt* toxins showed varied trends. These results support the idea that a single hypothesis is unlikely to explain all the interactions between *Bt* toxins and lepidopteran larvae in the presence of antibiotics. The antibiotic cocktail had no effect on Vip3A pathogenicity, and this may be linked to differences in the mode of action of Vip3A compared with Cry toxins (section 2.1.3.2).

As discussed above, the present study shows that antibiotics, including ampicillin in artificial diet bioassays can have variable effects on mortality and moult inhibition in 1st and 3rd instar larval stages of *H. virescens*. The continued exclusion of antibiotics in artificial diet bioassays appears, therefore, to be appropriate to avoid potential effects on insect susceptibility.

Chapter 8

Summary and General Discussion

8.1 Summary of experimental findings

Chapter 4: Susceptibility of *H. virescens* to Vip3A, Cry1Ab and Cry1Ac.

- There was little variability in the natural susceptibility to Vip3A, Cry1Ab and Cry1Ac in all tested populations of *H. virescens* based on LC_{50} and MIC_{50} data.
- The toxicity of Vip3A was much lower compared with Cry1Ab and Cry1Ac.
- Larval instars responded differently to Vip3A and Cry1Ab, with 1st to 4th instars all equally susceptible to Cry1Ab, whereas 4th instar larvae were much more tolerant to Vip3A compared with earlier instars.

Chapter 5: Vip3A selection, cross-resistance and genetics of resistance.

- Successful selection of a Vip3A resistant population (WF06-Vip3ASEL) within 13 selected generations.
- Vip3A resistance appeared to be relatively stable under laboratory conditions.
- Little or no cross-resistance in Vip3A resistant population to Cry1Ab or Cry1Ac.
- F_1 reciprocal crosses between WF06-Vip3ASEL and WF06-UNSEL indicated a possible paternal influence on inheritance of resistance, which ranged from almost completely recessive to incompletely dominant.
- Bioassays on F_2 from a backcross of F_1 progeny with WF06-Vip3ASEL using two discriminating concentrations of Vip3A indicated that resistance was polygenic.

Chapter 6: Fitness studies on Vip3A resistance.

- Vip3A resistant populations showed both a fitness benefit (reduced larval development time) and fitness costs (reduced pupal weight, survival to adult eclosion, reduced egg viability, and reduced male mating success).
- There were no apparent fitness costs or benefits in relation to fecundity, pupal development time and no change in adult sex ratio.

Chapter 7: Antibiotic effects on larval susceptibility to *Bt* toxins.

- The effect of antibiotics on *H. virescens* larval susceptibility to *Bt* toxins varied depending on antibiotic treatment, the *Bt* toxin used and the larval instar tested.
- An antibiotic cocktail (gentamicin, penicillin, rifampicin and streptomycin) had an antagonistic trend on Dipel toxicity with 3rd instar larvae and a synergistic trend on Dipel toxicity with 1st instar larvae.
- The antibiotic cocktail had a synergistic effect on Cry1Ab toxicity with 1st instar larvae and showed a similar trend with 3rd instar larvae.
- The antibiotic cocktail had no effect on Vip3A toxicity with 1st instar larvae, with antagonistic effects of individual treatments of gentamicin and penicillin apparently masked by the cocktail mixture.
- Individual antibiotic treatments (streptomycin and gentamicin) had synergistic effects with Dipel and Cry1Ab on moult inhibition, but no effects on mortality were apparent with the exception of antagonistic effects of ampicillin on Cry1Ab and of streptomycin on Dipel.

8.2 General Discussion

In the present study, susceptibility to Vip3A in field-derived populations of *H. virescens* demonstrated little variability between populations ranging from different geographical locations, for example, North Carolina, Mississippi and Arkansas, all of which are *Bt* cotton growing states, and a standard laboratory population. This

suggested that the frequency of resistant alleles to Vip3A in the field populations was below a level that would cause an immediate problem for control. This observation is supported by field studies on *H. virescens* with *Bt* cotton expressing Vip3A and a pyramided variety expressing both Vip3A and Cry1Ab (Mascarenhas *et al.*, 2005; Adamczyk and Mahaffey, 2008; Bommireddy and Leonard, 2008).

Control of *H. virescens* has been successful in *Bt* cotton varieties expressing Vip3A, with very few larvae surviving 14 days after infestation (Mascarenhas *et al.*, 2005). Survival up to 7 days was found to be greater (Adamczyk and Mahaffey, 2008; Bommireddy and Leonard, 2008). In all field trials, development of surviving larvae was severely inhibited, suggesting that it was unlikely that any larvae would have survived to pupation.

The variation in expression of Cry1Ac and Vip3A in plant structures and the trend of reduced efficacy of toxins towards the end of the season (Greenplate, 1999; Adamczyk *et al.*, 2001b; Llewellyn *et al.*, 2007; Bommireddy and Leonard, 2008), could present a window of opportunity for later instar larvae to survive and hence undergo selection for resistance. The potential for this to occur was demonstrated in the present study by the increased tolerance of 4th instar larvae of *H. virescens* to Vip3A compared with earlier instars. However, in the planned commercialisation of *Bt* cotton expressing Vip3A, this toxin will be pyramided with Cry1Ab (McCaffery *et al.*, 2006; Kurtz *et al.*, 2007) and field trials with such double constructs of *Bt* cotton have given very good control of *H. virescens,* with few survivors and little variation between plant structures. Successful control with Vip3A-Cry1Ab cotton has also been demonstrated against *H. zea*, *S. frugiperda* and *S. exigua* (Adamczyk and Mahaffey, 2008; Bommireddy and Leonard, 2008).

The use of pyramided *Bt* crops gives better control of insect pest complexes, taking advantage of the potential for one species that may be less susceptible to one of the toxins, to be susceptible to the other toxin and be controlled. This combination in a high dose plus refuge strategy (Gould, 1998; Roush, 1998; Zhao *et al.*, 2003; Bates *et al.*, 2005; Bravo *et al.*, 2008) should also lead to greater durability of the pyramided variety as compared to a single expression variety. However, for this to occur it is

critical that there is no cross-resistance between the two expressed toxins in the pyramided variety.

The selection of a Vip3A resistant *H. virescens* population in the present study enabled an assessment of cross-resistance and showed little or no evidence of cross-resistance to either Cry1Ab or Cry1Ac. These findings reinforce previous, reciprocal studies where a Cry1Ac resistant *H. virescens* and Cry1Ac *H. zea* population showed no cross-resistance to Vip3A (section 5.4). The novel properties of Vip3A, with structural differences and no sequence homology with known *Bt* Cry toxins, and distinct binding sites and pore formation (Estruch *et al.*, 1996; Yu *et al.*, 1997; Lee *et al.*, 2006) also suggests that cross-resistance mechanisms between Vip and Cry toxins are less likely to occur than between different Cry toxins.

The observed lack of cross-resistance demonstrates the potential value of using Vip3A for insect control alongside other *Bt* crops that currently express Cry toxins, varieties available include *Bt* cotton expressing Cry1Ac alone, pyramided Cry1Ac and Cry2Ab, and pyramided Cry1Ac and Cry1F, and *Bt* corn expressing Cry1Ab (McCaffery *et al.*, 2006). *Helicoverpa zea* is less susceptible to Cry1Ac compared with *H. virescens* and there is some evidence, albeit disputed, for field-evolved resistance to Cry1Ac (Moar *et al.*, 2008; Tabashnik *et al.*, 2008a; Tabashnik *et al.*, 2008b). The introduction of Vip3A could thus help to prevent *Bt* cotton field failures. *Spodoptera frugiperda* and *S. exigua* are also relatively tolerant to Cry1Ab but are highly susceptible to Vip3A (Adamczyk and Mahaffey, 2008). Field resistance to *Bt* crops has been reported in *S. frugiperda* against *Bt* corn expressing Cry1F in Puerto Rico, resulting in field failure and the immediate discontinuation of commercial cultivation (Moar *et al.*, 2008). There is also evidence of resistance in *B. fusca* populations to *Bt* corn expressing Cry1Ab in South Africa, although field failure has not been reported (van Rensburg, 2007).

The current IRM strategy in use with *Bt* crops combines high doses (expression) of toxin with the provision of refugia (non-Bt crop areas) (Gould, 1998; Roush, 1998; Zhao *et al.*, 2003; Bates *et al.*, 2005; Bravo *et al.*, 2008). There are several assumptions that this strategy follows regarding the development of resistance: that inheritance of resistance is recessive; that random mating occurs between resistant

and susceptible insects; and that high dose will kill all resistant heterozygotes and susceptible insects (taken as 25 times the concentration needed to kill all susceptible insects) (Bates *et al.*, 2005).

In order to develop and maintain IRM strategies it is essential to gain an understanding of the genetics of resistance to *Bt* toxins. In the present study, the inheritance of resistance to Vip3A suggested a paternal influence, with resistant males of *H. virescens* showing an incompletely dominant to incompletely recessive mode of inheritance but resistant females showing an almost completely recessive mode of inheritance. If this pattern of resistance occurred in the field, random mating between susceptible and resistant insects could lead to the survival of heterozygote resistant insects on *Bt* crops and thus increase the rate of development of resistance.

However, the present study also found moult inhibition of larvae associated with resistance to Vip3A. When moult inhibition data is taken into account, resistance to Vip3A is incompletely recessive, meeting the assumption of recessive resistance required for an effective high dose plus refuge strategy. Resistance risk assessment modelling has indicated that if resistance to Vip3A is recessive, the frequency of resistance alleles should not increase more than 10-fold within 15 years, suggesting the risk of resistance is low (McCaffery *et al.*, 2006). The fitness costs linked with Vip3A resistance in the present work would also have the potential to maintain the effectiveness of an IRM strategy

The apparent fitness benefit of faster development time for Vip3A resistant larvae of *H. virescens* is unlikely to be a significant fitness benefit in the field, due to the likelihood of overlapping generations and the observation that resistant males did not develop to adult eclosion faster than susceptible males and females. Diet incorporated Vip3A bioassays also demonstrated moult/development inhibition in some surviving insects.

Other laboratory Cry resistant insect populations vary in their ability to survive on *Bt* crops. For example, resistant populations of *P. xylostella*, *P. gossypiella* and *H. armigera* completed development on *Bt* crops, whereas *O. nubilalis* and *L. decemlineata* did not survive on *Bt* crops and a *H. virescens* population did not

survive on *Bt* cotton or non-*Bt* cotton (Tabashnik *et al.*, 2003). The reasons for poor survival on *Bt* crops may include the prolonged exposure to the toxin and higher toxin concentrations with *Bt* crops, the expression of the toxin, whether in active or protoxin form, and the change in diet. Plant chemistry may also affect survival, for example, the allelochemical gossypol, which accumulates in glands present throughout most of the vegetative and reproductive tissues of cotton, has been shown to impart plant resistance to *H. virescens*, *H. zea* and *P. gossypiella* (Gannaway, 1994; Romano and Scheffler, 2008; Tabashnik *et al.*, 2003).

8.3 Future Work

The present work has provided information on the development of resistance, cross-resistance, and fitness effects of resistance, and the effect of antibiotics on *Bt* toxicity. Further work should be conducted to build upon the knowledge and understanding gained thus far, including:

- studies on baseline susceptibility to Vip3A in field derived *H. virescens* populations and other target insects through annual monitoring programmes;
- investigation of the mechanism of resistance to Vip3A; for example using BBMV binding assays to determine whether loss of binding is involved;
- utilization of genetics and fitness information in IRM computer models to look at potential resistance scenarios and the effectiveness of the current IRM strategy;
- determination of cross-resistance to Cry1F and Cry2A, two other *Bt* toxins currently expressed in commercial *Bt* cotton;
- studies on the fitness cost of resistant male mating success; determination of the presence or absence of spermatophores in mated adult females when paired with resistant males; investigate the randomness of mating between resistant and susceptible individuals (Zhao *et al.*, 2008);
- investigate the ability of the Vip3A resistant population to survive on non-*Bt* and *Bt* cotton (Tabashnik *et al.*, 2003);

- determination of the presence of gut microbiota in *H. virescens* larvae to further understand the effects of antibiotics on *Bt* toxicity.

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Appendix 1

Comparative toxicity of *Bt* toxins against different larval instars of the NCSU and WF06 populations

Table A.1.1: LC_{50} values of the NCSU population for 1st, 2nd, 3rd and 4th instar *Heliothis virescens* larvae to Vip3A in a diet incorporation assay after 7 days (linked to Figure 4.1).

Larval instar	LC_{50} µg ml^{-1}	95 % CI[1]	Slope (± se)	n^2
1	1.69	0.98 – 2.92 a	0.52 (± 0.10)	336
2	1.77	1.40 – 2.23 a	1.47 (± 0.25)	240
3	1.50	1.20 – 1.87 a	1.52 (± 0.21)	288
4	5.11	3.77 – 6.93 b	1.05 (± 0.17)	288

[1] Values followed by same letter are not significantly different (P>0.01).

[2] Number of larvae used in bioassay, including control.

Table A.1.2: MIC_{50} values of the NCSU population for 1st, 2nd, 3rd and 4th instar *Heliothis virescens* larvae to Vip3A in a diet incorporation assay after 7 days (linked to Figure 4.1).

Larval instar	MIC_{50} µg ml^{-1}	95 % CI[1]	Slope (± se)	n^2
1	0.18	0.04 – 0.96 a	0.38 (± 0.12)	336
2	1.77	1.40 – 2.23 b	1.47 (± 0.25)	240
3	1.44	1.14 – 1.81 b	1.49 (± 0.21)	288
4	3.41	2.81 – 4.13 c	1.92 (± 0.29)	240

[1] Values followed by same letter are not significantly different (P>0.01).

[2] Number of larvae used in bioassay, including control.

Table A.1.3: LC_{50} values of the NCSU population for 1st, 2nd, 3rd and 4th instar *Heliothis virescens* larvae to Cry1Ab in a diet incorporation assay after 7 days (linked to Figure 4.2).

Larval instar	LC_{50} µg ml^{-1}	95 % CI[1]	Slope (± se)	n^2
1	0.080	0.058 – 0.110 a	1.04 (± 0.13)	288
2	0.071	0.036 – 0.137 a	0.70 (± 0.19)	120
3	0.060	0.041 – 0.088 a	1.44 (± 0.31)	120
4	0.092	0.057 – 0.153 a	0.86 (± 0.14)	240

[1] Values followed by same letter are not significantly different (P>0.01).

[2] Number of larvae used in bioassay, including control.

Table A.1.4: MIC_{50} values of the NCSU population for 1st, 2nd, 3rd and 4th instar *Heliothis virescens* larvae Cry1Ab in a diet incorporation assay after 7 days (linked to Figure 4.2).

Larval instar	MIC_{50} µg ml^{-1}	95 % CI[1]	Slope (± se)	n^2
1	0.039	0.030 – 0.050 a	1.57 (± 0.19)	288
2	0.029	0.017 – 0.049 a	1.09 (± 0.25)	120
3	0.032	0.022 – 0.045 a	2.15 (± 0.47)	96
4	0.030	0.017 – 0.052 a	0.86 (± 0.14)	240

[1] Values followed by same letter are not significantly different (P>0.01).

[2] Number of larvae used in bioassay, including control.

Table A.1.5: LC_{50} values of the WF06 population for 1st, 2nd, 3rd and 4th instar *Heliothis virescens* larvae to Vip3A in a diet incorporation assay after 7 days (linked to Figure 4.3).

Larval instar	LC_{50} µg ml^{-1}	95 % CI[1]	Slope (± se)	n^2
1	2.63	1.87 – 3.70 a	0.83 (± 0.10)	384
2	2.91	2.36 – 3.60 a	1.60 (± 0.19)	336
3	3.76	2.46 – 5.77 a	0.76 (± 0.10)	288
4	> 100[3]		0.86 (± 0.14)	240

[1] Values followed by same letter are not significantly different (P>0.01).

[2] Number of larvae used in bioassay, including control.

[3] Mortality at 100µg ml^{-1} = 13 %.

Table A.1.6: MIC_{50} values of the WF06 population for 1st, 2nd, 3rd and 4th instar *Heliothis virescens* larvae Vip3A in a diet incorporation assay after 7 days (linked to Figure 4.3).

Larval instar	MIC_{50} µg ml^{-1}	95 % CI[1]	Slope (± se)	n^2
1	0.90	0.69 – 1.18 a	1.10 (± 0.12)	384
2	2.07	1.68 – 2.56 b	1.60 (± 0.18)	336
3	1.88	1.39 – 2.56 b	1.08 (± 0.12)	336
4	9.21	5.18 – 16.4 c	0.78 (± 0.16)	240

[1] Values followed by same letter are not significantly different (P>0.01).

[2] Number of larvae used in bioassay, including control.